Generation of recombinant CHO cells towards bioengineered heparin and heparan sulfate production

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Introduction

1.1 Overview of Heparin

1.1.1 Discoveries and milestones

Heparin, a naturally occurring polysaccharide, has a rich history spanning over a century, marked by scientific exploration and medical advancements (Mulloy et al., 2016; Onishi et al., 2016). The journey of heparin research traces back to the early 20th century when researchers initially observed the anticoagulant properties in extracts derived from animal tissues (**Fig. 1-1**) (Aláez-Versón et al., 2017; Contejean, 1895; Hemker, 2016; Mulloy et al., 2016; Onishi et al., 2016; Torri and Naggi, 2016; Wardrop and Keeling, 2008). In 1916, Jay McLean, a student at Johns Hopkins University, identified this property in an extract from canine liver (McLean, 1916). It was not until 1922 that Howell and Holt demonstrated the effectiveness of this extract, naming it "heparin" and establishing its role in preventing blood clotting (Best, 1959; Hemker, 2016).

Throughout the 20th century, researchers made significant progress in isolating and characterizing heparin from various animal sources, such as beef lung and porcine intestine. The development of purification techniques, such as alcohol precipitation and ion-exchange chromatography, enabled the production of pure heparin suitable for clinical use. These advancements facilitated the introduction of heparin as a crucial anticoagulant therapy during the 1930s, saving numerous lives (Brinkhous et al., 1939). In the 1950s, scientists elucidated the relationship between heparin and antithrombin (AT), leading to the isolation of highly purified AT by Abildgaard in 1968 (Abildgaard, 1968; Monkhouse et al., 1955; Waugh and Fitzgerald, 1956). Further investigations in the early 1980s revealed a unique pentasaccharide within heparin that enhances its therapeutic efficacy by binding to the AT binding site (Jean Choay et al., 1981; Thunberg et al., 1982). Heparin has since become a cornerstone of modern medicine, aiding in extracorporeal therapy, surgeries, and preventing conditions like deep vein thrombosis (Thacker et al., 2022).

Despite its widespread use, challenges have emerged in heparin production and safety. At the beginning, the outbreak of bovine spongiform encephalopathy (BSE) in the UK during the 1990s significantly impacted heparin production, although South American countries like Brazil and Argentina continue to produce bovine-derived heparin (Brown et al., 2001; Szajek et al., 2015; van der Meer et al., 2017). Between 2007 and 2008, the US faced severe adverse reactions and fatalities due to adulterated heparin contaminated with oversulfated chondroitin sulfate (OSCS) from China (Kishimoto et al., 2008; Mans et al., 2015; McMahon et al., 2010; Szajek et al., 2015; Zhu et al., 2019). Additionally, global heparin supply disruptions occurred during events like the African

swine fever pandemic since 2018 (Normile, 2018; Vilanova et al., 2019). Recurrent supply shortages and safety concerns have prompted regulatory agencies like the FDA (U.S. Food and Drug Administration) and the European Pharmacopeia to recommend stringent measures, including polymerase chain reaction (PCR) as well as physicochemical and immunological analyses, to detect any contaminants and ensure the purity of heparin (Pharmacopeia, 2017; Services, 2013; van der Meer et al., 2017). Despite these challenges, heparin remains an important medication, as recognized by the World Health Organization (WHO), underscoring its vital role in modern healthcare (WHO, 2017).

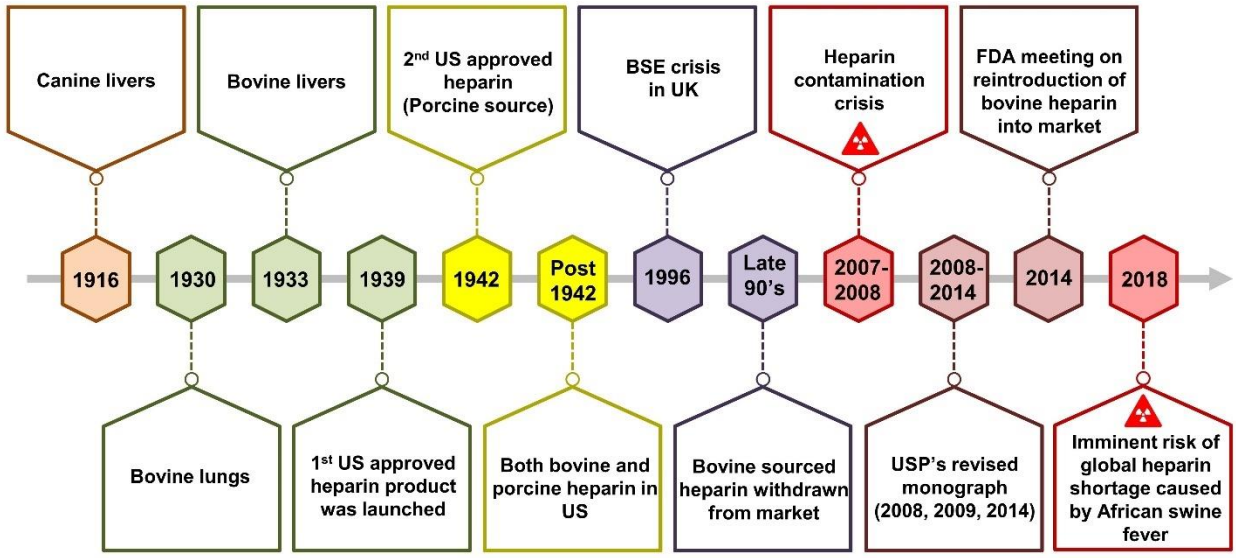

Fig. 1-1 A timeline of the historical development of therapeutic heparin. USP: United States pharmacopeia, BSE: bovine spongiform encephalopathy, FDA: Food and drug administration.

1.1.2 Structure of heparin

Heparin is a linear polysaccharide composed of repeating sulfated disaccharide units, where α-D-glucosamine (GlcN) and β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) are linked by 1,4 glycosidic bonds. This molecule is highly sulfated, with the D-glucosamine residues being N-sulfated, 3-O-sulfated, and 6-O-sulfated, while the D-glucuronic acid and L-iduronic acid residues are 2-O-sulfated (**Fig. 1-2**). A characteristic feature of heparin is its potent anticoagulant effect, which arises from a specific pentasaccharide sequence. This sequence, rich in sulfate groups, binds to AT and significantly enhances its activity by several thousand-fold.

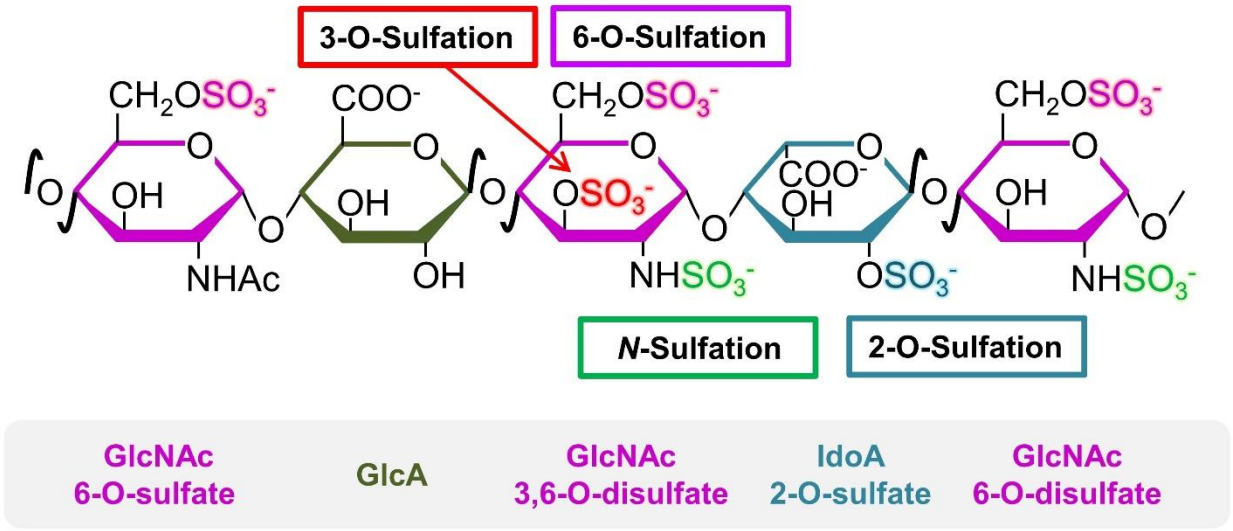

Fig. 1-2 Antithrombin-specific binding pentasaccharide sequence. GlcNAc: *N*-acetyl glucosamine, GlcA: Glucuronic acid, IdoA: Iduronic acid.

1.1.3 Heparin and its anticoagulant mechanism

(I) Blood coagulation reactions

When a blood vessel is damaged, the hemostatic process is initiated in two stages: primary and secondary hemostasis. Primary hemostasis involves the adhesion and aggregation of platelets to endothelial collagen, forming a platelet plug. Secondary hemostasis involves a cascade of reactions among coagulation factors, leading to the formation of a stable fibrin clot through the action of thrombin and factor XIIIa.

Here, we will outline the secondary hemostasis process, where heparin exerts its effects. There are two main pathways involved: the intrinsic pathway, activated by contact with a negatively charged surface, and the extrinsic pathway, triggered by tissue factors (TFs) released at the injury site (**Fig. 1-3**). The intrinsic pathway begins when factor XII comes into contact with negatively charged surfaces, such as phospholipids exposed at the site of vascular injury. This contact leads to the autoactivation of factor XII to factor XIIa. Factor XIIa subsequently activates factor XI, which then activates factor IX. The extrinsic pathway, also known as the tissue factor pathway, is initiated when tissue factor (TF), a membrane-bound protein, interacts with plasma factor VII. This interaction forms the TF-VIIa complex, which activates both factor IX and factor X. Both pathways converge to activate factor X. In the intrinsic pathway, factor IXa forms a complex with factor VIIIa, calcium ions, and phospholipids, known as the tenase complex, which activates factor

X to Xa. In the extrinsic pathway, the TF-VIIa complex directly activates factor X. Once factor X is activated to Xa, it forms the prothrombinase complex with factor Va, calcium ions, and phospholipids. This complex significantly accelerates the conversion of prothrombin (factor II) to thrombin (factor IIa). While factor Xa can catalyze prothrombin activation alone, the presence of factor Va greatly enhances the reaction rate. Thrombin production begins with the low-level activity of factor Xa and prothrombinase complexes. Thrombin then amplifies its own production by providing positive feedback that activates factors V, VIII, and XI. The generated thrombin converts soluble fibrinogen into insoluble fibrin and activates factor XIII, which cross-links fibrin, stabilizing the clot.

Heparin exerts its anticoagulant effect during secondary hemostasis. It enhances the activity of antithrombin-III (AT) by binding to it, which in turn inhibits thrombin and other serine proteases involved in the coagulation cascade, such as factors Xa and IXa. This action effectively prevents the formation of fibrin clots, highlighting heparin's crucial role in anticoagulant therapy.

(ii) Control of blood clotting

The regulation of blood coagulation is critical to prevent widespread activation of the coagulation system and excessive fibrin deposition. For effective functioning, the system must activate only at the site of vascular injury and remain active just long enough to produce sufficient fibrin to close the wound. This is achieved through various regulatory mechanisms, one of which involves the control of coagulation via heparin and AT.

AT is a serine protease inhibitor that can inhibit several activated coagulation enzymes. Key enzymes in the coagulation cascade, including factors IXa, Xa, the TF-VII complex, and thrombin, are swiftly bound and neutralized by AT. Heparin greatly enhances the inhibitory activity of AT, which underlies the anticoagulant action of pharmaceutical heparin. When thrombin binds to AT, it forms a stable antithrombin-thrombin (TAT) complex that is quickly removed from circulation. In intact blood vessels, AT binds to endothelial heparin proteoglycans, allowing it to inactivate factor Xa and thrombin and thereby prevent thrombus formation. Conversely, in damaged blood vessels, this mechanism is thought to specifically promote thrombus formation and clotting.

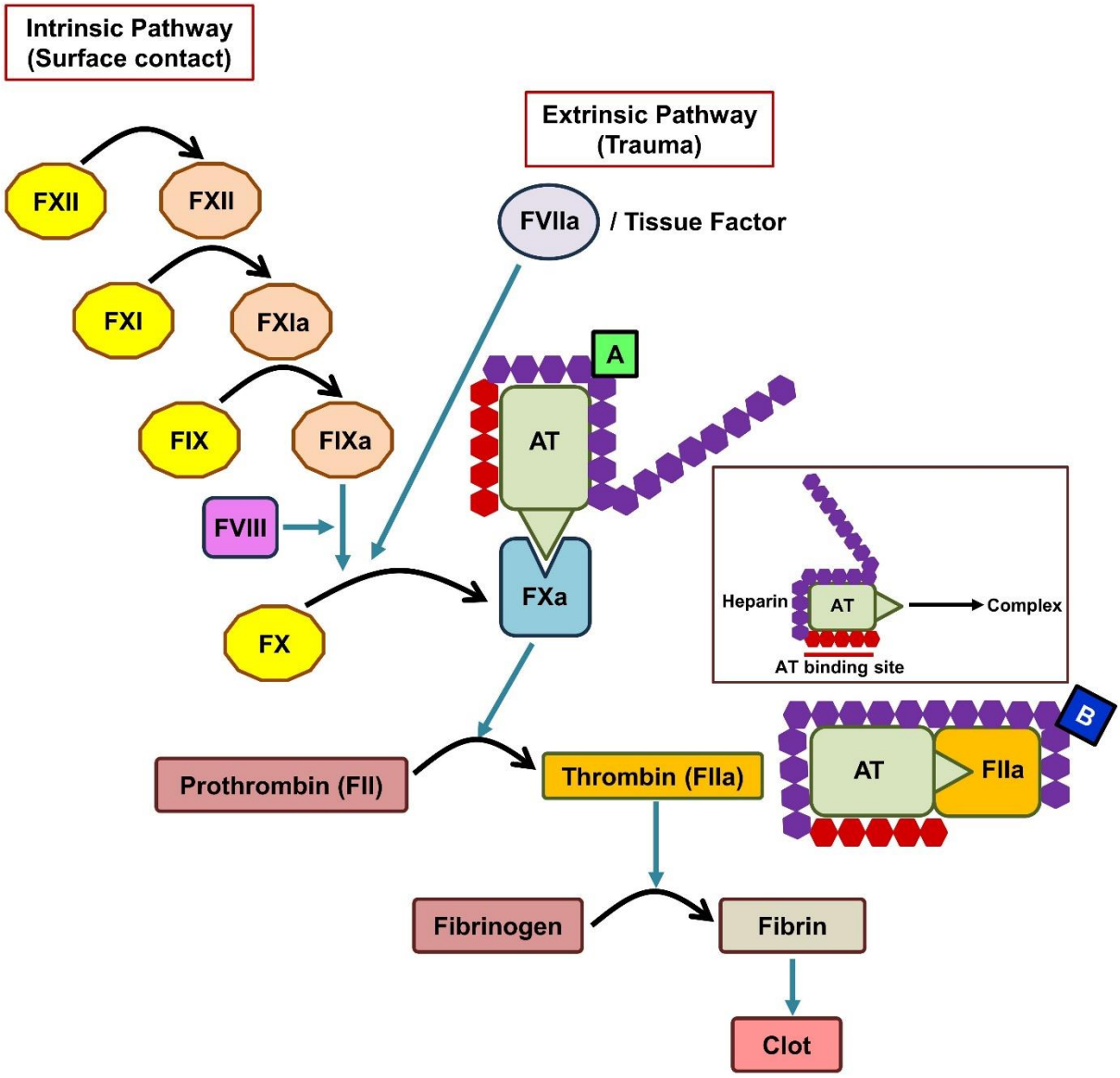

Fig. 1-3 Heparin and its mechanism of action in coagulation cascade. Within the coagulation cascade, heparin operates through intricate interactions with antithrombin-III (AT). In the depicted scheme, represented by the green box (Box A), AT bound with heparin fragments containing the unique pentasaccharide sequence efficiently inhibits Factor Xa (FXa). Conversely, illustrated in the blue box (Box B), AT must engage with heparin chains exceeding 18 disaccharide units to inhibit thrombin (Factor IIa).

1.1.4 Derivatives of heparin

The anticoagulant effect of heparin is achieved by binding to AT and enhancing its activity approximately 1000-fold. The specific coagulation factors targeted by the heparin-AT complex are influenced by the length (molecular weight) of the heparin chain (**Fig. 1-4**). Consequently, heparin is fractionated into various components based on its molecular weight distribution to optimize its anticoagulant efficacy. Therefore, the FDA categorizes heparin into three different forms based on its molecular weight distribution. Unfractionated heparin (UFH), characterized by an average molecular weight (MW_{avg}) in the range of 15–19 kDa, is tailored for intravenous administration. Conversely, low-molecular-weight heparin LMWH, with an MW_{avg} ranging from 3.5 to 8 kDa, is designed for subcutaneous use. Similarly, ultra-low-molecular-weight heparin (ULMWH), with an MW_{avg} below 2 kDa, is also intended for subcutaneous administration (Qiao et al., 2020; Wang et al., 2022).

(i) Unfractionated heparin

Unfractionated heparin (UFH), the initial pharmaceutical-grade form of heparin, is derived from various animal sources. Commercial UFH production involves a complex series of extraction and purification steps, including sourcing and stabilization of materials, extraction of heparin from proteoglycans, heparin capture, filtration, heparin oxidation, and finally isolation and drying (Baytas and Linhardt, 2020; Jeske et al., 2019; van der Meer et al., 2017). Despite rigorous safety protocols, achieving pure UFH without co-extracting structurally distinct GAGs such as HS, CS, and DS is challenging. Concerns about potential pathogens and bioactive compounds in animal-derived UFH necessitate thorough contamination risk analyses (Baytas and Linhardt, 2020). In addition, UFH is utilized in the management of renal failure and during cardiopulmonary bypass procedures in heart-lung and dialysis machines due to its potent anticoagulant properties. Despite UFH's primarily hepatic metabolism, its use in renal patients requires careful consideration and monitoring to mitigate the risk of accumulation and associated complications (Aláez-Versón et al., 2017; Baytas and Linhardt, 2020). Heparin can induce heparin-induced thrombocytopenia (HIT) in approximately 5% of patients, while the incidence with LMWH is around 1%. It is important to note the distinction between HIT and immune HIT; it is immune HIT that is life threatening and has a much lower incidence (Liu and Linhardt, 2014; Walenga, 2005).

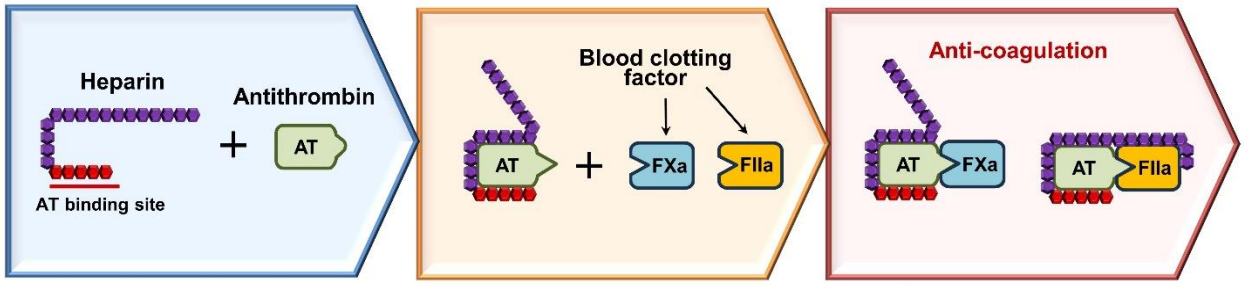

Fig. 1-4 Mechanism of heparin-mediated inactivation of coagulation factors. Heparin inactivates coagulation factors by binding to AT through a high affinity pentasaccharide sequence, which induces a conformational change in AT, accelerating its interaction with thrombin (FIIa) and FXa. UFH can inactivate both FIIa and FXa, requiring high molecular weight to effectively bind thrombin. In contrast, LMWH primarily catalyzes the inactivation of FXa by antithrombin, needing at least 18 saccharide units for effective catalysis. Consequently, LMWH exhibits less inhibitory activity against thrombin compared to UFH.

(ii) Low-molecular-weight heparin

Low-molecular-weight heparin (LMWH), the second generation of heparin characterized by shorter chains of 16–22 monosaccharide units, is originally synthesized through controlled depolymerization or fractionation of larger UFH chains using chemical or enzymatic processes (Baytas and Linhardt, 2020; Cosmi and Palareti, 2012). This synthesis involves various partial depolymerization reactions, such as peroxidative cleavage, nitrous acid cleavage, and chemical and enzymatic ß-elimination (Higashi et al., 2012). These reactions result in structural variations in polydispersity, degree of sulfation, and proportion of pentasaccharide binding regions, which in turn cause notable differences in both pharmacokinetics and pharmacodynamics (Onishi et al., 2016). LMWH variants derived by these techniques include dalteparin, enoxaparin, certoparin, nadroparin, bemiparin, reviparin, parnaparin, and tinzaparin (Hao et al., 2019; Qiu et al., 2021). Despite the efficacy of LMWH, challenges such as higher production costs and lower yields have prompted researchers to explore novel approaches to address these issues (**Table 1-1**) (Baytas and Linhardt, 2020; Shen et al., 2019; Sun et al., 2018; Zhi et al., 2019).

Table 1-1 Recent advances in LMWH preparation methods

Approaches	Strategies	Products	References
Photodepolymerization of UFH	The use of catalyst (i.e., titanium oxide) to produce hydroxyl radicals.	Highly pure LMWH	(Higashi et al., 2012)
Sono-Fenton process	Combination of physical ultrasound treatment with a chemical Fenton reaction.	LMWH with significantly higher APTT (activated partial thromboplastin time) and anticoagulant activity	(Zhi et al., 2019)
Ultrasound treatment	Radical hydrolysis reaction assisted by an ultrasonic wave and catalyzed by hydrogen peroxide.	A variety of depolymerize polysaccharides	(Achour et al., 2013; Petit et al., 2007)
Physicochemical depolymerization	Combination of H_2O_2/ascorbic acid free radical reaction with ultrasonic wave.	Large scale production of LMWH	(Shen et al., 2019)

The primary goals of bioengineered heparin include ensuring safety, establishing a reliable supply chain, potentially reducing the risk of thrombocytopenia, and achieving better consistency. The pursuit of these objectives remains central to improving patient outcomes and ensuring the availability of high-quality heparin products. Recent studies highlight the development of bioengineered heparin, which is chemically and biologically similar to porcine-derived counterparts, demonstrating the ability to be converted to LMWH (Douaisi et al., 2024). Shorter LMWH chains offer numerous advantages over UFH, including reduced binding to plasma and other proteins (except AT), improved bioavailability and half-lives, consistent dosage response, lower incidence of HIT and osteopenia, and reduced need for laboratory monitoring (Onishi et al., 2016). During anticoagulant therapy, a subset of LMWHs, comprising 25-50% of the total, contains the 17-18 sugar units necessary to form a ternary complex with AT and FIIa. This specific subset influences the inhibitory activity against thrombin. LMWHs can still inhibit factor X activation, as only 5 sugar units are required for FXa inactivation (**Fig. 1-4**) (Qiu et al., 2021; Masuko and Linhadrt, 2012).

(iii) Ultra-low-molecular-weight heparin

Ultra-low-molecular-weight heparin (ULMWH), characterized by 5–10 monosaccharide units, primarily consists of a pentasaccharide sequence (Chandarajoti et al., 2016). Typically, ULMWH is considered a specialized FXa inhibitor with minimal anti-FIIa activity (Petitou and van Boeckel, 2004). Due to its short chain length, the pentasaccharide form of ULMWH exhibits minimal incidence of HIT (Liu and Linhardt, 2014). However, its clinical use remains limited due to its

potent anticoagulant activity and irreversibility. In contrast, UFH is reversible, and LMWHs are partially reversible. Despite offering advantages such as improved structural integrity, extended lifespan, and consistent quality control, the availability of ULMWH is restricted primarily due to its cost, time-consuming manufacturing process, and limited clinical applications. Consequently, ULMWH accounts for only a modest percentage of total utilization compared with LMWH and UFH (Sun et al., 2018).

1.1.5 Diverse applications beyond anticoagulation

The versatile protein interactions of heparin have sparked considerable interest in its potential applications beyond its traditional role as an anticoagulant. Here, I offer a glimpse into some of these applications (Wang et al., 2022), which will be further explored in detail in **Chapter 2**.

(i) Infectious disease

In the initial stages of infection, certain viruses, bacteria, and fungi rely on binding and invading heparan sulfate present on the host cell surface. Heparin, with a structure akin to heparan sulfate, competes with pathogenic proteins for binding to the cell surface, potentially serving as a clinical intervention in infectious disease treatment or prevention. Leveraging this characteristic, an extracorporeal hemoperfusion device has been devised to trap bacteria and viruses from the bloodstream, exhibiting promising results in preclinical studies, with reported reductions of up to 87% for Zika virus and up to 62% for adenovirus (Seffer et al., 2021; Wang et al., 2022).

(ii) Drug delivery

The high affinity binding of heparin to growth factors (Kd = 1-10^{-3} nM) has spurred numerous investigations aimed at preserving and prolonging the activity of these factors. One approach involves immobilizing them onto biocompatible materials like hydrogels and nanofibers (Meher et al., 2024; Zare et al., 2024). For instance, the immobilization of heparin onto polyethylene glycol hydrogel has been shown to facilitate sustained release of vascular endothelial growth factor (VEGF) both in vitro and in vivo, promoting angiogenesis in mouse subcutaneous graft models (Tae et al., 2006).

1.1.6 Supply and demand of heparin

Heparin, a cornerstone of anticoagulant therapy, holds a crucial position on the WHO's esteemed list of “essential medicines”, signifying its indispensability in healthcare. Traditionally sourced from porcine small intestine mucosal tissue, both UFH and most LMWHs have long been relied upon for their therapeutic efficacy. However, the industry faced a significant setback in 2008 when

contamination of OSCS during heparin production led to widespread adverse reactions and fatalities among patients (Guerrini et al., 2008). Moreover, the recent outbreak of African swine fever in China, resulting in a substantial decline in the pig population, has further underscored the vulnerability of supply chain of heparin (Mans et al., 2015).

With an annual demand requiring approximately 1 billion pigs, the reliance on animal-derived heparin poses inherent risks of future shortages (Raedts et al., 2013). Efforts to diversify sourcing methods, including revisiting bovine-derived heparin, have been proposed as potential solutions. However, safety concerns persist regarding animal-derived products, compounded by variations in activity and composition compared to porcine heparin.

While alternative anticoagulants like fondaparinux and direct oral anticoagulants (DOACs) have emerged, their widespread adoption is hindered by cost implications and a dearth of long-term usage data. Consequently, the imperative for novel heparin production technologies remains paramount, ensuring a stable and sustainable supply of this critical therapeutic agent.

1.2 Glycosaminoglycans

Glycosaminoglycans are essential, negatively charged linear polysaccharides composed of repeating structures of amino saccharides (D-glucosamine, D-galactosamine) and uronic acids (D-glucuronic acid, D-galactose, L-iduronic acid). Based on their specific disaccharide compositions, GAGs are classified into four main groups: heparin/heparan sulfate (HS), keratan sulfate (KS), chondroitin/dermatan sulfate (CS/DS), and hyaluronic acid (HA). Unlike HA, which exists independently within the extracellular matrix (ECM), the other GAGs are covalently attached to ECM proteins, forming complex structures known as proteoglycans. GAG is biosynthesized in the Golgi apparatus of animal cells and undergoes sulfation modification to form sulfated glycosaminoglycans (sGAGs). sGAGs are indispensable in numerous physiological processes, such as signal transduction, where they mediate cellular communication; blood coagulation, by regulating clot formation and dissolution; the complement cascade, influencing immune responses; cell adhesion, facilitating cell-to-cell and cell-to-matrix interactions; and pathogen recognition, by binding to and neutralizing various pathogens. The biological significance and functional diversity of GAGs are heavily influenced by their molecular structure, including the specific monosaccharides, the sequence of sugar units, and the nature of their glycosidic linkages. These structural features determine the specific binding affinities and interactions of GAGs with a variety

of proteins and other molecules. In the following, we will focus on HS, CS, and KS, which are classified as sGAGs.

1.2.1 Classification of GAGs

(i) Heparan sulfate

Heparan sulfate (HS) is a linear polysaccharide closely analogous to heparin, characterized by a repetitive structure of sulfated disaccharide units where uronic acid and GlcNAc are connected via 1,4 glycosidic bonds. Unlike heparin, which is exclusively synthesized in connective tissue mast cells, heparan sulfate is produced ubiquitously across mammalian cells.

The principal disparity between heparin and heparan sulfate lies in their sulfation patterns (**Table 1-2**). Heparin typically exhibits a sulfation level of approximately 1.8 to 2.4 sulfate groups per disaccharide unit, contrasting with lower sulfation level of heparan sulfate of around 0.6 to 1.8 sulfate groups per disaccharide unit (Glass, 2018).

Table 1-2 Difference between heparan sulfate and heparin (Xu and Esko, 2014)

Characteristics	**Heparan sulfate**	**Heparin**
Cells to produce	Virtually all cells	Connective tissue mast cells
Core proteins	Many (~17)	Serglycine
Binding to cell membranes	Yes	Nothing
Molecular size	20-100 kDa	7-20 kDa
Replace NS/NAc domain	Yes	Minimum
Average sulfate/disaccharide	0.6-1.8	1.8-2.4
Percentage of IdoA	20-50%	≥80%
N-sulfation	30-60%	≥80%
AT binding region	0-0.3%	~30%

Heparin and heparan sulfate are believed to interact with proteins, binding to specific sites within tissues. This interaction can induce the formation of oligomers and serve as a scaffold for facilitating protein-protein interactions.

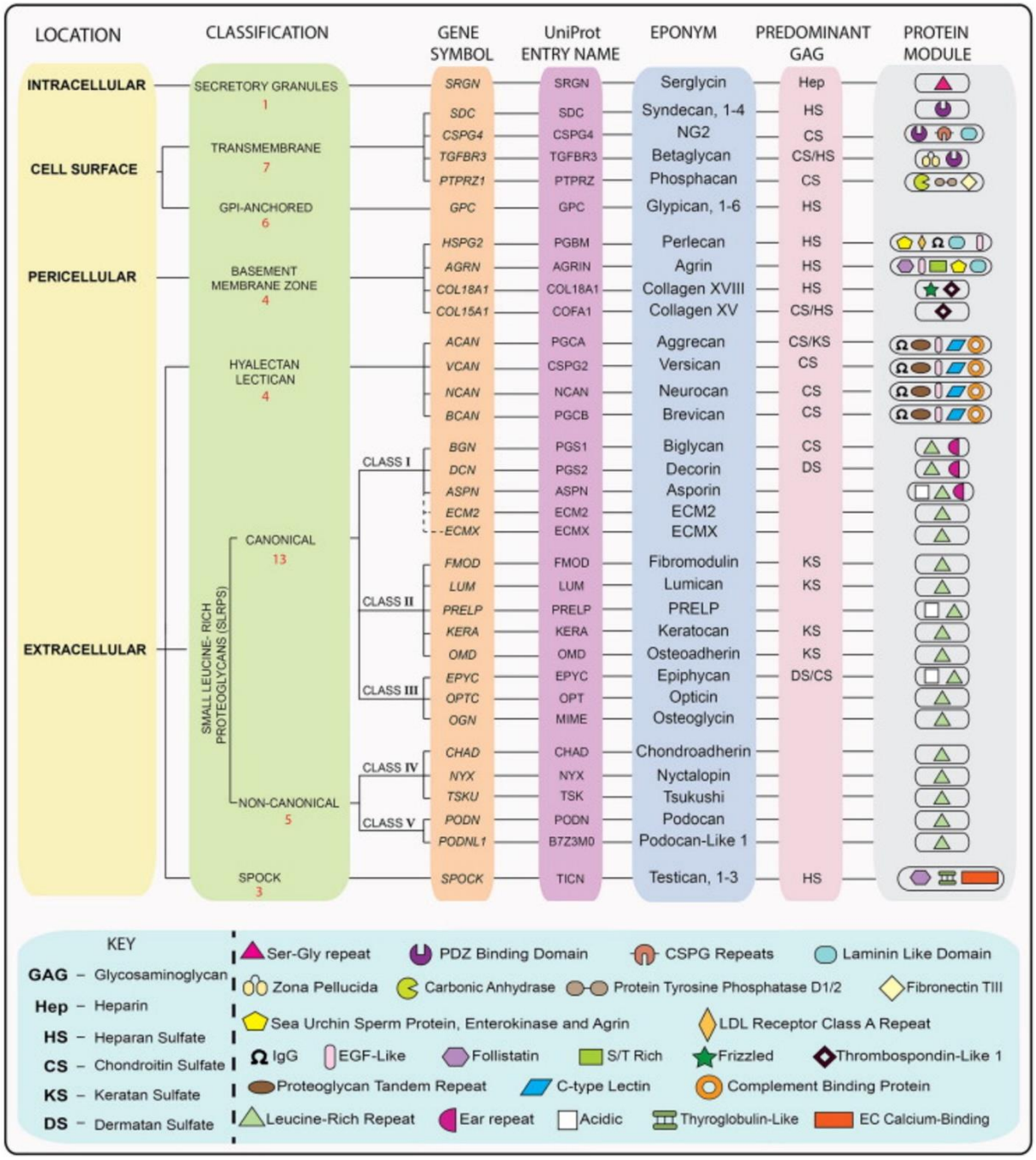

Fig. 1-5 Comprehensive classification of proteoglycans. Proteoglycans are categorized into four families based on their cellular and subcellular localization, homology at the protein and genomic levels, and the presence of unique protein modules that are often shared among members of each

class. A key for identifying the various modules is provided in the accompanying panel below (Iozzo and Schaefer, 2015).

(ii) Chondroitin/dermatan sulfate

Chondroitin sulfate (CS) is a polysaccharide with a repetitive structure composed of a disaccharide unit, where uronic acid and *N*-acetylgalactosamine (GalNAc) are bonded via a 1,3-glycosidic linkage. It is classified into five types-A through E-based on variations in the type of uronic acid and the sulfate group attachment to GalNAc. Particularly noteworthy is chondroitin sulfate B, also known as dermatan sulfate (DS), where glucuronic acid is converted to iduronic acid.

CS is widely distributed in the connective tissues of mammals and plays essential roles in organizing extracellular matrices, contributing significantly to the structural integrity of tissues such as cartilage and bone. Beyond its structural functions, CS is involved in various biological processes, including inflammation regulation, immune response modulation, infection defense mechanisms, and wound healing processes (Volpi, 2019). The biosynthesis of CS involves a series of enzymatic reactions, depicted in **Fig. 1-5**, where enzymes like glycosyltransferases, sulfotransferases, and epimerases catalyze the assembly and modification of CS chains (Mikami and Kitagawa, 2013). These enzymes play critical roles in determining the specific structure and properties of CS molecules, thereby influencing their biological functions and physiological effects in various tissues and biological processes.

(iii) Keratan sulfate

Keratan sulfate (KS) is a GAG composed of repeating disaccharide units of D-galactose (Gal) and GlcNAc. Unlike other GAGs like CS and HS, KS does not contain uronic acid in its disaccharide structure. It is widely distributed throughout the body, particularly in connective tissues, epithelial tissues, and the central and peripheral nervous systems (Funderburgh, 2002). One of its notable locations is in the cornea, where it contributes to the transparency and structural integrity of the tissue (Caterson, 2018). In addition to its structural role, KS also participates in various biological processes such as cell adhesion, migration, and tissue development (Ljubimov and Saghizadeh,

2015). Its presence and functions make it a crucial component in maintaining the health and functionality of various tissues and organs in the body.

1.2.2 Biosynthesis of sulfated glycosaminoglycans

The biosynthesis of sulfated glycosaminoglycans (sGAGs) takes place in the Golgi apparatus of nearly all animal cells. Specifically, heparin is synthesized in connective tissue mast cells, while heparan sulfate undergoes biosynthesis in the Golgi apparatus (Ghiselli, 2017). Here, we elucidate the biosynthesis process of heparin and heparan sulfate.

(i) Heparin biosynthesis

Heparin, a complex polysaccharide with a diverse array of monomeric units, undergoes a natural biosynthetic process within the endoplasmic reticulum (ER) and Golgi apparatus. This process follows a highly structured pathway involving more than 22 distinct enzymes (Fig. 2) (Baytas and Linhardt, 2020). The biosynthesis of the heparin proteoglycan core protein, serglycin, initiates within the ER (Stevens and Adachi, 2007). This process begins with the attachment of a tetrasaccharide linker to a serine residue in the core protein, composed of xylose, two galactose units, and glucuronic acid. The coupling process is facilitated by xylosyltransferase (XylT)-1 or -2, followed by the sequential addition of two galactose units by galactosyltransferase (GalT) -1 and -2, and the incorporation of glucuronic acid by glucuronosyltransferase (GlcAT) (Sugahara and Kitagawa, 2002). Once the linker construction is complete, monosaccharide addition to its non-reducing end is achieved by three isoforms of the EXT glycosyltransferase family. The polysaccharide chains are polymerized by sequentially adding a GlcA residue followed by a GlcNAc residue to the chain through the actions of the EXT1 and EXT2 enzymes (Farrugia et al., 2015).

The biosynthesis further progresses with the involvement of *N*-deacetylase/*N*-sulfotransferase (NDSTs) as the initial step, followed by glucuronyl C5-epimerase (GLCE), 2-O-sulfotransferases (HS2ST1), 6-O-sulfotransferases (HS6STs), and finally, 3-O-sulfotransferases (HS3STs). Interplay between these enzymes is expected. Initially, *N*-acetyl groups are replaced with *N*-sulfo groups by NDST isoforms, particularly NDST-2, which plays a crucial role in modifying the GAG chains on the serglycin core protein, essential for mast cell heparin synthesis (Kreuger and Kjellen, 2012). The maturation phase involves intricate modifications to sugar units, primarily orchestrated by Golgi-associated sulfotransferases from various families. This includes epimerization of GlcA to IdoA residues by GLCE, the transfer of sulfate groups to the C-2 position of most IdoA and

some GlcA by HS2ST1, and the attachment of sulfate groups to either GlcNAc or GlcNS residues at the C-6 position by HS6STs. Finally, GlcNAc and GlcNS residues undergo 3-O-sulfation by HS3STs (**Fig. 1-6**) (Liu and Linhardt, 2014).

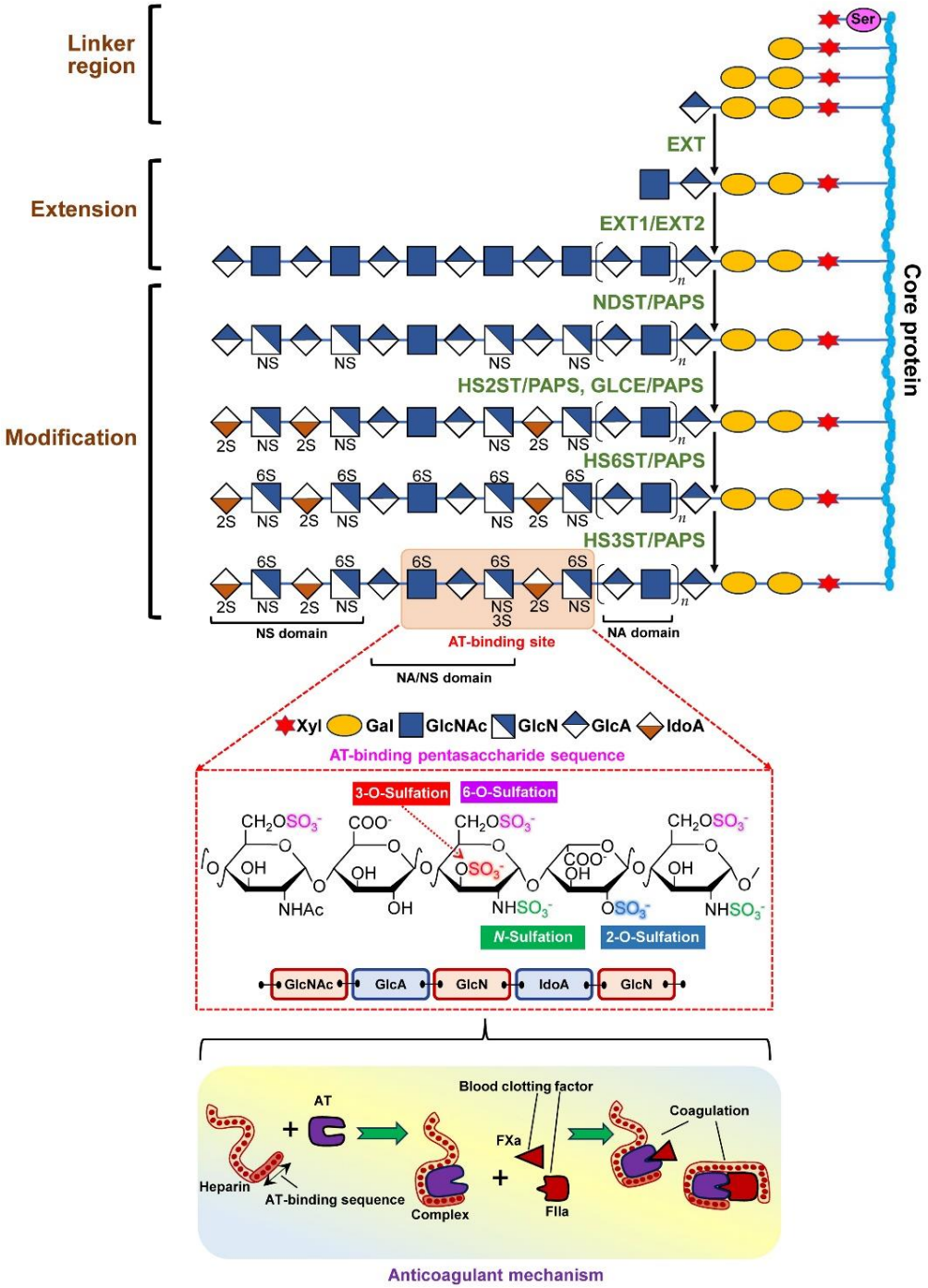

Fig. 1-6 Biosynthetic steps of heparin/HS within the endoplasmic reticulum (ER) and the Golgi apparatus (Baytas and Linhardt, 2020). Synthesis is initiated by the sequential addition of four monosaccharides, -Xyl-Gal-Gal-GlcA-, to a serine residue of the serglycin core protein. Subsequent chain elongation involves the alternating addition of GlcNAc and GlcA. Further modification of the chain is facilitated by epimerase and sulfotransferase enzymes, resulting in a diverse structure with AT-binding sites. Ser: serine, Xyl: xylose, Gal: galactose, EXT: exotosin, NDST: N-sulfotransferase, GLCE: glucuronyl C5-epimerase, HS2ST: 2-O-sulfotransferase, HS6ST: 6-O-sulfotransferase, HS3ST: 3-O-sulfotransferase, PAPS: 3'-phosphoadenosine 5'-phosphosulfate, GlcA: β-D-glucuronic acid, GlcN: α-D-glucosamine, GlcNAc: N-acetylglucosamine, IdoA: α-L-iduronic acid. Subsequently, the anticoagulation mechanism was elucidated, revealing that the anticoagulant effect of heparin arises from a specific interaction between a unique pentasaccharide sequence and AT, an endogenous anticoagulant glycoprotein that impedes several coagulation factors. AT: antithrombin-III, FXa: factor-Xa, FIIa: factor-IIa**.**

(ii) Elongation of glycans

Mammalian glycosyltransferases, known as EXTLs, are omnipresent and include two exostosins (EXT1 and EXT2) and three exostosin-like enzymes (EXTL1-3). Except for EXTL1, all these enzymes are ubiquitously expressed (Busse-Wicher et al., 2014). Both EXT1 and EXT2 are essential for the transfer of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc). Co-expression studies have demonstrated that EXT1 and EXT2 can form a complex, which enhances their transfer activity. EXTL2, on the other hand, exhibits transfer activity to the tetrasaccharide-binding regions of α-linked GlcNAc or α-linked *N*-acetylgalactosamine (GalNAc). However, studies have shown that knocking out EXTL2 does not affect heparan sulfate (HS) synthesis in CHO cells. Current models suggest that both transitions occur in the state of 2-O phosphorylated xylose within the linker tetrasaccharide, which leads to the termination of glycan elongation. Consequently, EXTL2 is considered a negative regulator of HS synthesis (Marques et al., 2022). Conversely, knocking out EXTL3 has been reported to significantly suppress HS synthesis in CHO cells, indicating that EXTL3 plays a critical role in the initiation of HS biosynthesis (Chen et al., 2018).

(iii) Sulfation modification

In the sulfation of GAGs such as heparin, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) serves as a crucial coenzyme in sulfotransferase reactions. The sulfation process of GAGs involves four

key steps: a) transport of sulfate ions into cells via sulfate transporters, b) conversion of sulfate ions to PAPS by PAPS synthase, c) transport of PAPS from the cytoplasm to the Golgi apparatus by PAPS transporters, and d) Transfer of sulfate groups from PAPS to GAGs by sulfotransferases (**Fig. 1-7**) (Kamiyama and Nishihara, 2004). This sequence ensures the proper sulfation of GAGs, which is essential for their biological functions.

(a) Biological source of sulfuric acid

The sulfuric acid used in the synthesis of PAPS in living organisms is primarily sourced from sulfate ions directly taken up into cells. Additionally, sulfur-containing amino acids, such as methionine and cysteine, are metabolized within cells to produce sulfate ions, which are then utilized in the synthesis process.

(b) Sulfate ion transporter

Sulfate, being a highly dissociative divalent hydrophilic anion, cannot freely pass through the phospholipid bilayer of the cell membrane and thus depends on specific transport systems, known as sulfate ion transporters, to enter cells and organelles. Among these transporters, the Solute carrier family 13 member 1 (SLC13A1, NaSi-1), isolated in 1993 using the African clawed frog oocyte expression cloning system, is a sodium ion-dependent sulfate transporter. Its stable expression in MDCK cells resulted in a four-fold increase in Na^+-sulfate ion co-transport, primarily on the apical cell surface. Another significant transporter, Solute carrier family 13 member 4 (SLC13A4, Sut-1), was isolated from human high endothelial venules (HEVs). Similar in function to Solute carrier family 26 member 1 (SLC26A1, Sat-1), an anion channel from a rat liver cDNA library, SLC13A4 enhances sulfate ion uptake when expressed in African clawed frog oocytes. Additionally, Solute carrier family 26 member 2 (SLC26A2, DTDST) shares amino acid similarity with SLC26A1 and is crucial for GAG synthesis, as mutations in this gene lead to reduced sulfation of CS. These transporters are essential for sulfate ion homeostasis and the proper synthesis and function of GAGs (Girard et al., 1999; Markovich, 2001; Rossi et al., 1996).

(c) PAPS synthesis

Once inside cells, sulfate ions are converted into the high-energy form PAPS by PAPS synthase. In plants, bacteria, fungi, and yeast, PAPS synthase consists of two distinct enzymes: ATP sulfurylase, which produces adenosine-5'-phosphosulfate (APS) from ATP and sulfate ions, and APS kinase, which phosphorylates APS using another ATP molecule to generate PAPS. Due to its structural instability, APS exists only briefly as an intermediate in the PAPS synthesis pathway. In

contrast, mammals utilize bifunctional enzymes, PAPSS1 and PAPSS2, for PAPS synthesis. These enzymes combine both the ATP sulfurylase and APS kinase domains. Notably, the introduction of PAPSS1 into CHO cells has been shown to increase PAPS synthesis significantly (Girard et al., 1999).

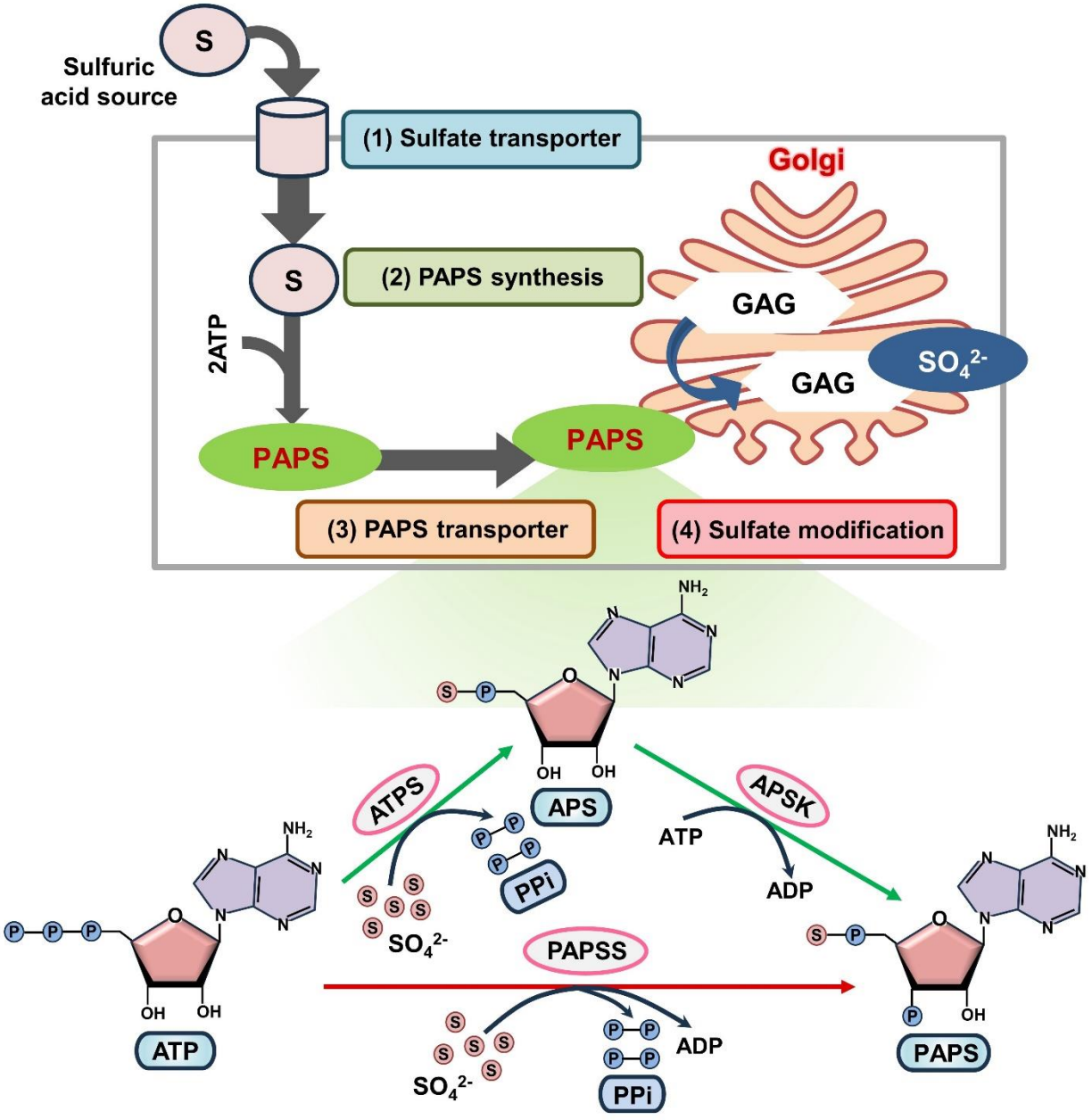

Fig. 1-7 Schematic overview of the GAG sulfation process. (a) Sulfate ions are transported into the cell via specific sulfate transporters. (b) Inside the cell, sulfate ions are converted to PAPS by PAPS synthase. (c) PAPS is then transported from the cytoplasm to the Golgi apparatus by PAPS

transporters. (d) Finally, sulfotransferases transfer sulfate groups from PAPS to GAGs, ensuring proper sulfation necessary for GAG biological function (Kamiyama and Nishihara, 2004). The lower section illustrates the metabolic pathway of PAPS in different organisms. The pathway is shown for animals and plants (red arrows) and microorganisms (green arrows), highlighting the distinct bio-chemical routes for PAPS synthesis, which is crucial for the sulfation process of heparin and HS. PAPS: 3'-phosphoadenosine 5'-phosphosulfate, ATP: adenosine -5'-triphosphate, APS: adenosine -5'-phosphosulfate, PPi: pyrophosphate, APSK: APS kinase, ATPS: ATP sulfurylase, PAPSS: PAPS synthase, S: sulfate group.

(d) Incorporation of PAPS into the Golgi apparatus

PAPS synthesized in the cytoplasm is transported to the Golgi apparatus by PAPS transporters, specifically PAPST1 and PAPST2 (known as SLC35B2 and SLC35B3 in mice). Knockdown of these genes in mouse embryonic stem cells has been reported to cause a 30-40% reduction in HS and CS sulfation, along with an approximately 20% decrease in PAPST activity (Sasaki et al., 2009).

(e) Sulfate group modification by sulfotransferase

Using PAPS transported into the Golgi apparatus, a group of sulfotransferases anchored to the Golgi membrane catalyze the sulfation of glycosaminoglycans. In heparin and HS, the sulfation process follows a specific sequence: N-sulfation, epimerization and 2-O-sulfation, 6-O-sulfation, and finally 3-O-sulfation. The enzymes responsible for catalyzing these reactions are listed in **Table 1-3**.

Table 1-3 Enzymatic machinery in heparin and HS biosynthesis

Abbr.	Enzyme	Gene	Localization	Source	Expression	Function
NDST	*N*-deacetylase/*N*-sulfotransferase	Ndst 1~4	Golgi apparatus	*Rattus norvegicus*/ *Mus musculus*	*Saccharomyces cerevisiae*	Initiates *N*-deacetylation and *N*-sulfation of GlcN residues
C5 epi	Glucuronyl C5 epimerase	Glce	Golgi apparatus	*Cricetulus griseus*	*Pichia pastoris*	Converts GlcA to IdoA in the biosynthesis of heparin and HS
Hs2st	Heparan sulfate 2-O-sulfotransferase	Hs2st1	Golgi apparatus	*Cricetulus griseus*	*Cricetulus griseus*	Catalyzes the addition of sulfate groups to the 2-O position of UA residues in HS
Hs3st	Heparan sulfate 3-O-sulfotransferase	Hs3st 1~3	Golgi apparatus	*Mus musculus*	*Komagataella pastoris*	Catalyzes the addition of sulfate groups to the 3-O position of GlcN residues in HS

Hs6st	Heparan sulfate 6-O-sulfotransferase	Hs6st 1~6	Golgi apparatus	*Mus musculus*	*Komagataella pastoris, Escherichia coli*	Catalyzes the addition of sulfate groups to the 6-O position of GlcN residues in HS

(f) Desulfation and glycosylation

In vivo, GAGs are subject to not only biosynthesis and sulfation but also desulfation and degradation mechanisms. Specific enzymes have been identified for the removal of each sulfate group, the promotion of *N*-acetylation, and the cleavage of glycosidic bonds in disaccharides (Kowalewski et al., 2012). The enzymes involved in the desulfation of HS are detailed in **Table 1-3** and **Fig. 1-8**, while those involved in the degradation of HS are listed in **Table 1-4**.

Table 1-4 Heparin/HS desulfation enzymes

Enzyme name	Function
IDS	De-2-O sulfation of IdoA
ARSG	De-3-O-sulfation
SGSH	De-*N*-sulfation of non-reducing terminal of GlcN
ARSK	De-2-O-sulfation of GlcA
GNS Sulf1 Sulf2	De-6-O-sulfation
HSNAT	*N*-acetylation of sulfated glucosamine

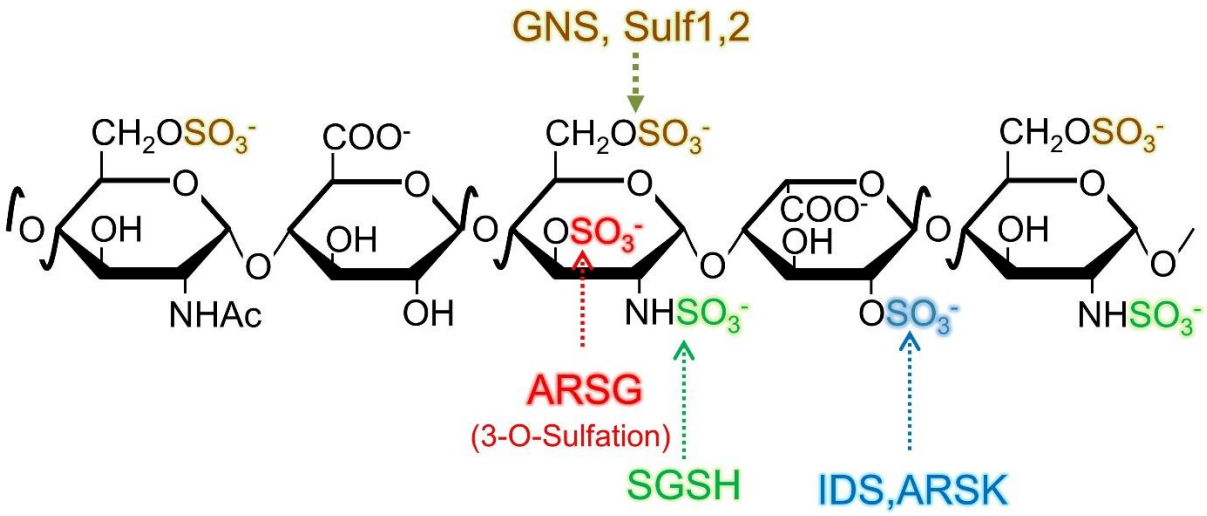

Fig.1-8 Overview of heparin/HS desulfation mechanism.

Table 1-5 Heparin/HS degrading enzymes

Enzyme name	Function
IDUA	Degradation of non-sulfated α-L- iduronosidic bond
NAGLU	Hydrolysis of GlcNAc residues
GUSB	Degradation of non-sulfated β-D-glucuronyl bond
Heparanase	Degradation of sulfated β-D-glucuronyl bond

1.3 Proteoglycans

GAGs interact with specific residues of membrane proteins, leading to the formation of glycoproteins known as proteoglycans. These proteoglycans encompass a diverse group of molecules, with over 20 species identified to date (**Fig. 1-5**). They are categorized into four types based on their localization within the cell: intracellular, cell surface, pericellular, and extracellular (Iozzo and Schaefer, 2015). Proteoglycans play a crucial role in the composition of the extracellular matrix, with heparan sulfate proteoglycans (HSPGs) particularly implicated in facilitating the transport of growth factors into cells in vivo (Glass, 2018). In this context, the primary membrane proteins comprising HSPG are delineated.

3.1 Extracellular matrix proteoglycans

Extracellular matrix proteoglycans (ECMPGs), crucial components of tissue architecture, contribute to structural integrity and cellular signaling. They are involved in various physiological processes, including tissue development, wound healing, and inflammation regulation (Iozzo and Schaefer, 2015). Notably, ECM proteoglycans such as aggrecan and perlecan serve as reservoirs for growth factors and cytokines, modulating their availability and activity within the ECM (Theocharis, 2016). Heparin, occasionally found in ECM proteoglycans, may further regulate these processes by interacting with growth factors and cytokines, influencing their signaling pathways (Lin et al., 2004).

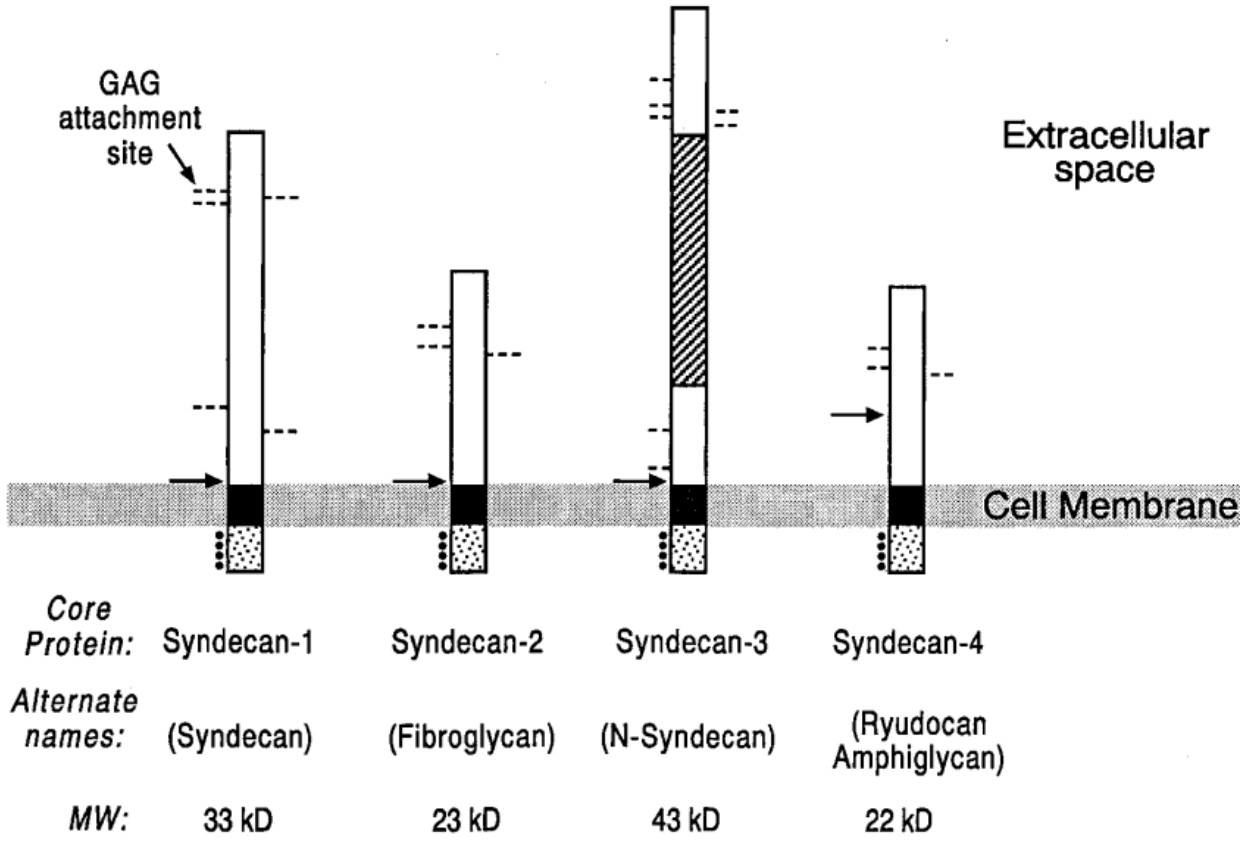

Fig. 1-9 Schematic Diagram of the Syndecan family. The diagram illustrates the Syndecan family, highlighting the homologous transmembrane domain (black) and the intracellular domain (stippled) with conserved tyrosines (dots). Additionally, it indicates potential dibasic cleavage sites (arrows) and the Thr, Ser, Pro-rich domain of Syndecan-3 (crosshatched) (Rosenberg, et al., 1997).

3.2 Cell surface proteoglycans

Cell surface proteoglycans (CSPGs), including syndecans and glypicans, play diverse roles in cellular adhesion, migration, and signal transduction (Couchman et al., 2012). They are crucial for mediating cell-cell and cell-matrix interactions and are involved in processes such as cell proliferation, differentiation, and tissue morphogenesis (Tkachenko et al., 2005). Heparan sulfate chains present on cell surface proteoglycans contribute to ligand binding and receptor activation, thereby modulating downstream signaling events (Filmus et al., 2008). Additionally, heparin moieties on these proteoglycans may regulate cellular responses by modulating ligand-receptor interactions and signaling cascades (Eslo et al., 2001). Syndecans, core proteins of proteoglycans, are transmembrane proteins consisting of one to four domains, including extracellular, transmembrane, and intracellular domains (**Fig. 1-9**) (Rosenberg, 1997). All GAG-binding sites

are located within the extracellular domains of syndecans, facilitating interactions with various extracellular matrix components, growth factors, and cytokines. This positioning enables syndecans to mediate critical cellular processes such as adhesion, migration, and signal transduction.

3.3 Secretory granule proteoglycans

Secretory granule proteoglycans (SGPGs), exemplified by serglycin, are essential for the storage and release of bioactive molecules, including heparin (Kolset, 2011). Serglycin is predominantly expressed in hematopoietic cells and is involved in granule maturation and the packaging of various proteases, cytokines, and growth factors within secretory granules (Schick et al., 2001). Heparin, stored within secretory granules, plays a critical role in processes such as blood coagulation, inflammation, and immune responses (Lindahl U, 2020). Upon appropriate stimulation, serglycin-bound heparin is rapidly released, thereby exerting its physiological effects (Kolset, 2008).

1.4 Approaches of synthesis and modifications

Innovative approaches in heparin research have led to significant advancements in synthesis and modification techniques, enabling the production of structurally defined heparin derivatives with tailored properties for diverse biomedical applications. This section explores key methodologies, including chemical synthesis, chemoenzymatic strategies, and advances in bioengineering modifications of heparin and HS.

1.4.1 Chemical synthesis

Exploring heparin oligosaccharides in biomedical science is a journey filled with both challenges and exciting possibilities (Hogwood et al., 2023). Natural heparin, sourced from pig intestines and composed of approximately 60 sugar units, is too complex for simple chemical synthesis (Baytas and Linhardt, 2020; Jiang et al., 2023). However, a significant breakthrough occurred with the discovery of a specific pentasaccharide sequence that tightly binds to AT (Petitou et al., 1999). This paved the way for developing synthetic anticoagulant oligosaccharides, with fondaparinux being the standout drug that effectively inhibits coagulation enzymes (Ding et al., 2017; Linhardt and Liu, 2012). Despite these advances, the chemical synthesis of oligosaccharides faces two significant hurdles: protecting functional groups and achieving stereoselective glycosylation (Dey et al., 2020; Ding et al., 2017; Jin et al., 2016). These issues often result in complex synthetic steps and minimal yields (Baytas and Linhardt, 2020; Dey et al., 2020; Ding et al., 2017; Zhao et al.,

2024). However, new approaches have begun to tackle these challenges head-on. One promising strategy involves using 'building blocks' derived from natural polysaccharides such as heparin or HS (Lu et al., 2006; Pawar et al., 2019). This method allows researchers to create customized oligosaccharides with specific structures and functions. Additionally, innovative techniques such as the reversible addition-fragmentation chain transfer (RAFT) method offer alternative solutions to synthesis complexities, especially regarding the protection of hydroxyl groups during the creation of long chains. This technique has led to the development of heparin-mimic polymers with molecular weights of 5-50 kDa (Nahain et al., 2019). Polymers incorporating sodium 4-styrene sulfonate as the monomer, or copolymers of sulfonated monomers and acrylic acid in an equal ratio, show enhanced anticoagulant activity (Nahain et al., 2019). Synthetic heparin oligosaccharides are also crucial for studying the biological activities of heparin and HS. Microarray studies using precisely crafted synthetic constructs with specific sulfation patterns have unveiled complex binding interactions between various chemokines, growth factors, and HS sequences (Avizienyte et al., 2016; Jayson et al., 2015; Zong et al., 2017). These discoveries underscore the significant impact of structural details on protein function, offering valuable insights for therapeutic strategies. In summary, while synthesizing heparin oligosaccharides remains a challenging task, recent breakthroughs have significantly advanced the field (Baytas and Linhardt, 2020). These developments allow for the engineering of oligosaccharides with tailored functionalities, holding immense promise for deepening our understanding of glycobiology and transforming therapeutic interventions in biomedical science.

1.4.2 Chemoenzymatic synthesis

The field of chemoenzymatic synthesis has witnessed remarkable progress in the production of GAGs, particularly LMWH analogs, offering a scalable and efficient approach (Gottschalk and Elling, 2021; Wang et al., 2020; Zhang et al., 2019). Researchers have successfully employed recombinant biosynthetic enzymes to convert precursor heparosan polysaccharides into heparin-like structures, enabling gram-scale production of heparin derivatives with promising anticoagulant activity (Baytas and Linhardt, 2020; Li et al., 2021; Na et al., 2020; Nehru et al., 2021; Roy et al., 2021; Sheng et al., 2024). Additionally, strains engineered for simultaneous heparosan and enzyme production streamline synthesis, facilitating one-pot production of heparin products (Bhaskar et al., 2015). One of the most recent breakthroughs is a scalable method for creating bioengineered heparin that is comparable to porcine heparin using biosynthetic enzymes

on an inert platform. *N*-sulfoheparosan with analogous *N*-sulfation levels is required for this reaction. This heparin can be converted into LMWH, comparable to USP enoxaparin (Douaisi et al., 2024). In another study conducted by Deng et al., the focus was on synthesizing *N*-sulfated heparin polysaccharides, pivotal for the production of anticoagulant heparin (Deng et al., 2024). The methodology employed metabolically engineered *E. coli* K5 in conjunction with protein engineered NST (Deng et al., 2024). This strategic combination allowed for a streamlined approach to biosynthesis, bypassing the need for NDST and reducing production costs. This optimized process not only facilitated the direct generation of potent anticoagulant heparins but also ensured economic efficiency and sustainable operations (Deng et al., 2024). Despite these promising developments, achieving industrial-scale production remains a significant challenge in the field of chemoenzymatic synthesis of heparin.

1.4.3 Advances in bioengineering

The advancement of heparin oligosaccharides and polysaccharides through sophisticated chemical and chemoenzymatic synthesis techniques presents a highly promising alternative to traditionally sourced heparin from animal and bacterial origins (Deng et al., 2024; Sun et al., 2022; Zhao et al., 2024). However, despite their potential, the existing production capacity of these techniques fails to meet the global demand for heparin active pharmaceutical ingredient (API). Given this challenge, there is a growing interest in engineering custom cell factories capable of manufacturing non-animal derived heparin analogs, leveraging advancements in synthetic biology. While substantial progress has been achieved in producing K5 heparosan (Jiang et al., 2023), a precursor to heparin, limited attention has been paid to developing modified heparin analogs with altered structures and anticoagulant properties. Notably, investigations have explored the utility of Chinese hamster ovary (CHO) cells and yeast platforms for this purpose. CHO cells are particularly favored due to their innate heparin/HS modification enzymes, rendering them suitable hosts for biosynthesis. Augmenting the expression of specific enzymes, such as Hs3st1 and Ndst-2, in CHO cells has resulted in the production of HS with improved anticoagulant efficacy, albeit inferior to pharmaceutical heparin (Baik et al., 2012a, 2012b). Furthermore, efforts have focused on enhancing enzyme localization within CHO cells to optimize activity (Datta et al., 2013b). Yeast, characterized by its cost-effectiveness and ease of cultivation, emerges as an appealing alternative to mammalian cells for heparin production. Through the genetic manipulation of *Pichia pastoris*, a methylotrophic yeast strain, and the establishment of a cell-free enzymatic system, significant

strides have been taken in generating heparin analogs with properties akin to those derived from animals (Zhang et al., 2022a). Additionally, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology facilitates the regulation of enzyme expression in the heparin biosynthetic pathway through efficient genome editing (Cress et al., 2015). As researchers continue to optimize the expression of remaining heparin biosynthetic enzymes, the prospect of large-scale production of metabolically engineered heparin using mammalian cells grows closer (**Fig. 1-10**) (Baik et al., 2012a; Datta et al., 2013c, 2013a; Leyh et al., 1988; Thacker et al., 2022; Zhang et al., 2022; Zhao et al., 2024). Overcoming current obstacles in enzyme arrangement and cooperation within the cellular environment is essential for realizing this goal. Nevertheless, ongoing progress in synthetic biology offers hope for overcoming these challenges. With a deeper understanding of enzyme regulation mechanisms and collaborative efforts, the future establishment of cell factories dedicated to mass-producing heparin appears increasingly feasible. Ultimately, this advancement would represent a transformative achievement in bioengineering heparin, revolutionizing the model of anticoagulant production and enhancing patient care worldwide.

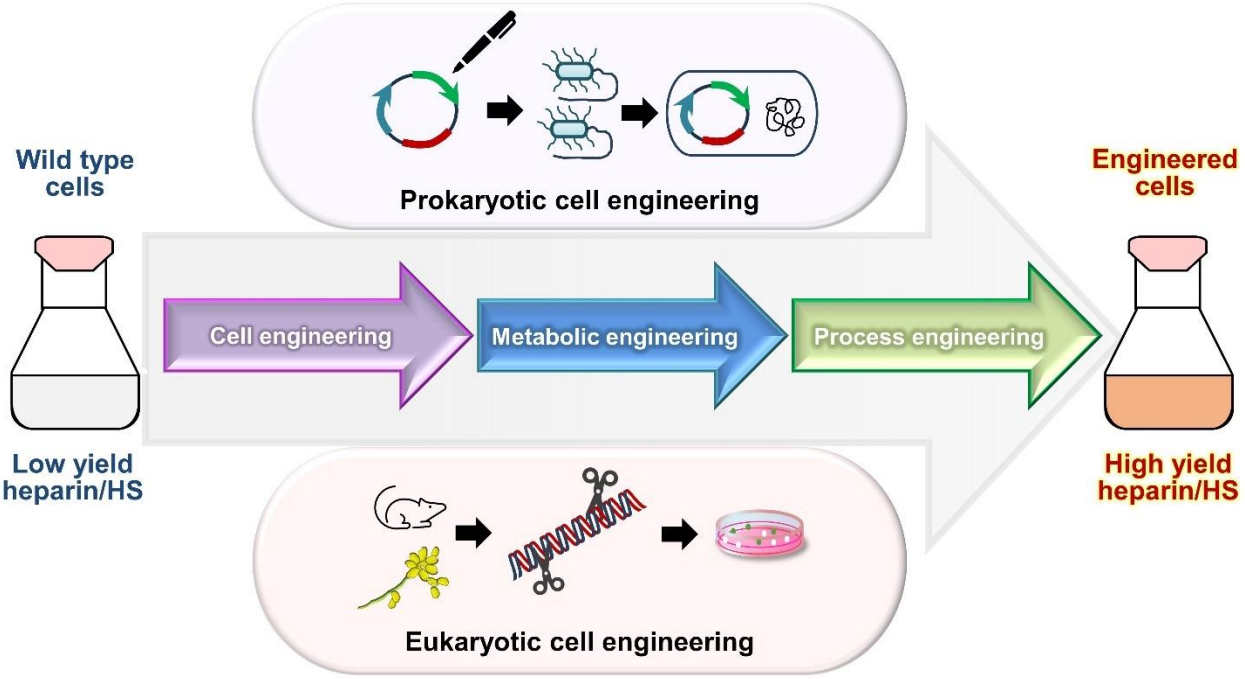

Fig. 1-10 Overview of advanced bioengineering processes in eukaryotic and prokaryotic expression systems, followed by modifications to generate heparin/HS.

1.4.3.1 Genetic recombination technology

The introduction of genes into living organisms represents a fundamental tool for dissecting various biological phenomena. Advancements in gene transfer technologies have paved the way for the development of genetically modified crops, genetic therapies, the production of valuable substances like pharmaceuticals utilizing genetically engineered animal cells, and the creation of induced pluripotent stem (iPS) cells, which have garnered significant interest for their potential applications in regenerative medicine. During gene transfer processes, foreign genes are initially introduced into the organism's cells and subsequently integrated into the chromosomes. This manipulation enables cells to acquire novel traits and functionalities.

(i) Gene transfer methods

Gene transfer methods can be broadly categorized into three main approaches: scientific methods, physical methods, and biological methods (Kim et al., 2017). Scientific methods encompass techniques such as calcium phosphate precipitation, DEAE dextran-mediated transfection, and lipofection (Gulick). Physical methods involve direct physical delivery of genetic material into cells, such as microinjection and electroporation. Biological methods, on the other hand, utilize viral vectors, including retroviral vectors, lentiviral vectors, and adenovirus vectors, to deliver genetic material into target cells. Here is an overview of these methods:

(a) Lipofection

Lipofection involves the formation of complexes between cationic artificial lipids known as liposomes and DNA through electrostatic interactions, facilitating their uptake by cells through processes such as endocytosis or membrane fusion. Its advantages include relatively low cytotoxicity and no restriction on the size of the nucleic acid to be introduced.

(b) Electroporation

Electroporation is a technique where brief electric pulses are applied to the cell membrane, creating small pores through which DNA or RNA can be introduced. By optimizing parameters such as voltage, duration of pulses, temperature, cell/DNA concentration, gene introduction can be achieved conveniently and rapidly, making it widely used due to its versatility (Grosser, 2016).

(c) Virus vectors

Virus vectors involve the introduction of genetically modified viruses, wherein harmful components such as replicative ability and pathogenicity are removed, into target cells to deliver the desired gene. In animal cells, retrovirus vectors are predominantly utilized due to their ability

to efficiently integrate into chromosomes, a process that is challenging to achieve with physical or chemical methods.

(ii)Integration of foreign genes into chromosomes

To achieve stable expression of introduced genes within cells using the aforementioned methods, except for retro/lentivirus vectors, it becomes imperative to integrate the gene into the chromosomes of the target cells. This integration can occur randomly throughout the chromosome (random integration) or at specific sequences (targeted integration). Here are some summarized approaches:

(a) Accidental integration

In animal cells, although infrequent, foreign DNA material may integrate accidentally into chromosomes during cell proliferation. This phenomenon can be exploited to integrate genes into chromosomes without the need for specific sequences to be incorporated into the plasmid vector.

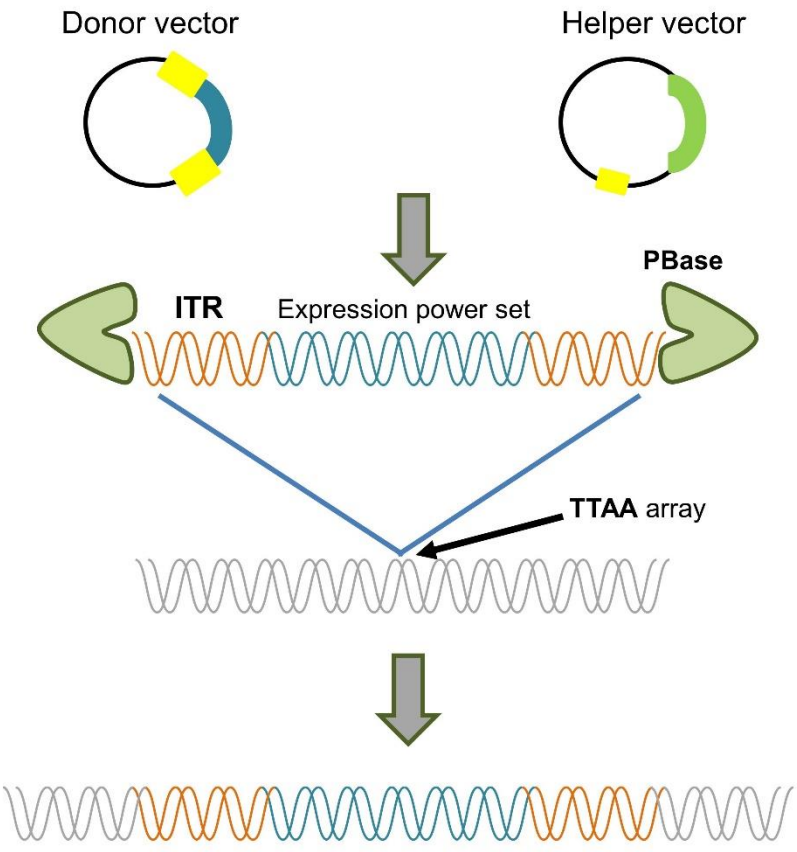

Fig.1-11 Integration of target genes by PiggyBac transposon.

(b) PiggyBac transposon

Transposons are genetic elements that perform cut-and-paste or copy-and-paste operations in the genome, contributing to genetic diversity and mutation. The PiggyBac (PB) transposon, derived from the insect Trichoplusia ni, incorporates donor vectors containing inverted terminal repeat (ITR) sequences upstream and downstream of the expression cassette, along with a helper vector containing the PB transposase (PBase). Upon co-introduction, PBase recognizes the ITR and excises the expression cassette, facilitating a cut-and-paste transposition into TTAA sequences within the genome. Subsequently, the open DNA ends are repaired via the non-homologous end-joining (NHEJ) pathway of the double-strand break repair system, completing the integration into the genome (**Fig. 1-11**) (Matasci et al., 2011; Wei et al., 2022).

(c) Genome editing tools

Genome editing tools include Zinc-finger nucleases (ZFN), TAL effector nucleases (TALEN), and the more recent Clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9). The CRISPR/Cas9 system functions as a bacterial adaptive immune system where the Cas9 nuclease cleaves foreign DNA and incorporates it into the CRISPR region to produce guide RNA (gRNA). When the same foreign DNA invades again, the gRNA

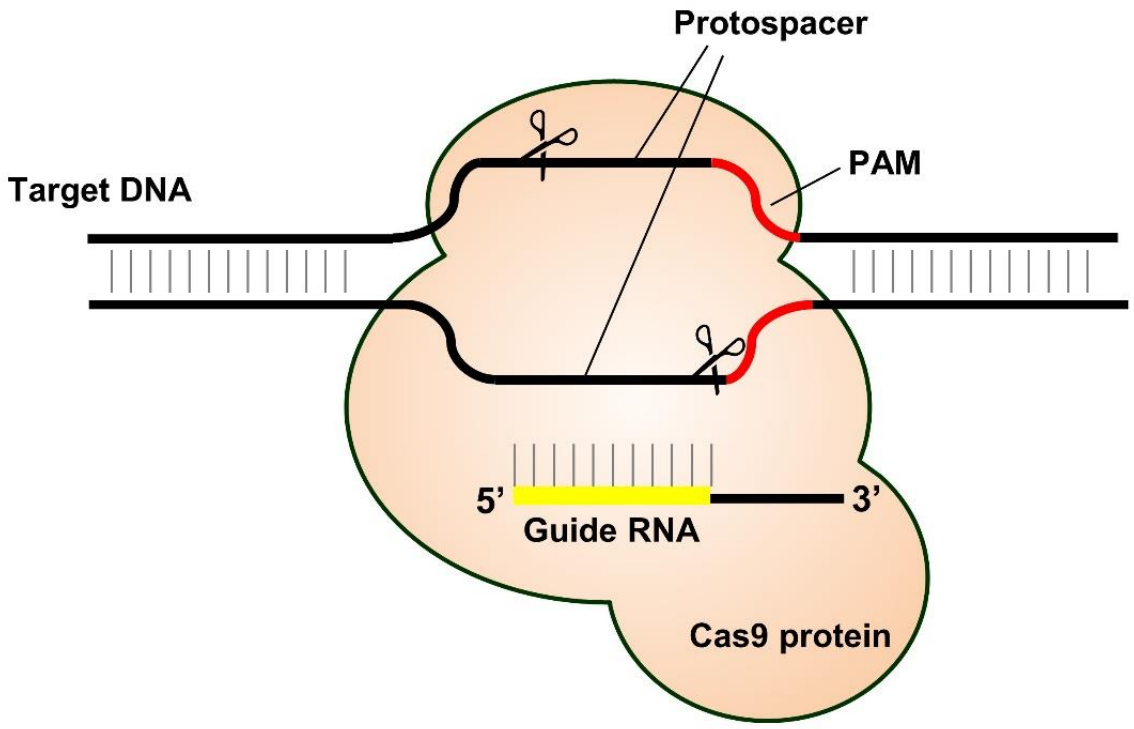

Fig. 1-12 Knockout of target genes by the CRISPR/Cas9 system.

forms a complex with Cas9, which then recognizes and binds to the protospacer adjacent motif (PAM) sequence downstream of the target DNA, leading to DNA cleavage. In genome editing, introducing gRNA and Cas9 results in a double strand break in the target DNA, repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ often introduces gene insertions or deletions, causing frameshift mutations and gene knockouts (**Fig. 1-12**) (Hsu et al., 2014; Zhang et al., 2019). For gene transfer, homologous sequences flanking the expression cassette facilitate HR for gene knock-in, although HR efficiency is low. Recent advancements have improved integration efficiency without relying on homologous sequences (Suzuki et al., 2016).

1.5. Previous research on heparin production using animal cells

Recent years have seen significant growth in biopharmaceutical production using animal cells. Advances in genetic modification technology and culture methods have increased protein production per cell from about 50 mg/L in the 1980s to 10 g/L today (Hacker et al., 2009; Huang et al., 2010). Heparin production requires complex glycosylation and advanced sulfation, making it difficult to achieve through simple organic synthesis, enzymatic reactions, or microbial fermentation alone. Consequently, researchers have turned to animal cells for producing heparin-like glycans. For instance, CHO cells, which initially lacked the expression of several genes necessary for heparin biosynthesis, were genetically modified to include heparan sulfate glucosamine 3-sulfotransferase 1 (Hs3st1) and heparan sulfate *N*-deacetyl/*N*-sulfotransferase 2 (NDST2) (Baik et al., 2012b). These modifications enabled the production of glycans with anticoagulant activity. Furthermore, expressing Hs3st1 on the Golgi membrane, its natural location, improved 3-O-sulfation of the product (Datta et al., 2013b). Additionally, HEK293 cells were genetically engineered to express serglycin, a core protein involved in heparin PG formation. Culturing these cells in a medium with 25 mM glucose resulted in the secretion of heparin-like glycans exhibiting 1/7th of the anticoagulant activity of UFH (Lord and Whitelock, 2014).

Research using mouse mast cell tumor (MST) cells, which naturally produce highly sulfated heparan sulfate, has also provided insights into heparin biosynthesis. By knocking out enzymes involved in chondroitin sulfate biosynthesis and introducing genes crucial for synthesizing AT-binding pentasaccharide sequences, researchers have produced glycans with anti-FXa activity comparable to heparin (Thacker et al., 2022). However, mass production of glycans achieving both anti-FXa and anti-FIIa activity equivalent to UFH remains elusive.

1.6 Dissertation goal

The goal of this dissertation is to provide a thorough and insightful analysis of heparin, a naturally occurring polysaccharide renowned for its extensive therapeutic applications. This study will explore diverse biological properties of heparin, focusing on its established role as a crucial anticoagulant and its emerging potential in anti-inflammatory, antiviral, and anti-tumorigenic therapies. By examining the historical context and current use of heparin in treating thrombotic disorders and other medical conditions, the research will underscore its evolving significance, particularly in combating infectious diseases like COVID-19.

Additionally, this dissertation will investigate recent advancements in nanotechnology and bioengineering that have augmented heparin's therapeutic potential. It will highlight innovations in nano-drug delivery systems and nanomaterials that enhance the efficacy and functionality of heparin. The study will also address the challenges associated with sourcing heparin from animal tissues, such as variability and adulteration, and discuss cutting-edge solutions offered by metabolic engineering and synthetic biology for producing bioengineered heparin.

The research objectives include analyzing metabolic engineering strategies in prokaryotic systems, reviewing advancements in bioengineered heparin development, and exploring innovative approaches to improving heparin production in eukaryotic systems, particularly through recombinant CHO cells. By examining these areas, the dissertation aims to assess future potential of heparin in bioengineering and nanotechnology, offering new insights into enhancing patient outcomes and therapeutic interventions.

Furthermore, we have successfully used CHO cells to produce heparin-like polysaccharides with anticoagulant activity in our laboratory. Initially, we generated CHO cells that overexpressed heparan sulfate glucosamine 3-sulfotransferase 1 (Hs3st1) and heparan sulfate glucosamine *N*-deacetylase/*N*-sulfotransferase 2 (NDST2), leading to the successful production of heparin-like polysaccharides (CHO/2F) (Sagawa. K., 2017). Subsequently, by genetically introducing the extracellular domain of syndecan-1 (SDC1), a membrane protein, as a secretion carrier, we confirmed the secretion of heparin-like substances into the culture supernatant (CHO/2F-SDC) (Sagawa. K., 2019). Furthermore, to enhance the degree of sulfation of the secreted polysaccharides, we introduced mouse heparan sulfate glucosamine 6-sulfotransferase 3 (mHs6st3), achieving a significant improvement in anticoagulant activity (anti-FXa specific activity) (CHO/3F-S) (Akiyama. K., 2021). Additionally, we successfully reduced the secretion of

sGAGs while maintaining anti-FXa activity by knocking out CS *N*-acetylgalactosaminyltransferase 2 (CSGALNACT2) using the CRISPR/Cas9 system. This enhanced the specific activity of anti-FXa. mHs3st1, a mouse-derived heparan sulfate 3-sulfotransferase 1, is involved in the 3-O-sulfation of *N*-sulfoglucosamine, crucial for synthesizing antithrombin binding sites (Thacker, 2014). Although 3-O-sulfation is rare in HS modification, it is essential for specific binding with antithrombin. Mice with the Hs3st1 gene knocked out exhibit reduced anticoagulant activity of heparin-like substances without showing procoagulant properties and suffer from developmental delays leading to postnatal lethality. This suggests that 3-O-sulfation by Hs3st1 has physiological roles beyond anticoagulant activity (Shworak, 2002). hNDST2, a human-derived heparan sulfate glucosamine *N*-deacetylase/*N*-sulfotransferase, is involved in *N*-deacetylation and *N*-sulfation, the initial modifications after the formation of the basic structure of glucosamine and uronic acid repeats. These modifications are essential for subsequent 2-O-sulfation and other sulfate group modifications. Mice with the NDST2 gene knocked out show reduced numbers of connective tissue-type mast cells, decreased intracellular granules, and reduced negative charge of heparin within cells (Forsberg, 1999).

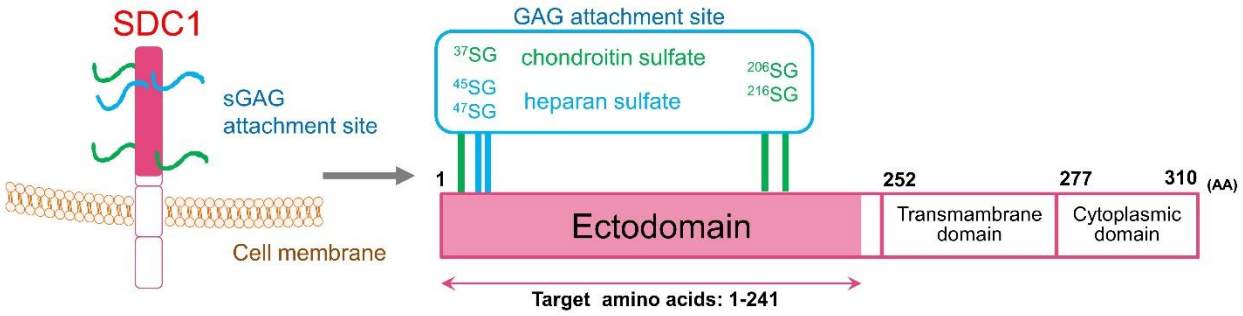

Fig. 1-13 Schematic diagram of Syndecane 1 (Manon-Jensen et al., 2013).

hSDC1, a member of the syndecan family derived from humans, is a core protein with five GAG binding sites. Given previous reports on MMP cleavage sites (**Fig. 1-13**) (Manon-Jensen, 2013), we constructed a synthetic gene avoiding MMP cleavage sites near the membrane in the extracellular domain. When this gene was introduced into cells expressing Hs3st1 and NDST2, heparin-like substances were confirmed to be secreted into the culture supernatant. In contrast, when the core protein serglycin (SRGN), primarily binding heparin, was similarly introduced, it was found to be unsuitable for secretion into the extracellular space.

mHs6st3, a mouse-derived heparan sulfate 6-sulfotransferase 3, is involved in the 6-O-sulfation of *N*-acetylglucosamine and *N*-sulfoglucosamine. Similar to 3-O-sulfation, 6-O-sulfation is heterogeneous. Its characteristics include modification of *N*-acetylglucosamine and occurrence even in NAc domains where *N*-sulfation is insufficient. This modification contributes to the formation of antithrombin-specific binding pentasaccharides and sequences that specifically bind fibroblast growth factor (FGF) (Masri, 2017). Among the 17 genes related to heparin/heparan sulfate sulfation transiently introduced into CHO/2F-SDC, Hs6st3 showed a significant improvement in anti-FXa specific activity, leading to its stable expression and marked enhancement of specific activity. This created CHO/3F-S cells that secrete highly anticoagulant heparin-like polysaccharides.

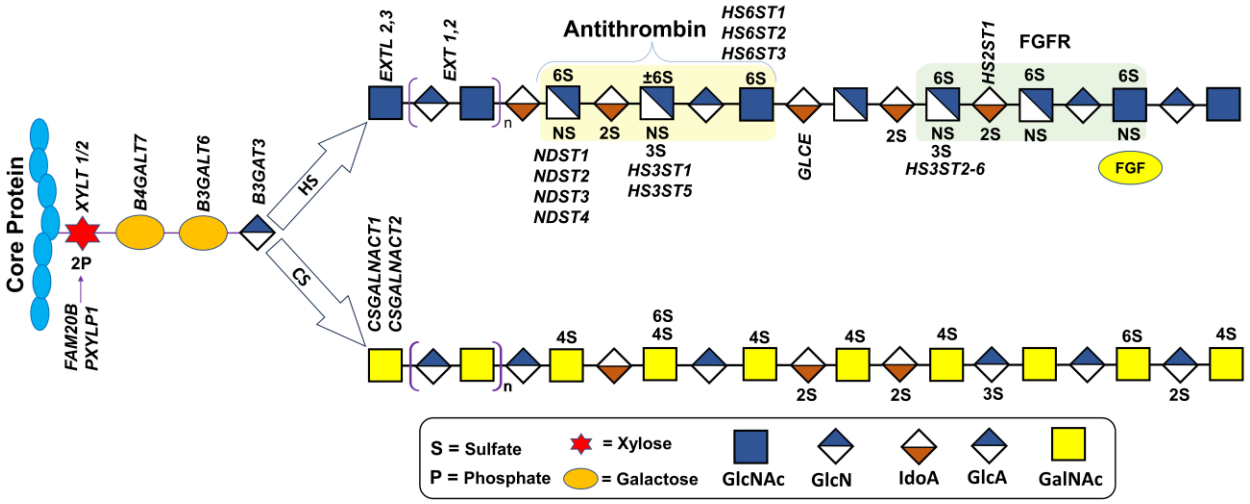

Fig. 1-14 Biosynthesis of glycosaminoglycans. This illustration represents HS (upper chain) and CS (lower chain) structures with antithrombin binding pentasacchairde and enzymes responsible for HS and CS biosynthesis.

This study aims to further enhance the anticoagulant activity and promote the synthesis of target polysaccharides secreted by CHO/3F-S cells. We performed gene disruption of HS desulfatases and enzymes involved in CS biosynthesis. We found that disrupting the CSGALNACT2 (CS2) gene, responsible for initiating CS chain elongation by transferring GalNAc to the tetrasaccharide linker of core protein, increased anticoagulant activity (**Fig. 1-14**) (Mikami and Kitagawa, 2013). This led to the creation of CHO/3F-S_CKO cells.

In essence, this dissertation aspires to provide a detailed profile of heparin, evaluate its therapeutic utilities, and explore future advancements, thereby contributing significantly to the fields of biomedical research and clinical practice.

1.7 Research strategies

This dissertation employs a targeted approach to enhance heparin production using recombinant CHO cells, focusing on genetic manipulation and bioprocess optimization. Firstly, we will inactivate HS desulfation enzymes using CRISPR-Cas9 to prevent sulfate removal and promote the synthesis of highly sulfated heparin-like polysaccharides. Additionally, by disrupting the CS2 gene, we aim to eliminate the CS biosynthetic pathway, redirecting cellular resources towards heparin production. Furthermore, we will investigate the influence of temperature shifts (TS) on heparin productivity, determining optimal conditions that enhance protein expression and cellular metabolism. Finally, we will use analytical techniques to evaluate the quality and efficacy of the produced heparin and develop scalable bioreactor protocols to ensure process stability and economic viability in large-scale production.

1.8 Outline of the dissertation

In Chapter 1, an overview of heparin, glycosaminoglycans, and proteoglycans is provided, alongside discussions on synthesis approaches and modifications. Additionally, the chapter reviews previous research on heparin production using animal cells, while also establishing the background, dissertation goals, and research strategies.

In Chapter 2, the focus shifts to an in-depth exploration of expanding therapeutic potential of heparin beyond its conventional applications. This includes advancements in bioengineering and nanotechnology that enhance its efficacy in clinical settings. The chapter examines recent developments in heparin biosynthesis and its diverse clinical applications, highlighting both opportunities and challenges in fully harnessing its therapeutic capabilities. From inflammation modulation and antiviral therapies to cancer treatment and tissue regeneration, multifaceted roles of heparin in modern medicine are thoroughly examined, aiming to redefine therapeutic interventions and improve patient outcomes.

In Chapter 3, the focus is on exploring recent advancements in bioengineered heparin production across both prokaryotic and eukaryotic systems. This chapter comprehensively analyzes innovative approaches and achievements in metabolic engineering, highlighting their implications for future applications. Specifically, it delves into the potential of recombinant CHO cells as a promising platform for synthesizing heparin, discussing recent breakthroughs, ongoing challenges, and opportunities for improvement. The goal is to assess these technologies critically, aiming to advance the development of safer, more sustainable, and clinically effective heparin therapeutics.

In Chapter 4, an enhancement of heparin production using recombinant CHO cells with a targeted approach centered on genetic manipulation and bioprocess optimization. The chapter employs CRISPR-Cas9 technology to deactivate HS desulfation enzymes, thereby preserving sulfate groups and promoting the synthesis of highly sulfated heparin-like polysaccharides. Additionally, disruption of the CS2 gene aims to eliminate the CS biosynthetic pathway, redirecting cellular resources towards heparin production. The chapter also investigates the influence of temperature shifts (TS) on heparin productivity, optimizing conditions to enhance protein expression and cellular metabolism. Analytical techniques are employed to evaluate the quality and efficacy of

the produced heparin, while scalable bioreactor protocols are developed to ensure process stability and economic viability for large-scale production.

In Chapter 5, presents a summary of the key findings from the previous chapters and proposes further enhancements in bioengineered heparin production.

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Chapter 2

Multifaceted heparin: Diverse applications beyond anticoagulant therapy

2.1 Background

Heparin, a highly sulfated therapeutic agent with a long history, has been acknowledged for its remarkable anticoagulant properties (Chen, 2021; Hao et al., 2019; Qiu et al., 2021; Wang et al., 2022a; Zang et al., 2022). It was first discovered in extracts from animal tissues in the early 20th century. Jay McLean identified its anticoagulant effect in canine liver in 1916 (McLean 1916). It was later named "heparin" by Howell and Holt in 1922 and became pivotal in medical practice by the 1930s following advancements in purification techniques (Hemker 2016; Best 1959; Brinkhous et al., 1939). Its interaction with antithrombin was elucidated in the 1950s, leading to enhanced therapeutic understanding (Waugh et al., 1956; Monkhouse et al., 1955; Abildgaard et al., 1968). A brief history of heparin is presented in Fig. 1 (Hogwood et al., 2023; Mulloy et al., 2016). Since its discovery, this GAG has played a pivotal role in preventing and treating thrombotic disorders, saving countless lives globally (McLean 1916; Thacker et al., 2022). However, beyond its established role in anticoagulation, emerging research has unveiled a plethora of novel therapeutic pathways, including anti-inflammatory, antiviral/bacterial, anti-thrombotic, anti-metastatic, anti-hypolipidomic, and anti-angiogenesis activities (Hao et al., 2019; Qiu et al., 2021a; Wang et al., 2022a; Aláez-Versón et al., 2017; Folkman et al., 1983; Lever et al., 2002; Lindahl., 2002; Shi et al., 2021; Torri and Naggi., 2016).

Currently, the COVID-19 pandemic remains a critical global health challenge (Hippensteel et al., 2020). Ongoing research, including hypotheses, clinical data, and retrospective cohort studies, suggests that UFH and LMWH may reduce mortality in COVID-19 patients by leveraging their anticoagulant, antiviral, and anti-inflammatory properties (Hippensteel et al., 2020; Dutch et al., 2021; Ennemoser et al., 2021; Know et al., 2022; Seffer et al., 2021; Thachil et al., 2020; Jiang et al., 2023). Consequently, the versatile nature of heparin has prompted thorough exploration of its diverse applications across various medical fields.

The development of LMWH was driven by the need to create heparin derivatives that could effectively inhibit factor Xa without the extensive anticoagulant effects associated with thrombin inhibition (Lindahl et al., 1979; Dementiev et al., 2004; Petitou et al., 2004; van Boeckel et al., 1993). Research indicates that factor Xa inhibition by heparin relies predominantly on a specific

pentasaccharide sequence, while effective thrombin inhibition involves additional interactions with a longer polysaccharide chain (Rosenberg et al., 1994, 1979). Understanding these distinct mechanisms was crucial in the development of LMWHs. LMWHs are derived from heparin through depolymerization, resulting in shorter chains that preferentially inhibit factor Xa over thrombin. This structural modification retains the antithrombotic efficacy of heparin while reducing the risk of bleeding complications, making LMWHs a vital therapeutic option in various clinical settings for preventing and treating thromboembolic disorders.

Recent years have witnessed a profound expansion in therapeutic potential of heparin, driven by advancements in comprehending its complex structure-function relationships and interactions with biological molecules (Hao et al., 2019; Kim et al., 2017; Fu et al., 2019; Capila et al., 2002; Casu et al., 2010; Gulberti et al., 2020). Traditional extraction methods from animal origins have yielded heparin with a complex structure and mechanism of action that have captivated researchers for decades (Baytas and Linhardt., 2020; Bhaskar et al., 2012; Oduah et al., 2016; Suflita et al., 2015; Carlsson et al., 2012; Lidholt et al., 1988). Its sulfated polysaccharide backbone imparts a unique molecular architecture, facilitating interactions with a range of biological molecules, from growth factors to cell surface receptors (Qiu et al., 2021; Wang et al., 2022; Chen., 2021; Vaidyanathan et al., 2017; Glass., 2018; Zare et al., 2024). While its anticoagulant effects remain its hallmark feature, the elucidation of the complex pharmacology of heparin has revealed a wealth of therapeutic opportunities across numerous therapeutic areas (Vaidyanathan et al., 2017; Zare et al., 2024). Moreover, the integration of heparin with nanomaterials has initiated a new era of drug delivery and functionalization, offering innovative pathways to enhance its therapeutic efficacy and bioavailability (Zare et al., 2024; Rodriguez-Torres et al., 2018; Meher et al., 2024; Kumar et al., 2023; Saurav et al., 2024; Kemp et al., 2009; Sultana et al., 2024; Olczyk et al., 2005).

2.2 Aim

This review aims to provide a comprehensive overview of the expanding therapeutic potential of heparin beyond its traditional role. It underscores the pivotal role of heparin in contemporary therapeutic strategies, bolstered by cutting-edge advancements in bioengineering and nanotechnology. By exploring recent advances in understanding its biosynthesis process and clinical applications, we seek to illuminate the diverse opportunities and challenges in harnessing the full therapeutic repertoire of this remarkable molecule. From inflammation modulation to antiviral therapy, from cancer treatment to tissue regeneration, heparin stands at the forefront of

modern medicine, offering numerous possibilities to improve patient outcomes and redefine therapeutic interventions.

2.3 Diverse applications of heparin

2.3.1 Heparin in anti-inflammatory therapies

Heparin, long recognized for its role as an anticoagulant, has recently garnered attention for its remarkable anti-inflammatory properties. Inflammation plays a crucial role in various diseases, and recent research has elucidated the multidimensional role of heparin in modulating inflammatory pathways. Extensive reviews have shed light on how heparin interacts with key proteins involved in inflammation, including those within the complement system, selectins, and chemokines (Casu et al., 2010; Linhardt and Toida, 2004; Mousavi et al., 2015; Poterucha et al., 2017; Qiu et al., 2021a; Severin et al., 2012; Yan et al., 2017). These interactions not only inhibit neutrophil activation and vascular smooth muscle cell proliferation but also modulate the expression of inflammatory mediators by engaging with vascular endothelial cells (Gilotti et al., 2014). Beyond its traditional use as an anticoagulant, heparin demonstrates promising anti-inflammatory effects across diverse conditions such as arthritis, inflammatory bowel disease, and bronchial asthma (Abdelaty and Abd-Elsalam, 2007; Baumgart, 2010; Bendstrup and Jensen, 2000; Cai et al., 2020; Ceranowicz et al., 2008; Ghonim et al., 2018; Granell et al., 2003; Malhotra et al., 2004; Poterucha et al., 2017; Qi et al., 2016; Shute, 2023; Shute et al., 2018). Exciting developments include novel formulations like LMWH MMX (CB-01-05-MMX), a novel oral Parnaparin sodium, which show potential in treating ulcerative colitis with favorable safety profiles observed in clinical trials (Baumgart, 2010). Moreover, inhaled heparin emerges as a potential adjunctive therapy for chronic obstructive pulmonary disease and asthma, offering both anti-inflammatory and mucolytic benefits (Fath et al., 1998; Shute et al., 2018). In acute pancreatitis cases characterized by elevated triglyceride levels, combination therapy involving LMWH calcium and insulin has demonstrated efficacy in improving immune function and coagulation parameters (Cai et al., 2020). Sepsis, a severe and life-threatening condition triggered by gram-negative bacteria, has drawn intense scrutiny due to its high mortality rate (Chen, 2021b). The interactions of heparin with pro-inflammatory factors and involvement in procoagulant cascades hold promise in mitigating inflammation and coagulopathy associated with sepsis (Derhaschnig et al., 2003; Li and Ma, 2017; Poterucha et al., 2017).

Recent investigations underline the potential of heparin to disrupt high mobility group box 1 protein-lipopolysaccharide interactions, yielding significant anti-inflammatory effects (L. Li et al., 2015; Tang et al., 2021). Additionally, heparin and selectively desulfated heparin have shown efficacy in binding to positively charged histones, thereby attenuating their inflammatory actions (Hogwood et al., 2020; Wang et al., 2020). Recent findings suggest that altered forms of heparin derivatives possess the ability to bind to positively charged histones, thereby mitigating histone-induced inflammation (Wildhagen et al., 2014). Specifically, selectively desulfated heparin, with diminished anticoagulant properties, retains its effectiveness as an anti-histone agent. For instance, a derivative known as anti-thrombin affinity depleted heparin (AADH), lacking anticoagulant activity, directly binds to histones, reducing histone-mediated cytotoxicity and mortality in inflammation and sepsis models in mice without heightening bleeding risk (Wildhagen et al., 2014). Moreover, during sepsis, oligosaccharides of heparin or HS may influence patient cognitive functions (Zhang et al., 2019). Therapeutically, doses of UFH protect the glycocalyx from shedding by reducing inflammation in models of septic shock. In treating sepsis, LMWH serves as a vital component of supportive therapy (Li and Ma, 2017; Yini et al., 2015). It helps improve multiple organ dysfunction syndrome and reduces the mortality rate within a shorter period. Recent assessments of the effectiveness of UFH highlight a notable decrease in mortality rates over 28 days, accompanied by minimal bleeding risks (Li et al., 2021). Unlike LMWH, UFH provides a spectrum of effects beyond its anticoagulant properties. It can disrupt nuclear factor NF-κB activation, block the movement of chemokines and monocytes, bolster the endothelial barrier, and regulate the angiopoietin (Ang)/Tie2 axis (Fu et al., 2022). Although the anti-inflammatory properties of heparin are separate from its blood-thinning effects, concerns about bleeding limit its use as an anti-inflammatory treatment. Therefore, refining heparin to minimize or eradicate its anticoagulant activity holds promise in enhancing its efficacy against inflammatory diseases.

2.3.2 Heparin in COVID-19 and other infectious diseases

Exploring the potential of heparin as a COVID-19 treatment, particularly in severe cases with clotting issues and organ damage, has sparked interest (Jiang et al., 2023a; Qiu et al., 2021a). Recent research suggests that heparin may effectively target multiple aspects of COVID-19, including thrombosis prevention, inhibition of viral entry, and reduction of cytokine activity, supported by both clinical observations and experimental data (Wang et al., 2022b). The presence of coagulation abnormalities in COVID-19 poses an omnious threat, often leading to high

mortality rates; however, studies suggest that administering UFH or LMWH could potentially reduce mortality in critically ill patients by preventing blood clot formation (Dutch and Thrombosis, 2021; Kyriakoulis et al., 2022; Poli et al., 2022; Tang et al., 2020; van Haren et al., 2022). Initial recognition of the anticoagulant properties of heparin in COVID-19 stemmed from a retrospective study that included nearly 500 patients in Wuhan, China (Zhai et al., 2019). Subsequent studies across the US and Italy further supported the link between heparin usage and reduced mortality rates, particularly among elderly patients (Poli et al., 2022; Rentsch et al., 2021). These findings emphasize the importance of early prophylactic anticoagulation upon hospital admission for COVID-19 patients, as recommended by the International Society for Thrombosis and Hemostasis (ISTH) guidelines (Thachil et al., 2020).

Apart from its function as an anticoagulant, heparin exhibits direct antiviral effects by attaching to SARS-CoV-2 proteins, impeding viral attachment and replication (Clausen et al., 2020; Mycroft-West et al., 2020b; Zhang et al., 2020). This interference with the virus-host cell interaction suggests heparin's potential in fighting COVID-19 and similar coronaviruses. Additionally, heparin displays encouraging signs in blocking the main protease of SARS-CoV-2, a key process in viral replication (Li et al., 2022). These findings highlight the potential of heparin as a first-line antiviral treatment for COVID-19 (Mycroft-West et al., 2020b). Moreover, heparin shows positive effects in reducing inflammation linked to COVID-19, particularly in cases of cytokine storm syndrome (CSS) observed in severe infections (Cron et al., 2023). By interacting with inflammatory cytokines and pathways, heparin could potentially alleviate the systemic inflammatory response often seen in severe COVID-19 cases. Additionally, there is promising evidence suggesting that heparin may be effective in managing COVID-19-associated myocarditis, thereby broadening its therapeutic utility (Yu, 2022). Sun and colleagues observed a significant increase in a protein called heparin-binding protein (HBP) in severely ill COVID-19 patients as their condition worsened. This observation emphasizes the potential involvement of HBP in the systemic inflammatory response associated with severe COVID-19. The correlation between HBP levels and the deterioration of COVID-19 symptoms suggests that HBP may serve as a crucial disease indicator (Xue et al., 2021). Furthermore, recognizing HBP as a potential therapeutic target in COVID-19 underscores its significance in developing targeted treatment strategies aimed at mitigating the severe inflammatory response observed in critically ill patients. In summary, heparin provides a wide array of benefits for COVID-19 patients, serving as both an anticoagulant, antiviral, and anti-

inflammatory treatment. However, to fully harness its potential, further research is needed to fine-tune the dosage, timing, and duration of heparin administration, especially for patients with unique medical profiles (Thachil, 2020). As researchers delve deeper into the therapeutic potential of heparin, it becomes increasingly clear that it holds significant promise in combating COVID-19.

In addition to ongoing research into COVID-19, heparin and its derivatives exhibit promising abilities to hinder the adhesion and invasion of various viruses such as dengue virus (DENV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), influenza virus, Zika virus and human papillomavirus 16 (HPV16) (Angeletti, 2017; Copeland et al., 2008; Ghezzi et al., 2017; Kim et al., 2019; S. Y. Kim et al., 2017; Lin et al., 2002; Nassar et al., 2012; Plochmann et al., 2012; Skidmore et al., 2015; Urbinati et al., 2021). Heparin also directly impacts viruses and bacteria, reducing iron levels in human macrophages to control Mycobacterium tuberculosis bacterial replication and partially impeding Zika virus replication (Abreu et al., 2018; Ghezzi et al., 2017). This dual action prevents virus-induced cell necrosis and apoptosis without harming uninfected cells, while simultaneously activating cell survival signaling pathways (Hills et al., 2006).

Malaria, a significant infectious epidemic, demonstrates the antimalarial potential of heparin as it specifically binds to Plasmodium-infected red blood cells (pRBCs) and liver circumsporozoites (Leitgeb et al., 2011; Marques et al., 2014; Sinnis et al., 2007). Nanomedical applications of heparin have emerged to transport drugs to the mosquito stages of malaria parasites (Aláez-Versón et al., 2017). A recent study introduces dendronized hyperbranched polymers (DHPs) designed for loading antimalarial agents and coated with heparin to target red blood cells infected with Plasmodium falciparum. These DHP-heparin complexes exhibit both the inherent antimalarial properties of heparin, with an IC50 of around 400 nM, and specific targeting towards P. falciparum-infected erythrocytes compared to uninfected ones. This innovative approach presents a promising addition to the limited repertoire of structures available for effectively loading and delivering antimalarial agents (San Anselmo et al., 2022). In Lyme disease (LD), heparin derivatives like non-anticoagulant heparin (NACH) impede Borrelia burgdorferi sensu lato (Bbsl) attachment to mammalian cells and boost antibody immune responses to thwart LD (Lin et al., 2020; Steere et al., 2016). Moreover, Exploiting the natural attraction between bacteria or viruses and heparin offers promising avenues for capturing and eliminating them from circulation. This strategy is particularly useful in extracorporeal medical devices such as the Seraph® 100 Microbind®

Affinity Blood Filter (Axelsson et al., 2010). This pioneering device integrates polyethylene beads immobilized with heparin, authorized for reducing and eradicating pathogens from the bloodstream. Preliminary trials demonstrate its efficacy in diminishing viral loads, including Zika virus, adenovirus, and SARS-CoV-2, indicating its potential as adjunct therapy for severely ill COVID-19 patients (Buitrago-Pabón et al., 2024; Seffer et al., 2021b).

2.3.3 Heparin in oncology

Since 1865, when Armand Trousseau linked superficial migratory thrombophlebitis (SMT) to underlying malignancy, the intricate relationship between cancer and thrombosis has been acknowledged (Goubran et al., 2013; Metharom et al., 2019). Among cancer-assisted thrombotic events, venous thromboembolism (VTE) including deep vein thrombosis (DVT) and pulmonary embolism (PE) reigns supreme, often necessitating treatment with LMWH (Cosmi, 2021; Gervaso et al., 2021; Smorenburg and Van Noorden, 2001). Recent insights assembled from reviews and meta-analyses suggest that heparin and its derivatives extend beyond their anticoagulant role, exerting a direct influence on tumor biology pathways, including inflammation, angiogenesis, and metastasis (Falanga and Marchetti, 2023; Ma et al., 2020). One notable target in cancer therapy is heparanase, an endoglycosidase prevalent in malignant tumors (Coombe and Gandhi, 2019; Kaur et al., 2021; Lindahl U, 2020). Heparin and its mimetics show promise in selectively inhibiting heparanase expression, while also targeting essential mediators such as fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) involved in tumor angiogenesis (Kilarski and Bikfalvi, 2007; Lanzi et al., 2017; Ma et al., 2020). Furthermore, modified heparin derivatives demonstrate potential in blocking galectin 3-mediated cancer cell adhesion and angiogenesis, offering a new frontier in anti-metastasis and anti-cancer drug development (Duckworth et al., 2015).

Despite encouraging preclinical data, clinical trials evaluating the efficacy of heparin and its derivatives in cancer treatment encounter obstacles (Atallah et al., 2020). For instance, in lung cancer phase III trials, LMWH failed to significantly improve survival rates (Ek et al., 2018; Gezelius et al., 2019). Similarly, the non-anticoagulant heparin analog necuparanib did not achieve the anticipated efficacy in Phase II clinical trials for pancreatic cancer, highlighting the challenges in developing effective non-anticoagulant heparin-based therapeutics for oncology applications (O'Reilly et al., 2017). Moreover, adverse effects, including bleeding-related complications, have been observed with heparin mimetics such as PI-88 (Atallah et al., 2020). Nonetheless, ongoing

research ventures into novel heparin mimetics such as PG545 (pixatimod), which boast immunomodulatory and antiangiogenic properties (Chhabra et al., 2022). Phase I trials have exhibited promising safety profiles and encouraging disease progression control, particularly in advanced solid tumors (Dredge et al., 2018). With PG545 now undergoing phase II trials in combination with nivolumab (Chhabra et al., 2022), further exploration is warranted to unlock the potential of heparin and its derivatives in cancer treatment.

Subcutaneous LMWH administration has limitations, including adverse reactions at the injection site, discomfort, bruising, and bleeding, which can significantly affect patients' quality of life (Khorana et al., 2017). However, recent advancements have led to the emergence of direct oral anticoagulants (DOACs) as a viable alternative for treating cancer-associated thrombosis in specific patient cohorts. Supported by the results of numerous high-quality randomized controlled trials (RCTs), DOACs have gained recognition as an effective and safe option, receiving strong recommendations in clinical guidelines (Agnelli et al., 2020; McBane et al., 2020; Planquette et al., 2022; Raskob et al., 2018; Schrag et al., 2023; Young et al., 2018). Chronologically, these findings have progressed alongside the understanding of thromboprophylaxis after cancer-related surgery. Specifically, DOACs, such as apixaban and rivaroxaban, have demonstrated equivalence to LMWH in preventing postoperative venous thromboembolism (VTE). Consequently, oral DOACs are increasingly considered as potentially effective and safe alternatives to subcutaneous LMWH for thromboprophylaxis in patients undergoing cancer surgery (Zhou et al., 2024).

Several methods are available to address the neutralization of overdosed heparin-based anticoagulants, each with its own limitations. Protamine sulfate is the primary agent used to neutralize UFH by forming a stable complex with heparin, effectively reversing its anticoagulant effects. However, when used for LMWHs, protamine only partially neutralizes their activity, typically reversing about 60-80%, leaving residual anticoagulant effects that can pose a clinical challenge, especially in cases of significant overdose. Additionally, protamine sulfate can cause adverse reactions, such as hypotension, bradycardia, and anaphylaxis, particularly in patients with fish allergies or previous exposure to protamine. Alternative approaches, such as hemodialysis or the use of agents like recombinant FVIIa or activated prothrombin complex concentrates, have been explored for reversing heparin effects, but they are less effective and carry their own risks. These limitations underscore the complexity of managing heparin overdoses and highlight the need

for careful monitoring and individualized treatment plans to mitigate the risks associated with both heparin use and its neutralization (Frackiewicz et al., 2023).

Above all, while evidence suggests the promising role of heparin and its derivatives in cancer therapy, a deeper understanding of their mechanisms of action is imperative (Ma et al., 2020). Furthermore, the innovation of heparin mimetics with diminished anticoagulant properties, coupled with the refinement of dosing protocols, is imperative for improving therapeutic effectiveness and safety (Mohamed and Coombe, 2017a; Wang et al., 2022b). Extensive clinical investigations into their anti-cancer effects, both standalone and in combination with existing therapies, offer hope for advancements in cancer treatment.

2.3.4 Heparin in nephropathy

In renal medicine, heparin holds a vital position as a potent anticoagulant, valued for its efficacy in preventing blood clot formation and mitigating thrombotic risks. Its role extends across diverse renal conditions, including nephrotic syndrome, diabetic nephropathy, and hemodialysis, where it serves as a reliable ally in patient management. Nephrotic syndrome, a condition characterized by the injury of vascular endothelial cells, triggers a hypercoagulable state in the body, increasing the risk of complications such as lower limb venous thrombosis, particularly prevalent in children (Yoshikawa et al., 2015). Clinical management often involves a comprehensive approach encompassing anticoagulants, anti-infective, and vasodilator drugs (Huang et al., 2019; Medjeral-Thomas et al., 2014; Muso et al., 2015; Sinha et al., 2015; Yoshikawa et al., 2015). Among these, LMWH emerges as a prominent therapeutic option, exhibiting robust anticoagulant properties. LMWH effectively mitigates secondary pathological hypercoagulability by augmenting blood viscosity and inhibiting kidney blood clot formation, thus minimizing the likelihood of thrombosis-related complications and bleeding incidents (Medjeral-Thomas et al., 2014). Additionally, LMWH demonstrates anti-inflammatory and antiproliferative effects, fostering extracellular matrix repair and endothelial cell restoration, ultimately alleviating kidney damage. This treatment modality holds promise in addressing the complex interplay of coagulation abnormalities and renal dysfunction, offering significant therapeutic potential in the management of nephrotic syndrome (Huang et al., 2019).

Diabetic nephropathy (DN) represents a significant challenge among individuals with diabetes, posing a substantial burden on health outcomes. Recent clinical investigations underscore the promising role of sulodexide, a LMW glycosaminoglycan, in ameliorating proteinuria in DN

patients, even when co-administered with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor antagonists, suggesting its potential for renal protection (R. Li et al., 2015; Stopschinski et al., 2020). The proposed mechanism of sulodexide involves the inhibition of heparanase activity, thus preserving the integrity of HS within the glomerular capillary wall and restoring the ionic permselectivity of the glomerular basement membrane (GBM) (Weiss et al., 2007). Ongoing clinical trials seek to further elucidate the renal protective properties of sulodexide in DN (Lewis and Xu, 2008). Additionally, exogenous administration of GAGs induces notable chemical and anatomical alterations in renal tissues, with both heparin and LMWH exhibiting suppressive effects on inflammatory responses within diabetic glomeruli (Abbadi et al., 2020; Wang et al., 2014). Experimental investigations in rodent models indicate that altered heparin with decreased anticoagulant efficacy efficiently alleviates glomerular and tubular matrix deposition, suppresses the expression of transforming growth factor beta (TGF-β1), and diminishes albuminuria. These findings imply a promising prospect for GAG therapy in thwarting diabetic glomerulosclerosis by mitigating TGF-β1 overexpression (Ceol et al., 2000). Current therapeutic strategies for DN emphasize the identification of pivotal molecular targets, with emerging evidence suggesting a correlation between kidney injury and receptor for advanced glycation end products (RAGE) gene expression. LMWH emerges as a promising RAGE antagonist, exerting notable renoprotective effects by alleviating albuminuria, augmenting glomerular cell count, and attenuating mesangial expansion, thus highlighting the therapeutic promise of RAGE antagonists in DN management (Myint et al., 2006; Yamamoto et al., 2007).

In the field of allergic purpura nephritis among pediatric patients (TIAN Yan, 2016), LMWH calcium stands out as a crucial treatment option. Its therapeutic significance extends beyond its anticoagulant properties, encompassing the prevention of thrombosis, modulation of blood viscosity, and facilitation of basement membrane reconstruction (Chen and Mao, 2015). By addressing these diverse aspects, LMWH plays a vital role in alleviating the severity of nephritis in affected children. These observations underscore the various functions of LMWH in treating kidney diseases, suggesting a hopeful path for improving patient outcomes in different clinical situations.

Currently, the practice of hemodialysis is widely used in clinical settings to prevent against acute renal failure. Nonetheless, ensuring uninterrupted treatment necessitates the prevention of blood coagulation (CAO Lei, 2019). Although heparin is commonly employed to inhibit clot formation

in hemodialysis apparatus, its efficacy in minimizing the risk of bleeding during hemodialysis remains limited. In response, LMWH has gained widespread adoption in clinical practice to mitigate this concern. LMWH serves to exert an anticoagulant effect, thus reducing the likelihood of bleeding events in patients undergoing hemodialysis, while also boasting an extended duration of action (Wong et al., 2016). In summary, heparin, particularly LMWH, plays a critical role in the management of renal diseases by preventing thrombosis and reducing the risk of thrombotic complications. Its use is guided by the specific renal condition and individual patient factors, highlighting the importance of personalized treatment approaches in renal care.

2.3.5 Heparin in cardiopathy

Heparin serves as a cornerstone in the management of acute coronary syndrome (ACS), a critical condition characterized by acute myocardial ischemia due to thrombosis triggered by unbalanced atherosclerotic plaque rupture or erosion within the coronary arteries (National Health and Family Planning Commission Expert Committee on Rational Drug Use, 2018). ACS encompasses a spectrum of events, including ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI), and unstable angina (UA), all with the potential to progress to acute myocardial infarction or sudden cardiac death.

During the acute phase of ACS, primary percutaneous coronary intervention (PCI) stands as the preferred approach for treating STEMI, while intravenous thrombolysis offers an alternative for patients ineligible for immediate PCI (Ibanez et al., 2018). Thrombolysis, commonly utilizing urokinase or streptokinase, is complemented by anticoagulant therapy, typically involving UFH or LMWH (Expert committee on rational drug use of the national health and family planning commission Chinese pharmacists association Fu Xianghua Huo Yong, 2019). LMWH, exemplified by enoxaparin, has emerged as a favored option, offering comparable or superior efficacy to UFH with a more favorable safety profile. In the management of coronary heart disease (CHD) with ACS, anticoagulant therapy plays a central role in preventing thrombotic complications and optimizing outcomes. Heparin acts as an adjunct to both PCI and thrombolytic therapy in STEMI cases and forms a cornerstone in early conservative strategies for NSTEMI (Cohen et al., 2006; Eikelboom et al., 2000). During PCI procedures, intravenous anticoagulation with either heparin or bivalirudin, coupled with antiplatelet agents, is standard practice (Bikdeli et al., 2023; Centurión, 2016; Erlinge et al., 2017; Patel et al., 2019). While bivalirudin exhibits similar efficacy and safety to heparin, the latter offers substantial cost advantages (Deharo et al., 2018; Rashid et al., 2019).

In a recent examination of randomized controlled trials, Al-Abdouh et al. conducted a systematic review and meta-analysis, which included the landmark BRIGHT-4 (Bivalirudin with prolonged full-dose infusion during primary PCI vs. heparin) trial. Their findings revealed that bivalirudin, administered during PCI for MI, resulted in reduced rates of major bleeding and cardiovascular mortality compared with unfractionated heparin. Surprisingly, there were no significant differences observed in major adverse cardiovascular events, all-cause mortality, MI, stroke, or stent thrombosis between the two treatment groups (Al-Abdouh et al., 2024). These results closely correspond with the outcomes of several other meta-analyses conducted on similar subjects (Capodanno et al., 2016). Furthermore, LMWH, particularly enoxaparin, has captured interest as a preferred post-PCI anticoagulant owing to its superior efficacy and safety compared to UFH, eliminating the need for routine anticoagulant intensity monitoring (Centurión, 2016; Collet and Thiele, 2020). Emerging evidence advocates for the use of LMWH-heparin combination therapy in complex coronary artery disease cases undergoing PCI, highlighting the evolving landscape of anticoagulation strategies in the management of CHD.

2.3.6 Heparin in neuroprotection

Heparin has demonstrated promising neuroprotective effects in neurodegenerative diseases and traumatic brain injury by modulating neuroinflammation, promoting neuronal survival, and enhancing neuroregeneration (Bergamaschini et al., 2009; Ma et al., 2007). This multifaceted mechanism of action highlights its potential as a therapeutic intervention for conditions such as Alzheimer's disease (AD), Parkinson's disease (PD) and stroke. AD poses a significant burden on elderly healthcare systems worldwide, characterized by cognitive decline and dementia, leading to diminished quality of life and increased mortality rates among affected individuals. The development of AD is characterized by the gradual buildup of amyloid plaques and neurofibrillary tangles, which are considered hallmark features of the condition (Knopman et al., 2021). This accumulation occurs progressively within the brain, representing a slow and irreversible process that poses significant challenges for therapeutic interventions. These pathological features not only underscore the complexity of AD but also impede the development of effective treatments.

Recent research has elucidated the role of heparin sulfate proteoglycans in AD pathogenesis (Snow et al., 2021), influencing the formation of plaques and tangles while facilitating pathogen entry into brain cells. This understanding has prompted exploration into the therapeutic potential of heparin and its derivatives in AD management. Studies have demonstrated their capacity to

mitigate various aspects of AD pathology, including the reduction of amyloid peptide levels, inhibition of tau phosphorylation, and attenuation of inflammatory responses (Bergamaschini et al., 2009; Ma et al., 2007). Notably, Enoxaparin, a LMWH, has emerged as a promising candidate, showing efficacy in reducing beta-amyloid plaque accumulation and enhancing cognitive function in AD animal models (Timmer et al., 2010). Additionally, heparin oligosaccharides have exhibited neuroprotective properties by modulating amyloid precursor protein secretion (Leveugle et al., 1998) and impeding the uptake of tau aggregates, offering novel avenues for AD therapy (Zhao et al., 2020). Despite the challenges posed by the anticoagulant activity of heparin, researchers are investigating marine-derived heparin analogs with enhanced therapeutic profiles, targeting key enzymes implicated in AD pathology without significant anticoagulant effects (Mycroft-West et al., 2021, 2020a, 2019). Moreover, synthetic heparin oligosaccharides such as SN7–13 have shown promise in blocking tau protein uptake and inhibiting tau aggregate formation, presenting innovative strategies for addressing tauopathies associated with AD (Weiss et al., 2007). Recent research findings propose that increased expression of the HS3ST1 gene might facilitate the propagation of tau pathology, revealing a novel target for potential therapeutic interventions in AD (Z. Wang et al., 2023). On the other hand, another neurodegenerative disorder, PD, is marked by the progressive loss of dopaminergic neurons situated in the substantia nigra area of the brain. This loss results in evident clinical symptoms, such as involuntary tremors during periods of rest, sluggish movement (bradykinesia), and challenges in maintaining normal posture and walking (Shin et al., 2022). In a recent study conducted by Wang and his team, they examined the effectiveness of LMWH and LMWCS in treating PD. The research found that LMWH helped alleviate mitochondrial dysfunction due to its antioxidant properties, while LMWCS was effective in reducing neuroinflammation by blocking platelet activation (Wang et al., 2024). As research progresses, heparin-based therapies continue to emerge as promising modalities for addressing the adaptable challenges posed by AD, PD and related neurodegenerative diseases.

2.3.7 Heparin in nanomedical research and drug delivery systems

In recent years, the landscape of nanomedicine has been transformed by the remarkable integration of heparin, once solely known for its anticoagulant properties. Its journey from an anticoagulant to a multi-dimensional player in the nanotechnology arena has been propelled by its unique attributes, including biocompatibility, biodegradability, and intricate interactions with biological molecules. This transformation has steered us into a new era, where the utilization of heparin as a

coating material for nanoparticles has become particularly noteworthy, enriching their stability and biocompatibility for a diverse array of biomedical applications. Additionally, the advent of heparin-functionalized nanoparticles has heralded a promising avenue for precise drug delivery, offering targeted treatment with minimized off-target effects and systemic toxicity (Kumar et al., 2023; Rodriguez-Torres et al., 2018). Moreover, the immunomodulatory properties of heparin have attracted attention in nanomedicine, particularly in inflammation-associated diseases such as cancer, cardiovascular diseases, and autoimmune disorders. By facilitating the delivery of anti-inflammatory agents to targeted sites, heparin-coated nanoparticles offer potential in effectively attenuating inflammatory responses (Meher et al., 2024; Zare et al., 2024b).

In addition, advancements in polymer-based nanocomposites have driven progress in biomedical applications, with heparin playing a central role (Meher et al., 2024; Saurav et al., 2024). Serving as a highly bioactive polymer, heparin enhances the biotic competence of nanocomposites, expanding their utility across diverse clinical scenarios. The incorporation of structural and chemical derivatives of heparin enables the fabrication of resourceful nanocomposites tailored for specific clinical applications, including drug delivery, wound healing, tissue engineering, and biosensing (Meher et al., 2024; Zare et al., 2024b). Recent developments in heparin-oriented nanotechnology present promising avenues for advancing healthcare delivery and treatment modalities. This section underlines the successful integration of heparin and its derivatives into nanocomposites, emphasizing their potential in both laboratory research and clinical practice (**Fig. 2-1**). Understanding the advantages and challenges associated with heparin-based nanocomposites is crucial for fostering future innovations in this dynamic field (Meher et al., 2024).

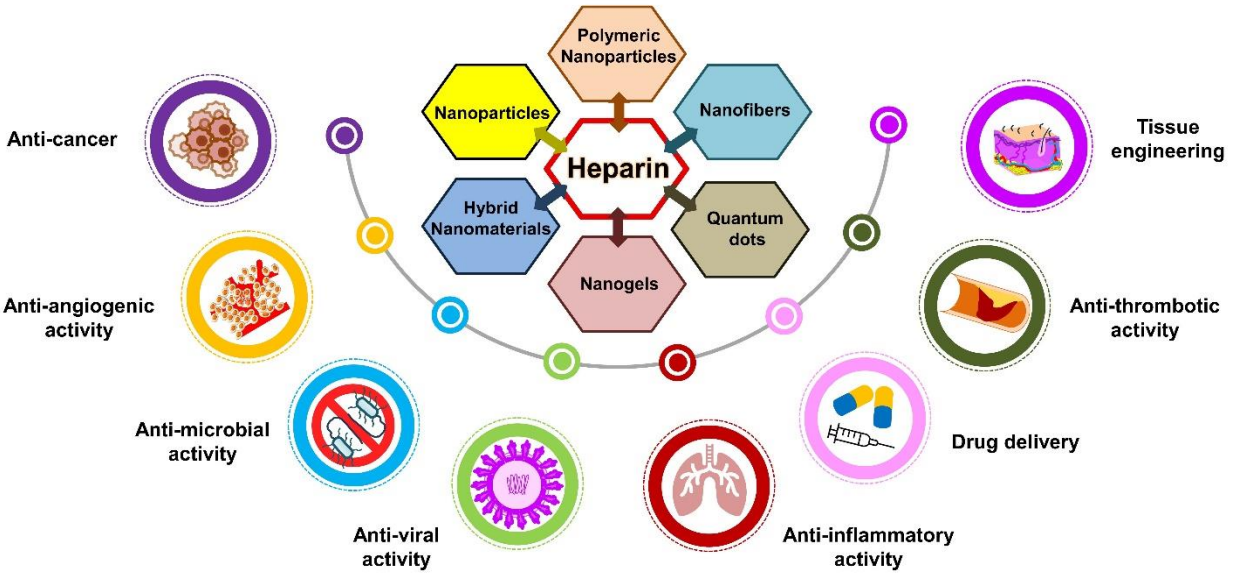

Fig 2-1 Nanotechnology-driven innovations in heparin-based therapeutics.

2.3.7.1 Suppressing cancer progression with heparin nanocomposites

Heparin is recognized for its diverse interactions with biomolecules involved in tumorigenesis, influencing both tumor development and metastasis. Specifically, LMWHs interact with growth factors such as VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor), interfering with their activity and inhibiting angiogenesis, thus slowing tumor progression (Falanga et al., 2013). Furthermore, heparin's inhibition of cell adhesion molecules such as P-selectins and integrins impacts crucial pathways in cancer advancement, including proliferation, metastasis, invasion, and angiogenesis (Ejaz et al., 2021). Exploiting these properties, various nanodrug delivery systems incorporate heparin and LMWH coatings to effectively combat metastasis (Wu et al., 2020). These methods have demonstrated heightened cytotoxicity against breast cancer cells and successful tumor management (Chen et al., 2020). Heparin-modified liposomes, particularly when carrying photosensitizer cargo, display enhanced anticancer and anti-metastatic effects by impeding platelet adhesion and diminishing migration and invasion in breast cancer models (Cao et al., 2021; Mei et al., 2016; Wu et al., 2020). Moreover, heparin-modified graphene oxide nanocomposites deeply infiltrate tumors, inducing hypoxia, promoting vascular normalization, and counteracting the overexpression of pro-oncogenic markers (Du et al., 2022). This positions them as potential agents for suppressing both tumor growth and metastasis. Additionally, innovative strategies such as drug-loaded heparin-conjugated graphene oxide masked by cancer cell membranes offer distinct approaches for integrated photothermal and

chemotherapeutic immunotherapy against melanoma. In the realm of metastasis, tumor cells exhibiting heightened heparinase expression facilitate epithelial-mesenchymal transition (EMT), a pivotal process for hematogenous metastasis (Erpenbeck and Schön, 2010). LMWH-conjugated chlorin e6 in micelles has shown efficacy in inhibiting EMT in breast cancer stem cells, suggesting therapeutic potential in metastasis inhibition.

2.3.7.2 Targeting angiogenesis with heparin-functionalized nanoparticles

Heparin emerges as a key contributor in angiogenesis, navigating through a complicated path affected by chain length and sulfation position. Its dual role as both a promoter and inhibitor of blood vessel formation presents its significance in neovascularization (Norrby, 2006). Within nanocomposites, heparin engages in detailed conversations with angiogenic growth factors (AGFs) such as VEGF and FGF-2, directing endothelial cell responses (Akl et al., 2016; Kemp et al., 2009). Interestingly, nanoparticles linked with diaminopyridinyl-derivatized heparin show potential in halting FGF-2-induced angiogenesis, unveiling the therapeutic promise of heparin interventions (Kemp et al., 2009). Furthermore, heparin-functionalized nanocomposites, such as collagen or hydroxyapatite matrices, carefully regulate VEGF release, encouraging the emergence of new blood vessels (Knaack et al., 2014). In ischemic models, cryogel-based nanoscaffolds loaded with VEGF and heparin offer promise, while heparin-loaded hydrogels modulate angiogenesis through VEGF and cPGE pathways (Nawaz et al., 2022). Coating nanosheets with LHT7 boosts their antitumor effectiveness by suppressing angiogenesis, and heparin nanoparticles exhibit impressive ability in crossing the blood-brain barrier to hinder glioma proliferation (Wang et al., 2016). With surface-functionalized heparin nanoparticles delivering potent anti-cancer drugs, varied applications of heparin in angiogenesis modulation and cancer therapy take center stage, promising a new period in biomedical innovation.

2.3.7.3 Tailored heparin nanocomposites for enhanced regeneration

In tissue regeneration and wound healing, heparin plays a versatile role, expediting the healing process through various mechanisms. Heparin enhances the expression of crucial growth factors such as hepatocyte growth factor (HGF), nitric oxide (NO), granulocyte colony-stimulating factor (G-CSF) and vascular endothelial growth factor (VEGF), promoting endothelial and satellite cell proliferation while regulating myeloperoxidase activity and triggering anti-inflammatory responses at injury sites (Nourreddine et al., 2020). Extensive research validates the effectiveness of heparin in burn wound healing, attributed to its anti-inflammatory properties, immune

modulation, NO production, and accumulation of proangiogenic factors (Saliba Jr, 2001). Additionally, heparin-loaded nanofiber sutures and scaffolds show promising results in tissue regeneration, facilitating repair in Achilles tendons and nerves in animal models (Ye et al., 2021). These heparin-functionalized nanofibers not only support cell growth and nerve extension but also serve as excellent wound dressings, especially when combined with antimicrobial or anti-inflammatory agents. Nanocomposites incorporating heparin, such as heparin-polyvinyl alcohol@gold nanocomposite (H-PVA@Au), a highly porous bandage, enhance wound healing by promoting skin and collagen formation while exerting antimicrobial effects (Zhang and Zhao, 2022). Furthermore, heparin serves as a valuable scaffold material and functionalizing agent in other scaffolds and nanoformulations, improving cell adhesion and overall biocompatibility (Gulati et al., 2017; Liang and Kiick, 2014). Customized nanocomposites modified with heparin and growth factors such as recombinant human bone morphogenetic protein-2 (rhBMP-2) demonstrate enhanced bone regeneration capabilities, while heparin-loaded fibrous membranes facilitate mesenchymal stem cell attachment and proliferation (Duan and Wang, 2010). These collective findings draw attention to the diverse applications of heparin in tissue engineering and regenerative medicine, opening avenues for innovative biomedical interventions.

In addition, heparin exhibits remarkable versatility, functioning as both an effective anti-inflammatory agent and a potent antiviral compound, showcasing its broad therapeutic potential across various medical fields. Through its interactions with various immune components, heparin effectively neutralizes inflammatory chemokines and regulates complement factors, thereby curbing inflammatory responses (Conzelmann et al., 2020). Moreover, it disrupts leukocyte attachment by engaging with adhesion molecules and interferes with inflammatory transcription pathways (Hogwood et al., 2020; Ludwig, 2009; Zaferani et al., 2014). Recent studies have emphasized its ability to modulate multiple inflammation-related signaling pathways, highlighting its potential as a therapeutic agent for inflammatory conditions (Li et al., 2020). When incorporated into nanocomposite materials such as nanofiber matrices and nanoparticle formulations, heparin demonstrates potent anti-inflammatory properties with minimal side effects, further enhancing its therapeutic potential. In addition to its anti-inflammatory role, heparin exhibits strong antiviral activity against enveloped viruses and those utilizing HSproteoglycans as entry receptors. By impeding viral attachment, competing for coreceptor binding, and disrupting viral replication processes, heparin effectively suppresses viral propagation across a range of viruses (Rider, 1997;

Vitiello and Ferrara, 2023; Yu et al., 2021). While its mechanism against SARS-CoV-2 remains unclear, heparin presents promise as a therapeutic agent (Kim et al., 2020). Innovative approaches incorporating heparin into liposomes and engineered nanomaterials (Hendricks et al., 2015; Wasik et al., 2017) offer exciting prospects for combating viral infections and detecting viral pathogens.

2.3.7.4 Heparin in smart drug delivery systems

Heparin-based smart drug carriers have received considerable attention in recent years due to their potential to revolutionize chemotherapy drug delivery. These carriers exploit the unique properties of heparin to enhance drug loading, biocompatibility, and targeted delivery. By chemically linking heparin with hydrophobic drugs such as paclitaxel (PTX) (J. Wang et al., 2023) via pH-sensitive cis-aconitic bonds (Ye et al., 2014), researchers have created polymer drugs that can self-assemble into micelles (Ye et al., 2014). These micelles, which have a hydrophobic core and a hydrophilic, negatively charged shell, provide an optimal environment for drug encapsulation and controlled release. The versatility of heparin allows for further functionalization; for example, doxorubicin hydrochloride (DOX·HCl) and cationic folic acid (CFA) can be incorporated through electrostatic interactions, endowing the micelles with both passive and active tumor targeting capabilities. This dual pH sensitivity ensures that both PTX and DOX are released in the acidic tumor microenvironment, enhancing therapeutic efficacy while minimizing systemic toxicity (Li et al., 2016). Compared to traditional PCL-heparin conjugates (Yu et al., 2013), these heparin-based carriers offer higher drug loading capacity (DLC) and improved biocompatibility, addressing key challenges in chemotherapy such as drug resistance and side effects (Li et al., 2016). This innovative approach highlights the potential of heparin as a robust platform for the development of multifunctional drug carriers, paving the way for more effective and safer cancer treatments.

2.4 Challenges and opportunities

(i) Extending half-life for improved compliance: The short half-life of heparin necessitates frequent administration, resulting in poor patient compliance. However, ongoing efforts to develop extended-release formulations aim to address this challenge and potentially improve patient adherence to treatment regimens.

(ii) Extraction challenges and solutions: The complex extraction process of crude heparin from animal sources is a limitation compared to other biopolymers. However, advances in depolymerization, chemical modification, and synthetic production have paved the way for

overcoming these challenges (Thacker et al., 2021; Wang et al., 2022b), unlocking new opportunities for heparin utilization.

(iii) Management of bleeding risk and prevention of thrombocytopenia: While heparin is highly effective as an anticoagulant, it carries a risk of bleeding, especially in vulnerable populations such as the elderly or those with renal insufficiency (Qiu et al., 2021a). Strategies to monitor and mitigate the risk of bleeding, along with advances in anticoagulant therapy, offer opportunities to improve patient safety during heparin treatment. In addition, HIT is a major concern because of its potential to lead to severe complications, including thrombotic events (Liu and Linhardt, 2014). Understanding the mechanisms underlying HIT and developing novel approaches to prevent or mitigate its occurrence could significantly improve patient outcomes and safety during heparin therapy.

(iv) Advancing precision medicine approaches: The potential to optimize heparin therapy and reduce the risk of adverse reactions in individual patients can be realized by leveraging advances in personalized medicine, such as pharmacogenomics and biomarker identification. Tailoring treatment regimens based on patient-specific factors and genetic profiles could improve efficacy and safety while reducing the incidence of adverse events associated with heparin treatment.

(v) Environmental sustainability and improved bioavailability: In response to the escalating challenges of environmental pollution and climate change, there is an increasing demand for biodegradable and sustainable materials in nanocomposites. Heparin, acknowledged as a green polymer, offers a promising solution due to its biocompatibility and non-toxic properties. Furthermore, the minimal bioavailability of heparin and its low molecular weight counterparts is a significant challenge that can be effectively addressed by their incorporation into nanocomposites (Eidi et al., 2010). This strategic integration not only improves bioavailability, but also mitigates the generic toxicity of nanomaterials.

(vi) Clinical translation challenges: Despite significant progress, the translation of heparin nanocomposites from preclinical studies to human clinical trials remains challenging (Qiu et al., 2021a; Wang et al., 2022b)). Issues such as unpredictable dosing efficiency, target site accumulation and diffusion hinder their clinical translation (Meher et al., 2024) and require further research and optimization.

(vii) Monitoring and management of other adverse effects: While some adverse effects of heparin treatment, such as increased eosinophils or hyperkalemia, are relatively rare and reversible with

cessation of treatment, others, such as calcium deposition at injection sites in patients with chronic renal failure, require careful monitoring and management (Zhang and Zhao, 2022). The development of tailored approaches to address these specific adverse effects could improve the safety and tolerability of heparin therapy in affected patient population.

2.5 Advancing heparin research

The integration of heparin into multiple therapeutic modalities stands as an indication of the persistent pursuit of transformative breakthroughs in medical research, promising to revolutionize precision medicine. By tapping into the diverse properties of heparin, researchers are exploring innovative paths in targeted therapy, wound care, bioimaging, and infection control. Engineered heparins, synthesized through chemical, chemoenzymatic, and metabolic engineering approaches, have emerged as promising alternatives to animal-sourced heparin, addressing concerns over the fragility of the heparin supply chain and the recent contamination incidents (Baytas and Linhardt, 2020; Vaidyanathan et al., 2017). These engineered heparins offer the potential to fine-tune heparin-binding motifs and other molecular characteristics, enhancing therapeutic efficacy and reducing side effects (Paluck et al., 2016). For example, microneedle patches delicately infused with heparin exemplify a departure from traditional systemic anticoagulation methods, offering localized delivery that minimizes systemic side effects and improves patient adherence (Paluck et al., 2016). This targeted approach not only improves therapeutic efficacy but also alleviates the burden on patients undergoing anticoagulation therapy. Moreover, the synergy between heparin and electroconductive hydrogels presents an enticing avenue for advancing wound healing and nerve regeneration, with the incorporation of clotting modulation of heparin alongside the bioactive properties of polymers such as polyaniline and polypyrrole opening new frontiers for therapeutic interventions (Paluck et al., 2016; Yu et al., 2023). Compounds that perform similar functions as heparin, such as binding to the heparin-binding site on a protein, may also be characterized as heparin mimetics, and the synthesis of clinically useful heparin mimetics is a relatively recent achievement, with the prospect of developing mimetics that display higher relative potency and greater selectivity of action than their parent molecule being a major driving factor in this field of research (Mohamed and Coombe, 2017b). The fusion of heparin with cutting-edge nanotechnology further underscores its versatility, as heparin-based nanoplatforms demonstrate remarkable capabilities in enhancing imaging contrast and combating microbial proliferation, thereby offering a comprehensive solution to pressing medical challenges (Zare et

al., 2024b). As these innovations continue to mature and scale industrially, they hold the promise of not only improving clinical outcomes and elevating patient care standards but also broadening access to advanced therapeutic solutions. With ongoing research and innovation, the journey of heparin in medical science is poised to reveal unique possibilities for personalized and targeted therapies.

2.6 Conclusions and future outlook

In summary, beyond its traditional role as an anticoagulant, heparin's multifaceted utility spans a broad range of medical applications. This review has provided a comprehensive overview of its diverse therapeutic uses and its relevance in nanomedicine. Heparin's intricate interactions with biological components enable it to regulate fundamental processes critical to both health and disease. From its involvement in inflammation and wound healing to its significance in cancer therapy and infectious disease management, heparin is emerging as a key player in both basic and clinical research.

To advance these applications, several challenges must be addressed, such as managing heparin's anticoagulant side effects and elucidating its mechanisms and roles in various diseases. Research should focus on optimizing engineered heparins with improved safety, stability, and bioavailability, and explore the potential of inhaled forms of heparin for antiviral and anti-inflammatory therapies. The development of advanced heparin-based systems, including microneedles, hydrogels, and nanoplatforms for gene delivery, requires overcoming limitations such as thrombogenicity and short half-life. Additionally, addressing concerns related to biosafety, immunogenicity, and stability is critical for improving the clinical translation of heparin-based nanocomposites. Expanding research into alternative heparin sources and synthetic methods is essential to ensure a reliable and cost-effective supply. Integrating these advancements will position heparin at the forefront of innovative therapeutic strategies and biomedical applications.

To balance clinical benefits with potential adverse effects, it is crucial to optimize drug regimens and refine the structure-activity relationships to enhance therapeutic efficacy and safety. Moreover, there is a need for more robust clinical trials to provide reliable evidence for informed medical decision-making. Thus, heparin, an esteemed drug with a century-long legacy, has transcended its conventional role as a simple anticoagulant and is poised for further advancement through continued research and optimization efforts.

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Chapter 3

Bioengineered heparin: Advances in production technology

3.1 Background

Glycosaminoglycans (GAGs), a family of negatively charged acidic polysaccharides (Zhang et al., 2019) renowned for their structural diversity, play a crucial role in medicine (Awofiranye et al., 2020; Casale and Crane, 2023; Qui et al., 2021; Jayatilleke and Hulett, 2020; Liang et al., 2016; Linne et al., 2021). GAGs are typically categorized into distinct types: hyaluronan (hyaluronic acid, HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, and keratan sulfate (KS) (Liu et al., 2009; Mende et al., 2016). These GAGs are characterized by the composition of their constituent monosaccharides, the configuration of their glycosidic bonds, and the intricate patterns of sulfation that they exhibit (Mende et al., 2016). Among them, heparin is an anticoagulant that has been used clinically for almost a century (Mulloy et al., 2016; Onishi et al., 2016). It was discovered in 1916 by Jay McLean and William Henry Howell (Hao et al., 2019b; McLean, 1916). Heparin is used worldwide in the treatment of thrombosis, which can occur in various clinical settings such as cardiovascular disease, strokes, and post-operative surgery. As an anticoagulant, heparin is also used as a coating in medical equipment such as dialysis tubing and blood collection tubes (Thacker et al., 2022).

Heparin is predominantly sourced from Chinese porcine intestinal mucosa and is a complex GAG with extensive sulfate patterns (Baytas and Linhardt, 2020; Hogwood et al., 2023; van der Meer et al., 2017). The heparin backbone features a disaccharide unit comprising α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA) and α-D-glucosamine (GlcN), with the predominant repeating unit being the trisulfated (TriS) disaccharide GlcNS6S-IdoA2S (Linhardt, 2003). As with all animal-derived products, there are concerns regarding animal welfare, disease, and product contamination (Al-Hakim, 2021; Bussey et al., 2004; McCarthy et al., 2020; van der Meer et al., 2017). In 2008, the adulteration of heparin had serious consequences, resulting in the deaths of hundreds of individuals who received contaminated batches. The contaminant was identified as oversulfated chondroitin sulfate (OSCS) (Guerrini et al., 2008; Liu et al., 2009). Furthermore, outbreaks of African swine fever since 2018 have increased the vulnerability of the global heparin supply chain (Normile, 2018; Vilanova et al., 2019).

The growing concern over the availability and safety of commercially sourced GAGs has prompted a shift toward biomanufacturing methods, leveraging synthetic biology for pathway refinement (Oduah et al., 2016). Progress has been made in producing certain GAGs, but challenges remain in terms of productivity, efficiency, and cost. However, there is optimism about alternative methods, such as the production of heparin using heparosan as a starting material. Through microbial engineering and enzyme-driven modifications, heparosan offers a promising approach to producing lab-derived heparin and HS (Kuberan et al., 2003a; Kusche et al., 1991; T. Wang et al., 2020). Heparosan is an acetylated polysaccharide produced by bacteria, which differs significantly from heparin, a highly sulfated polysaccharide. Despite these differences, heparosan serves as a valuable precursor for heparin production through chemical and chemoenzymatic methods (Higashi et al., 2011; Fu et al., 2016; Chavaroche et al., 2013; Lindahl et al., 2005; Datta et al., 2021).

The applications of heparin have expanded beyond its traditional use as an anticoagulant, prompting significant efforts in industrial heparin production to meet the growing demand (**Fig. 3-1**). Recent advancements in bioengineered heparin production, exploring prokaryotic and eukaryotic systems, offer promising alternatives to traditional methods (Baik et al., 2012a; Bhaskar et al., 2015; Datta et al., 2013a, 2013b; Leyh et al., 1988; Liu and Linhardt, 2014; Thacker et al., 2022; Zhang et al., 2022). Commercial GAG production, which relies on extraction from animal tissues, faces quality control challenges, including difficulties in isolating GAGs with the specific chain lengths and sulfation patterns required for particular therapeutic uses. In addition, chemical and enzymatic synthesis methods offer alternatives but face hurdles such as isomer separation and complexity (Gottschalk et al., 2019; Mende et al., 2016; Petitou et al., 1999; Xu et al., 2011). Advances in metabolic engineering and synthetic biology offer potential for microbial biosynthesis, mammalian biosynthesis, and chemoenzymatic biosynthesis of GAGs, promising high-purity products (Baytas and Linhardt, 2020; Fu et al., 2016; Liu and Linhardt, 2014; T. Wang et al., 2020; Xu et al., 2011; Zhang et al., 2008).

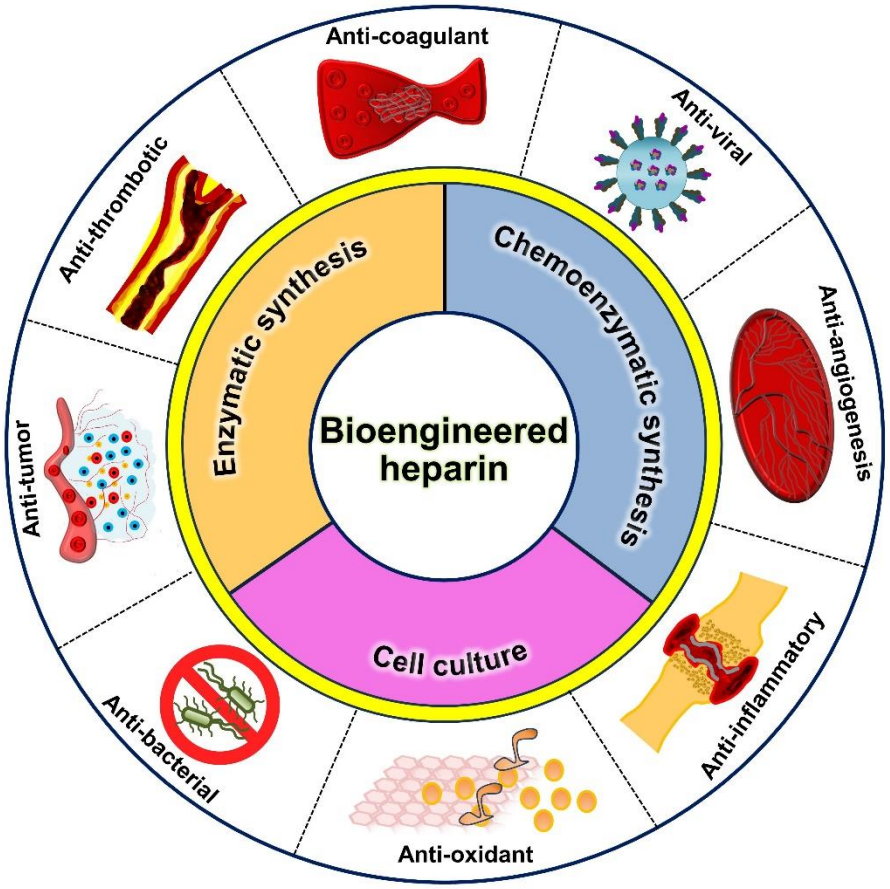

Fig. 3-1: Progress in the production of bioengineered heparin and its diverse applications.

3.2 Aim

This review offers a thorough examination of recent progress in prokaryotic and eukaryotic systems for bioengineered heparin production, including innovative approaches, advancements in metabolic engineering, and future prospects. Additionally, it delves into the potential of recombinant Chinese hamster ovary (CHO) cells for synthesizing heparin, discussing recent advancements, obstacles, and prospects (Qiu et al., 2018; Chen et al., 2018; Karlsson et al., 2021).

3.3 Advancements in prokaryotic systems

Recent advancements in using prokaryotic systems for bioengineered heparin synthesis have involved genetic modifications of various bacterial strains employing diverse strategies to efficiently produce heparin-like glycan compounds (**Table 3-1**). The primary focus lies in heparosan production, which serves as the precursor for both heparin and HS, dictating the backbone structure and polymer length. Subsequent sections will provide an overview of recent

research on enhancing pathways in bacteria naturally synthesizing heparosan, followed by a concise summary of each catalytic step required to convert heparosan into customized heparin-based molecules.

3.3.1 Biosynthesis of heparosan

Heparosan, a linear acidic polysaccharide, is naturally synthesized in the capsules of potential pathogens such as *Escherichia coli* and *Pasteurella multocida*. The polymerization of heparosan involves the alternating transfer of two distinct monosaccharide uridine 5'-diphosphate (UDP) precursors to an acceptor molecule (DeAngelis, 2015). In addition, fermentation yield and molecular weight are important factors affecting heparosan, which are influenced by the polymerization process catalyzed by heparosan synthases. Beyond its potential as a precursor for bioengineered heparin (Chavaroche et al., 2013), heparosan and its derivatives have shown promise in various biological functions. Moreover, owing to its remarkable biocompatibility, non-immunogenicity, and non-toxic nature, heparosan has been extensively used in the development of drug delivery vehicles in biotechnology (Lane et al., 2017). Recently, heparosan has emerged as a viable alternative to HA in the design of a new class of biopolymer-based drug carriers for anticancer therapy (Rippe et al., 2019). The aforementioned studies highlight the importance of heparosan development in the pharmaceutical industry.

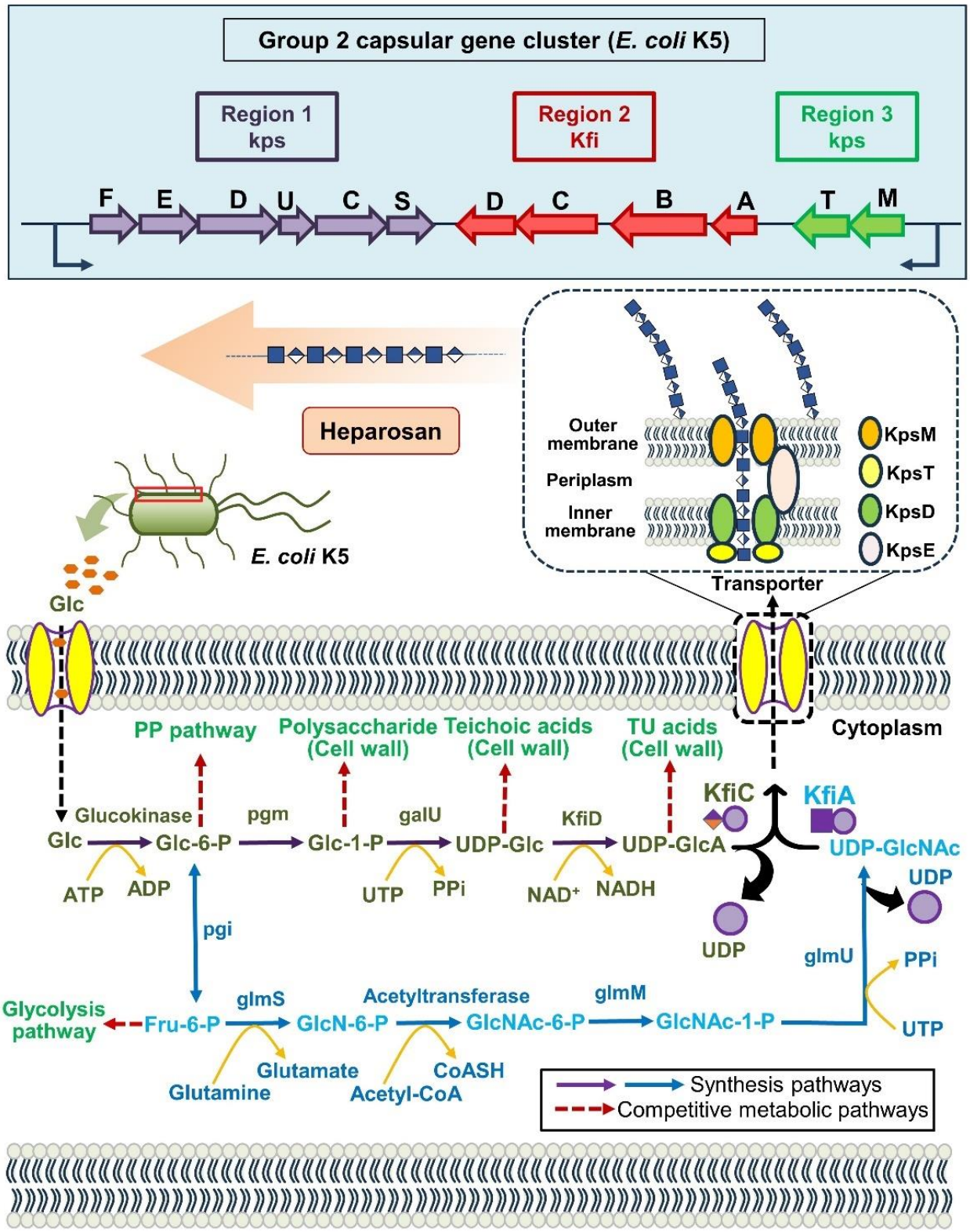
Group 2 capsular gene cluster (E. coli K5)
Region 1 kps
Region 2 Kfi
Region 3 kps
F E D U C S D C B A T M
Heparosan
E. coli K5
Glc
Outer membrane
Periplasm
Inner membrane
KpsM
KpsT
KpsD
KpsE
Transporter
Cytoplasm
PP pathway
Polysaccharide (Cell wall)
Teichoic acids (Cell wall)
TU acids (Cell wall)
KfiC
KfiA
Glucokinase
pgm
galU
KfiD
Glc
Glc-6-P
Glc-1-P
UDP-Glc
UDP-GlcA
UDP-GlcNAc
ATP
ADP
UTP
PPi
NAD+
NADH
UDP
UDP
pgi
glmU
PPi
UTP
Glycolysis pathway
Fru-6-P
glmS
GlcN-6-P
Acetyltransferase
GlcNAc-6-P
glmM
GlcNAc-1-P
Glutamine
Glutamate
Acetyl-CoA
CoASH
Synthesis pathways
Competitive metabolic pathways

Fig. 3-2 Illustration of the gene cluster and the microbial biosynthetic pathway of heparosan in native *E. coli* K5. This process involves the synthesis of two key precursors, UDP-GlcA and UDP-GlcNAc, indicated by violet and dark blue arrows, respectively. Heparosan polymer assembly occurs in the cytoplasm via GTases KfiA and KfiC, followed by transport across the inner membrane facilitated by the ABC transporter system consisting of the proteins KpsM and KpsT. KpsM forms the transmembrane domain, while KpsT forms the nucleotide-binding domain of the transporter. The completed heparosan polymer accumulates in the periplasmic space, facilitated by KpsE. Subsequently, KpsD, which forms an outer membrane channel, exports the heparosan polymer to the cell surface. Simultaneously, competing cellular pathways (indicated by dark red arrows) share precursors and carbon flux with heparosan biosynthetic pathways. Fru-6-P: fructose 6-phosphate, galU: UDP-glucose pyrophosphorylase, glmM: phosphoglucosamine mutase, glmS: amidotransferase, Glc: glucose, Glc-6-P: glucose-6-phosphate, Glc-1-P: glucose-1-phosphate, KfiA: GlcNAc-transferase, KfiC: GlcA-transferase, KfiD: UDP-glucose dehydrogenase, pgm: phosphoglucomutase, pgi: phosphoglucoisomerase, PP: pentose phosphate, PPi: pyrophosphate, TU acid: teichuronic acid, UDP: uridine-5'-diphosphate, UDP-Glc: UDP-glucose, UDP-GlcA: UDP-glucuronic acid, UDP-GlcNAc: UDP-*N*-acetylglucosamine.

3.3.1.1 Native producers of heparosan

The primary component of the extracellular capsular polysaccharide (CPS) of *E. coli* K5 serovar O10:K5:H4, commonly known as K5 capsule or heparosan, shares similarities with the desulfated key precursor of heparin or HS (Blundell et al., 2009). Other native producers of heparosan include *P. multocida* type D (DeAngelis and White, 2002; Pandit and Smith, 1993; Rimler, 1994), *Avibacterium paragallinarum* genotype II (Wu et al., 2010), and *E. coli* Nissle 1917 (EcN) (Cress et al., 2013; Datta et al., 2021). The metabolic pathway for heparosan synthesis is illustrated in Fig. 3. To produce CPS, various bacterial species require exogenous, inexpensive, and simple carbon sources for the synthesis of UDP precursors. For example, wild-type *E. coli* K5 can use glucose and glycerol as carbon sources (Liu et al., 2012; Wang et al., 2010), while recombinant *E. coli* strains can also use fructose and mannose (Yan et al., 2015). These precursors are typically derived from glucose-6-phosphate and fructose-6-phosphate. Initially, optimal fermentation processes were employed to achieve the highest heparosan production, followed by the application of optimal feeding strategies to further increase heparosan production in *E. coli* K5 (Liu et al., 2012; Wang et al., 2010). The maximum documented yield of heparosan production in *E. coli* K5 is 15

g/L (Wang et al., 2010). In addition, other native producers such as *P. multocida* type D (DeAngelis and White, 2002) and *A. paragallinarum* genotype II (Wu et al., 2010) are capable of synthesizing heparosan CPS. However, research into their potential commercial development has not yet been performed.

3.3.1.2. Metabolically engineered microbial cell factories for heparosan production

Currently, heparosan production relies on the *E. coli* K5 strain; however, concerns about pathogenicity and potential endotoxin production have prompted the exploration of alternative, safer platforms derived from non-pathogenic strains. Because heparosan serves as the initial carbon backbone for the economically feasible synthesis of bioengineered heparin, its availability is of paramount importance (Cress et al., 2013; Wang et al., 2011, 2010). The development of synthetic biology has facilitated the creation of non-pathogenic generally regarded as safe (GRAS) microbial cell factories through metabolic engineering approaches. Over the past 15 years, several microbial strains have been metabolically engineered to produce heparosan, as shown in **Table 3-1**. The metabolic pathway for heparosan synthesis involves three main steps: (i) synthesis of monosaccharide precursors [namely, UDP-glucuronic acid (UDP-GlcA) and UDP-*N*-acetylglucosamine (UDP-GlcNAc)] through intracellular metabolic conversion of inexpensive substrates (e.g., glucose, glycerol, sucrose); (ii) polymerization of monosaccharide precursors into heparosan chains via enzymatic action; and (iii) transport of synthesized heparosan chains to the extracellular membrane via transporters (DeAngelis, 2012; Kang et al., 2018). To enhance heparosan production by microbial cell factories in response to these processes, various strategies have been implemented, which are categorized in this review into the following classes: (a) modulation of the metabolic pathway of UDP-sugar precursors; (b) engineering of heparosan synthases; (c) synergy of metabolic pathways; (d) engineering fermentation processes; and (e) acceleration of heparosan export.

(a) Modulating the metabolic pathway of UDP-sugar precursors

Microbial strains naturally produce UDP-GlcA and UDP-GlcNAc, which serve as the basic building blocks for heparosan synthesis. An abundant supply of UDP precursors is essential for effective heparosan synthesis. Modulation of the initial UDP-precursor sugar concentration plays a pivotal role in determining heparosan chain length and polymerization (Chavaroche et al., 2010). However, an unbalanced and insufficient supply of UDP-sugar precursors can directly impair polysaccharide chain elongation (Wang et al., 2010). Furthermore, a significant amount of these

precursors is also used during metabolic synthesis for cell wall construction and biomass accumulation (Chen et al., 2017). Therefore, (i) identifying metabolic bottleneck; (ii) harmonizing the provision of UDP-sugar building blocks; and (iii) optimizing metabolic flux constitute the modular approach to precursor metabolic pathway synthesis (**Fig. 3-3**).

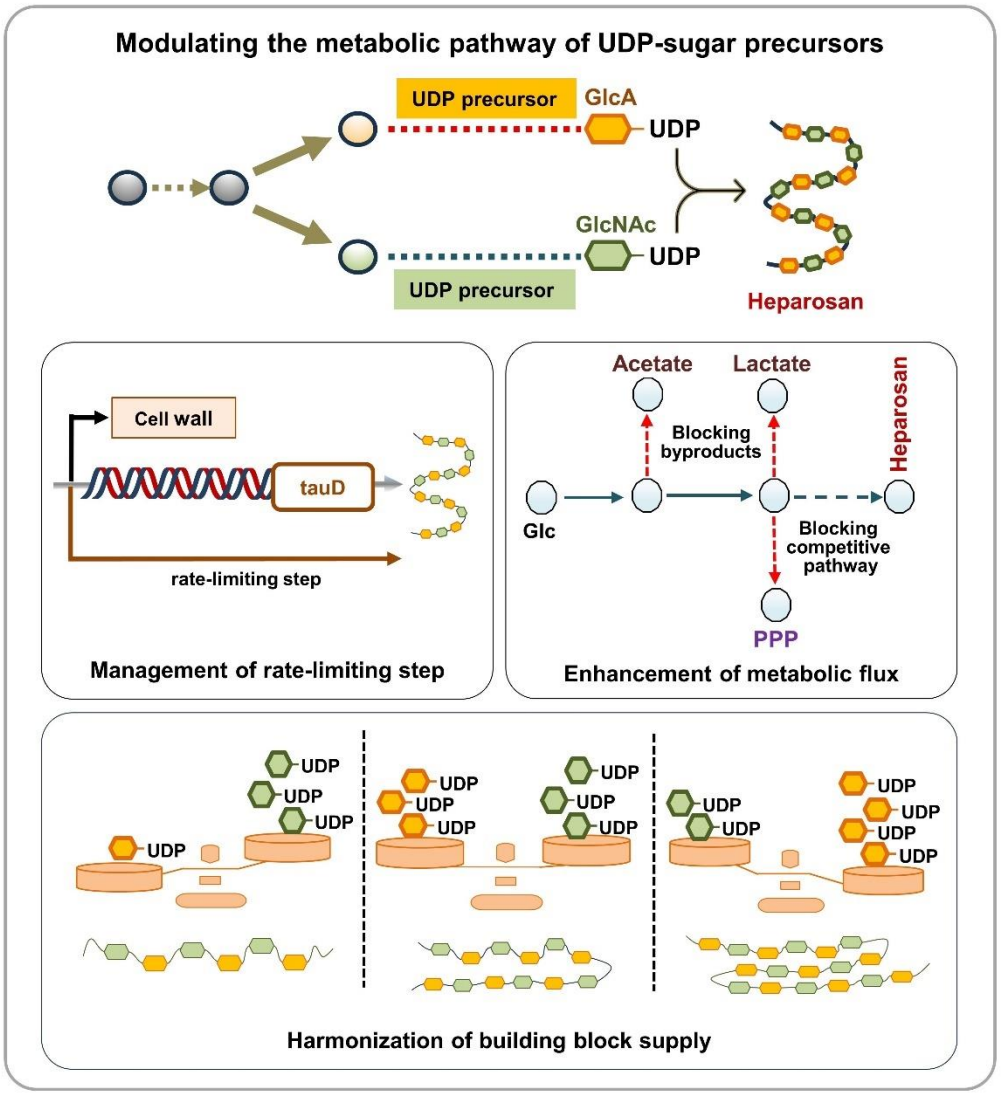

Fig. 3-3 Modulation of the metabolic pathway of UDP precursors. The process involves managing rate-limiting steps to overcome bottlenecks, coordinating the supply of building blocks to regulate sugar chain polymerization, and balancing intracellular metabolism by redirecting carbon flux from by-product formation to polysaccharide synthesis. Additionally, it requires suppressing competitive metabolic pathways to minimize the waste of carbon flux towards precursor

production. GlcA: glucuronic acid, GlcNAc: *N*-acetylglucosamine, tauD: UDP-glucose dehydrogenase, PPP: pentose phosphate pathway, Glc: glucose, UDP-uridine-5'-phosphate.

(i) Identifying metabolic bottleneck

A detailed analysis of UDP precursor biosynthetic pathways is necessary to construct an efficient microbial cell factory for heparosan production. Identification of metabolic bottlenecks during UDP precursor biosynthesis is crucial, as they can significantly hinder heparosan production. For example, the tauD-encoded UDP-glucose dehydrogenase is considered a potential rate-limiting enzyme that catalyzes the oxidation of UDP-glucose to UDP-GlcA (Roman et al., 2003). Deficiency of this enzyme can directly affect UDP-GlcA metabolism, limiting metabolic flux (Chen et al., 2017). Overcoming this issue involves addressing the rate-limiting step in precursor biosynthesis, which can be achieved by enhancing the expression of the rate-limiting enzyme, integrating the rate-limiting gene from external sources, or co-expressing the rate-limiting gene alongside other genes in the metabolic pathway (S. Hu et al., 2022; Jin et al., 2016; Zhang et al., 2018). For example, endogenous upregulation of tauD in *Bacillus subtilis* 168 significantly increased the titer of heparosan by 55%, reaching 2.65 g/L in batch experiments (Jin et al., 2016). Furthermore, overexpression of tauD also significantly impacted the molecular weight and polydispersity of heparosan production. Thus, enhancing the expression of the rate-limiting enzyme not only improves heparosan elongation in microbial cell factories but is also critical for the synthesis of cell wall polysaccharides (Jin et al., 2016). In a rare case, the native *E. coli* K5 strain exhibited constant heparosan chain length despite reduced heparosan synthesis due to dehydrogenase overexpression (Roman et al., 2003). Conversely, *Pichia pastoris*, despite possessing an endogenous UDP-glucose dehydrogenase gene, was incapable of heparosan production. Therefore, multiple tauD-encoded UDP-glucose dehydrogenase genes from various organisms were selected and independently expressed in recombinant *P. pastoris* strains. As a result, the strain modified with the *B. subtilis* tauD gene achieved the highest heparosan production titer (**Table 3-1**) (Zhang et al., 2022). In addition, another critical enzyme, glucose-1-phosphate uridylyltransferase, is required for CPS biosynthesis in *E. coli* (Bonofiglio et al., 2005) and provides the precursor UDP-glucose for the rate-limiting step in heparosan biosynthesis (Roman et al., 2003; Wang et al., 2011). Enhancement of heparosan production by combinatorial overexpression of UDP-glucose pyrophosphorylase (galU) with tauD has shown significant improvements (S. Hu et al., 2022).

During heparosan biosynthesis, UDP-GlcNAc serves not only as a complement to UDP-GlcA in precursor functions but also as a central target for a singular increase in phosphoglucosamine mutase (glmM) activity. This deliberate modification significantly elevates heparosan production from modest to remarkable levels (S. Hu et al., 2022). Furthermore, the synchronized upregulation of UDP-glucose dehydrogenase (KfiD), glmM, and galU has boosted heparosan synthesis to an impressive 0.80 g/L in shake flask experiments (**Table 3-1**) (S. Hu et al., 2022). Thus, co-overexpression of the rate-limiting enzyme with other metabolic enzymes of the UDP-sugar precursor pathway has a clear impact on heparosan production.

(ii) Harmonizing the provision of UDP-sugar building blocks

Once the rate-limiting step is mitigated, synchronizing the supply of UDP precursors becomes essential for regulating heparosan polymerization. Maintaining a balanced ratio between UDP-GlcA and UDP-GlcNAc is critical, as an imbalance in their concentrations can directly inhibit chain elongation (Wang et al., 2011). Previous studies have shown that the rate and length of heparin polymerization are influenced by the relative proportions of these UDP precursors in mammalian heparin production (Lidholt et al., 1988; Roman et al., 2003). Moreover, excess UDP-GlcNAc could engage in competitive interaction with UDP-GlcA for binding to the UDP-GlcA transporter, potentially reducing its translocation to the Golgi apparatus (Lidholt et al., 1988).

Chemoenzymatic approaches regulate UDP-precursor synthesis by controlling precursor amounts in reaction systems (Li et al., 2020), while metabolic engineering strategies manage precursor ratios in biological processes (Kang et al., 2018). Various strategies have been developed to regulate the supply of UDP precursors, including maintaining substrate availability and increasing critical precursor concentrations. During heparosan production, substrate concentration can affect the molecular weight (MW) and polydispersity of polymerization. For example, purification of cryptic *P. multocida* heparosan synthase 2 (PmHS2) in vitro allows for the synthesis of longer heparosan polymers with higher MW, suggesting that lower substrate concentrations result in higher-MW heparosan chains with lower polydispersity index, while higher substrate concentrations have the opposite effect.

In particular, a single precursor may play a critical role in regulating MW in different hosts. Therefore, enhancing the synthesis of essential precursors by coordinating the expression of precursor pathway enzymes proves to be more efficient in controlling MW. In the native *E. coli* K5 strain, UDP-GlcA content has a greater influence on heparosan MW than UDP-GlcNAc content.

The synthesis and MW of heparosan in *Bacillus megaterium* were significantly enhanced by coordinated overexpression of key precursor UDP-GlcA pathway enzymes, such as tauD and GalU, resulting in increased metabolic flux toward the UDP-GlcA synthesis pathway. In addition, Hu et al. observed similar trends in their studies and found that increased UDP-GlcA concentrations enhanced heparosan polymerization (S. Hu et al., 2022). Furthermore, higher UDP-GlcA levels may promote continuous polymerization and the formation of residual higher-molecular-weight heparosan due to competition with endogenous cellular biosynthesis (Chen et al., 2017). Further investigation revealed that higher UDP-GlcNAc levels can impair heparosan polymerization and decrease its molecular weight, while elevated UDP-sugar levels mitigate these effects (Lidholt et al., 1988). Because UDP precursors are the building blocks of cell wall synthesis, decreasing the rate of cell wall synthesis helps to reduce UDP precursor consumption while still providing sufficient precursors for glycosaminoglycan synthesis (Zhou et al., 2018).

(iii) Controlling metabolic flux to enhance production

Controlling metabolic flux through metabolic engineering is critical to maximize heparosan production. In heparosan biosynthesis, the upregulation of enzymes involved in producing both UDP precursors has a significant impact. Interestingly, co-expression or combinatorial expression of these enzymes has been observed to yield diverse results (**Table 3-1**) (Chen et al., 2017; S. Hu et al., 2022; Jin et al., 2016; Nehru et al., 2020). Given the intricate nature of metabolic pathways, maintaining intracellular metabolic balance is crucial for effectively regulating pathways and achieving efficient polysaccharide synthesis (Zhang et al., 2012). Numerous strategies have been developed to enhance metabolic flux for the synthesis of various polysaccharides in microbial cell factories. These strategies include inhibiting competing pathways (Jin et al., 2016), implementing modular pathway engineering (Zhang et al., 2018), manipulating cofactors (Puvendran and Jayaraman, 2019), and using transcription factor-based approaches (Wu et al., 2013). In the context of heparosan production, optimizing metabolic flux through the regulation of combinatorial enzyme expression, fermentation process engineering, pathway engineering, and enhancing the availability of precursor metabolites has become a focal point for researchers. In EcN, fine-tuning metabolic flux via the simultaneous enhancement of bsgalU, eckfiD, and ecglmM led to a notable enhancement compared with solely overexpressing eckfiD (S. Hu et al., 2022). These findings also confirm the importance of balanced biosynthesis of the two precursors in heparosan biosynthesis.

This observation is consistent with previous studies conducted on *Corynebacterium glutamicum* (S. Hu et al., 2022; Nehru et al., 2020) and *B. megaterium* (Y. Wang et al., 2020b).

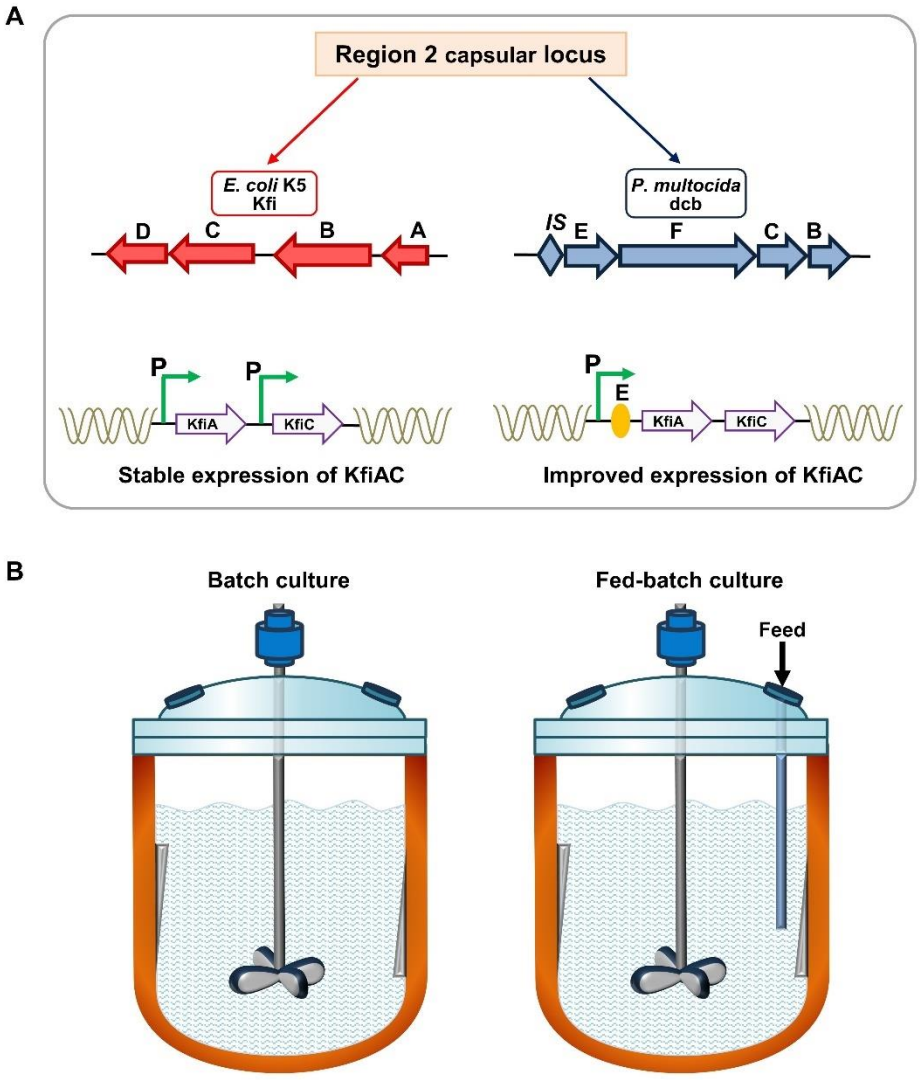

Fig. 3-4 Engineering heparosan synthases and refining fermentation processes. (A) Regulating the expression of heparosan synthase entails the stable expression of KfiAC through a dual promoter system and augmenting expression levels using enhancers. E: enhancer, KfiA: GlcA transferase, KfiC: GlcNAc transferase, **P**: promoter. (B) Enhancing fermentation processes by managing fermentation parameters, nutrient conditions, and specific fermentation mode such as batch and fed-batch.

(b) Engineering heparosan synthase

The synchronized functions of heparosan synthases and glycosyltransferases (GTases) are essential for the effective synthesis of heparosan with distinct structural and functional properties (Sugiura et al., 2010; Wang et al., 2011). Heparosan synthase catalyzes the intricate polymerization of UDP-GlcA and UDP-GlcNAc into heparosan chains, orchestrating the meticulous elongation of the polysaccharide backbone through the sequential addition of monosaccharide units in a highly specific and regulated manner (Kane et al., 2006). In contrast, GTases represent a diverse ensemble of enzymes that facilitate the transfer of sugar moieties from activated donor molecules to specific acceptor substrates, thereby forming glycosidic linkages (Lairson et al., 2008). Consequently, these enzymes have become important targets for metabolic engineering efforts aimed at improving heterologous heparosan biosynthesis in microbial cell factories (**Table 3-1**). Typically, the roles of GTases and heparosan synthases are critical in determining both the degree of polymerization and the molecular weight of the heparosan produced. This requires a deep understanding of regulatory mechanisms borrowed from other organisms to achieve precise modulation of gene expression (Alper et al., 2005; Michalodimitrakis and Isalan, 2008; Pitera et al., 2007).

Derived from E. coli K5

Various research efforts have focused on manipulating the degree of polymerization of heparosan sugar chains through synthase engineering. These studies have aimed to increase synthase expression levels and to explore natural enzymes. In wild-type *E. coli* K5, the kps locus, which encodes proteins that regulate heparosan biosynthesis and export, consists of three regions: region 1, region 2, and region 3 (Yan et al., 2015). The genes within the serotype-specific region 2 encode glycosyltransferases, including the four essential heparosan biosynthetic genes KfiA, KfiB, KfiC, and KfiD. These genes are surrounded by two conserved regions: region 1 (kpsFEDUCS cluster) and region 3 (kpsMT) (Whitfield, 2006; Whitfield and Roberts, 1999). The gene products of KfiABCD are responsible for heparosan biosynthesis (Petit et al., 1995). The kpsFEDUCS cluster encodes conserved transport proteins critical for chain initiation, while the kpsMT genes play a role in heparosan export to the cell surface (Cress et al., 2014; McNulty et al., 2006). As mentioned above, each distinct region has a key function in the process of heparosan biosynthesis. In particular, region 2 emerges as a key contributor due to its role in facilitating the assembly of hetero-oligomeric protein complexes located on the inner surface of the cytoplasmic membrane

(Petit et al., 1995). Among the four glycosyltransferases located in region 2, KfiA and KfiC form a complex known as KfiAC. This complex is responsible for the elongation of the heparosan chain at its non-reducing end (Hodson et al., 2000; Li et al., 2013). Consequently, the levels and activities of these two genes can potentially limit heparosan production, highlighting the importance of carefully balancing their overexpression.

Several systems-level strategies, such as the introduction of translational enhancers and the implementation of dual promoter plasmid systems, have been developed to increase synthase expression levels (Nehru et al., 2021, 2020). According to the results reported by Hu et al., optimization of UDP sugar expression coupled with the co-expression of the KfiAC complex resulted in enhanced heparosan production (S. Hu et al., 2022). In addition, increasing KfiA expression using a translational enhancer significantly increased production by 61% (Fig. 5A). Conversely, in the case of KfiC, the use of an enhancer did not significantly improve yield. This study highlights the importance of upregulating and balancing heparosan synthase expression to increase heparosan production (S. Hu et al., 2022). Another investigation revealed that the simultaneous expression of two GTase complexes in a polycistronic manner led to markedly reduced levels of KfiA expression compared with KfiC. This disparity could arise from the disproportionate expression levels of KfiA and KfiC proteins when both genes are regulated by a single promoter (Nehru et al., 2020). To address this issue, a dual promoter plasmid system was developed, resulting in significantly increased expression of both glycosyltransferase genes (**Fig. 3-4A**) (Nehru et al., 2021).

The remaining two glycosyltransferases—KfiB and KfiD—play a supporting role during heparosan polymerization in conjunction with the GTase complex (Roman et al., 2003). KfiD, which is a UDP-glucose dehydrogenase, catalyzes the oxidation of UDP-glucose to form UDP-GlcA (Roman et al., 2003), whereas the precise role of KfiB remains unclear. However, Leroux and Priem investigated the function of KfiB and proposed that it contributes to the stabilization and scaffolding of the KfiA–KfiC complex. In addition, KfiB assists in the transport of this complex to the inner membrane, where chain initiation occurs (Griffiths et al., 1999; Leroux and Priem, 2016). For example, the introduction of both KfiA and KfiC into *E. coli* BL21 (DE3) resulted in heparosan production of 63 mg/L and subsequent co-expression of the KfiB gene from *E. coli* K5 further increased the yield (100 mg/L) and molecular weight (~91.47 kDa) (Zhang et al., 2012). This observation highlights the essential role of KfiB in polysaccharide chain synthesis

(Hodson et al., 2000). Alternatively, in the absence of KfiB, fusing the *E. coli* trigger factor with KfiC effectively stabilized KfiC, resulting in significant heparosan production of 1.5 g/L (**Table 3-1**) (Leroux and Priem, 2016). Because KfiB facilitates the localization of the enzyme complex to the cell membrane, it plays an important role in the efficient export of heparosan.

Derived from P. multocida type D

Another potential natural producer, *P. multocida* type D, also possesses a capsular polysaccharide gene locus that shares substantial homology with the group 2 CPS loci of *E. coli* and *Neisseria meningitidis*, consisting of three distinct regions (Willis and Whitfield, 2013). The central CPS-specific region contains the *hssA* gene, encoding PmHS1, which is surrounded by two regions, dcbC and dcbE, known to encode a UDP-glucose dehydrogenase and an *E. coli* KfiB homolog, respectively (Cress et al., 2014; DeAngelis and White, 2002). Little information is available about most of the proteins encoded by region 2, except for the well-studied dcbF (DeAngelis and White, 2004; Kane et al., 2006). Remarkably, in GAG-producing strains of *P. multocida* types A, D, and F, the gene encoding PmHS2 (*hssB*) shares 70% identity with PmHS1 and is located outside of the putative CPS gene locus (DeAngelis and White, 2004). In ongoing research, the exploration of natural enzymes has emerged as a promising and accessible avenue for the production of MW-tailored heparosan during the metabolic engineering process. For example, the use of PmHS2 sourced from *P. multocida* facilitated the production of a heparosan variant characterized by a relatively high molecular weight, typically in the range of 200–300 kDa. Conversely, the use of PmHS1, also derived from *P. multocida*, resulted in the production of a heparosan species with a lower molecular weight, typically in the range of 39–53 kDa (Chen et al., 2017; Williams et al., 2019). Although synthases derived from *E. coli* K5 are more commonly used than those derived from *P. multocida*, this observation highlights the importance of modulating the duration of enzyme expression as a means of controlling the molecular weight of heparosan.

(c) Metabolic pathway synergy

The concept of metabolic pathway synergy encompasses a comprehensive methodology that involves the systematic integration of diverse engineering strategies to optimize all metabolic processes collectively, leading to synergistic enhancements (Deng et al., 2021). Efforts to synchronize the expression levels of pathway enzymes using rudimentary methods such as overexpression or the selective deletion of one or more genes have demonstrated considerable complexity and difficulty within the field of metabolic engineering (Deng et al., 2021). In the

current landscape of biotechnological advances, the exploitation of metabolic synergy has gained considerable traction as a favored approach for the development of engineered microbial cell factories. Nowadays, some microbes such as *P. pastoris* hold great promise as versatile microbial platforms for the scalable and cost-effective production of heparosan. Recent investigations into engineered *P. pastoris* for heparosan biosynthesis provide a compelling example of the advantages derived from the integration of multiple genetic modifications (Zhang et al., 2022). Indeed, these modifications include the coordination of transcriptional and translational processes that aim to achieve a harmonious balance and cohesive enzyme expression within the respective pathway. Optimization of the coding sequences for 2A peptides significantly enhanced the expression of KfiA, KfiC, and tuaD genes, resulting in an almost five-fold increase in heparosan production to 164.55 mg/L (Zhang et al., 2022). In another study, it was observed that antibiotic-driven evolution facilitated the addition of multiple copies of the 19-kb kps locus responsible for controlling heparosan synthesis into the chromosome of EcN (Yu et al., 2023). This process, coupled with the deletion of recA to enhance strain stability during continuous culture, resulted in a significant increase in heparosan production, reaching a remarkable 9.1 g/L. Notably, this production level exceeded that of wild-type EcN, highlighting the efficacy of the genetic interventions employed to increase heparosan yield (**Table 3-1**) (Yu et al., 2023) . This research demonstrates the tactical advantage gained through the fusion of genetic techniques, aimed at enhancing both the number and the longevity of genes. Against this background, dynamic control techniques offer a promising avenue to enhance the development of robust and highly productive microbial cell factories. These techniques allow the flexible regulation of metabolic fluxes and pathway gene expression levels at different metabolic stages. Such advances are critical for the efficient production of valuable commodities such as heparosan.

(d) Engineering fermentation process

Upon closer examination of fermentation methods for glycosaminoglycan synthesis, it became apparent that extracellular microenvironmental factors exert a remarkable influence on the molecular weight of GAGs produced by microbial cell factories (Datta et al., 2021; Roy et al., 2021). Typical influencing factors include (i) the precise adjustment of fermentation induction parameters, (ii) the intricate variations in nutrient conditions, and (iii) the specific fermentation mode employed, each of which influences the dynamics and outcomes of the fermentation process (**Fig. 3-4B**).

Fine-tuning of fermentation induction parameters is essential to match the host physiology and the designed pathway. Research by Zhang et al. highlights the importance of heparosan biosynthesis, which was observed to inhibit cell growth. This highlights the potential to increase heparosan production by finely controlling the suppression of cell growth (Zhang et al., 2012). For example, in designed strains of *B. subtilis* and *B. megaterium*, early induction resulted in increased heparosan yields, while delayed induction resulted in lower yields (Chen et al., 2017; Nehru et al., 2021). In addition, early induction of eliminase led to the cleavage of freshly produced heparosan, which produced smaller oligosaccharides, as mentioned above (Roy et al., 2021). Nevertheless, heparosan production was reduced by induction that was either too rapid or too premature. This reduction can be attributed to either depleted precursors in the case of late induction or insufficient biomass in the case of extremely early induction (Nehru et al., 2021). Therefore, modulation of the induction approach is essential to obtain MW-tailored heparosan and higher yields.

The complex variation in nutrient conditions significantly influences heparosan production, with defined minimal media being preferred due to their better nutrient management and simpler processing. Conversely, the use of a complex medium (containing tryptone and yeast extract) led to a reduction in titer and presented hurdles during the purification process (Yu et al., 2023). However, the transition to defined media supplemented with glucose resulted in improved yields and reduced costs (Wang et al., 2011; Yu et al., 2023). The choice of carbon source has a dual effect, simultaneously affecting the production and molecular weight of heparosan. Commonly used primary carbon sources include glucose, glycerol, and sucrose, while yeast extract and tryptone are often used as primary nitrogen sources. Surprisingly, non-pathogenic modified *E. coli* strains produced heparosan with reduced MW in a nutrient-rich medium, while M9 minimal medium cultures showed a broader MW distribution (Roy et al., 2021). Although glucose inhibits heparosan synthesis, it simultaneously promotes significant biomass accumulation (Yu et al., 2023). Glycerol has emerged as the preferred carbon source for heparosan production, increasing productivity in certain strains (Liu et al., 2012; Zhang et al., 2012). However, despite the associated increase in acetate accumulation, glucose may be preferred from an economic perspective (Restaino et al., 2013, 2011). Therefore, the process of selecting a carbon source requires careful evaluation of strain performance, yield potential, and economic feasibility. However, despite continuous research efforts, the precise influence of carbon sources on heparosan production remains elusive.

To date, researchers have extensively studied two popular fermentation modes, batch and fed-batch fermentation, with particular emphasis on their effects on heparosan production in engineered strains. In particular, the superiority of fed-batch culture over batch culture in this regard is highlighted. The transition from batch to fed-batch mode results in a significant increase in heparosan yields, as demonstrated in both *E. coli* BL21 and *B. megaterium* (**Table 3-1**) (Nehru et al., 2020; Zhang et al., 2012). Achieving high cell densities, which are critical for successful large-scale production, depends on factors such as dissolved oxygen levels and the provision of carbon sources (Yu et al., 2023). The feeding strategy employed in fed-batch culture has a significant impact not only on optimizing heparosan yield, but also on mitigating the accumulation of toxic by-products (Nehru et al., 2021). To maximize yield, various carbon source feeding methods, including pH and DO (dissolved oxygen) stat feeding, have been carefully investigated (Liu et al., 2012). In particular, DO stat feeding has been shown to be very promising, demonstrating the highest heparosan titers in *E. coli* K5, driven by DO feedback (Liu et al., 2012). A study has also highlighted the importance of synchronizing nitrogen supplementation with carbon source availability to prolong the exponential growth phase, a conclusion supported by experimental results (Cimini et al., 2010).

(e) Accelerating heparosan transport

Recent advances in heparosan production using recombinant microbial strains have led to significant progress, yielding polymers with high molecular weights (**Fig. 3-5**) (Roy et al., 2021). The long polymers, unsuitable for clinical applications requiring bioengineered heparin, have led researchers to explore the use of KflA (K5A phage eliminase) or elmA (K5 polysaccharide eliminase). This research aims to degrade heparosan and generate lower-molecular-weight oligosaccharides, thus allowing the regulation of heparosan production (Clarke et al., 2000; Legoux et al., 1996; Manzoni et al., 2004).

Despite considerable efforts, the control of heparosan hydrolysis by elmA has proven challenging, resulting in the formation of a heterogeneous mixture of oligosaccharides with different degrees of polymerization (Barreteau et al., 2012). To modulate the size of heparosan oligosaccharides and obtain size-defined oligosaccharides, a recent study described a technique involving differential activation of biosynthetic genes and elmA (Roy et al., 2021). Despite promising results, the inclusion of KflA has added complexity to the regulation of the molecular weight properties of heparosan, resulting in increased polydispersity (Wang et al., 2011, 2010). Therefore, strategies

such as careful adjustment of fermentation conditions and exploration of photochemical depolymerization are being implemented to overcome these challenges and improve product quality (Higashi et al., 2011).

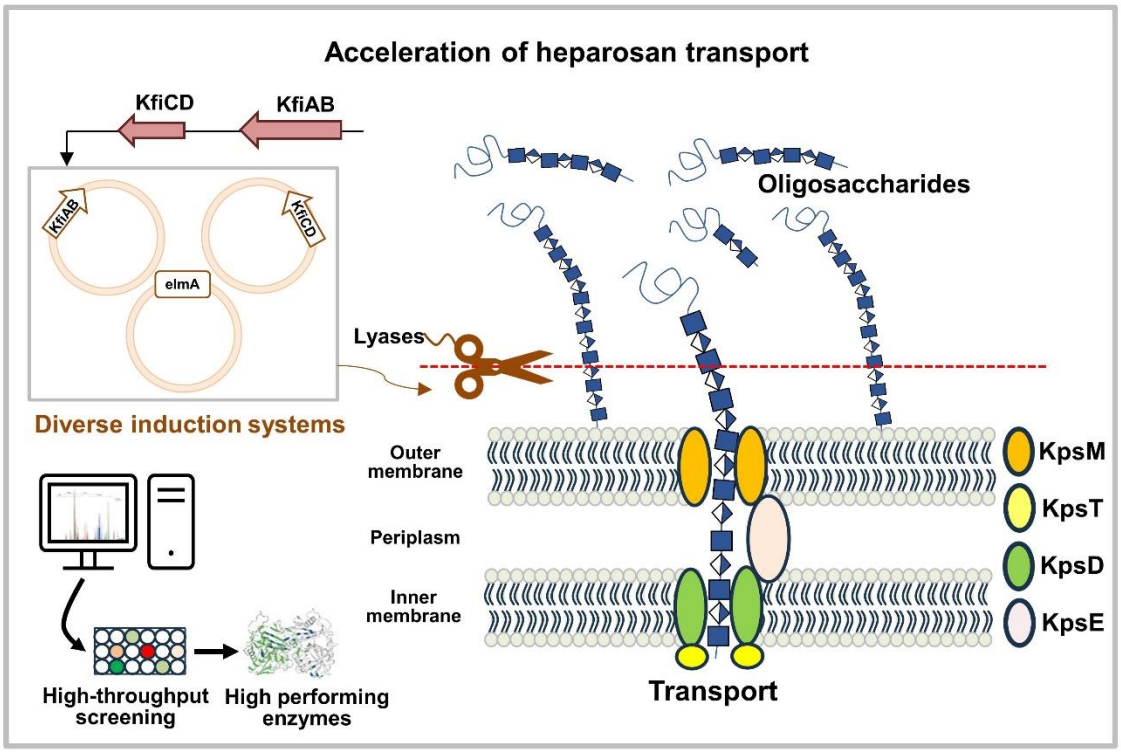

Fig. 3-5. Schematic diagram for the regulation of heparosan transport. The process employs two strategies. First, distinct induction systems are used to control the expression of heparosan biosynthetic genes and elmA. elmA: K5 polysaccharide eliminase, KfiA: GlcA transferase, KfiC: GlcNAc transferase. Second, lyases are engineered to produce molecular weight-tailored heparosan.

The integrity of the entire cellular membrane is critical to protect intracellularly synthesized heparosan from external shear forces, thereby preserving its structural stability during increased accumulation (Liu et al., 2023). Effective strategies such as the "membrane shield strategy" implemented in *C. glutamicum* have proven influential in strengthening membrane integrity and increasing precursor supply, resulting in the robust production of heparosan (**Table 3-1**) (L. Hu et al., 2022). Furthermore, the spatiotemporally synchronized depolymerization-polymerization strategy facilitates the controlled production of heparosan oligosaccharides (5.8 g/L) with precise sizes (S. Hu et al., 2022). While previous studies emphasized the importance of export genes in

heparosan synthesis (Bronner et al., 1993), recent discoveries suggest that their function may primarily involve the regulation of polymer size rather than being essential for synthesis itself (Barreteau et al., 2012). Future investigations are aimed at engineering recombinant cell factories with different enzyme activities to produce heparosan variants with different molecular weights, thereby optimizing production efficiency and meeting specific application requirements (Sheng et al., 2024).

3.3.2. Heparosan to bioengineered heparin

The first step in designing customized heparin-based oligo- and polysaccharides is to control the elongation of heparosan chains, which determines both the polymer length and the sugar unit backbone composition. However, owing to the complex nature of heparin and HS glycosaminoglycans, additional steps are essential to ensure the biological activity of the molecules, requiring extensive control measures.

The enzymatic conversion of heparosan to heparin and HS involves *N*-deacetylation/*N*-sulfation, O-sulfation, C5 epimerization, and depolymerization reactions, as illustrated in **Fig. 3-6A** (Bhaskar et al., 2015; Lu et al., 2018). An increase in overall yield could be achieved by replacing key enzymatic steps such as de-*N*-acetylation/*N*-sulfation, O-sulfation, and depolymerization with chemical methods (Cress et al., 2019; Naggi et al., 2001). Further investigation of the pharmaceutical properties of these products is imperative, although numerous research groups have used this method to generate modified heparosan with anticoagulant properties (Kuberan et al., 2003b; Lindahl et al., 2005).

3.3.2.1. Through enzymatic modification

Chemoenzymatic synthesis, a method that mimics the natural production pathway of heparin, combines chemical and enzymatic approaches to effectively address the challenges encountered in chemical synthesis. Enzymes act as catalysts in carbohydrate synthesis, providing precise stereoselectivity and regioselectivity without the need for protecting group manipulation in glycosylation reactions (Gijsen et al., 1996; Karst and Linhardt, 2003). This method uses a variety of heparin/HS biosynthetic enzymes under mild conditions, yielding structurally diverse heparin polysaccharides and uniform LMWH and ULMWH oligosaccharides (Chen et al., 2005; Xu et al., 2014; Zhang et al., 2008). The process typically starts with heparosan derived from the K5 strain of *E. coli*, which is modified in vitro with recombinant enzymes to replicate the heparin biosynthetic pathway (Chappell and Liu, 2013). As a result, these recombinant biosynthetic

enzymes can be efficiently produced on a large scale and serve as effective biocatalysts in heparin and HS synthesis (Raedts et al., 2013; Restaino et al., 2013).

N-Deacetylase/*N*-sulfotransferases

The potency of *N*-deacetylase/*N*-sulfotransferases (NDSTs), particularly NDST-1 and NDST-2 (Li et al., 2018), represents a significant hurdle in the development of bioengineered heparin. Although four human NDST isoforms have been identified to date and are distributed in various tissues and cells, the enzymatic synthesis of heparin has primarily been attributed to the NDST-1 and NDST-2 isoforms (Li et al., 2018). The *N*-sulfotransferase (NST) domain of NDST is efficiently expressed in *E. coli* and functions without *N*-deacetylase activity. Further evidence for the efficient catalysis of *N*-deacetylation/*N*-sulfation processes at the microgram level was provided by NDST-2 generated by baculovirus and rat NDST-1 in *Saccharomyces cerevisiae* (Saribas et al., 2004; Kuberan et al., 2003). However, the limited expression levels of recombinant NDSTs in yeast or insect cells hinder their utility as biocatalysts (Dou et al., 2015; Saribas et al., 2004). Chemoenzymatic heparin synthesis provides an alternative strategy, often integrating chemical *N*-deacetylation and *N*-sulfation reactions. In this process, heparosan undergoes *N*-deacetylation upon treatment with NaOH, followed by treatment with trimethylamine-sulfur trioxide for *N*-sulfation (Kuberan et al., 2003; Wang et al., 2011). This sequential process results in the formation of the intermediate *N*-sulfoheparosan (Wang et al., 2011). The proportion of *N*-sulfation to *N*-acetylation in the synthesized heparin derivatives can be precisely managed by varying the chemical reaction time, enabling the achievement of *N*-sulfation levels similar to those in therapeutic UFH (Wang et al., 2011). For example, the group led by Douaisi successfully produced *N*-sulfoheparosan (NSH) with a MW range comparable to that of porcine intestinal heparin by employing chemical *N*-deacetylation, depolymerization, and *N*-sulfation of heparosan (Douaisi et al., 2014). Moreover, the *N*-deacetylation step involving NaOH treatment resulted in a notable reduction in the molecular weight of the initial material, which facilitated the depolymerization of the synthesized heparin derivatives (Wang and Li et al., 2011; Wang and Yang et al., 2011). Another recent advancement was made by Zhang et al., utilizing the *P. pastoris* expression system to successfully achieve active expression of NDST-1 and demonstrate enzymatic heparin production (Zhang et al., 2022). Analysis of the variant Mut02 with multiple mutations revealed an impressive sulfation level of 82.87% in the *N*-sulfated heparosan product, indicating its enhanced capability for heparin biomanufacturing compared to the wild-type MBP–hNST (Xi et al., 2023).

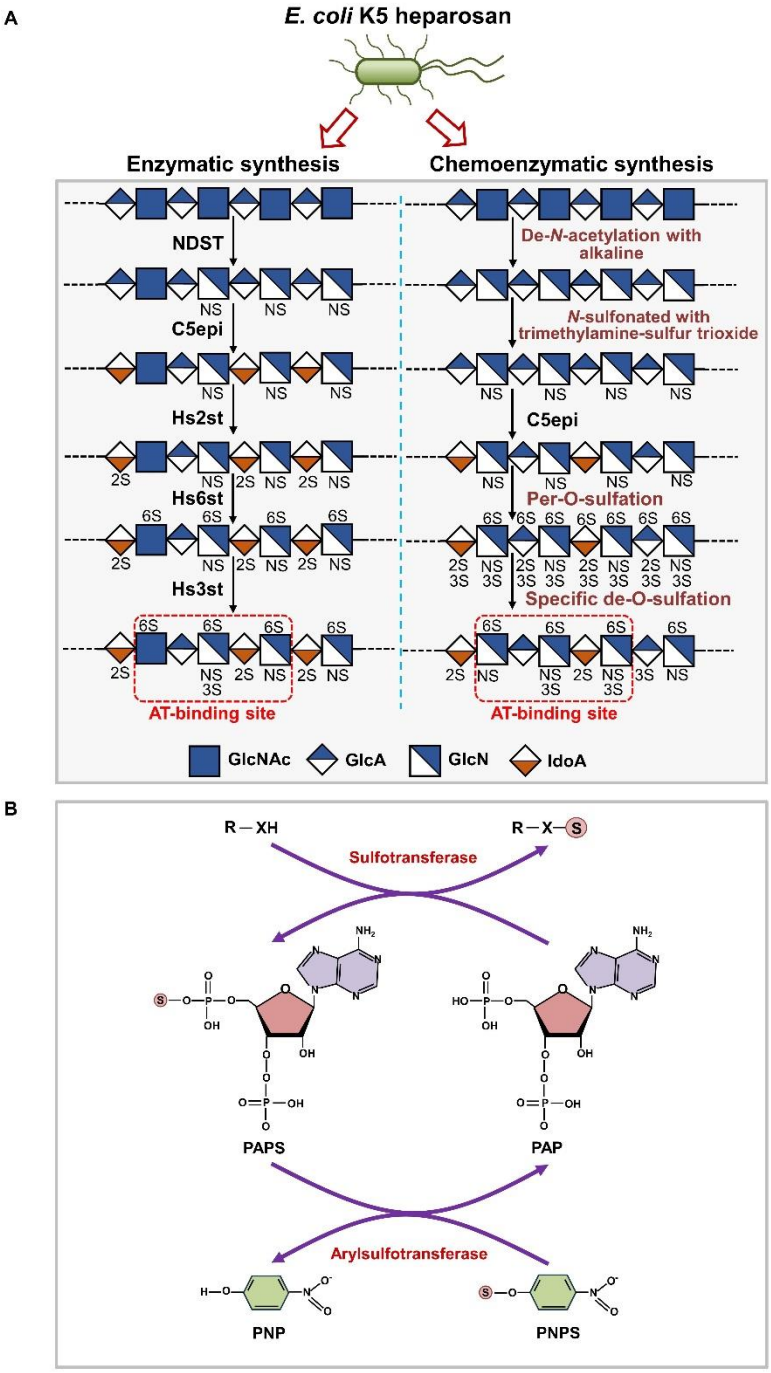

Fig. 3-6. Enzymatic and chemoenzymatic approaches to heparin synthesis. (A) In vitro biosynthetic pathway of heparin by modification of heparosan with recombinant enzymes. NDST: *N*-deacetylase/*N*-sulfotransferase, C5epi: glucuronyl C5-epimerase, Hs2st: heparan sulfate 2-O-sulfotransferase, Hs3st: heparan sulfate 3-O-sulfotransferase, Hs6st: heparan sulfate 6-O-

sulfotransferase, GlcA: β-D-glucuronic acid, GlcN: α-D-glucosamine, GlcNAc: *N*-acetylglucosamine, IdoA: α-L-iduronic acid. (B) Chemoenzymatic *N*-sulfation scheme of heparins with a PAPS regeneration system catalyzed by aryltransferase (R = sugar or peptide or small cytosolic molecules; when X = O and when X = NH/NR). PAPS: 3'-phosphoadenosine 5'-phosphosulfate, PAP: 3'-phosphoadenosine 5'-phosphate, PNP: p-nitrophenol, PNPS: para-nitrophenyl sulfate, S: sulfo group.

C5-Epimerization

The C5-epimerization process, catalyzed by C5epi, is a critical step in the biosynthesis of heparin and HS derivatives. This enzyme converts GlcA to IdoA within the GAG chain, introducing structural diversity essential for biological function. While C5-epimerization typically proceeds in one direction, from GlcA to IdoA, earlier studies suggested that the reaction could be reversible under specific conditions (Li et al., 2001; Li, 2010). However, subsequent experimental evidence demonstrated that the reversibility of this process strictly depends on the arrangement of *N*-acetylation and O-sulfation within the heparin oligosaccharide (Sheng et al., 2012; Hagner-McWhirter et al., 2004; Qin et al., 2015).

The molecular context at the epimerization site (EPS) significantly influences C5epi activity. A GlcNS at the -1 position relative to the EPS facilitates enzyme binding and epimerization. In contrast, the presence of GlcNAc at this position blocks enzyme binding, inhibiting epimerization. Additionally, reversible epimerization between GlcA and IdoA is possible when GlcNS, GlcN, or an unoccupied site is located at the -3 position. However, if GlcNAc is present at the -3 position, this reversibility is suppressed, directing the process unidirectionally from GlcA to IdoA. Once IdoA is formed, subsequent modification by 2-O-sulfotransferase (2-OST) results in the formation of 2-O-sulfated IdoA (IdoA2S), effectively locking the residue in place. This sulfation prevents reverse epimerization, even in the presence of conducive conditions at other positions (Paul et al., 2012), leading to the accumulation of stable IdoA2S within the heparin structure (Sheng et al., 2012; Hagner-McWhirter et al., 2004; Qin et al., 2015). NMR studies further confirm that 2-O-sulfated IdoA, along with 6-O-sulfated or 3-O-sulfated GlcN residues, inhibits further epimerization by altering enzyme-substrate interactions (Debarnot et al., 2019). Moreover, recent findings underscore the significance of post-translational modifications in the functionality of C5epi. *N*-linked glycosylation and disulfide bond formation have been identified as critical for maintaining the enzyme's structural integrity and catalytic efficiency. In a study by Cui et al. (2023),

the recombinant expression of human C5epi via a lentiviral system yielded high levels of active enzyme, with a specific activity of up to 1.6 IU/mg, highlighting its potential application in heparin biosynthesis.

O-Sulfotransferases

O-Sulfation is critical for enhancing the anticoagulant properties of heparosan, which requires the involvement of enzymes such as 6-O-sulfotransferase (6-OST) and 3-O-sulfotransferase (3-OST) in the chemoenzymatic synthesis of heparin and HS. These enzymes are essential due to their efficiency in effective conversion rates with mild reaction conditions (Lange et al., 2016). In human biology, three isoforms of 6-OST (HS6ST-1, HS6ST-2, and HS6ST-3) and seven isoforms of 3-OST (HS3ST-1–6) are responsible for transferring sulfate groups to the C-6 and C-3 positions of GlcN, respectively. Because of their similar substrate specificity patterns, combinations of these isoforms, such as 6-OST-1 and 6-OST-3, are often used to catalyze 6-O-sulfation. Sulfotransferase enzymes, specifically 6-OST-1 and 6-OST-3, have been successfully produced by fed-batch fermentation in *E. coli* (Restaino et al., 2013; Zhang et al., 2015). Importantly, GlcA residues adjacent to GlcNS6S and GlcNAc6S moieties escape recognition by C5epi, preventing further modification of heparosan treated with 6-OST-1 or 6-OST-3 prior to the epimerization reaction. Upon the completion of C5-epimerization and subsequent 2-O-sulfation, the modified IdoA2S-containing heparosan can be further tailored by 6-OSTs to yield the major TriS structure of heparin (Sterner et al., 2014). A significant advancement was achieved by integrating the precursor synthesis pathway and all modification enzymes into a single *P. pastoris* strain, enabling the production of HS from the C1 compound methanol in a 3-L fermentation vessel (Zhang et al., 2022).

The final step in the production of anticoagulant heparin involves 3-OST treatment, which is critically mediated by the 3-OST-1 isoform for 3-O-sulfation. This process forms a unique pentasaccharide AT-binding site that is essential for potent anticoagulant activity (Wang et al., 2017). Monitoring 3-OST-1 sulfation is critical during bioengineered heparin synthesis because of its impact on broader biological functions such as tissue formation and neuronal growth (Thacker et al., 2014; Xu and Esko, 2014). A high-throughput sensing platform facilitates real-time monitoring of 3-OST sulfation in the final stage of bioengineered heparin production (Lin et al., 2019). In addition, an integrated strategy for the analysis of 3-O-sulfated HS structures has been

developed, supporting the development of improved heparins with the potential for fewer side effects and advancing the understanding of the biological functions of HS (Karlsson et al., 2021).

3.3.2.2. Through PAPS regeneration system

PAPS: an exclusive sulfur donor

A critical element of this chemoenzymatic strategy is the incorporation of the PAPS regeneration system into enzymatic modification processes (Burkart et al., 2000) (**Fig. 3-6B**). Intracellularly, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) plays a critical role in the sulfation of GAG chains, but its prohibitive cost undermines its utility in the in vitro production of CS and heparin/HS (Carlsson and Kjellén, 2012; Zhang et al., 2022). Sulfotransferase enzymes facilitate the transfer of sulfur groups from PAPS to GAG substrates. PAPS biosynthesis depends on ATP sulfurylase (ATPS) and APS kinase (APSK), which are dependent on the availability of inorganic sulfate (Koprivova and Kopriva, 2016). While ATPS catalyzes the conversion of ATP and SO_4^{2-} to APS and pyrophosphate, APSK mediates the subsequent conversion of APS and ATP to PAPS and AMP (Zhou et al., 2011). Owing to its high-energy nature and rapid turnover, PAPS is maintained at minimal concentrations in cells, posing a significant challenge to the large-scale production of sulfated GAGs and highlighting the need for an efficient and cost-effective PAPS regeneration system (Zulueta et al., 2013). In response to this challenge, a PAPS recycling system using arylsulfotransferase IV (AST IV) has been developed, enabling a streamlined one-pot chemoenzymatic approach for bioengineered heparin production (Bhaskar et al., 2015). Recent research has focused on the identification and investigation of different sources of AST IV (Kaysser et al., 2010; Malojcic et al., 2014). Co-expression of ATPS, APSK, and pyrophosphatase in non-pathogenic *E. coli* facilitates economic PAPS production (Zhou et al., 2011). Identification of Nudix hydrolase NudJ (Bao et al., 2015) and establishment of a defined method for PAPS preparation after purification improve understanding and control of the in vitro enzymatic synthesis of PAPS. In addition, an ATP regeneration system based on chemically synthesized monopotassium phosphoenolpyruvate ($PEPK^+$) helps achieve a high rate of conversion (98%) of ATP to PAPS (An et al., 2017). Combining ATPS and APSK to create a bifunctional enzyme streamlines the acquisition of PAPS synthase and increases the efficiency of synthesis (Xu et al., 2021). Recent findings have revealed the overexpression of human-derived phosphoadenosine phosphosulfate synthase, a bifunctional enzyme, in *E. coli* (Monterrey et al., 2023). Against this background, the successful construction holds promise for the establishment of an artificially

efficient PAPS biosynthetic system that facilitates the enzymatic production of sulfated glycosaminoglycans and their oligosaccharides (Kang et al., 2018).

3.3.2.3. Through substrate controls

The formation of glycosidic linkages between monosaccharides, dependent on high-energy nucleotides such as UTP (uridine-5'-triphosphate), is critical for glycoconjugate synthesis, including GAG formation. GAG synthesis occurs in the ER and Golgi compartments, in contrast to UDP-sugar synthesis in the cytosol. Nucleotide sugar antiporters, independent of cellular energy, facilitate the transport of activated sugar donors into these organelles while shuttling nucleotide monophosphates back to the cytosol (Berninsone and Hirschberg, 1998; Caffaro and Hirschberg, 2006). Modulation of UDP-sugar synthesis or transport has the potential to significantly affect HS/heparin biosynthesis. In addition, essential UDP-sugars such as UDP-GlcNAc, UDP-GlcNTFA, and UDP-GlcA required for in vitro enzymatic heparosan polysaccharide backbone synthesis can be synthesized by chemoenzymatic methods (Masuko et al., 2012). Alternatively, chemical synthesis of these UDP sugars and their analogs is feasible but involves a laborious process with limited yields (Masuko et al., 2012).

3.3.3. Significant progress in bioengineered heparin development

The development of one-pot synthesis techniques for heparin production represents a significant advancement in the field, providing a more efficient and cost-effective alternative to traditional methods (Bhaskar et al., 2015; Chen et al., 2011; Polat and Wong, 2007). Unlike the previously employed stepwise conversion processes, this innovative approach enables direct heparin production without the need for labor-intensive isolation, purification, and characterization of intermediates. By bypassing these complex steps, the process is streamlined, increasing both efficiency and cost-effectiveness. In addition, the staged addition of enzymes ensures precise substrate conversion, further optimizing the synthesis process. However, continuous optimization efforts are required to ensure the equivalence of the final product to pharmaceutical-grade heparin, especially when using homogeneous substrates to prevent potential product variations (Bhaskar et al., 2015).

In addition to the one-pot synthesis approach, the use of immobilized biosynthetic enzymes represents another significant advancement in heparin synthesis. By immobilizing key enzymes involved in the process, such as those responsible for PAPS cofactor recycling, researchers have achieved enhanced stability and activity, resulting in higher yields and lower costs (Xiong et al.,

2013). The simplified purification process, made possible by enzyme immobilization and the efficient removal of spent cofactors through dialysis, results in a cleaner and more cost-efficient synthesis approach (Xiong et al., 2013).

Researchers have successfully synthesized ULMW and LMW heparins with well-defined, homogeneous structures through chemoenzymatic approaches (Baytas and Linhardt, 2020; Liu et al., 2010; Lu et al., 2018; T. Wang et al., 2020; Xu et al., 2014, 2011). These bioengineered heparins demonstrate promising therapeutic potential, characterized by high yields and structural consistency. Notably, ULMW heparins exhibit comparable efficacy to existing pharmaceuticals like Arixtra®, but with a more streamlined synthesis process (Xu et al., 2011). Furthermore, enhancing LMW heparins with specific domains has improved their biological properties, rendering them suitable for a broad spectrum of therapeutic applications (Xu et al., 2014). Their potential for hepatic clearance also offers advantages for patients with renal impairment, broadening their clinical applicability. In summary, these advancements mark significant progress in anticoagulant therapy, promising more targeted treatments and broadening the scope of therapeutic options in the realm of bioengineered heparins.

3.3.4. Summary and prospects

Significant progress has been achieved in leveraging microbial cell factories as an innovative approach for heparin production. Key strategies for developing robust industrial platforms include regulating metabolic flux, identifying production bottlenecks, creating gene regulatory tools, enhancing PAPS synthesis, elucidating enzyme mechanisms, and modulating sulfotransferase activity. Ultimately, the integration of various techniques to optimize pathways, tailor host organisms, and refine production processes will enable the engineering of microbes capable of using cost-effective resources to produce LMW heparosan. This advancement meets the increasing demand for essential anticoagulant medications.

3.4. Advancements in eukaryotic systems

In recent years, the field of bioengineered heparin production has seen significant advancements, especially through developments in eukaryotic systems (**Table 3-2**). Using yeast and mammalian cell cultures, these systems produce complex glycosaminoglycans with enhanced bioactivity and therapeutic efficacy. In the following sections, we will explore the implementation of various approaches to enhance bioengineered heparin production. These approaches include metabolic pathway engineering, optimization of enzyme expression, use of advanced genome editing

techniques, precise control of modification processes, *de novo* synthesis, and bioprocess engineering.

3.4.1. Engineering heparin/HS metabolic pathway

Metabolic engineering, which aims to improve specific gene pathways to produce desired products while suppressing competing pathways, typically involves the transfer of enzymes or entire metabolic pathways from less adaptable organisms to engineered microorganisms (Jones and Koffas, 2016; Xu et al., 2013). This manipulation allows the efficient production of a variety of valuable products (Bhan et al., 2013; Keasling, 2010; Wang et al., 2016). While traditional techniques such as promoter engineering play a role in this, modern methods such as dynamic balancing and compartmentalization are also used to optimize metabolic pathways and increase product output (Cress et al., 2015; Jones et al., 2015). Synthetic biology tools continue to contribute to pathway optimization and metabolic engineering. Safety concerns have prompted a shift away from conventional animal-based approaches to GAG production, leading to the development of recombinant technologies for the synthesis of polysaccharides such as heparin and CS (Chen, 2015; Liu et al., 2009; Qui et al., 2018; Chen et al., 2018). This involves the fermentation of precursor molecules such as heparosan and chondroitin, followed by modification using enzymes in the biosynthetic pathway to yield the desired final product (Suflita et al., 2015). CHO cells, extensively used in biotechnology, are emerging as a preferred host for the production of therapeutic proteins due to their safety profile and proficient glycosylation machinery. Their ability to express a variety of glycosylation enzymes makes them well suited for the synthesis of glycosaminoglycans (Karlsson et al., 2021; Baik et al., 2012a) . In addition, CHO cells exhibit the ability to biosynthesize HS, a precursor of heparin, via a biosynthetic pathway similar to that of heparin, suggesting the feasibility of using CHO cells for heparin production (Lord et al., 2016; Stevens and Austen, 1989). Using genetic manipulation techniques, researchers enhanced heparin synthesis in CHO cells and achieved a remarkable ~100-fold increase in the anticoagulant potency of HS by introducing human NDST2 and mouse Hs3st-1 genes (**Table 3-2**) (Baik et al., 2012b, 2012a). A comparative analysis with rat mast cells identified gaps in the expression profiles of essential biosynthetic enzymes in CHO cells, particularly the absence of Hs3st1 and NDST2. Addressing this deficiency through the overexpression of NDSTs, Hs6sts, and Hs3sts in CHO cells could transform HS into a heparin-like product (Baik et al., 2012a). The complexities associated with assessing 3-O-sulfation levels and coordinating gene expression highlight the intricacy of

engineering heparin production in CHO cells (Baik et al., 2012a; Glass, 2018; Karlsson et al., 2021).

The production of bioengineered heparin in CHO cells depends on achieving a delicate balance in both the expression levels and modification processes of various enzymes. Studies have shown that precise targeting of Hs3st-1 to the Golgi apparatus results in increased expression levels of Hs2st, Hs6st, and NST, which further enhances anticoagulant activity (Datta et al., 2013a). The above investigations highlight the directional movement of engineered glycosaminoglycans toward the extracellular space, indicating the need to enhance core protein expression for improved transport and yield of bioengineered HS/heparin (Baik et al., 2012a; Karlsson et al., 2021). Metabolic engineering interventions have increased pathway flux and facilitated purification without cell lysis. However, refinement of pathway dynamics and elevation of enzyme expression levels are essential to achieve a pharmaceutical-grade bioengineered HS product. The implementation of an inducible system shows promise for optimizing NDST2 and Hs3st1 expression simultaneously (Baik et al., 2012a).

3.4.2. Recombinant expression of proteoglycans

The presence of proteoglycans is essential for the production of bioengineered heparin, as they act as natural scaffolds that facilitate heparin biosynthesis (Annaval et al., 2020). Serglycin, a prominent core protein found in various cell types such as mast cells and endothelial cells, becomes a proteoglycan when it is decorated with GAGs (Gasimli et al., 2014). Harnessing the biosynthetic machinery within host cells by expressing serglycin or analogous proteoglycans enables the production of heparin-like molecules. These proteoglycans provide the basic scaffold to which heparin chains are attached via enzymatic modifications (Lord and Whitelock, 2014). However, the main challenge of this approach is to understand the regulatory mechanisms of HS/heparin biosynthetic enzymes to ensure that recombinant forms are decorated with heparin rather than CS/DS. Furthermore, overcoming the obstacle of scaling up production beyond the milligram scale is another major challenge (Lord and Whitelock, 2014). The absence of proteoglycan expression would hinder the synthesis of bioengineered heparin due to the lack of a scaffold. In a notable experiment, introducing serglycin expression in HEK-293 cells led to the generation of a proteoglycan that included both chondroitin sulfate (CS/DS) and heparan sulfate (HS/heparin). The glycosaminoglycan composition was influenced by the glucose concentration in the medium: high and low glucose levels favored CS production, while optimal HS/heparin production was

achieved at 25 mM glucose, without affecting cell proliferation. The resultant heparin-like HS demonstrated approximately one-seventh the anticoagulant activity of unfractionated heparin, suggesting the potential for serglycin-mediated heparin production. This pioneering effort marks the first successful bioengineering of human heparin-like HS using serglycin, offering safety and accessibility benefits compared with traditional mammalian cell-based approaches (**Table 3-2**) (Lord et al., 2016; Chen et al., 2018). Further research should aim to evaluate the anticoagulant efficacy of HS in various assays and to scale up production to satisfy market demands (Lord et al., 2016).

3.4.3. Multiplex genome editing

Recent advancements in synthetic biology have paved the way for the use of engineered mammalian cells as a new source for the production of heparin (Thacker et al., 2022). Unlike protein synthesis, which is often governed by the expression of a single gene, the synthesis of heparin involves a metabolic pathway regulated by a multitude of enzymes, making multiplex genome engineering techniques a necessity (Glass, 2018; Kreuger and Kjellén, 2012). Heparin differs from HS in its sulfation patterns and levels of anticoagulant activity (Xu and Esko, 2014). Traditionally, heparin production has been confined to mast cells, but the difficulty of culturing mast cells at a commercial scale has limited its feasibility (Thacker et al., 2022). In contrast, HS can be synthesized in a variety of cell types already employed for recombinant protein production, presenting a promising avenue for cell-based engineered heparin production (Oduah et al., 2016). Efforts to enhance the anticoagulant activity of heparin focus on increasing the expression of specific sulfotransferases to improve the AT-binding pentasaccharide motif, a key determinant of its efficacy (Glass, 2018). Several groups have successfully initiated heparin production in the lab, whether this be by chemical synthesis, chemoenzymatic synthesis, or genetic engineering of a cell within the heparin biosynthetic pathway, and the cellular approach has been applied to numerous cell lines (Qiu et al., 2018; Chen et al., 2018; Karlsson et al., 2021; Deng et al., 2024; Weiss et al., 2020).

One study has used mastocytoma cells, which are cancerous tumors of mast cells, and through multiplex genome engineering, managed to produce heparin/heparan sulfate on the mastocytoma cell surface that is as potent as porcine-derived heparin (**Table 3-2**). This approach includes strategic pathway adjustments to prevent CS contamination and tailoring the gene expression profile to yield HS compositions similar to those derived from porcine heparin (Thacker et al.,

2022). The successful adaptation to serum-free medium further highlights the potential of this method for large-scale commercial heparin production (Thacker et al., 2022).

While mammalian cells synthesize both CS and HS, their co-production poses purification challenges due to potential contamination (Thacker et al., 2022). Notably, MST cells exhibit significant levels of CS in addition to HS (Gasimli et al., 2014). Enzymatic analysis quantified CS at approximately 20% of the total purified glycosaminoglycans from MST cells (Thacker et al., 2022). To address this, Thacker and his group genetically excised the genes governing CS biosynthesis from the cell lines using CRISPR/Cas9 technology, resulting in a CS-deficient cell line (Thacker et al., 2022). This modification led to an increase in HS production. However, the exact effects of these genetic alterations on the functionality of enzymes involved in HS biosynthesis have yet to be fully elucidated (Thacker et al., 2022).

This study also aims to increase the anti-FXa activity of HS from MST cells for pharmaceutical use. Comparative gene expression analysis between MST cells and mast cells revealed lower levels of Hs3st1 in MST cells (Thacker et al., 2022). In addition, MST cells showed significant expression of other HS sulfotransferases. The obtained cells were then genetically engineered to express different sulfotransferase genes, resulting in the identification of colonies with increased anti-FXa activity. The HS extracted from these colonies showed variable anti-FXa activity, with some samples exceeding 200 U/mg, particularly in cell pellets (Thacker et al., 2022). Therefore, this study using multiplex genome engineering represents a significant advance in the synthesis of recombinant HS from genetically engineered mammalian cells. The resulting HS exhibits anticoagulant efficacy comparable to or superior to that of pharmaceutical heparin, highlighting the potential role of synthetic biology in replacing animal-derived heparin.

3.4.4. De novo biosynthesis using cell-free enzymatic system

The development of a cell-free enzymatic system for heparin production represents a significant leap forward in biotechnology. This approach aims to improve scalability, efficiency, and control by operating within an environment free from living cells. Through the application of genetic engineering techniques, such as N-terminal engineering and Tag-fusion expression, Zhang et al. have achieved remarkable progress (**Table 3-2**) (Zhang et al., 2022). They have successfully engineered *P. pastoris* strains to efficiently produce enzymes crucial for heparin biosynthesis, achieving a sulfation level of 2.4 sulfate groups per disaccharide and producing 2.08 g/L of bioengineered heparin in fed-batch cultures. By co-culturing these engineered *P. pastoris* strains,

they managed to place enzymes essential for heparin synthesis together within a single bioreactor. This strategy lays the foundation for a cell-free catalytic platform, advancing towards the establishment of a *P. pastoris* cell factory dedicated to bioengineered heparin production. This underscores the suitability of *P. pastoris* as an exemplary host for the synthesis of complex natural products (Zhang et al., 2022) . Nevertheless, continued research is crucial for optimizing the biosynthesis of precursors, rejuvenating the sulfate group donor, and improving the catalytic efficiency of sulfotransferase in *P. pastoris*, thereby unlocking additional possibilities for bioengineered heparin production.

3.4.5. Bioprocess research

In efforts to maximize the yields and activity levels of bioengineered heparin, researchers are fine-tuning metabolic engineering and refining bioprocesses. Baik and colleagues have shown how adjustments in fermentation conditions, feeding techniques, and medium components can significantly increase product yields (Baik et al., 2015). For example, adding cysteine to engineered CHO-S cell line cultures increased their anticoagulant activity. Despite progress, differences between bioengineered and pharmaceutical heparin compositions suggest that further refinements are needed (Baik et al., 2015). Another study suggests that, to meet commercial standards, cells need to be grown in serum-free medium, which elevates polysaccharide quantity and anti-FXa activity (Thacker et al., 2022). It was observed that clones transduced with specific genes exhibited sustained high activity and sulfate content, suggesting potential effects of serum components (Thacker et al., 2022). Moreover, the concentration of glucose in the culture medium was found to influence glycosaminoglycan synthesis and its modifications, calling for a deeper exploration of how glucose levels affect heparin production (Lord et al., 2016). Consequently, strategic manipulations in bioprocessing are critical for achieving desirable yields and maintaining the quality attributes of the product.

3.4.6. Recombinant CHO cells: a promising alternative

Recombinant CHO cells offer a compelling platform for engineered heparin production, leveraging their established role in biotherapeutic protein synthesis. These cells are preferred due to their efficient protein folding, adaptability to serum-free culture, and viral resistance, making them suitable candidates for HS/heparin production (Baik et al., 2012a; Tihanyi and Nyitray, 2020). Despite promising early efforts to engineer the HS biosynthetic pathway, significant challenges remain. CHO-derived HS exhibits sulfation patterns distinct from porcine heparin, which are

critical for its anticoagulant function (Baik et al., 2012a). While overexpression of key enzymes has enhanced sulfation levels, replicating the precise patterns required for fully functional heparin remains difficult. Nevertheless, advancements in metabolic engineering and multiplex genome editing have shown potential to generate highly sulfated HS with anticoagulant properties comparable to, or even exceeding, those of conventional heparin (Thacker et al., 2022). However, significant barriers to commercial application persist. Scale-up efforts and purification processes in recombinant systems, including mastocytoma cells, continue to face technical challenges (Thacker et al., 2022). Additionally, economic feasibility remains an issue, as production efficiency must be improved to compete with traditional animal-derived heparin. Despite these limitations, recombinant heparin holds potential for niche markets, particularly in applications such as cardiopulmonary bypass, where enhanced quality could justify higher production costs (Thacker et al., 2021). Although challenges remain, recombinant CHO cells represent a promising avenue for scalable production of bioengineered heparin for biotherapeutic applications (**Fig. 3-7**).

Upregulation of sulfation level

Enhancing sulfation in recombinant CHO cells is crucial for improving the therapeutic properties and efficacy of bioengineered heparin. Sulfation significantly influences the anticoagulant activity of heparin by modulating its interaction with AT, resulting in potent anticoagulant effects. Additionally, sulfation is essential for maintaining the proper structure and functionality of heparin, influencing its binding affinity to various proteins and affecting its biological activities. Therefore, optimizing sulfation levels through genetic and metabolic engineering is key to producing high-quality bioengineered heparin. This involves a multifaceted approach, including the upregulation of enzymes in the GAG sulfation pathway and the implementation of effective PAPS regeneration systems (**Fig. 3-7**). Overexpression of enzyme genes involved in sulfation, such as NDST2 and Hs3st1, offers a promising strategy for improving sulfation levels (Baik et al., 2012a). Developing a highly stable and productive heparin production technology, which includes optimizing medium components and enhancing genetic modifications, is essential to achieve these goals. Such advancements could revolutionize anticoagulant therapy by providing bioengineered heparin with enhanced efficacy and safety profiles, leading to significant innovations in biopharmaceuticals.

Enhancing anticoagulant efficacy

The enzyme Hs3st1 plays a crucial role in enhancing anti-FXa activity by regulating 3-O-sulfation (Atha et al., 1985) and has been studied in relation to growth conditions and genetic variations

(Thacker et al., 2022). The absence of Hs3sts in CHO cells underscores the necessity of investigating the overexpression of specific isozymes to meet heparin production standards (Baik et al., 2012a). Additionally, the contribution of Hs6st1 and Hs6st2 transduction, their substrate specificities, and the critical role of NDST2 in heparin biosynthesis have been thoroughly examined (Forsberg et al., 1999; Humphries et al., 1999; Kreuger and Kjellén, 2012; Thacker et al., 2022). The discovery of sulfotransferase inhibition by a zinc finger protein provides valuable insights into the regulatory processes governing heparin biosynthesis (Weiss et al., 2020). Together, these studies offer comprehensive insights and strategies for the development of recombinant CHO cells aimed at improving the production of heparin and its anticoagulant properties.

Production via extracellular secretion

The production of bioengineered heparin via extracellular secretion poses several challenges related to secretion optimization, purification, and yield enhancement due to the complexity of the biosynthesis and purification processes. However, scalable production in bioreactors offers potential solutions to these challenges through genetic and metabolic engineering interventions, which could help meet the medical demand for bioengineered heparin. To overcome this, recombinant expression of extracellular HS proteoglycans such as syndecan, glypican, and serglycin has been proposed to aid in extracellular secretion (**Fig. 3-7**) (Farrugia and Melrose, 2023; Lord and Whitelock, 2014). In addition, the adaptation of cells to a serum-free medium for bioengineered heparin production presents other challenges, including optimizing culture parameters and reducing production costs (Thacker et al., 2022). Despite these obstacles, this transition represents a critical advancement in improving biomanufacturing systems for bioengineered heparin, ultimately enhancing its clinical applications and therapeutic efficacy.

Elimination of CS biosynthetic pathway

Inactivation of the CS pathway represents a strategic way to enhance bioengineered heparin production (Thacker et al., 2022). This method redirects metabolic flux toward heparin biosynthesis, ensuring that precursor molecules and enzymes prioritize heparin production over CS. In addition, inactivation of the CS pathway can increase cellular sulfation capacity by upregulating enzymes involved in heparin sulfation. By reducing competition for resources and enzymes, this approach minimizes diversion to CS production, thereby increasing the efficiency of heparin synthesis. However, this strategy faces challenges, such as potential effects on cellular metabolism and the need for selective pathway inactivation to avoid disruption of essential

processes. Understanding long-term effects and ensuring cell viability and productivity are critical to scalability. Nevertheless, overcoming these challenges promises significant advances in bioengineered heparin production in recombinant CHO cells.

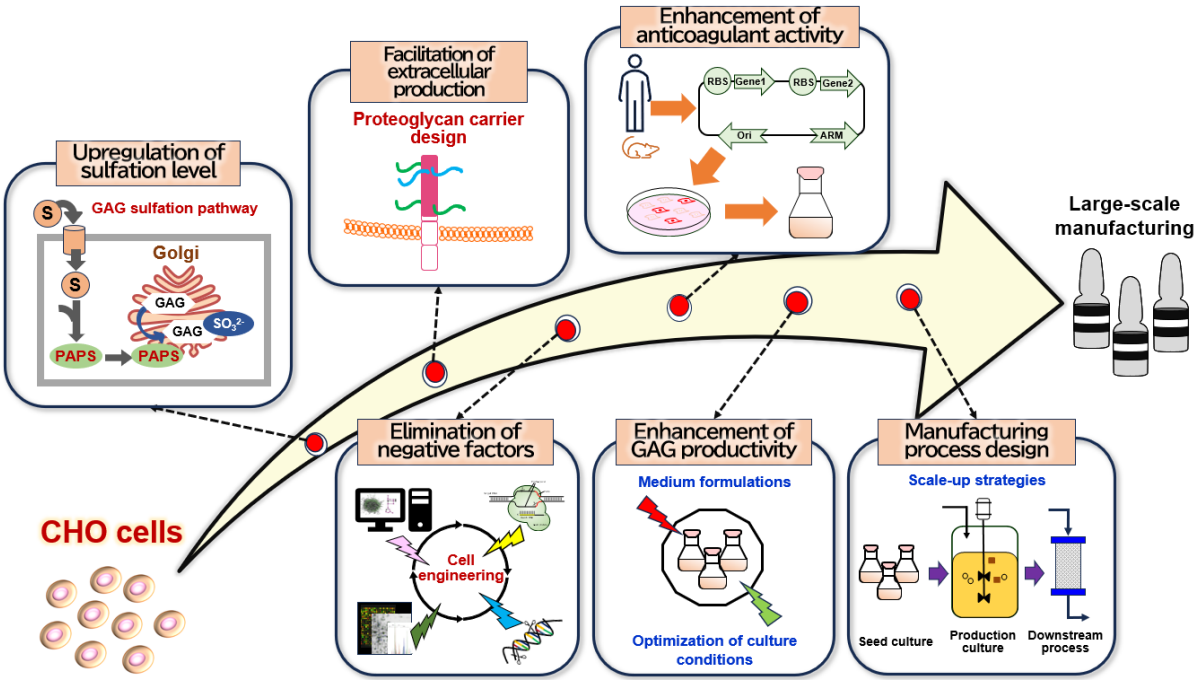

Fig. 3-7. Metabolic strategies for the production of bioengineered heparin using recombinant CHO cells.

Inactivation of desulfation enzymes

Desulfation enzymes, including sulfamidases, glycosidases, and sulfatases, play a crucial role in bioengineered heparin production by regulating sulfation levels in mammalian cells, which directly impacts the quality and function of heparin. Investigating these enzymes, which are involved in both the synthesis and the degradation of HS, has been indispensable not only for uncovering related genetic abnormalities but also for understanding the complex structures of HS and heparin (Griffin and Gloster, 2017; Kowalewski et al., 2021). To address these challenges, novel methods such as microarray analysis and computational techniques have emerged as powerful tools for identifying key desulfation enzymes (**Fig. 3-7**). Furthermore, advanced techniques such as CRISPR/Cas9-mediated targeting and knockdown have significant potential for inhibiting these enzymes and enhancing HS production, offering a promising direction for improving bioactivity. Therefore, a comprehensive understanding and strategic modulation of desulfation enzyme activity are critical for optimizing the production of bioengineered heparin to meet rigorous quality and efficacy standards.

Scale-up strategies

Scaling up the production of bioengineered heparin is essential to meet the growing demand for this alternative anticoagulant therapy, especially as its acceptance in medical practice increases (Baik et al., 2015). Large-scale production allows for rigorous quality control measures to maintain product consistency and purity. It also supports ongoing research and development efforts to refine manufacturing processes and explore new therapeutic applications. The comprehensive approach to scaling up production using recombinant CHO cells includes optimization of bioreactor parameters, customization of medium formulations, use of genetic engineering techniques, and implementation of robust process monitoring and control methods, including agitation in bioreactors (**Fig. 3-7**). It also involves optimizing downstream processing, ensuring regulatory compliance, and effectively managing the supply chain. By integrating these strategies, manufacturers can address healthcare needs, ensure quality, and drive innovation in anticoagulant therapy.

3.4.7. Summary and prospects

Recent advancements in biotechnology have significantly improved the production of bioengineered heparin using eukaryotic systems for commercial applications. This progress is characterized by a notable shift toward *de novo* biosynthesis of heparin, a prominent trend in current research. Key areas of focus include strategic modulation of heparin biosynthetic enzymes, refinement and standardization of enzyme expression levels, implementation of targeted modifications to improve enzyme efficiency and robustness, augmentation of cellular PAPS pools critical for heparin biosynthesis, improvement of catalytic efficiencies of modified enzymes in cellular production systems, and optimization of bioprocess parameters to enhance heparin yield and quality. These collective efforts are critical for establishing robust industrial platforms for bioengineered heparin production, thereby opening up new therapeutic opportunities. In addition, among the range of promising mammalian cell lines, recombinant CHO cells are emerging as an attractive and predominant choice with considerable potential to make remarkable progress in the biotechnological advancement of heparin production.

3.5. Conclusions and outlook

The evolving role of heparin extends beyond its traditional use as an anticoagulant to reveal new therapeutic potentials. Recent advances in synthesis, biotechnology, and metabolic engineering have enabled the precise characterization of heparin derivatives such as LMWH, ULMWH, and

bioengineered heparins in both prokaryotic and eukaryotic systems. These developments offer promising opportunities to explore new therapeutic avenues. These innovations are deepening our understanding of the structural differences among animal-derived heparins and paving the way for safer alternatives. While pre-clinical and clinical studies are essential to evaluate the properties of these derivatives, the successful synthesis of heparin oligosaccharides is promising for improving therapeutic interventions in various diseases. However, despite significant progress in both systems, challenges remain in achieving precise control of molecular weight, optimal HS titer, and degree of sulfation. In addition, achieving high and cost-effective production levels remains a hurdle, requiring further bioprocess development and scale-up efforts. Although various mammalian cells have been used for production, challenges such as how to maximize production, optimize sulfation levels, improve purification methods, and resolve contaminant issues remain. In the future, the integration of multiple genes, process optimization, and the use of high-throughput screening methods in cell engineering should drive a new era of therapeutic innovation and accessibility.

Table 3-1

A review of heparosan production through metabolically engineered natural and recombinant host strains (HP: heparosan, P: phosphate, Glu: glucose, OS: oligosaccharide, MW: molecular weight)

Target and source	Engineering approaches	Encoded function	Hosts	Process (L)	Maximal Titer (g/L)	Duration (h)- Volumetric production rate (g/l/h)	MW (kDa)	References
Native host								
-	Fermentation process	-	*E. coli* K5	Fed-batch	10.2	-	-	(Viskov et al., 2008)
-	Fermentation process optimization	-	*E. coli* K5	Fed-batch (7) Shake flasks	15 0.07–0.5	37.5-0.4 4-(0.018-0.125)	84	(Wang et al., 2010)
-	Optimizing the effects of carbon sources and feeding strategies	-	*E. coli* K5	Fed-batch (3) Batch	8.63 0.82	11-0.785 20-0.041	-	(Liu et al., 2012)
Recombinant hosts								
PmHS2 (*P. multocida*)	Expression of recombinant PmHS2 in the presence of 5 mM of each UDP-sugar precursor	PmHS2: HP Synthase 2	*E. coli* BL21 (DE3)	Agarose gel electrophoresis	-	-	~103	(Chavaroche et al., 2010)
KfiA, KfiB, KfiC and KfiD (*E. coli* K5) elmA (*E. coli* K5)	Combinatorial co-expression of region 2 cluster and lyase genes	KfiA: GlcNAc-transferase KfiB: polymerase factor KfiC: GlcA-transferase KfiD: UDP-Glu dehydrogenase elmA:K5 polysaccharide eliminase	*E. coli* K12	Fed-batch (0.5 or 3)	1	50-0.02	DP-2 to 10 OS	(Barreteau et al., 2012)
KfiA, KfiB, KfiC and KfiD (*E. coli* K5)	Heterologous co-expression of four critical genes in a DO-stat fed-batch bioreactor	KfiA: GlcNAc-transferase KfiB: polymerase factor KfiC: GlcA-transferase KfiD: UDP-Glu dehydrogenase	*E. coli* BL21 (DE3)	Fed-batch (3) Shake flasks Batch	1.88 0.334 0.652	32-0.059 30-0.011 30-0.022	118.30-39.63	(Zhang et al., 2012)
KfiA, KfiC (*E. coli* K5)	Co-expression of KfiAC complex and fusion of KfiC with trigger factor from *E. coli* DH1	KfiA: GlcNAc-transferase KfiC: GlcA-transferase	*E. coli* BL21	Fed-batch (0.5)	1.5	24-0.063	-	(Leroux and Priem, 2016)

KfiA,KfiB, KfiC, KfiD (*E. coli K5*) elmA (*E. coli* K5)	Co-upregulation of four essential heparosan biosynthetic genes with or without the eliminase gene	KfiA: GlcNAc-transferase KfiB: polymerase factor KfiC: GlcA-transferase KfiD: UDP-Glu dehydrogenase elmA: eliminase	*E. coli* BL21 (DE3)	Fed-batch (6) Shake flasks	~0.48 ~0.28	40-0.012 24-0.012	~5 to >150	(Roy et al., 2021)
KfiA and KfiC (*E. coli K5*) tauD (*B. subtillis*)	Enhancement of precursor KfiA, KfiC; elevated expression of tauD	KfiA: GlcNAc-transferase KfiC: GlcA-transferase tauD: UDP-Glu dehydrogenase	*B. subtilis (168)*	Fed-batch (3) Shake flasks Shake flasks	5.82 1.71 2.65	72-0.080 48-0.036 48-0.055	39.72-67.70	(Jin et al., 2016)
PmHS1 (*P. multicida*) tuaD/gtaB module (*B. subtilis*) gcaD module (*B. subtilis*)	Co-expression of PmHS1; upregulation of UDP-sugar precursors and expression of synthetic pathway enzymes	PmHS1: HP Synthase 1 tauD: UDP-Glu dehydrogenase gtaB:UDP-Glu pyrophosphorylase gcaD:GlcNAc-1-P uridyltransferase glmM:Phosphoglucosamine mutase	*B. subtilis (168)*	Shake flasks	0.238	4-0.059	39-53	(Chen et al., 2017)
PmHS2 *(P. multocida)*	Utilizing T7 RNA polymerase expression system and overexpression of PmHS2	PmHS2: HP Synthase 2	*B. megaterium (MS941)*	Fed-batch (1.5) Shake flasks	~2.74 ~0.25	13-0.211 48-0.005	~200–300 ~10–40	(Williams et al., 2019)
KfiA, KfiC *(E. coli K5)* tuaD, gtaB, gcaD and glmM (*B. megaterium* DSM319)	Heterologous expression of KfiAC; enhancement of UDP-sugar precursor pathway genes	KfiA: GlcNAc-transferase KfiC: GlcA-transferase tauD: UDP-Glu dehydrogenase gtaB:UDP-Glu pyrophosphorylase gcaD:GlcNAc-1-P uridyltransferase glmM:Phosphoglucosamine mutase	*B. megaterium (*DSM319*)*	Fed-batch(3) Batch	1.32 0.394	25-0.053 16-0.025	31–60	(Nehru et al., 2020)
galU (*E. coli* MG1655), PmHS2 *(P. multocida)*	Co-expression of galU and PmHS2; photoautotrophic production	PmHS2: HP Synthase 2 galU: UDP-Glu pyrophosphorylase	*S. elongatus* (PCC 7942)	Shake flasks Large scale plastic bags	2.8 µg/L 0.44µg/L	72-0.93 µg/L/day 120-0.09 µg/L/day	- -	(Sarnaik et al., 2019)
KfiA, KfiC *(E. coli K5)* tauD and gtaB (*B. megaterium* DSM319)	Co-expression of KfiA and KfiC using dual promoter expression system; overexpressing tauD and gtaB	KfiA: GlcNAc-transferase KfiC: GlcA-transferase tauD: UDP-Glu dehydrogenase gtaB: UDP-Glu pyrophosphorylase	*B. megaterium* (DSM319)	Fed-batch (3) Shake flasks Batch	1.96 0.203 0.627	25-0.078 24-0.008 14-0.045	41.9 - 68.6	(Nehru et al., 2021)

-	Employing a high-density fermentation process	-	*E. coli* Nissle (1917*)*	Bioreactor (100L)	3	-	68	(Datta et al., 2021)
galU (*E. coli* MG1655) KfiD (*B. subtilis* 168) glmM (*C. glutamicum* ATCC 13032) KfiA and KfiC (*E. coli* Nissle 1917)	Enhancing the expression of synthetic pathway genes galU, KfiD, glmM and co-expression of KfiA and KfiC	galU: UDP-Glu pyrophosphorylase KfiD: UDP-Glu dehydrogenase glmM:Phosphoglucosamine mutase KfiA: GlcNAc-transferase KfiC: GlcA-transferase	*E. coli* Nissle (1917*)*	Fed-batch (3) Shake flask	11.50 0.80	72-0.160 24-0.033	312.39-410.84	(S. Hu et al., 2022)
KfiA and KfiC (*E. coli* Nissle 1917), galU, glmS, glmM, glmU and ugd (Han et al., 2014; Y. Wang et al., 2020a)	Using a membrane shield strategy: co-expression of KfiA and KfiC; overexpression of pathway enzymes galU, glmS, glmM, glmU and ugd	KfiA: GlcNAc-transferase KfiC: GlcA-transferase glmU: UDP-GlcNAc pyrophosphorylase/GlcN-1-P *N*-acetyltransferase glmM:Phosphoglucosamine mutase galU: UDP-Glu pyrophosphorylase glmS: L-glutamine-D-fructose-6-P aminotransferase ugd: UDP-Glu dehydrogenase	*C. glutamicum*	Fed-batch (3) Shake flasks	5.8 0.1	-	3000	(L. Hu et al., 2022)
KfiA, KfiC (*E. coli* K5) ugd, glum and pgma (*L. lactis* NZ9000)	Concurrent expression and screening of KfiA and KfiC, overexpression of ugd, glmu and pgma using Nisin control-based expression system	KfiA: GlcNAc-transferase KfiC: GlcA-transferase ugd: UDP-Glu dehydrogenase glmu: UDP-GlcNAc pyrophosphorylase/GlcN-1-P *N*-acetyltransferase pgmA: phosphoglucomutase	*L. Lactis* (SH6*)*	Fed-batch (3) Batch	1.263 0.754	30-0.042 -	10-20	(Guhan et al., 2022)
BstauD and BsytdA *(B. subtilis),* CgugdA2 *(C. glutamicum),* Ecugd *(E. coli),*Ppcugd *(P. putida),* KfiA and KfiC *(E. coli* Nissle 1917*)*	Under the control of two strong promoters, UDP-glucose dehydrogenase-encoding genes from distinct sources were separately expressed in conjunction with heparosan polymerase-encoding genes KfiA and KfiC	tauD, sytdA, ugdA, cugd: UDP-Glu dehydrogenase KfiA: GlcA-transferase KfiC: GlcNAc-transferase	*P. Pastoris (23 and 24)*	Shake flasks	0.101 (Pp23) 0.165 (Pp24)	-	-	(Zhang et al., 2022)

KfiA and KfiC *(E. coli* K5*)*	Enlarging the 19-kb kps locus via directed chromosome evolution.	KfiA: GlcA-transferase KfiC: GlcNAc-transferase	*E. coli* Nissle 1917	Fed-batch (5) Shake flasks Batch	9.1 104mg/l/OD	40-0.228	-	(Yu et al., 2023)

Table 3-2

An overview of heparin/HS production through metabolically engineered eukaryotic hosts (TriS: trisulfate)

Engineering approaches	Target and encoded enzyme function	Hosts	Maximal Titer	Product type	Anti-FXa activity and MW	Sulfation degree	References
Exogenously expressing two crucial biosynthetic enzymes	❖ NDST2, Hs3st1 NDST: *N*-deacetylation and sulfation of GlcNAc Hs3st: Sulfation of GlcN at C-3	CHO-S cells	173.2μg/ 5×10^7cells (Culture medium) 15.5 μg/ 5×10^7cells (Cell pellets)	HS/Heparin	--	**Cell pellets** NS-83.7% **Culture medium** 1. NS-97.5% 2. NS6S and TriS disaccharide (minor amount)	(Baik et al., 2012a)
Developing Golgi-targeted Hs3st1 and enhancing Hs3st1 expression in the Golgi apparatus to improve control over heparin/HS polymerization	❖ Hs3st1 HS3st: Sulfation of GlcN at C-3	CHO-S cells	3.68 μg/10^7 cells (Cell pellets)	HS/Heparin	~137 U/mg	**Cell pellets** 1. TriS 9.7%, 2. NS6S 1.1%, 3. NS2S 6.3%, 4. NS 17.1%, 5. 6S 1.5%, 6. 2S 2.7% and 7. 0S 61.6%. **Culture medium** 1. TriS 10.9%, 2. NS6S 1.7%, 3. NS2S 5.7%, 4. NS 14.0%, 5. 6S 1.8%, 6. 2S 2.1% and 7. 0S 63.8%.	(Datta et al., 2013a)
Increasing the expression of Hs3st1, a pivotal biosynthetic enzyme	❖ Hs3st1 Hs3st: Sulfation of GlcN at C-3	MST cells (MST-10H)	1.8 μg/10^7 cells (Cell pellets)	HS/Heparin	50-60 U/mg 10-12 kDa	**Cell pellets** 1.TriS 63.3%, 2. NS6S 20.6%, 3. NS2S 4.3%, 4. NS 7.2%, 5.6S 2.1%, 6.2S 0.7% and 7.0S 1.8%. **Culture medium** 1.TriS 30.8%, 2.NS6S 18.8%, 3.NS2S 11.4%, 4.NS 11.7%, 5.6S 4.5%, 6.2S 10.5% and 7.0S 12.3%.	(Gasimli et al., 2014)

Enhancing the impact of bioprocess variables on anticoagulant effectiveness and production yield	--	CHO-S cells	~90 µg/ml (Fed-batch 2L)	HS/Heparin	--	**Culture medium** NS structures, with small amounts of NS6S, NS2S and 0S	(Baik et al., 2015)
Biosynthesis of heparin-like HS is achieved through recombinant expression of serglycin in human cells	❖ Serglycin Serglycin: CS/DS and HS/heparin proteoglycan	HEK-293	8.4-25.1 µg/ml (Culture medium)	HS/Heparin	--	**Culture medium** 1.ΔUA,S – GlcNS,6S-19% 2.ΔUA,2S – GlcNAc,6S-10% 3. ΔUA – GlcNS,6S-10% 4. ΔUA,2S – GlcNS-14% 5. ΔUA – GlcNAc,6S-16% 6. ΔUA,2S – GlcNAc-8% 7. ΔUA – GlcNS -8%% 8. ΔUA –GlcNAc -15%	(Lord et al., 2016)
Implemented a multiplex genome engineering approach to overexpress genes and eliminate contaminating chondroitin sulfate	❖ Hs3st1, Hs6st1, Hs6st2, NDST2, Csgalnact1, Csgalnact2 and Chsy1 Hs3st: Sulfation of GlcN at C-3 Hs6st: Sulfation of GlcN at C-6 NDST: *N*-deacetylation and sulfation of GlcNAc	MST cells	6.0 mg/L (Cell pellets) 0.72 mg/L (Culture medium) (Multiple flasks1L)	HS/Heparin	259-304 U/mg 25.7-33.0 kDa	**Cell pellets** 1.0S: 4-5% 2.NS: 85-87% 3.2-O-S:63-65% 4.6-O-S: 89-90%	(Thacker et al., 2022)
Enzymes responsible for heparin biosynthesis are effectively expressed through Tag-fusion and N-terminal engineering strategies	❖ NDST, C5epi, 2-OST, 6-OST and 3-OST 3-OST: Sulfation of GlcN at C-3 6-OST: Sulfation of GlcN at C-6 NDST: *N*-deacetylation and sulfation of GlcNAc 2-OST: Sulfation of IdoA and GlcA at C-2 C5epi: C5 epimerase	*P. pastoris* (28)	2.08 g/L (Fed-batch 3L)	Heparin	79.9 ng/mL 349 kDa	**Culture medium** 1. ΔUA -GlcNS and ΔUA 2S-GlcNAc- 0.77% 2. ΔUA 2S-GlcNS and ΔUA -GlcNS6S-1.61% 3. ΔUA 2S-GlcNS6S and ΔUA 2S-GlcNS6S3S-1.99%	(Zhang et al., 2022)

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Chapter 4

Production of heparin-like polysaccharides using recombinant CHO cells

4.1 Background

Heparin, a centuries-old therapeutic agent, has long been recognized for its remarkable anticoagulant properties (Mulloy et al., 2016; Onishi et al., 2016). Since its discovery in 1916, this glycosaminoglycan has been influential in the prevention and treatment of thrombotic disorders, saving countless lives globally (Aláez-Versón et al., 2017; Contejean, 1895; Hemker, 2016; Mulloy et al., 2016; Onishi et al., 2016; Torri & Naggi, 2016; Wardrop & Keeling, 2008). However, beyond its well-established anticoagulant role, emerging research has uncovered a plethora of novel therapeutic applications for heparin. The escalating global demand for heparin anticipates a market expansion exceeding $14 billion within the coming decade, reflecting significant growth prospects in the industry (Oduah et al., 2016; Zhang et al., 2022).

Currently, porcine intestinal mucosa serves as the primary global source of heparin API (active pharmaceutical ingredient) and is the sole FDA (US Food and Drug Administration)-approved source (Douaisi et al., 2024). However, the extraction of heparin from animal tissues presents substantial challenges, encompassing concerns regarding adulteration, contamination by prions and viruses, as well as the limitations of supply chains that are often unreliable (Kishimoto et al., 2008; Mans et al., 2015; Thacker et al., 2022). Moreover, ethical and religious considerations are heightened due to the use of animal-derived heparin, which also increases the risk of transmitting zoonotic diseases (Douaisi et al., 2024). Hence, there is a continual and compelling need to explore efficient alternative methods for synthesizing heparin, which remains both constraint and fascinating.

Heparin, a complex polysaccharide with a diverse array of monomeric units, undergoes a natural biosynthetic process within the endoplasmic reticulum (ER) and Golgi apparatus. This process follows a highly structured pathway involving more than 22 distinct enzymes (Fig. 2) (Baytas and Linhardt, 2020). The biosynthesis of the heparin proteoglycan core protein, serglycin, initiates within the ER (Stevens and Adachi, 2007). This process begins with the attachment of a tetrasaccharide linker to a serine residue in the core protein, composed of xylose, two galactose units, and glucuronic acid. The coupling process is facilitated by xylosyltransferase (XylT)-1 or -2, followed by the sequential addition of two galactose units by galactosyltransferase (GalT) -1

and -2, and the incorporation of glucuronic acid by glucuronosyltransferase (GlcAT) (Sugahara and Kitagawa, 2002). Once the linker construction is complete, monosaccharide addition to its non-reducing end is achieved by three isoforms of the EXT glycosyltransferase family. The polysaccharide chains are polymerized by sequentially adding a GlcA residue followed by a GlcNAc residue to the chain through the actions of the EXT1 and EXT2 enzymes (Farrugia et al., 2015).

The biosynthesis further progresses with the involvement of N-deacetylase/N-sulfotransferase (NDSTs) as the initial step, followed by glucuronyl C5-epimerase (GLCE), 2-O-sulfotransferases (HS2ST1), 6-O-sulfotransferases (HS6STs), and finally, 3-O-sulfotransferases (HS3STs). Interplay between these enzymes is expected. Initially, N-acetyl groups are replaced with N-sulfo groups by NDST isoforms, particularly NDST-2, which plays a crucial role in modifying the GAG chains on the serglycin core protein, essential for mast cell heparin synthesis (Kreuger and Kjellen, 2012). The maturation phase involves intricate modifications to sugar units, primarily orchestrated by Golgi-associated sulfotransferases from various families. This includes epimerization of GlcA to IdoA residues by GLCE, the transfer of sulfate groups to the C-2 position of most IdoA and some GlcA by HS2ST1, and the attachment of sulfate groups to either GlcNAc or GlcNS residues at the C-6 position by HS6STs. Finally, GlcNAc and GlcNS residues undergo 3-O-sulfation by HS3STs (Liu and Linhardt, 2014). These steps are crucial for completing the heparin biosynthesis process and is highly conserved in vivo (**Fig. 4-1**). The heparin chains that result typically exhibit a molecular weight ranging from 60,000 and 100,000 Da.

Over the past two decades, diverse strategies have been explored to find alternative sources for heparin. These efforts encompass reintroducing bovine or ovine heparin (Jeske et al., 2019), which, as animal-derived options, exhibit lower anticoagulant activity compared to commercial heparin and raise concerns related to prion-mediated diseases (Tovar et al., 2012; Vilanova et al., 2019). Chemical synthesis has successfully yielded fondaparinux (Arixtra), an ultra-low-molecular-weight-heparin (ULMWH) analog offering precise structural control despite challenges such as low yields and high costs (Walenga, 2005). In the realm of animal-free synthesis, focus has been on de novo chemical synthesis, with a specific emphasis on producing low-molecular-weight-heparins (LMWHs), alongside biosynthetic approaches and metabolic engineering in both prokaryotic and eukaryotic systems (Lord & Whitelock, 2014). Metabolic engineering endeavors

in yeast and mammalian cells have led to heparin variants that differ from those outlined by US Pharmacopeia (USP) standards, thereby limiting scalability and yield.

Chinese hamster ovary (CHO) cells have emerged as a preferred host system for bioengineered heparin production. CHO cells offer several advantages, including their well-established culture systems, robust growth characteristics, and compatibility with recombinant protein expression (Thacker et al., 2021; Thacker & Sharfstein, 2018). Furthermore, CHO cells provide a mammalian cell environment conducive to the proper folding and post-translational modifications required for heparin biosynthesis (Baik, Gasimli, et al., 2012; Baik, Wang, et al., 2012). This choice of host organism ensures the production of high-quality, bioactive heparin suitable for therapeutic use. Additionally, recent advancements in genome editing technologies, such as CRISPR-Cas9, have facilitated the generation of engineered CHO cell lines with enhanced productivity and improved product quality, further optimizing the bioengineering process for heparin production (Cress et al., 2015).

Previous studies have aimed to engineer the biosynthetic pathway of HS in CHO cells by introducing bifunctional NDST2 and Hs3st1 genes, leading to the production of heparin-like polysaccharides (Baik, Gasimli, et al., 2012). Despite these efforts, the anticoagulant activity of the engineered CHO-derived heparan sulfate remained approximately 50-fold lower than that of porcine-derived heparin (Baik, Gasimli, et al., 2012). This indicates a need for further optimization of the biosynthetic pathway to enhance heparin-like activity. To address this, we focused on the inactivation of desulfation enzymes (**Fig. 4-1**) and the elimination of the chondroitin sulfate (CS) biosynthetic pathway. CS production competes with HS for precursor metabolites and must be removed by exhaustive enzymatic digestion (Thacker et al., 2022). Inactivation of CS biosynthetic pathways has been shown to increase HS production with anticoagulant activities exceeding pharmaceutical-derived heparin (Thacker et al., 2022).

Our research utilized CRISPR/Cas9 technology to generate multiple knockout (KO) cell lines targeting genes responsible for CS biosynthesis. Specifically, we disrupted the CSGALNACT1 and CSGALNACT2 genes (**Fig. 4-2**), which play crucial roles in catalyzing the transfer of GalNAc residues onto core proteins, initiating CS chain formation. This genetic modification aimed to redirect the metabolic flux towards HS biosynthesis, enhancing the production of heparin-like polysaccharides with improved anticoagulant properties.

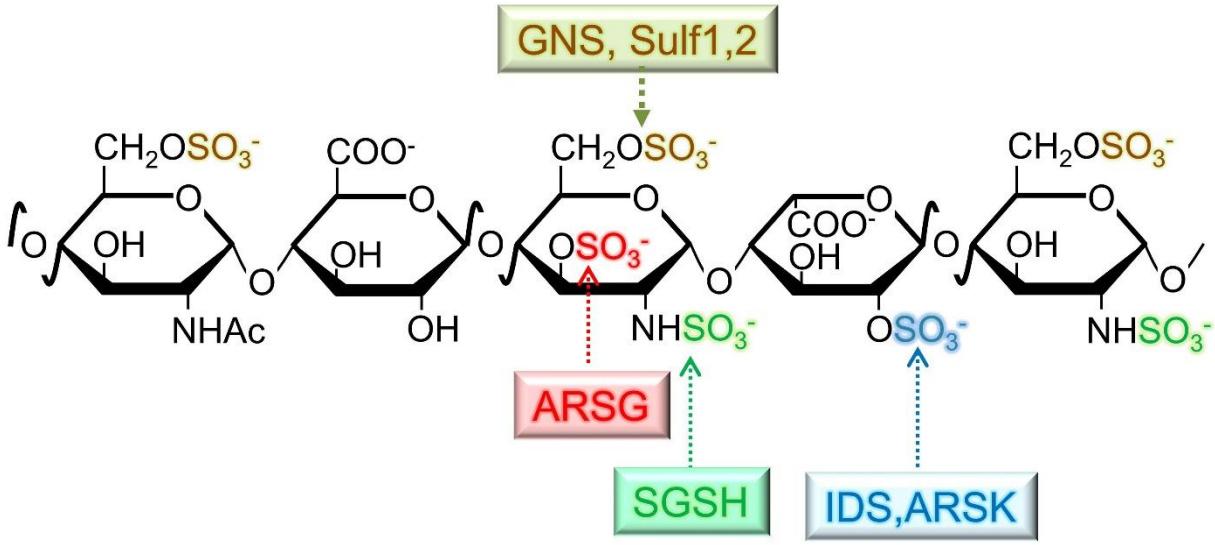


Fig. 4-1 Overview of heparin/HS desulfation mechanism.

We further investigated the effects of culturing these engineered cell lines at lower temperatures, as shifts from the typical culture temperature of 37°C to 33-30°C have been shown to enhance recombinant protein production by improving mRNA stability, transcription levels, and protein folding (Al-Fageeh et al., 2006; McHugh et al., 2020; Nguyen et al., 2020). Additionally, lower temperatures reduce total cell metabolism, decrease the production of toxic substances, and increase cell viability.

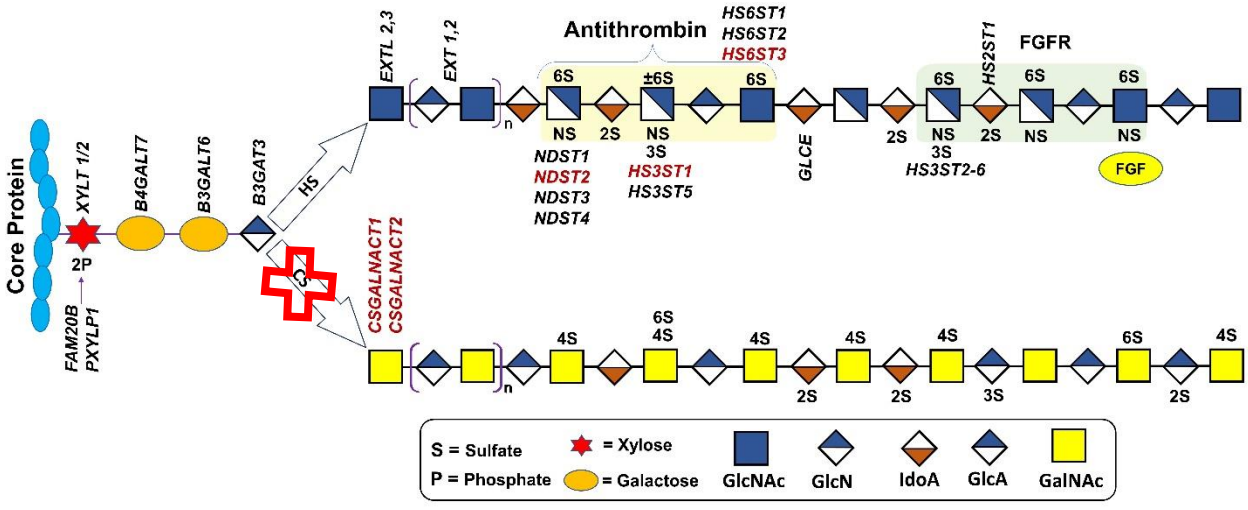


Fig. 4-2 Biosynthesis of glycosaminoglycans. This illustration represents HS (upper chain) and CS (lower chain) structures with antithrombin binding pentasacchairde and enzymes responsible for HS and CS biosynthesis.

4.2 Aim

In this study, we present the construction and characterization of multiple KO cell lines, the optimization of culture conditions, and the resulting improvements in heparin-like polysaccharide production and anticoagulant activity. Our findings highlight the potential for recombinant CHO cells to serve as a viable platform for the industrial production of bioengineered heparin and HS.

4.3 Methods and materials

4.3.1 Cells and media

CHO-S (#R80007; Invitrogen, Waltham, MA, USA) and recombinant CHO cells were cultured in F12 medium (#N6760; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (BioWest, Nuaillé, France), 100 U/mL Benzylpenicillin potassium (#021-07732; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 100 mg/L Streptomycin sulfate (#194-08512; Fujifilm Wako Pure Chemical Corporation) and 8 mM L-glutamine (#074-00522; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). For bio-engineered heparin production in serum-free cultures, FreeStyle CHO Expression Medium (#12651014, Invitrogen) containing 8 mM L-glutamine and antibiotics (penicillin-streptomycin) was used. Cells were cultured in a 5% CO2 incubator at 37°C as usual. Cells were maintained in F12 medium containing FCS using 100-mm cell culture dishes (BioLite #130182; Thermo Scientific, Waltham, MA, USA). For serum-free suspension culture, cells were incubated using bioreactor tubes (#87050; TPP Techno Plastic Products AG, Trasadingen, Switzerland) placed in a shaker (Model #0081704-000; Taitec, Koshigaya, Japan) with a 45° angle at 37°C or 33°C in a 5% CO2 incubator.

4.3.2 Plasmid construction

For the construction of sulfation-related enzyme expression vectors, mouse Hs3st1, human NDST2 and mouse Hs6st3 genes were amplified by PCR using MGC cDNA clone library (#MMM1013-202761171 for Hs3st1 and #MHS6278-202802041 for NDST2, Horizon Discovery, Cambridge, UK) and Mouse Tagged ORF Clone (#MR217272 for Hs6st3, Origene Technologies, Rockville, MD, USA) as a template, respectively. The Hs3st1- and NDST2-encoding DNA fragments obtained by PCR were inserted into EcoRI-XhoI-digested pcDNA4/myc-His A (#V863-20; Invitrogen) and BglII-NotI-digested pIRES2-DsRed-Express (#632463; Invitrogen) to generate pcDNA4/Hs3st1 and pCMV/NDST2, respectively.

To generate a SDC-expression vector as a secretory carrier of polysaccharide chains, DNA fragment encoding an extracellular domain of human SDC1 (1-229 amino acid, Accession number,

NP_001006947) was chemically synthesized (GeneArt, Thermo Fisher Scientific). To establish recombinant CHO cells co-expressing SDC and EGFP genes as a reporter, an IRES-EGFP gene was amplified by PCR from pQMSCV/IRES-EGFP (Akiyama et al., 2010) and inserted into a PiggyBac transposon vector (#PB513B-1; System Biosciences, Palo Alto, CA, USA) to generate PB/IRES-EGFP-2A-Puro. Chemically synthesized SDC gene together with Chinese hamster-derived EF1α promoter from PB/chEF1α/GEV_Hyg (Raman et al., 2023) were inserted into PB/IRES-EGFP-2A-Puro to generate PB/chEF1α/SDC1-IRES-EGFP-2A-Puro.

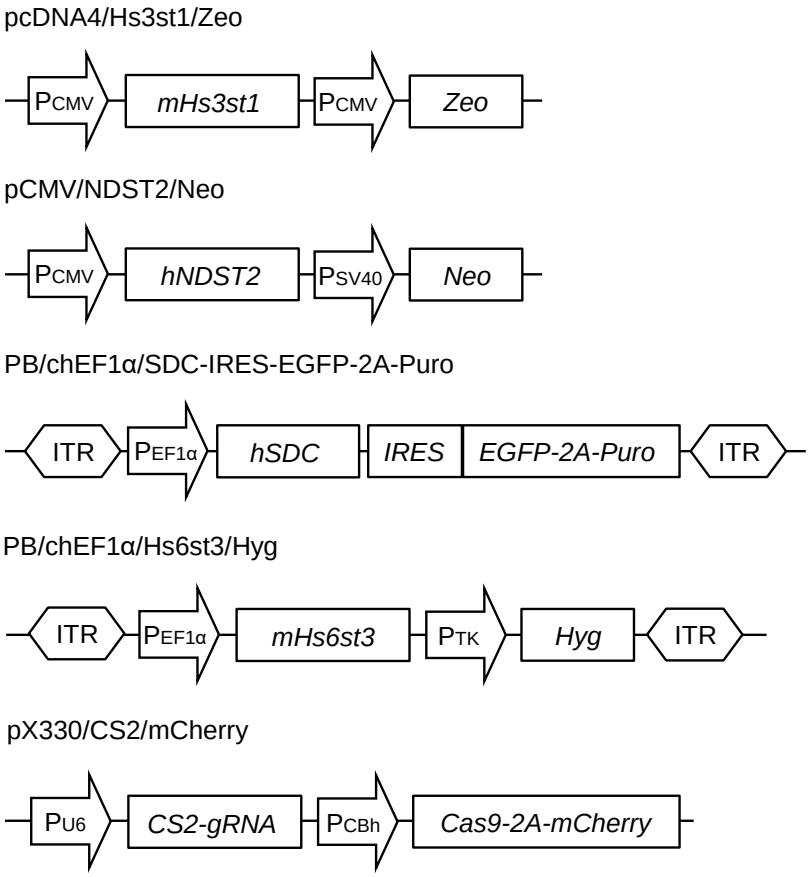


Fig. 4-2 Schematic representation of all expression plasmids. PCMV, Cytomegalovirus promoter; mHs3st1, mouse 3-O-sulfotransferase; hNDST2, human *N*-deacetylase/*N*-sulfotransferase 2; hSDC, human syndecan; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein derived from Aequorea Victoria; ITR, inverted terminal repeats, mHs6st3, mouse 6-O-sulfotransferase 3; U6, a type III RNA polymerase III promoter; CS2, chondroitin sulfate *N*-acetylgalactosaminyltransferases 1; gRNA, guide RNA; mCherry, bright red monomeric fluorescent protein; Zeo, zeocin; Neo, neomycin; Hyg, hygromycin.

A DNA fragment encoding Hs6st3 was inserted into XbaI-NruI-digested PB/chEF1α/GEV_Hyg to generate PB/chEF1α/Hs6st3/Hyg. To construct Cas9 and mCherry co-expression vector plasmid, a DNA fragment encoding Cas9-2A-mCherry was obtained from pU6-(BbsI)CBh-Cas9-T2A-mCherry (#64324, addgene) and inserted into BglII-EcoRI-digested pX330-U6-Chimeric_BB-CBh-hSpCas9 (#42230, addgene) to generate pX330/mcherry. Two oligo DNAs encoding CS2 guide RNA (gRNA) were chemically synthesized (Thermo): 5'-CAC CGA ACT TCA GCT CTG TCG ATC T-3' and 5'-AAA CAG ATC GAC AGA GCT GAA GTT C-3'. Annealed oligo DNA fragments were inserted into BbsI-digested pX330/mCherry to generate pX330/CS2/mCherry.
All PCRs were conducted using KOD plus Neo DNA polymerase (#KOD-401, Toyobo, Tsuruga, Japan) with primers shown in Table 4-1. The sequences derived from PCR products were confirmed by Sanger sequencing.

Table 4-1 Primers for PCR cloning and oligos for annealing

Gene	**Sequence (5'→3')**	
mHs3st1	F	AGATCTGAATTCGCCGCCACCATGACCTTGCTGCTCCTGGG
	R	AAACTCGAGAGATCTTCAGTGCCAGTCGAATGTTCTG
hNDST2	F	AAAAGATCTGCCGCCACCATGCTCCAGTTGTGGAAGGT
	R	AAAAGCGGCCGCGGATCCTCAGCCCAGACTGGAATGCT
mHs6st3	F	AAAATCTAGAGCCGCCACCATGGATGAAAGGTTCAACAAGTGGCTGC
	R	TTTAAATCGCGATTATCACCATCTGACCACTTGGCTGTTGTAGTCC
IRES-GFP	F	AAAGCGGCCGCGCCCCTCTCCCTCCCCC
	R	TTTTCGCGACCCTTGTACAGCTCGTCCATGCCGAG

4.3.3 Generation of recombinant CHO cells

To establish recombinant CHO cells expressing the Hs3st1 and NDST2 genes (CHO/2F), CHO-S cells were seeded in a 24-well plate at 2.0 x 10^5 cells/well using F12 medium containing 10% FBS to attach the cells onto the plate before day of transfection. Then, PvuI-lineralized pcDNA/Hs3st1 and ApaLI-digested pCMV/NDST2 expression vector plasmids (each 400 ng) were co-transfected

into CHO-S cells using Lipofectamine 2000 (#11668019, Invitrogen) according to the manufacturer's procedure. After 48 h, cells were plated in 100 mm dishes (BioLite #130182, Thermo Fisher Scientific) to begin corresponding drug selection (200 mg/L Zeocin (#R25001, Invitrogen) and 400 mg/L G418 (#A1720; Sigma-Aldrich). The drug-resistant cells were passaged for three times. Stable transformed cells expressing Hs3st1 and NDST2 were obtained. Cell clones (CHO/2F) were established by a limiting dilution method.

Next, to generate SDC-expressing CHO/2F cells, the cells (1.0×10^5 cells) were prepared for transfection using a Neon transfection system as described previously (Kawabe et al., 2015) expect for a modification of pulse voltage (1,600 V). The SDC expression vector plasmid (850 ng PB/chEF1α/SDC-IRES-EGFP-2A-Puro) were transiently co-transfected with a PiggyBac transposase expression vector plasmid (170 ng pPBase, #PB210PA-1, System Biosciences) into CHO/2F cells. After 7-day culture of transfection, the transfectants were seeded onto the 6-well tissue culture plates (BioLite #180184, Thermo Fisher Scientific) and selected using 50 mg/L Puromycin (#A1113803, Invitrogen) for 7 days with 3 passages to obtain stable transformed cells. Cell clones (CHO/2F-SDC) were established with a limiting dilution method.

For creating Hs6st3-expressing CHO/2F-S (SDC) cells, CHO/2F-S cells were seeded at the density of 1.2 x 10^6 cells/well into 6-well tissue culture plate. The next day, Hs6st3 expression vector plasmid (PB/chEF1α/Hs6st3/Hyg) was transiently transfected into CHO/2F-SDC without pPBase using a lipofection method as described above. After 48 hours of post-transfection, the cells were screened with 400 mg/L Hygromycin (Fujifilm Wako Pure Chemical Corporation) for 14 days with several passages. The stable expression cells were subjected to limiting dilution to establish cell clone (CHO/3F-SDC).

Finally, CS knock-out cell clones were established as follows. The gRNAs for exons of the specific target gene were designed using CRISPRdirect software (http://crispr.dbcls.jp) (Naito et al., 2015) and the details are provided in Table 4-2. The gRNA/Cas9 expression vector plasmids (pX330/CS2/mCherry) were transiently transfected into CHO/3F-SDC cells using a similar lipofection method in 24-well plate scale as noted above. After 2 days' culture, mCherry-positive cells were sorted using a cell sorter (Model #SH800, Sony, Tokyo, Japan). Limiting dilution were conducted for the obtained pooled cells to establish cell clone (CHO/3F-SDC-CKO). To confirm the targeted gene disruption, genomic DNAs were extracted from CHO/3F-SDC-CKO cell clone using a commercially available kit (#NPK-401, Toyobo) and genomic PCR analysis was

performed using TaKaRa Ex Premier DNA polymerase (#RR370A, Takara, Kusatsu, Japan) with primer pairs: 5'-TTC TTC ACA GCC GGA CCC AG-3' and 5'-CAC ATG CAC ACA CGC CAC AG-3'. PCR products were evaluated by Sanger sequencing.

Table 4-2 gRNA sequences targeting each gene

Gene	Accession No.	Function	gRNA/PAM	Position
SGSH	NW_003614274	De-*N*-sulfation	GACCAGCAGCACCGCGCAGC/AGG	exon1
GNS	NW_003615106	De-6-O-sulfation	GAGTGTTGTTCACGACGTGA/TGG	exon2
CS1	NW_003613760	GlcNAc transition	GGTACATGCAGGCGTCAAAC/TGG	exon1
CS2	NW_003614048	GlcNAc transition	AACTTCAGCTCTGTCGATCT/GGG	exon1

4.3.4 Measurement of anti-coagulant activity and sulfated GAG

For producing bio-engineered heparin, cells were seeded with 10 mL in a bioreactor tube at a density of 1.0×10^6 cells/mL at 37°C for 8 days as a batch culture. For culturing continuous production at high-cell-density (33°C and 30°C), the cells were seeded at a seeding density of 2.0×10^7 cells/mL and culturing for 30 days at both 33°C and 30°C. In case of 33°C, the medium was exchanged to fresh medium every other day, and the cells were re-seeded at the same initial density after counting the number of cells at that time. However, in case of 30°C, the medium was exchanged to fresh medium every third day. This reduced frequency of medium exchange at 30°C is likely due to the slower metabolic rate and reduced growth rate of the cells at the lower temperature, which results in lower nutrient consumption and waste production (Bárcenas-Moreno et al., 2009). As a result, the medium remains viable for a longer period, necessitating less frequent changes.

Anti-coagulant activities were measured using commercially available kits (Anti-Factor Xa, #221005; Anti-Factor IIa, #220005; both from HYPHEN BioMed, Neuville-sur-Oise, France). sGAG concentration was analyzed by Blyscan Glycosaminoglycan Assay Kit (#B1000; Biocolor Ltd., Co Antrim, UK) with some modification as follows: To enhance the precipitations, supernatant samples after adding a dye solution were stored at 4°C for overnight. Heparan sulfate (#H7640, Sigma-Aldrich) was used for standard curve. Specific anti-coagulant activities were calculated based on anti-coagulant activities and sGAG concentration.

4.3.5 Disaccharide analysis

The structural analysis and functional evaluation of heparin-like polysaccharides produced by various CHO cells were conducted in collaboration with the Laboratory of Pathological Analytical

Chemistry at the Graduate School of Pharmaceutical Sciences, Tokyo University of Science. The analyses were performed by Kyohei Higashi, Ph.D., Associate Professor, Physical pharmaceutical science, and the work is still ongoing**.**

4.4 Results

4.4.1 Establishment of different producer CHO cell lines

All mammalian cells generate HS, though the sulfation patterns and the ratios of IdoA to GlcA acid differ across cell types. Typically, HS has a significantly lower sulfation degree compared to heparin. Previous studies aimed to engineer the biosynthetic pathway of HS in CHO cells by introducing bifunctional NDST2 and Hs3st1 genes to produce heparin-like polysaccharides (Baik et al., 2012). However, despite enhancements, the engineered CHO HS exhibited about 50 times less anticoagulant activity than porcine-derived heparin. To address this issue, we initially developed a parental cell line designated as CHO/2F. Next, two additional engineered cell lines, named CHO/2F-S (SDC) and CHO/3F-S were previously established in our laboratory. Details of the culturing process were elaborated in section 4.3.3 of the materials and methods. These two engineered cell lines successfully enhanced anticoagulant activity, albeit lower compared to native heparin. Therefore, to enhance production of heparin-like polysaccharides further, we have established several knockout cell lines. Details about establishment of knock out cell clones were also discussed in materials and methods 4.3.3 section and further will discuss in section 4.4.2 of the results.

4.4.2 Construction of multiple knockout cell pool

We have constructed multiple knockout cell pools aimed at enhancing anticoagulant and anti-FIIa activities in recombinant CHO cells. To enhance anticoagulant activity and anti-FIIa activity, our focus centered on two pivotal strategies: the inactivation of desulfation enzymes and the elimination of the CS biosynthetic pathway. To deactivate the desulfation enzymes, we have chosen both SGSH and GNS, which are responsible for de-O-sulfation. In addition, we found that mammalian cells also produce CS alongside HS, requiring extensive enzymatic digestion for removal during purification. This CS production competes with HS for essential precursor metabolites as observed in previous studies on MST cells (Gasimli et al., 2014). Recently, Thacker et al. reported that inactivating the CS biosynthetic pathway resulted in the highest levels of HS production (Thacker et al., 2021; Thacker et al., 2022). The biosynthesis of HS and CS initiates

with the assembly of a linkage tetrasaccharide attached to serine residues on core proteins. Subsequently, CS biosynthesis diverges through the action of specialized CS biosynthetic enzymes (**Fig. 4-3**). Thus, we focused on CS1 and CS2, both are playing crucial roles in CS biosynthesis by catalyzing the transfer of GalNAc residues onto core proteins, initiating the formation of CS chains. Details regarding the characteristics of the targets were presented in Table 4-2. In our unpublished work, established engineered cell line (CHO/3F-S) already produced significant amount of heparin like polysaccharides in cell suspension. Thus, we utilized CRISPR/Cas9 to target SGSH, GNS, CS1 and CS2 in engineered CHO/3F-S, resulting in the generation of four distinct types of KO cell pools. Details regarding generation of KO cell pools and clones were discussed in the materials and methods section 4.3.3. To determine the optimal knockout KO cell pool, we assayed for sGAG concentration, Anti-FXa activity, and Anti-FXa specific activity. Among the four KO cell pools, pool CS2 demonstrated lower Anti-FXa activity compared to both the parental cell line and the other knockout pools (**Fig. 4- 3A**). Furthermore, a notable decrease in sGAG concentration was observed (**Fig. 4-3 B**). However, the exact influence of CS knockout on this reduction remains unclear. Interestingly, pool CS2 exhibited an increase in Anti-FXa specific activity, approximately 1.6-fold higher than that of the parental cell line (**Fig. 4-3 C**). Hence, we selected CS2 cell pool as a target gene. Following transfection, we isolated single-cell clones from CS2 cell populations using limiting dilution. Screening 30 clones by assays identified 3 clones with highest activity. CHO/3F-S_CKO#24, selected based on its enhanced Anti-FXa specific activity, underwent further validation. Next-generation sequencing of PCR amplicons from the mutated region of CS2 revealed a prevalent DNA sequence characterized by an 8-base deletion at the expected position (**Fig. 4-3 D**). Finally, we successfully established a stable CHO/3F-S_CKO cell line for further experimental analysis.

Ensuing verification, we compared this cell line with our previously developed engineered cell lines, CHO/2F-S and CHO/3F-S, focusing on sGAG concentration, Anti-FXa and Anti-FIIa activity, and their specific activities.

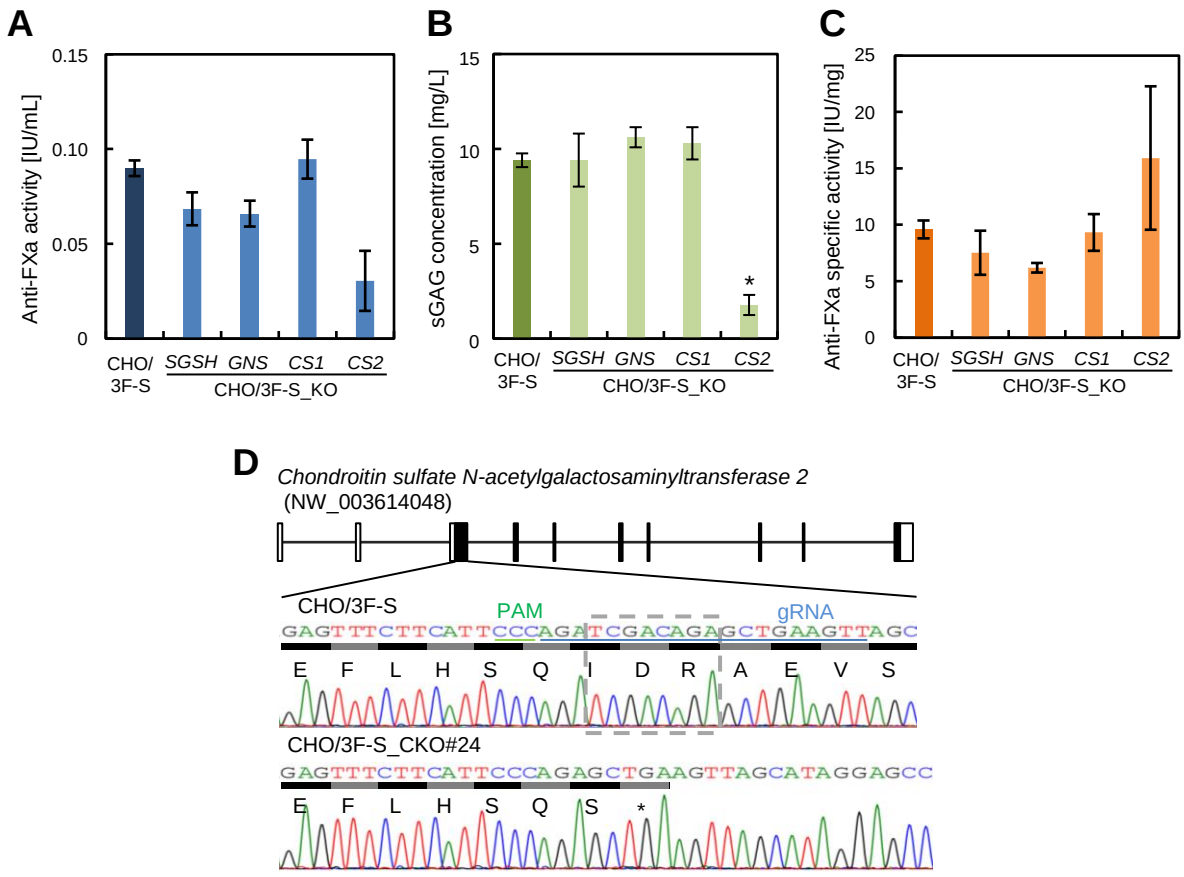


Fig. 4-3 Generation of different KO cell pools using gRNA/Cas9 expression plasmids and verification of desired KO cell clones. A. Anti-FXa activy; B. sGAG concentration; C. Anti-FXa specific activity; and D. Verification of CHO/3F-S_CKO # 24 clone.

The Anti-FXa and Anti-FIIa activities in the newly generated CHO/3F-S_CKO cell line remained at the same levels as before the gene disruption (CHO/3F-S) (**Fig. 4-4 A and B**). In contrast, the concentration of sGAG decreased by approximately 50% compared to pre-disruption levels (**Fig. 4-4 C**). This reduction is attributed to the disruption of enzyme genes involved in the early stages of CS synthesis, leading to a decrease in glycan production. As a result, the specific activity of Anti-FXa in the CHO/3F-S_CKO cell line was 1.6-fold higher than that of the parental cell line (**Fig. 4-4 D**), and the specific activity of Anti-FIIa was 1.9-fold higher (**Fig. 4-4 E**). These findings suggested that the genetic modifications not only successfully reduced the synthesis of competing chondroitin sulfate but also significantly enhanced the anticoagulant properties of the engineered cell line, making it a promising candidate for further heparin-like polysaccharide production.

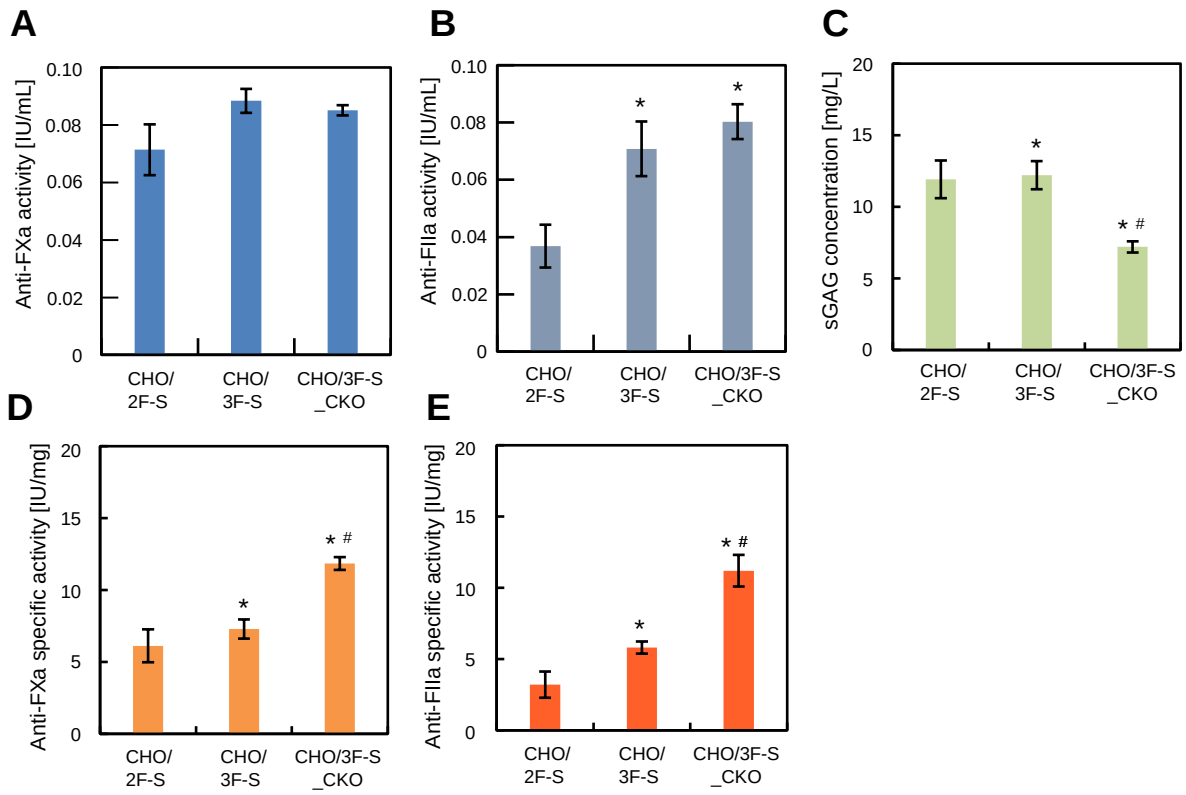


Fig. 4-4 Comparison of the newly generated CHO/3F-S_CKO cell line with the two previously established cell lines in the current laboratory. A**.** Anti-FXa activity; B. Anti-FIIa activity; C. sGAG concentration; D. Anti-FXa specific activity; and E. Anti-FIIa specific activity.

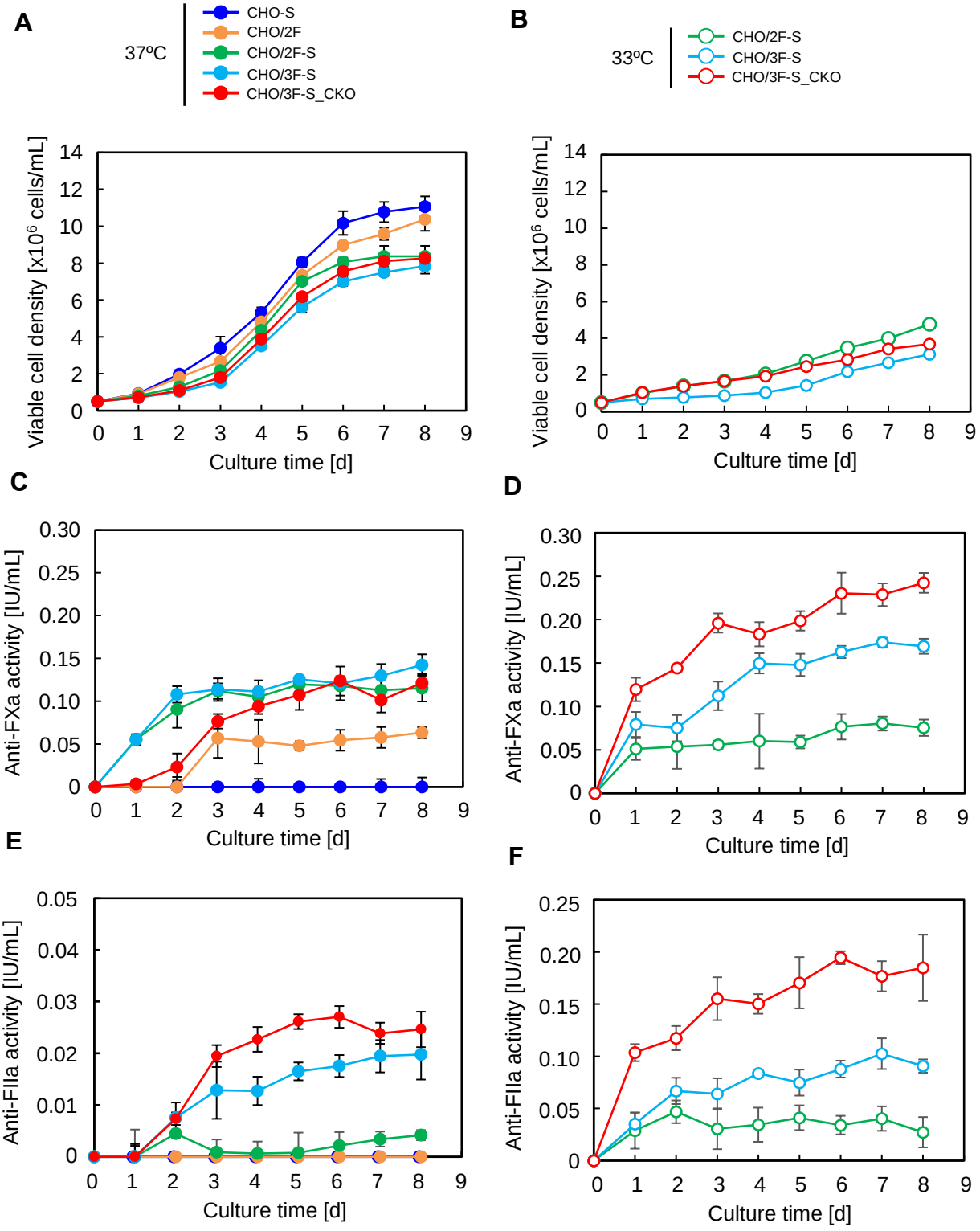
4.4.3 Heparin-like polysaccharide production in low-temperature cultures

In recent research, lowering the temperature for culturing CHO cells has proven effective in boosting industrial production of recombinant proteins (Al-Fageeh et al., 2006; McHugh et al., 2020; Nguyen et al., 2020). This approach, known as temperature shift (TS) (from 37°C to 33°C and 30°C), enhances specific productivity by stabilizing mRNA, increasing transcription rates, and improving protein folding. Moreover, it decreases overall cellular metabolism, which minimizes the production of harmful substances, adapts to nutrient limitations, and enhances cell survival (Kantardjieff et al., 2010; Shahabi et al., 2023a; Yee et al., 2009). However, the influence of the low-TS on heparin-like polysaccharide production in CHO cells has not been investigated. Therefore, we cultured CHO/3F-S_CKO alongside the previously developed two engineered and one parental cell lines at 33°C to assess their impact on bioengineered heparin/HS production. When cultured at 37°C, the growth curve exhibited a typical pattern with rapid logarithmic (**Fig. 4-5 A**) however, at 33°C, there was minimal growth observed throughout the entire culture period

(**Fig. 4-5 B**). In terms of Anti-FXa activity, CHO/3F-S_CKO achieved an activity of approximately 0.25 IU/ml on day 8 of culture at 33°C, showing a 2-fold increase compared with that upon cultivation at 37°C (**Fig. 4-5 C and D**).

Similar phenomena were observed with respect to Anti-FIIa activity at 37°C and 33°C (**Fig. 4-5 E and F**). In terms of sGAG concentration, CHO/3F-S_CKO exhibited a substantial reduction compared to the other established cell lines (CHO/2F-S and CHO/3F-S), measuring approximately 4 mg/L on day 8 at typical culture temperature (**Fig 4-5 G**), whereas at 33°C, the concentration was slightly higher than upon cultivation at 37°C (**Fig. 4-5 H**). The specific Anti-FXa and Anti-FXa activity of CHO/3F-S_CKO averaged 40 IU/mg and 70 IU/mg (**Fig. 4-5 I and J**), which was 2-fold higher than upon cultivation at 37°C on day 3. Furthermore, regarding Anti-FIIa specific activity, at 37°C, we observed approximately 9 IU/mg on day 3 (**Fig. 4-5 K**), whereas at 33°C, the activity was approximately 6.9-fold higher (~55IU/mg) compared to established cell lines (**Fig. 4-5 L**). In batch culture, where production can extend up to 8 days without medium replenishment, several dynamic factors influence product stability and yield. During this period, cellular metabolism depletes available nutrients and alters the culture environment, resulting in the accumulation of metabolites and by-products that may compromise product integrity. In the absence of fresh medium, identifying the optimal harvest time—day 3 at 33°C—is critical for minimizing product degradation. Importantly, this optimal harvesting window aligns with the cessation of cell growth, yet it does not correspond to a significant decrease in cell viability.

Heparin produced in this batch culture system may become increasingly susceptible to degradation over time, likely due to the action of cellular enzymes, such as desulfating or degrading enzymes, which disrupt its sulfation pattern and diminish Anti-FXa and Anti-FIIa activities. The absence of medium renewal over an extended culture period may exacerbate these enzymatic activities, highlighting the necessity for meticulous monitoring. This ensures that harvesting occurs at a time when heparin exhibits peak stability and bioactivity, with day 3 identified as the optimal point for maximizing yield and product quality. However, the precise cause of this observation remains unknown and requires further investigation to elucidate.


A
37ºC
CHO-S
CHO/2F
CHO/2F-S
CHO/3F-S
CHO/3F-S_CKO
B
33ºC
CHO/2F-S
CHO/3F-S
CHO/3F-S_CKO
Viable cell density [x10^6 cells/mL]
Culture time [d]
C
Anti-FXa activity [IU/mL]
D
E
Anti-FIIa activity [IU/mL]
F

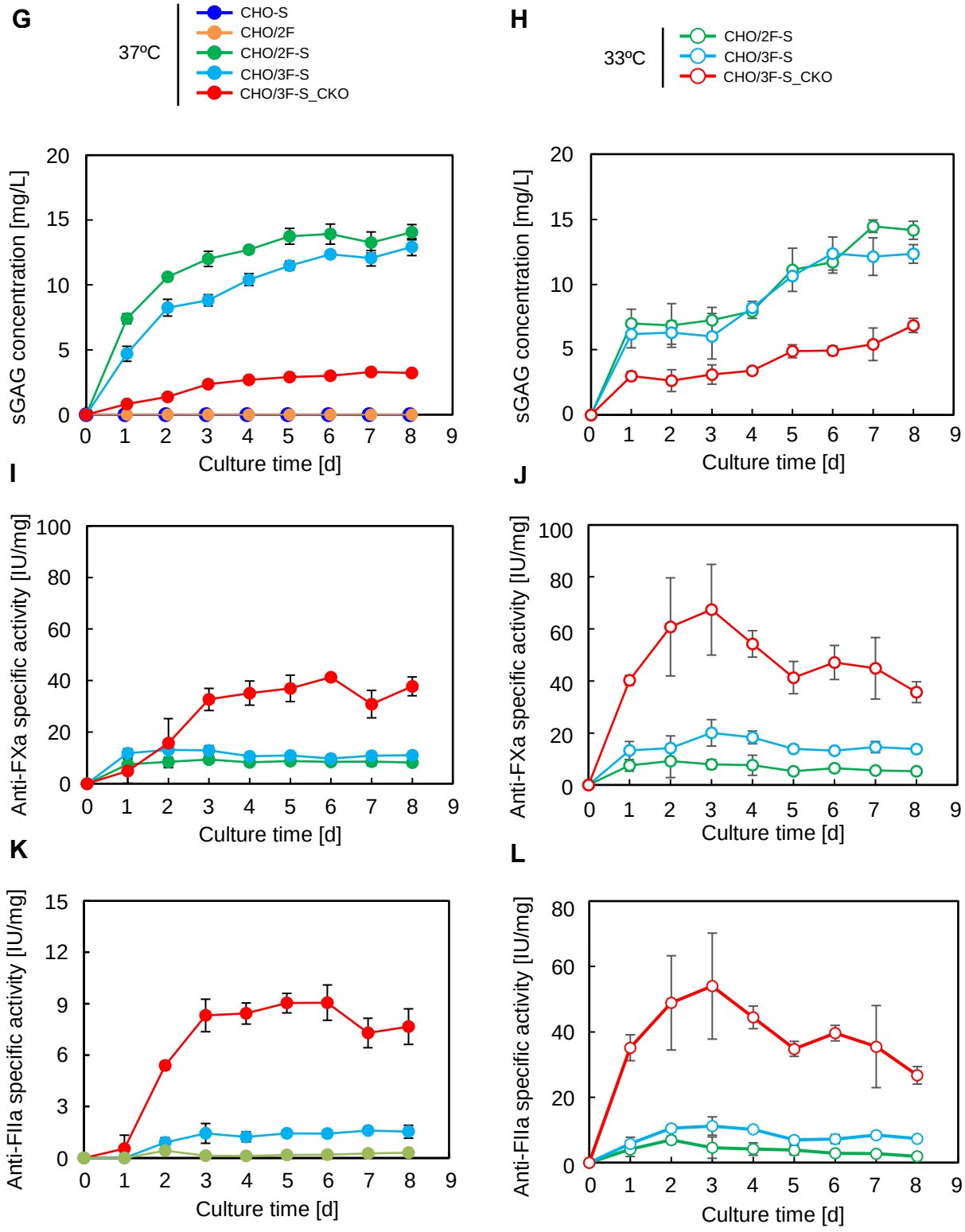


Fig. 4-5 Heparin-like polysaccharide production in low-temperature cultures. A. and B. Cell proliferation at 37°C and 33°C; C. and D. Anti-FXa activity at 37°C and 33°C; E. and F. Anti-FIIa

activity at 37°C and 33°C; G. and H. sGAG concentration at 37°C and 33°C; I. and J. Anti-FXa specific activity at 37°C and 33°C; and K. and L. Anti-FIIa specific activity at 37°C and 33°C.

The measurement results of glucose and lactate concentrations in CHO/3F-S_CKO culture are shown in **Fig. 4-6 A, B, C, D**. In CHO/3F-S_CKO cultures at 33°C, gradual glucose consumption was observed compared with other cell line, and lactate accumulation was also suppressed. This consequence suggested that lower temperature of 33°C not only results in more cost-effective nutrient usage but also reduces lactate production.

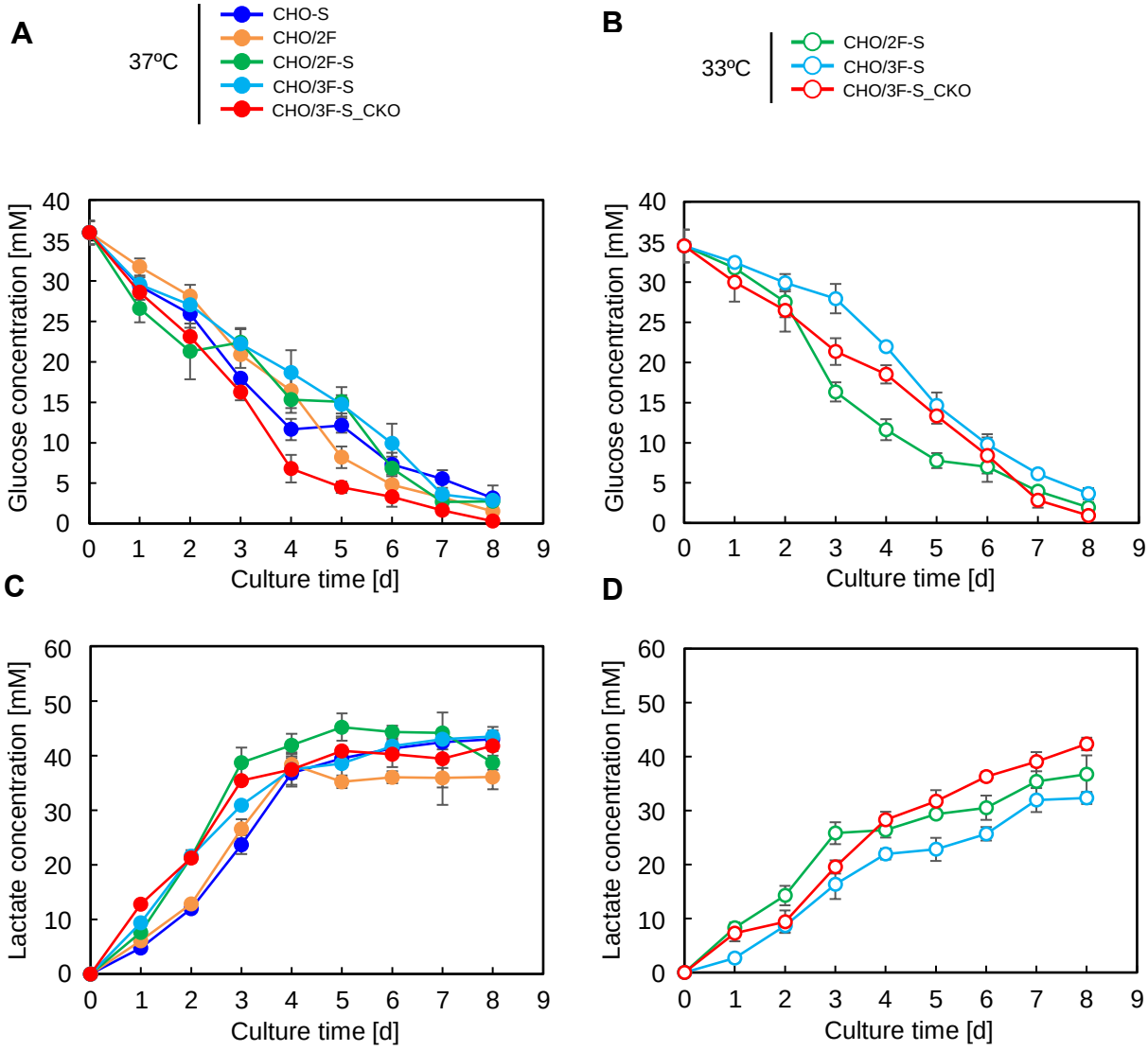


Fig. 4-6 Glucose and Lactate concentration. A. and B. Glucose concentration at 37°C and 33°C; and C. and D. Lactate concentration at 37°C and 33°C.

4.4.4 Bioengineered heparin-like polysaccharide production by CHO/3F-S_CKO cells in batch culture

Temperature shifts to lower culture temperatures are frequently employed in the manufacturing of protein therapeutics in mammalian cells to improve productivity, viability, and quality attributes (McHugh et al., 2020). In our study, three distinct temperature categories (37°C, 33°C, and 30°C) were evaluated to assess their impact on these parameters for established CHO/3F-S_CKO cell line.

When we cultured engineered CHO/3F-S_CKO cell line at 37°C, 33°C and 30°C, the most pronounced growth was observed at 37°C, while 33°C represents a moderate growth phase, and at 30°C almost no cell growth was observed (**Fig. 4-7 A**) but high production of Anti-FXa and Anti-FIIa activity, and their specific activities. Next, the concentration of sGAGs in the culture supernatant of the CHO/3F-S_CKO cell line was measured up to day 8, comparing temperatures of 37°C, 33°C, and 30°C. Analysis of sGAG concentrations revealed dynamic changes dependent on the temperature condition and time point. For the lower temperature culture at 30°C and 33°C, higher concentration of sGAG was observed compared to typical culture temperature (**Fig. 4-7 B**). The Anti-FXa and Anti-FIIa activity for established CHO/3F-S_CKO cell line was significantly enhanced, showing nearly a 4.5-fold improvement at 30°C compared to cultures maintained at 37°C (**Fig. 4-7 C and D**). This substantial increase suggests that the lower temperature fosters conditions favorable for heightened Anti-FXa and Anti-FIIa activity. In addition, regarding Anti-FXa and FIIa specific activity, on day 3 (72 hours), the activity peaked at approximately 200 units/mg in case of Anti-FXa (**Fig. 4-7 E**) and 175 units/mg in case of Anti-FIIa (**Fig. 4-7 F**), although after day 3, subsequently declined was observed. Such fluctuations could be attributed to factors such as substrate depletion, enzyme inhibition, or changes in environmental conditions like pH or temperature. Glucose and lactate concentration measurements revealed that at 30°C, gradual glucose consumption was observed with other conditions and lactate accumulation was also suppressed. Based on these findings, a temperature of 30°C is identified as optimal for heparin production, as it induces a metabolic slowdown that conserves energy and resources, thereby enhancing cellular viability under altered environmental conditions. This temperature not only supports the overall health of the cells but also facilitates an improved level of sulfation, which is crucial for maintaining the anticoagulant activity of heparin.

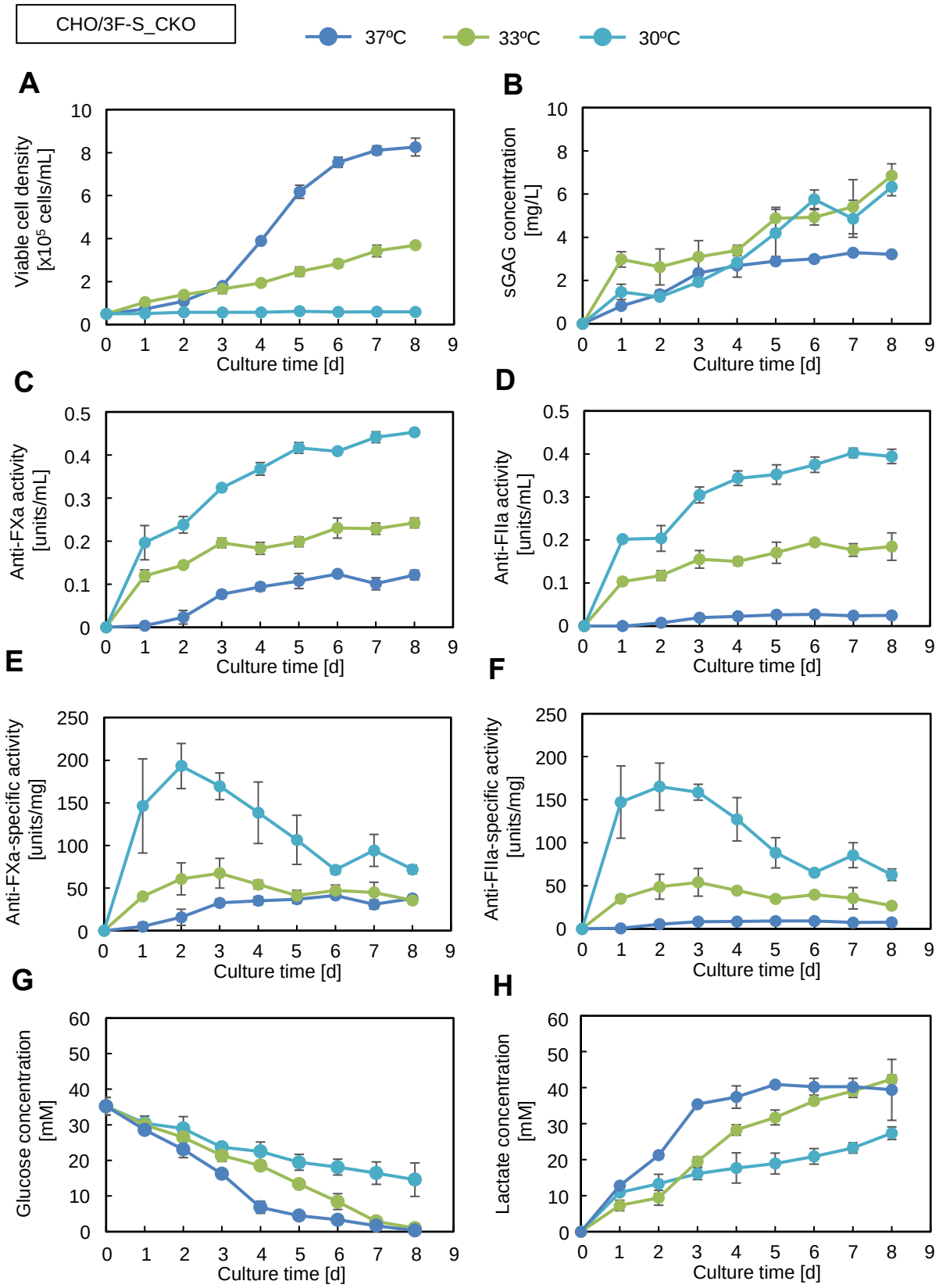


Fig. 4-7 Batch culture of established CHO/3F-S_CKO cell line at 37°C, 33°C and 30°C. A. Cell

proliferation; B. sGAG concentration; C. Anti-FXa activity; D. Anti-FIIa activity; E. Anti-FXa specific activity; F. Anti-FIIa specific activity; G. Glucose concentration and H. Lactate concentration.

Furthermore, a reduced metabolic rate at this temperature minimizes the production of toxic by-products that could otherwise compromise product integrity. Crucially, the optimal harvesting time at 30°C is day 2. Harvesting at this point allows for the maximal accumulation of heparin with the desired biochemical properties before the onset of degradation processes that may occur with prolonged culture. Collectively, these factors underscore the importance of temperature and timing in optimizing heparin production, ensuring that both yield and quality are maximized while minimizing potential degradation.

4.4.5 Bioengineered heparin/HS production by established cell lines in continuous culture

Continuous cultivation of cells at high density is essential for optimizing the production of bioengineered heparin. This approach ensures consistent and efficient synthesis of heparin over prolonged periods, supporting stable and predictable yields necessary for pharmaceutical applications. Maintaining high cell densities also enhances the productivity of heparin biosynthesis pathways, potentially reducing production costs while ensuring uniform quality in pharmaceutical manufacturing.

Therefore, we evaluated whether bioengineered heparin-like polysaccharide of established cell lines (CHO/2F-S, CHO/3F-S and CHO/3F-S_CKO) could be produced at a high concentration while maintaining a high specific production rate by culturing at a lower temperature (33°C) in continuous culture using a bioreactor tube with shaking (**Fig. 4-8**). The culture was conducted over 30 days, with complete medium replacement every second day. When seeded at a density of 2.0×10^7 cells/mL, comparable cell proliferation was observed between the engineered KO cell line and others (**Fig. 4-8 A**). In case of engineered CHO/3F-S_CKO cell line, we again noted a prominent reduction in sGAG concentration over time (**Fig. 4-8 B**) although the Anti-FXa activity was slightly higher compared to others (**Fig. 4-8 C**). Over the duration, there was a notable enhancement in Anti-FXa activity, showing an approximate 2.8-fold increase compared to the previously established CHO/3F-S cell line and a 6-fold increase compared to the CHO/2F-S cell line by day 18 (**Fig. 4-8 D**). Regarding Anti-FIIa activity, there was a noticeable increase over the study period for engineered CHO/3F-S_CKO cell line and showed heightened Anti-FIIa specific activity compared to other cell lines (**Fig. 4-8 E and F**).

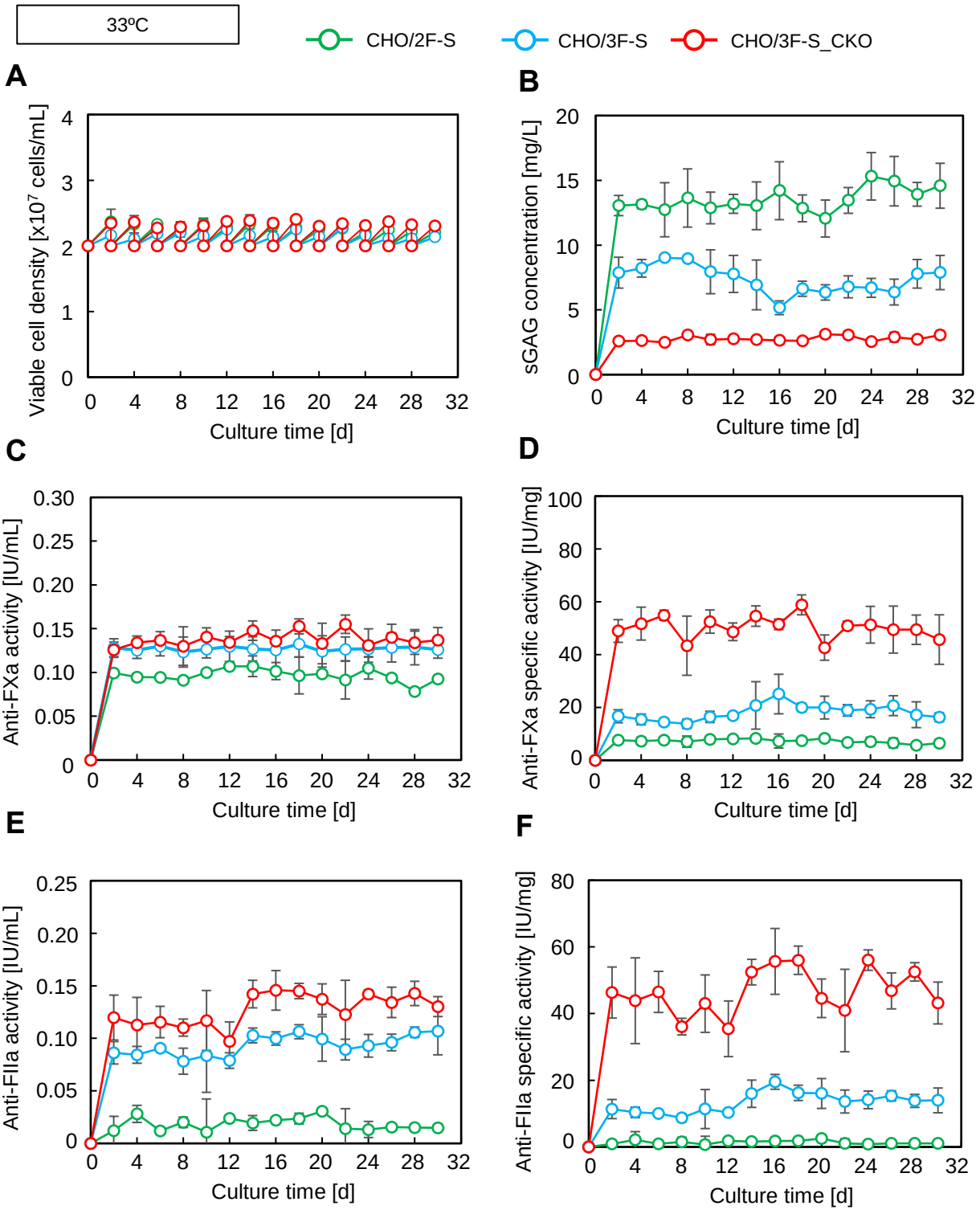

33ºC
CHO/2F-S
CHO/3F-S
CHO/3F-S_CKO
A
Viable cell density [x10^7 cells/mL]
Culture time [d]
B
sGAG concentration [mg/L]
Culture time [d]
C
Anti-FXa activity [IU/mL]
Culture time [d]
D
Anti-FXa specific activity [IU/mg]
Culture time [d]
E
Anti-FIIa activity [IU/mL]
Culture time [d]
F
Anti-FIIa specific activity [IU/mg]
Culture time [d]

Fig.4-8 Continuous culture of CHO/2F-S, CHO/3F-S and CHO/3F-S_CKO cell lines at low temperature. A. Cell proliferation; B. sGAG concentration; C. Anti-FXa activity; D. Anti-FXa specific activity; E. Anti-FIIa activity; and F. Anti-FIIa specific activity.

These results suggested that low-TS and inactivation of CS would enhance the anticoagulant production of engineered CHO/3F-S_CKO cell line.

4.4.6 Bioengineered heparin/HS production by CHO/3F-S_CKO cell line in continuous culture at low temperature

Next, we attempted continuous culture for the CHO/3F-S_CKO cell line over 30 days at both 33°C and 30°C. The seeding density and experimental setup were similar to previous conditions; however, at 30°C, complete medium replacement occurred every third day due to the reduced metabolic rate and slower nutrient consumption of the cells at this lower temperature. This slower metabolism results in less frequent depletion of nutrients and accumulation of waste products, making less frequent medium changes sufficient for maintaining optimal cell health and productivity. In contrast, at 33°C, the medium was exchanged every other day, aligning with previous findings to support cell growth effectively.

For the culture at 33°C, upon seeding at a density of 2.0 × 10^7 cells/mL, the engineered KO cell line proliferated similarly to previous conditions, whereas almost no growth was observed at 30°C (Fig. 4-9 A). Regarding sGAG concentration, cells cultured at the optimal temperature (30°C) exhibited higher sGAG levels compared to those cultured at 33°C (Fig. 4-9 B). The Anti-FXa activity showed approximately a 6-fold improvement (~0.6 IU/mL) (Fig. 4-9 C), and the specific Anti-FXa activity exhibited a 4-fold enhancement (~160 IU/mg) when cultured at the optimal temperature compared to 33°C (Fig. 4-9 D). Furthermore, Anti-FIIa activity increased around 3-fold, and specific activity increased nearly 1.8-fold at the optimal temperature compared to 33°C over the same period (Fig. 4-9 E and F). These findings underscore the significant impact of temperature on cell metabolism and heparin production. While the CHO/3F-S_CKO cell line demonstrated robust growth at 33°C, optimal sGAG synthesis and anticoagulant activities were notably enhanced at 30°C, despite the slower growth rate. This suggests that lower temperatures may create favorable conditions for maximizing product yield and quality by allowing for better sulfation patterns and improved biochemical activities. Overall, our results highlight the importance of optimizing culture conditions, including temperature and medium exchange frequency, to enhance the performance of recombinant cell lines in heparin production.

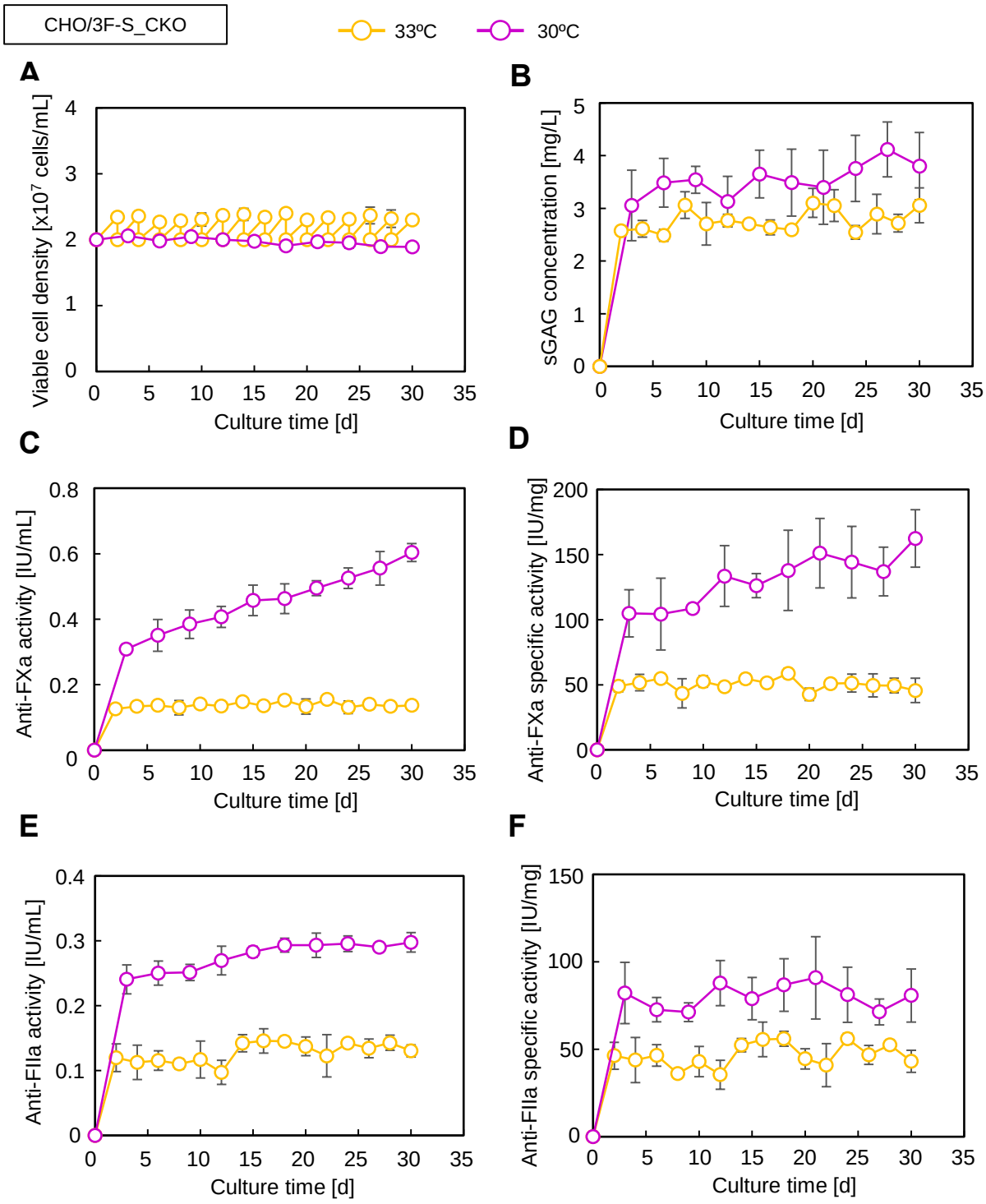


Fig. 4-9 Continuous culture of newly generated CHO/3F-S_CKO cell line at 33°C and 30°C. A. Cell proliferation; B. sGAG concentration; C. Anti-FXa activity; D. Anti-FXa specific activity; E. Anti-F-IIa activity; and F. Anti-FIIa specific activity.

These results suggest that culturing at the optimal temperature for CHO/3F-S_CKO cell line not only enhanced Anti-FXa and Anti-FIIa activities and their specific activities but also improved sGAG concentration.

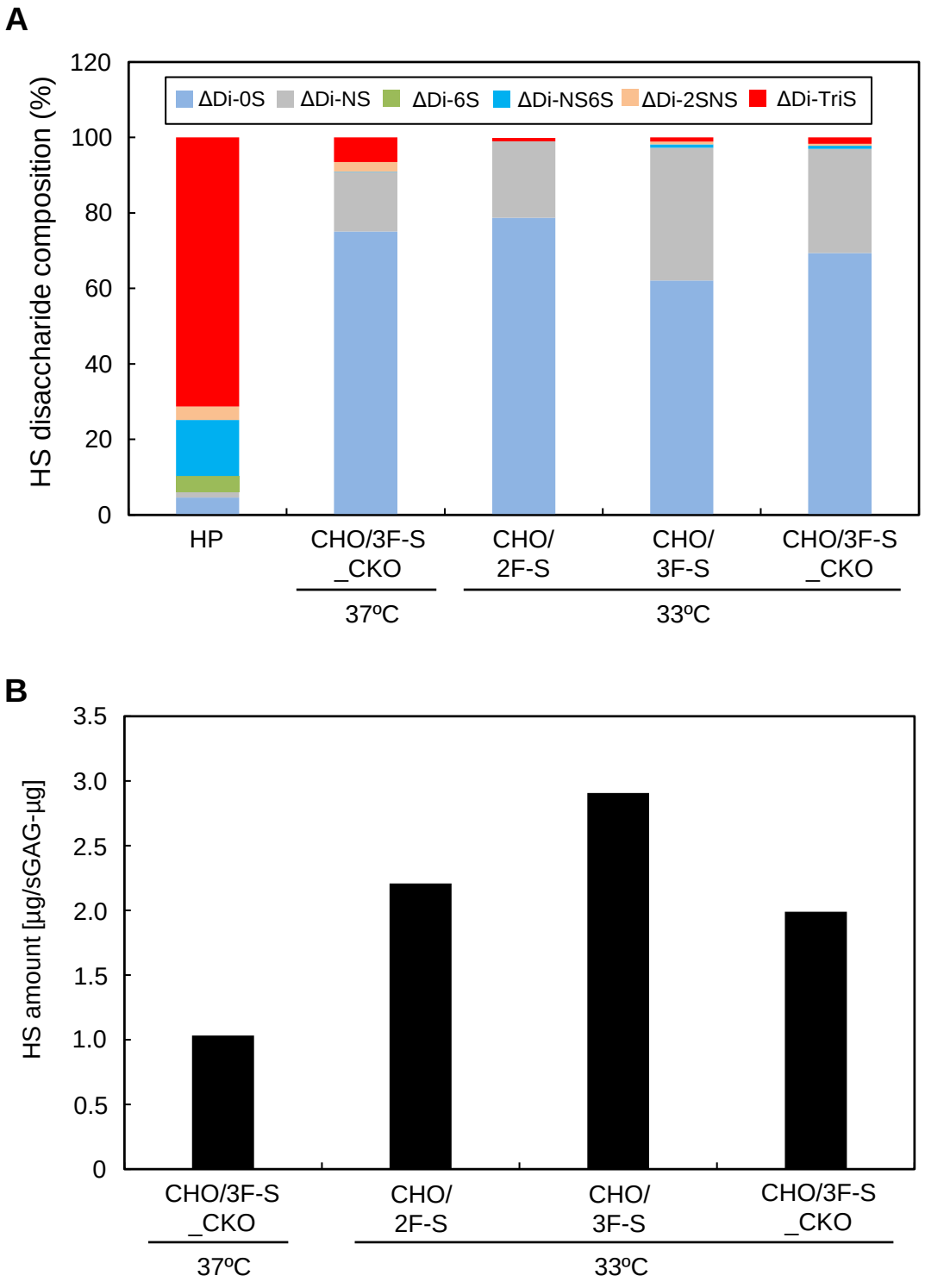


Fig. 4-10 Bioengineered heparin/HS characterization. A. HS disaccharide composition; and B. HS amount.

4.4.7 Characterization of bioengineered heparin-like polysaccharides

The structural analysis and functional evaluation of bioengineered heparin-like polysaccharides produced by different CHO cell lines were undertaken in collaboration with the Laboratory of Pathological Analytical Chemistry at the Graduate School of Pharmaceutical Sciences, Tokyo University of Science. The analyses were conducted by Dr. Kyohei Higashi, Associate Professor of Physical Pharmaceutical Science. This collaborative work remains ongoing. To analyze the composition of disaccharide, we observed engineered CHO/3F-S_CKO cell line showed nearly 70% Δ Di-0S, 10% Δ Di-NS, 5% Δ Di-2SNS and 15% Δ Di-TriS at 37°C. On the other hand, at 33°C, the following disaccharide composition was observed: approximatively 62% Δ Di-0S, 23% Δ Di-NS, 2% Δ Di-2SNS and 13% Δ Di-TriS (Fig. **4-10 A**). Further emphasis is required on the purification of the polysaccharide from the suspension, and it is anticipated that the knockout of CS may impact the sulfation pattern of the glycan structure overall. Given that the observed HS level was approximately 2 µg per µg of sGAG, which may not reflect an accurate measurement, it is essential to repeat the experiment to obtain a more reliable quantification of HS, particularly at 33°C and 30°C. Further investigation will be crucial to accurately assess the impact of these temperatures on both HS production and the sulfation efficiency of heparin-like polysaccharides (**Fig. 4-10 B**). These results indicated that lowering the temperature shift resulted in increased productivity as well as sulfation of CHO/3F-S_CKO cell line.

4.5 Discussion

CHO cells are integral to biotechnological research and industry for their robust growth and versatile capability in producing complex biomolecules(Thacker et al., 2021). Their stable genetic profile and efficient protein processing machinery ensure consistent, high-quality biopharmaceutical production, driving advances in therapeutic development and biotechnological innovation (Kim et al., 2012; Ritacco et al., 2018). Genetic engineering of CHO cells plays a pivotal role in enhancing productivity for biotherapeutics like proteins and antibodies (Kim et al., 2012). Techniques such as metabolic pathway optimization, protein expression elevation, and glycosylation pattern modification enable tailored production processes that meet diverse therapeutic needs. The scalability of CHO cell cultures in large bioreactors further supports

efficient, global-scale production with reliable yield and quality, essential for meeting demand in biologics markets (Baik et al., 2015).

In heparin synthesis, CHO cells have been genetically modified to produce HS, mimicking the biosynthetic pathway of heparin. Baik et al. achieved a significant 100-fold increase in HS anticoagulant activity by introducing human NDST2 and mouse 3OST1 genes into CHO cells, with further optimization achieving concentrations of approximately 90 μg/mL in stirred-tank bioreactors (Baik et al., 2015; Baik, Gasimli, et al., 2012a). Despite these advancements, bioengineered HS typically displays lower anticoagulant efficacy than natural heparin, likely due to complex interactions among heparin biosynthetic enzymes. Therefore, precise regulation of enzyme expression levels and modification processes is critical for maximizing bioengineered heparin production in CHO cells. Other studies emphasize targeting 3OST1 to the Golgi apparatus as a strategy to enhance the expression of key enzymes (2OST, 6OST, NST), showing promising prospects for improving bioengineered heparin yield and quality (Datta et al., 2013).

Our study demonstrates the transformative potential of genetic manipulation in enhancing heparin production using recombinant CHO cells. By targeting the CS biosynthesis pathway, we redirected cellular resources towards the biosynthesis of bioengineered heparin-like polysaccharides. This approach significantly increased bioengineered heparin and HS production from an engineered CHO/3F-S_CKO cell line with anticoagulant potency equaling pharmaceutical heparin. The structural analysis revealed a lower degree of sulfation in the bioengineered heparin compared to natural heparin. This discrepancy suggests ongoing challenges that require further investigation in collaboration with our partners. Potential factors contributing to this difference include the knockout of the CS biosynthesis pathway, the method of heparin collection from the cell suspension, and the procedures involved in the purification process. Addressing these issues comprehensively will be pivotal to advancing our understanding and resolving these structural disparities. Future efforts will focus on conducting extensive research to elucidate the underlying mechanisms and optimize the production process of bioengineered heparin to meet therapeutic standards effectively.

Furthermore, our investigation into the influence of temperature shift (TS) on heparin production highlighted its pivotal role in optimizing bioprocess efficiency. Lower TS conditions led to a substantial increase in heparin productivity, aligning with previous research on TS-mediated enhancements in protein expression and cellular metabolism (Al-Fageeh et al., 2006; Kantardjieff

et al., 2010; McHugh et al., 2020; Shahabi et al., 2023a, 2023b; Yee et al., 2009). This insight not only enhances our understanding of bioprocess optimization but also positions TS manipulation as a promising strategy for improving heparin yield in biopharmaceutical manufacturing. The results demonstrated that by seeding at 2.0 × 10^7 cells/mL and conducting low-temperature culture, heparin-like polysaccharide production was maximized. Notably, at the optimal lower temperature of 30°C, we successfully produced the bioengineered CHO/3F-S_CKO cell line with elevated sGAG concentration, Anti-FXa and Anti-FIIa activity, as well as their specific activities compared to commercial heparin. Additionally, this culture exhibited a gradual glucose consumption rate alongside significantly suppressed lactate accumulation, suggesting that the optimized culture conditions enable enhanced production of bioengineered heparin-like polysaccharides while reducing medium usage through strategic timing of medium replacement. Crucially, the optimal harvesting time identified at 30°C is day 2, which aligns with the peak accumulation of heparin before any significant degradation occurs. This timing is essential for maximizing both yield and product quality, ensuring that the heparin produced retains its biochemical properties. Mechanistic insights suggest that TS conditions influence glycosylation patterns and enzymatic activities critical for heparin biosynthesis, further emphasizing the necessity of optimizing bioprocess parameters, including harvesting times, to achieve consistent product quality and operational efficiency in heparin production.

In conclusion, our study integrates genetic manipulation and bioprocess optimization to establish a scalable and sustainable method for producing pharmaceutical-grade heparin. These findings contribute to advancing therapeutic glycosaminoglycan production, offering a pathway towards reliable and standardized heparin production that meets regulatory requirements and addresses current industry challenges. Although the sulfation level still lower compared to porcine derive heparin. Therefore, future research directions should focus on refining bioprocess conditions, exploring additional genetic engineering strategies, and expanding mechanistic insights to further enhance the commercial viability and therapeutic applications of bioengineered heparin.

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Chapter 5

Summary

5.1 Overview of the dissertation

This dissertation explores the multifaceted world of heparin, a crucial anticoagulant, and its derivatives, delving into their synthesis, applications, and production advancements. The work progresses from foundational concepts to cutting-edge bioengineering techniques, aiming to expand the therapeutic potential of heparin and improve its production efficiency and efficacy. Each chapter builds on the previous, weaving a comprehensive narrative that spans traditional applications to innovative bioengineering strategies.

Chapter 1 provides a foundational understanding of heparin, glycosaminoglycans, and proteoglycans, including their chemical properties and biological roles. It reviews various synthesis approaches and modifications of these molecules, focusing on their relevance in biomedical applications. The chapter also highlights previous research on heparin production using animal cells, setting the stage for the dissertation's goals and research strategies. By establishing this background, it frames the context for subsequent chapters that delve deeper into heparin's therapeutic potential and production advancements.

Chapter 2 shifts focus to the expanding therapeutic applications of heparin beyond its conventional use as an anticoagulant. It explores advancements in bioengineering and nanotechnology that enhance heparin's clinical efficacy. Recent developments in heparin biosynthesis are discussed, highlighting its diverse roles in modern medicine, including inflammation modulation, antiviral therapies, cancer treatment, and tissue regeneration. This chapter aims to redefine therapeutic interventions and improve patient outcomes by leveraging heparin's multifaceted properties.

Chapter 3 delves into recent advancements in the bioengineered production of heparin, examining both prokaryotic and eukaryotic systems. It provides a comprehensive analysis of innovative metabolic engineering approaches and their implications for future applications. The chapter emphasizes the potential of recombinant CHO cells as a promising platform for heparin synthesis, discussing breakthroughs, ongoing challenges, and opportunities for improvement. The critical

assessment aims to advance the development of safer, more sustainable, and clinically effective heparin therapeutics.

Chapter 4 focuses on enhancing heparin production through genetic manipulation and bioprocess optimization using recombinant CHO cells. Employing CRISPR-Cas9 technology, the chapter details efforts to deactivate HS desulfation enzymes to preserve sulfate groups, promoting the synthesis of highly sulfated heparin-like polysaccharides. Additionally, the disruption of the CS2 gene aims to eliminate the CS biosynthetic pathway, redirecting resources towards heparin production. The influence of temperature shifts on heparin productivity is also investigated. Analytical techniques evaluate the quality and efficacy of the produced heparin, while scalable bioreactor protocols ensure process stability and economic viability for large-scale production.

Chapter 5 consolidates findings from the previous chapters, emphasizing how genetic manipulation and bioprocess optimization contribute to enhanced heparin synthesis. It revisits the expanding therapeutic potential of heparin discussed in Chapter 2 and the bioengineering progress highlighted in Chapter 3, particularly the use of recombinant CHO cells. This chapter also presents proposals for further enhancements in bioengineered heparin production, aiming to push the boundaries of current methodologies and explore new avenues for research and development.

5.2 Perspectives

The production of bioengineered heparin using recombinant CHO cells offers a promising alternative to traditional animal-derived sources, addressing concerns related to supply consistency, contamination, and ethical considerations. Recent advances in genetic and metabolic engineering provide a solid foundation for overcoming the major challenges in this process. Key areas of focus include enhancing sulfation, improving anticoagulant efficacy, optimizing secretion and yields, and refining scale-up strategies (**Fig. 5-1**).

(i) Optimizing sulfation levels

Sulfation plays a crucial role in determining the anticoagulant activity of heparin. Efforts to enhance sulfation patterns in recombinant heparin involve upregulating key enzymes such as NDST2 and Hs3st1, which are involved in the biosynthesis and modification of heparin's structure (Baik et al., 2012). Increasing the availability of PAPS, the sulfate donor, through engineered

metabolic pathways is critical to replicating the high-sulfate content found in natural heparin. Success in this area would yield a more potent and therapeutically effective heparin product.

(ii) Enhancing anticoagulant efficacy

Ensuring the anticoagulant efficacy of recombinant heparin is closely linked to precise control over its structural features, particularly 3-O-sulfation, which is essential for antithrombin binding and inhibition of coagulation factors (Atha et al., 1985). The overexpression of enzymes like Hs6st1 and Hs6st2, coupled with a better understanding of their regulatory mechanisms, can help improve the functional properties of the produced heparin (Kreuger & Kjellén, 2012). This targeted approach could enhance heparin's therapeutic potential by mimicking or surpassing the activity of naturally derived products.

(iii) Improving extracellular secretion and yields

Increasing the efficiency of heparin secretion into the extracellular environment remains a technical challenge due to its binding to membrane proteins during biosynthesis (Sarrazin et al., 2011). Strategies to mitigate this involve expressing extracellular proteoglycans like syndecan and glypican, which may assist in releasing heparin into the culture medium (Farrugia & Melrose, 2023). Additionally, optimizing serum-free culture conditions can further improve yield and reduce production costs (Thacker et al., 2022). These enhancements are essential for scaling production to meet clinical and commercial demands.

(iv) Redirecting metabolic pathways

Redirecting metabolic fluxes to prioritize heparin biosynthesis over competing pathways, such as the CS biosynthetic pathway, presents another viable strategy for increasing production yields (Thacker et al., 2022). By downregulating or inactivating enzymes involved in CS production, resources can be redirected toward the synthesis of heparin. This metabolic engineering approach is essential for maximizing the cellular production of bioengineered heparin while maintaining cell viability and productivity.

(v) Managing desulfation enzyme activity

Controlling the activity of desulfation enzymes, such as sulfatases and glycosidases, is critical for maintaining the correct sulfation pattern of recombinant heparin. Inhibiting these enzymes through genetic modification, including the use of CRISPR/Cas9, offers a promising method for ensuring that the desired sulfation levels are retained throughout the production process (Griffin & Gloster,

2017). Fine-tuning the activity of these enzymes is essential for producing high-quality heparin with consistent biological activity.

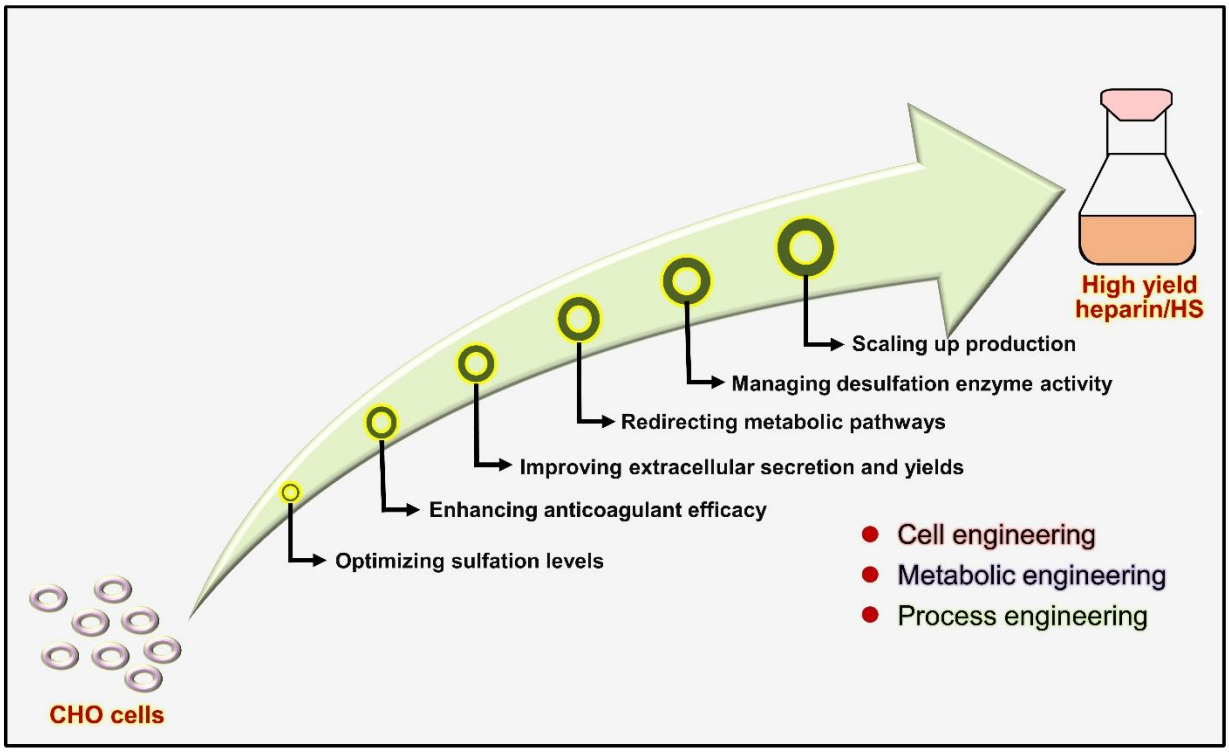

Fig. 5-1 Overview of bioengineered heparin production using recombinant CHO cells.

(vi) Scaling up production

Efficiently scaling up the production of bioengineered heparin in CHO cells is necessary to meet global demand, particularly for specialized medical applications. Optimizing bioreactor conditions, including controlling agitation and nutrient supply, will be key to ensuring robust cell growth and product yield (Baik et al., 2015). Advances in downstream processing techniques, including purification and quality control, are also required to ensure that the recombinant product meets regulatory standards. Scaling up will support broader accessibility to bioengineered heparin, potentially reducing reliance on animal-derived sources.

The future of bioengineered heparin production lies in overcoming technical challenges related to sulfation optimization, secretion efficiency, and large-scale production. Recombinant CHO cells present a viable platform for producing heparin with improved safety, consistency, and anticoagulant properties. Continued research into metabolic and genetic engineering strategies will

further refine the process, paving the way for recombinant heparin to become a reliable alternative in anticoagulant therapy (Zhang et al., 2022).

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