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Review

Quality control, safety assessment and preparation approaches of low molecular weight heparin

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ABSTRACT

Low Molecular Weight Heparins (LMWHs) are well-established for use in the prevention and treatment of thrombotic diseases, and as a substitute for unfractionated heparin (UFH) due to their predictable pharmacokinetics and subcutaneous bioavailability. LMWHs are produced by various depolymerization methods from UFH, resulting in heterogeneous compounds with similar biochemical and pharmacological properties. However, the delicate supply chain of UFH and potential contamination from animal sources require new manufacturing approaches for LMWHs. Various LMWH preparation methods are emerging, such as chemical synthesis, enzymatic or chemical depolymerization and chemoenzymatic synthesis. To establish the sameness of active ingredients in both innovator and generic LMWH products, the Food and Drug Administration has implemented a stringent scientific method of equivalence based on physicochemical properties, heparin source material and depolymerization techniques, disaccharide composition and oligosaccharide mapping, biological and biochemical properties, and *in vivo* pharmacodynamic profiles. In this review, we discuss currently available LMWHs, potential manufacturing methods, and recent progress for manufacturing quality control of these LMWHs.

1. Introduction

Heparin (HP) is complex, linear, and highly sulfated glycosaminoglycans (GAGs) found in cell granules, or in the extracellular matrix (Sasisekharan & Venkataraman, 2000). Heparin was first discovered in 1916 and has been used as a clinical anticoagulant product since the 1930s (Hogwood, Mulloy, Lever, Gray, & Page, 2023; McLean, 1916). Heparin is derived from a variety of animal species and tissues, and its structure varies with respect to its molecular weight distribution and sulfation pattern. Currently, porcine intestinal mucosa is the primary source of unfractionated heparin (UFH) used as an anticoagulant in clinical settings in the United States and Europe (Bangham & Woodward, 1970; Baytas & Linhardt, 2020). Heparin has a structure characterized by repeating disaccharide building blocks comprised of a uronic acid residue, either β-D-glucuronic acid (GlcA) or more commonly α-L-iduronic acid (IdoA) (1 → 4) glycosidically-linked to *N*-acetyl or *N*-sulfated α-D-glucosamine (GlcNAc, GlcNS). *O*-Sulfation typically takes place at the C2 position of IdoA, and the C3 and C6 positions of α-D-glucosamine (GlcN) (Casu, 1990; Casu, Naggi, & Torri, 2015; Zhao et al., 2020). Additionally, heparin contains distinctive pentasaccharide sequences (GlcNS/Ac6S(1 → 4)GlcA(1 → 4)GlcNS3S6S(1 → 4)IdoA2S(1 → 4)GlcNS6S) present within its polymer chain (Gallus & Coghlan, 2002; Zhang, Zhang, Tan, Pan, & Zhang, 2019).

The biosynthesis of heparin commences with the formation of the tetrasaccharide linker at the reducing end of the heparin chain (GlcA-Gal-Gal-Xyl) where it is covalently *O*-linked to the serine residues of a core protein within the endoplasmic reticulum (Turbbull & Gallagher, 1991; Weiss et al., 2020). The polysaccharide backbone composed of

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alternating GlcA and GlcNAc residues undergoes extension from the linkage region, driven by the polymerases exostosin glycosyltransferase (EXT). Subsequent modifications occur through de-*N*-acetylation and *N*-sulfation of many GlcNAc residues catalyzed by *N*-deacetylase/*N*-sulfotransferase (NDST). Additionally, C5-epimerase catalyzes the epimerization of many GlcA residues into IdoA, followed by 2-*O*-sulfation of IdoA, 6-*O*-sulfation of GlcNS and occasionally by 3-*O*-sulfation of GlcNS/GlcNS6S through the action of 2-*O*-, 6-*O*-, and 3-*O*-sulfotransferases in the Golgi (Fu, Suflita, & Linhardt, 2016; Mulloy, Hogwood, Gray, Lever, & Page, 2016). There are also minor residues, including GlcN and GlcA2S that have been identified in heparin (Douaisi et al., 2024).

Heparin is an important anticoagulant drug widely used in the clinical treatment of thrombosis and related conditions. Its anticoagulant activity is mediated by binding to the serine protease inhibitor antithrombin III (AT), indirectly interacting with factor Xa and factor IIa (Beurskens et al., 2020; Björk & Lindahl, 1982). The active pharmaceutical ingredient (API) of heparin is a polydisperse and heterogeneous polysaccharide with an average molecular weight of 16 kDa. The interaction between AT and heparin is mediated by a specific pentasaccharide sequence with a central 3-*O*-sulfo group, known as the AT binding site (Li, Johnson, Esmon, & Huntington, 2004; Onishi, Ange, Dordick, & Linhardt, 2016). In addition to its anticoagulant activity, heparin shows great potential as a biomolecule for treating inflammation, injury, tumors, and has antiviral activity (Banik, Yang, Kang, Lim, & Park, 2021; Cui, Sun, Wang, Zhang, & Wang, 2019; Kwon et al., 2020). Wang and colleagues reviewed the non-anticoagulant activity of heparin and its further development as an important drug (Wang et al., 2022).

Low molecular weight heparins (LMWHs), first introduced as heparin substitutes in the 1980s, were prepared using controlled enzymatic or chemical depolymerization of unfractionated heparin to produce chains with molecular weight of approximately 3–8 kDa (Salzman, 1986; Weitz, 1997). The different depolymerization methods utilized to prepare LMWHs contribute to variations in their pharmacokinetic properties and anticoagulant profiles Table 1 (Lopez, 1991; Iqbal & Sadaf, 2023; Merli & Groce, 2010). Enoxaparin is the most frequently used LMWH among all commercially available LMWHs. It is obtained by alkaline depolymerization treatment of heparin under specific conditions using sodium hydroxide (Saxena, Chaudhary, Chaudhary, & Aggarwal, 2022; Uzan, 1998). Controlled deaminative cleavage with nitrous acid followed by sodium borohydride is employed for depolymerizing heparin to obtain dalteparin and nadroparin (Cook et al., 2011; Pinna, Simula, & Zinellu, 2012). Tinzaparin is produced from the depolymerization of UFH by a highly purified heparin lyase of bacterial origin., Heparin lyase breaks the chains between the anomeric carbon of an *N*-sulfate-glucosamine and the glucuronic acid, resulting in an unsaturated uronic acid at its non-reducing end (Amerali & Politou, 2022) (Fig. 1).

The main difference between UFH and LMWH lies in their relative inhibitory effects on factor Xa and thrombin (factor IIa). While UFH displays equivalent inhibitory activity against both factors: anti-factor Xa/anti-factor IIa ratios of approximately 1, LMWHs have higher activity against factor Xa, but reduced activity against factor IIa: anti-factor Xa/anti-factor IIa ratios of approximately 4 (Hao, Sun, Wang, Zhang, & Wang, 2019). The impact of molecular weight distribution on the anticoagulant activity of heparin was recognized several decades ago (Barrowcliffe, Mulloy, Johnson, & Thomas, 1989; Yur'eva et al., 2021). Compared to UFH, LMWHs are subcutaneously bioavailable, have a longer half-life, more predictable anticoagulant response, and show a decreased risk of heparin-induced thrombocytopenia (HIT), a life-threatening adverse effect associated with heparin (Merli & Groce, 2010; Spadarella et al., 2020) (Table 2).

Currently, both UFH and LMWHs demonstrate numerous adverse effects and a predictable supply chain crisis. All heparin approved for medical use in the United States and Europe originates from porcine intestinal mucosa, with the majority originating from Chinese pigs (Al-Hakim, 2021). This dependence on a single species and a single major country of origin weakens the robustness of the heparin supply worldwide, particularly in light of recent swine plague outbreaks (Vilanova, Tovar, & Mourão, 2019). Alternative animal sources, such as bovine and ovine intestinal mucosa have been developed as new products of UFH and LMWH (Oliveira et al., 2022; Tovar et al., 2013). However, these animal source materials can contain infectious agents causing viral or prion diseases, including bovine spongiform encephalopathy and scrapie (Andrews et al., 2020). Modern capabilities in synthetic chemistry and biology indicate that these methods could offer an alternative source of LMWHs. Recently, various patents for low molecular weight heparin either degraded by chemically or enzymatically method lose market exclusivity worldwide, leading to generic or biosimilar products that capture market share from less expensive drug products (Brouwers, Roeters van Lennep, & Beinema, 2019; Iqbal & Sadaf, 2023). The approval of the first generic version of enoxaparin by the Food and Drug Administration in 2010 represents a significant development in US regulatory science and policy (Lee et al., 2013; Minghetti, Cilurzo, Franzé, Musazzi, & Itri, 2013).

The development and regulation of LMWH and its generic version is covered by several detailed reviews (Iqbal & Sadaf, 2023; Lee et al., 2013; Lever, Mulloy, & Page, 2012). This review provides a comprehensive review of the approach used to latest advances in LMWHs

Table 1
Commercially available LMWHs.

LMWH	Trade name	Characteristics	Method of preparation	Mean Mw (KD)	Anti-Xa/IIa ratio	Ref
Ardeparin	Normiflo	2-*N*-acetyl-6-*O*-sulfo-D-glucosamine, Labile glycosidic bonds	Peroxidative depolymerization	6.0	1.9	Troy et al., 1997
Dalteparin	Fragmin	2,5-anhydro-D-mannose at reducing terminus	Nitrous acid depolymerization	6.0	1.9–3.2	Lindahl et al., 1979; Pineo & Hull, 2004
Enoxaparin	Clexane/ Lovenox	4,5 unsaturated uronic acid at non-reducing terminus	Benzylation and alkaline depolymerization	4.2	3.3–5.3	Uzan, 1998; Mascellani et al., 2007; Fareed et al., 2003
Nadroparin	Fraxiparine	2,5-anhydro-D-mannose at reducing terminus	Nitrous acid depolymerization	4.5	2.5–4.0	Davis & Faulds, 1997
Reviparin	Clivarin	2,5-anhydro-D-mannose at reducing terminus	Nitrous acid depolymerization, chromatographic purification	4.0	3.6–6.1	Wellington, McClellan, & Jarvis, 2001
Tinzaparin	Innohep/ Logiparin	4,5 unsaturated uronic acid at non-reducing terminus	Heparin lyase digestion	4.5	1.5	Mousa, 2002; Amerali & Politou, 2022
Certoparin	Sandoparin	2,5-anhydro-D-mannose at reducing terminus	Deaminative cleavage with isoamyl nitrite	5.4	1.5–2.5	Barnett, 1982; Donadini, Ageno, Guasti, & Squizzato, 2013
Parnaparin	Fluxum	2-*N*, 6-*O*-disulfo-D-glucosamine at reducing terminus	Oxidative depolymerization with Cu^{2+} and hydrogen peroxide	5.0	1.5–3.0	Frampton & Faulds, 1994
Bemiparin	Beparine	2-*O*-sulfo-4-enepyranosuronic acid at non-reducing terminus	Alkaline degradation	3.6	8.1–9.7	Sánchez-Ferrer, 2010

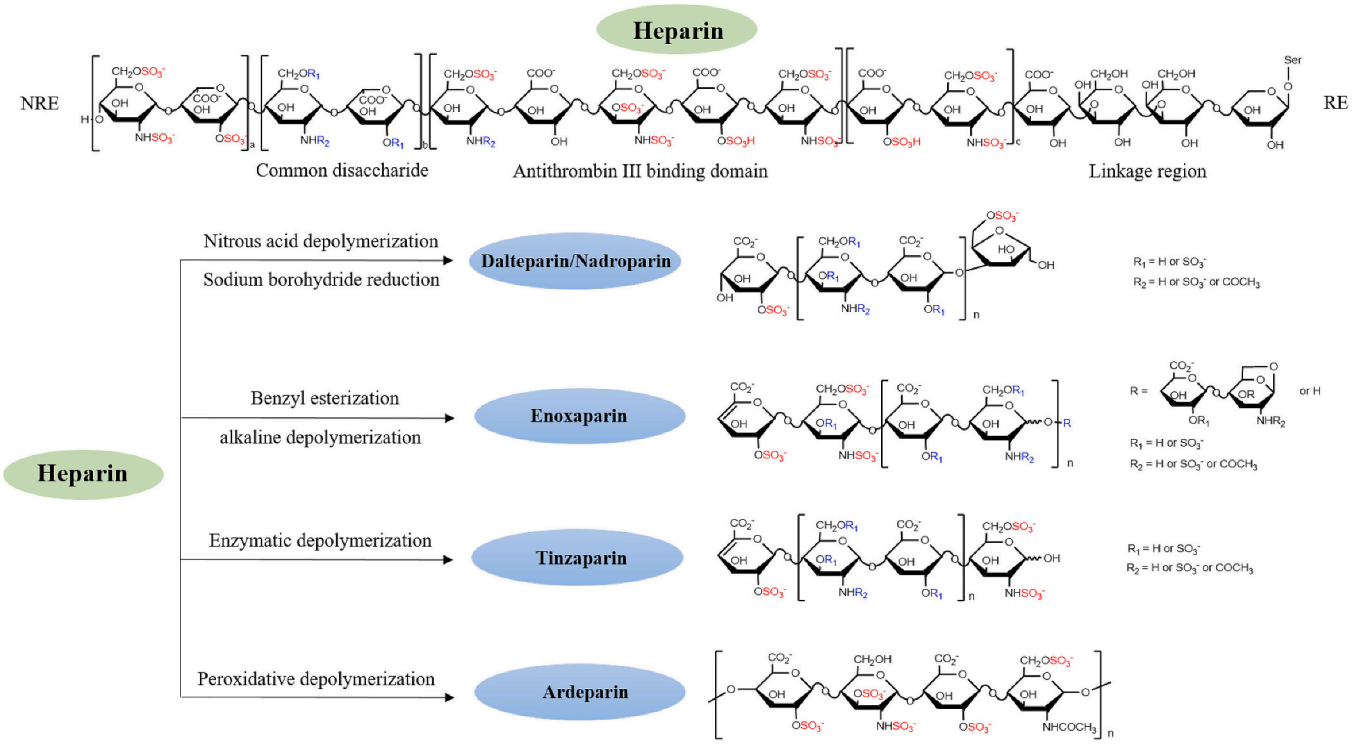

Fig. 1. Chemical structures of heparin and different LMWHs.

Table 2
Current limitations and clinical challenges of UFH and LMWHs.

Characteristics	UFH	LMWH
Cost	Lower	Higher
Half-life	Shorter (~1 h)	Longer (~3–6 h)
Mode of administration	Intravenous	Subcutaneous
Anticoagulant response	Less predictable	More predictable
Side effects	High	High
Reversibility	Rapidly reversible by PS	Partially reversible by PS
Risk of HIT	Higher	Lower

quality assurance and manufacture. The quality control of structural characteristics and bioactivity assay were evaluated. Next, we focused on the various modes of LMWH preparation, including synthesis and degradation methods, such as chemical, chemoenzymatic, bioengineered syntheses, and chemical degradation, enzymatic degradation, and physical degradation.

2. Quality control and compositional analysis

LMWHs are unique drugs with heterogeneous and complicated carbohydrate chains, making their manufacture and characterization challenging. Due to their special origin and physicochemical properties, assessing the quality and consistency of efficacy of their generic versions poses many challenges. Unlike small molecule drugs with relatively simple chemical structures, LMWHs are a complex mixture of oligosaccharide chains, the structure of which cannot be unambiguously confirmed by conventional means of structural confirmation alone, and the clinical indices are usually insensitive to the differences between products. Therefore, specific methods and indicators need to be established to characterize the consistency or similarity of their APIs. It is necessary to establish a comprehensive and systematic *in vitro* and *in vivo* evaluation methodology to assess the consistency of the characteristics of generic drugs to ensure that these products do not pose safety risks, with the most significant risk being immunogenicity.

Currently, the US Food and Drug Administration and the European Medicines Agency (EMA) have issued a series of guidelines and literature to guide the research, development and evaluation of generic LMWH formulation. The Food and Drug Administration defines equivalence criteria for enoxaparin that include physiochemical properties, heparin starting material, mode of depolymerization, disaccharide composition, fragment mapping and sequence of oligosaccharide species, biological and biochemical assays, and *in vivo* pharmacodynamics profile (Table 3). Generic LMWHs differ in anticoagulant activity, molecular weights and specific oligosaccharide sequences. For example, a series of analytical approaches including heparin lyase digestion, AT affinity chromatography, gel permeation chromatography, anion exchange chromatography, mass spectrometry and NMR spectroscopy have been used to assess potential differences between the original enoxaparin and generic enoxaparin (Mourier, Herman, Sizun, & Viskov, 2016) (Table 4).

Table 3
USP criteria for enoxaparin.

Category	Criteria
Anticoagulant potency	FX_a: NLT 90, NMT 125; FII_a: NLT 20, NMT 35, ratio of FX_a to FII_a is between 3.3 and 5.3
Mw distribution	Average Mw 4500 Da, range between 3800 and 5000 Da, 16 % Mw less than 2000 Da, range between 12 and 20 %, 74 % Mw between 2000 and 8000 Da, range between 68.0 and 82.0 %, no more than 18 % Mw higher than 8000 Da
Disaccharide building blocks	Degree of sulfation is no less than 1.8 per disaccharide unit
Oligosaccharide profile	4-enopyranose uronate at NRE, 20 % contain a 1,6-anhydro derivative on the RE, range between 15 and 25 %
Bacterial endotoxins	No more than 0.01 USP endotoxin unit per IU of FX_a
Nitrogen content	1.8–2.5 %
Heavy metals	No more than 0.0030 %
Sodium content	11.3–13.5 %
Molar ratio of sulfate to carboxylate	NLT 1.8
Benzyl alcohol	NMT 0.1 %

Table 4
Analytical methods used for quality control of LMWH.

Category	Methods	Characters	Ref
Mw distribution	Support vector machine computational technique	Mw determination with standards	Arnold et al., 2017
	SEC-MALS/RI	Mw determination in the absence of standards	Beirne, Truchan, & Rao, 2011; Ouyang et al., 2017
Disaccharide analysis	SAX-UV	Disaccharide analysis with high resolution, stability and quantitative accuracy	Sadowski, Gadzala-Kopciuch, & Kowalkowski, 2017
	Multiple heart cut (MHC) two-dimensional LC-MS/MS	Compatible with MS	Chen et al., 2021; Chen et al., 2022
	Ion-pair reverse-phase MS (IPRP-MS)	High resolution, efficient, convenient, reduction in column efficiency and ion-pair reagent may impact MS	Galeotti & Volpi, 2013; Yang et al., 2011; Zhang, Xie, Liu, Liu, & Linhardt, 2009
	Pre-column derivatization	High sensitivity using AMQC	Wang et al., 2021
	HILIC-LC-MRM-MS	Good compatibility with MS	Sun et al., 2016
	CE-MS	High resolution and sensitivity, unique interface to linked ESI-MS	Lin et al., 2017; Ouyang et al., 2019; Sun et al., 2016
Terminal groups and minor modifications	LC-UV, LC-MS	Linkage region oxidation, 2,3-epoxide and galacturonic acid GalA	Mourier & Viskov, 2004; Ozug et al., 2012; Gardini et al., 2021
	LC-MS	Quantitative analysis of the linkage region tetrasaccharides	Chen et al., 2017
	NMR combined with PCA, FDA, PLS-DA, LDA	Distinguish between different bands of LMWHs	Guerrini et al., 2015; Jiang, Li, Ma, Shi, & Wu, 2022; Monakhova, Diehl, & Fareed, 2018
	Quantitative HSQC	Percentage of monosaccharide and disaccharides	Mauri et al., 2017
	[^{1}H, ^{15}N] NMR and 2D NMR	2,5-anhydromannitol residue, amine groups of GlcN and GlcN3S	Langeslay et al., 2013; Beecher, Manighalam, Nwachuku, & Larive, 2016; Beecher & Larive, 2015
Oligosaccharide mapping	Top-down & bottom-up with GlycReSoft and GlycCompSoft software	Composition of oligosaccharides with different degrees of polymerization	Li, Zhang, Zaia, & Linhardt, 2012; Maxwell et al., 2012; Li et al., 2014; Sadowski, Gadzała-Kopciuch, & Buszewski, 2020; Wang et al., 2016
	Online cation suppressor SEC-MS	Reduce the number of false positive	Zaia et al., 2016
	SEC-MS with Glycommaping software	Improve the performance of SEC separation	Yan et al., 2022
	HepFarser	Decipher the major components of LMWHs	Wang et al., 2021
	MsPHep	LMWH databases using a theoretical database-based strategy	Xie, Bu, LaCava, & Chi, 2023
Antithrombin binding region	Exhaustive treatment with heparin lyase II	3-*O*-sulfated tetrasaccharide composition	Li et al., 2014; Chen et al., 2017
	[^{1}H, ^{15}N] HSQC NMR	Characterize the 3-*O*-sulfated tetrasaccharides	Beecher et al., 2016
Risk of HIT	SPR	Binding kinetics	Pattnaik, 2005; Zhang, Datta, Dordick, & Linhardt, 2020
	Electron microscopy	Complex size	Nevzorova et al., 2019
	Zeta potential	Complex charge	Bertini et al., 2017
	Size-exclusion chromatography and MS	Structure mechanism of PF4-LMWH interaction	Wu et al., 2019; Shi et al., 2023

Due to the unique structural aspects of LMWH products, properly designed clinical trials can be used to demonstrate that generic products are safe and effective, although clinical metrics are not sufficiently sensitive to distinguish chemical and activities differences among these drugs. For this reason, the Food and Drug Administration proposes that the consistency of the generic product and the reference product should be evaluated in five ways: 1. The physical and chemical properties should be the same; 2. The heparin raw material should be in compliance with the USP, and the quality standard of the heparin sodium API and the depolymerization pattern of the generic raw material should be consistent with that of the reference product; 3. The disaccharide composition, oligosaccharide fragment distribution and oligosaccharide sequence are consistent; 4. The *in vitro* biological and biochemical activities of the generic product and the reference product are consistent; 5. The human pharmacodynamic equivalence between the generic product and the reference product, mainly anti-Xa and anti-IIa activity, are consistent. In 2016, the Food and Drug Administration introduced immunogenicity-related considerations for LMWH. The most common adverse reaction to heparin is bleeding, the most serious of which is caused by heparin-induced thrombocytopenia (HIT) (Arepally, 2017). HIT is mediated by platelet factor 4 (PF4)-heparin complex antibody inducers, and impurities in LMWH may act as immune agonists or promote immunogenicity by altering the interaction of LMWH with PF4 (Sachais et al., 2012).

2.1. Animal sources

Porcine intestinal mucosa is the primary source for heparin, while bovine and ovine intestinal mucosa are alternative sources. There are drawbacks to using porcine intestinal mucosa as the exclusive source for heparin. With the increased demand for heparins and LMWHs, there is an emerging need for alternative sources to porcine intestinal heparin. Bovine intestinal heparin was considered potentially at risk and was withdrawn by manufacturers in the 1990s due to the bovine spongiform encephalopathy epidemic. New techniques have been developed to improve the safety of bovine intestinal heparin (Andrews et al., 2020; Szajek et al., 2015). As opposed to bovine intestinal heparin, ovine intestinal heparin has a comparable pharmacological profile to porcine intestinal heparin (Hoppensteadt et al., 2015; Olson et al., 2023).

Dalteparin-like LMWHs were obtained from bovine lung and ovine intestinal heparin. The effectiveness of these LMWHs as a substitute for porcine intestinal mucosa has been assessed (Xie et al., 2018). The heparin contamination crisis happened when crude porcine intestinal heparin was adulterated with oversulfated chondroitin sulfate (Liu, Zhang, & Linhardt, 2009). In addition, outbreaks of African swine fever have led to the death of millions of pigs, which severely threatened the global heparin supply chain. Therefore, reconsideration of the use of bovine and ovine intestinal heparins and depolymerized enoxaparin are being discussed at regulatory and pharmaceutical levels and at various stages of development (Sharma, Civelli, & Petersen, 2022). The key tests for LMWH quality assurance are the characterization of the parent heparin API. Recently, bovine and ovine intestinal heparins were compared to porcine intestinal heparin (Kouta et al., 2019; Kouta et al., 2021; Zhang, Shi, Li, Shi, & Chi, 2023). Douaisi and coworkers have recently prepared enoxaparin from chemoenzymatically synthesized heparin (Douaisi et al., 2024). Baytas et al. reported a β-elimination preparation of LMWH obtained from a remodeled heparin of bovine intestinal origin, which was treated with 3-*OST* (Baytas et al., 2021). LMWHs prepared from bovine intestinal and lung heparin, and ovine intestinal heparin, were also compared to branded enoxaparin using pharmacopeial potency, top-down, bottom-up, and compositional analysis. The results showed a comparable oligosaccharide distribution to commercially available enoxaparin (Chen et al., 2019; Hoppensteadt et al., 2015; Jeske et al., 2018; Liu et al., 2017).

2.2. Anticoagulant potency

The therapeutic effects of LMWHs are mainly due to their interactions with plasma proteins including factor Xa and thrombin. The anticoagulant mechanism of enoxaparin is attributed to its ability to bind to AT and formation of ternary complexes with coagulation factors, principally Xa and IIa. The anticoagulant activity is measured by clot-based methods such as activated clotting time (ACT), thrombin time (TT), activated partial thromboplastin time (aPTT), anti-factor Xa and anti-factor IIa assay, protamine sulfate (PS) neutralization studies, and thrombin generation assay (TGA). In the USP monograph, enoxaparin has a potency of no less than (NLT) 90 and no more than (NMT) 125 anti-factor Xa international units (IU)/mg, and NLT 20.0 and NMT 35.0 anti-factor IIa IU/mg, calculated on a dried basis. In addition, the ratio of anti-factor Xa activity to anti-factor IIa activity is between 3.3 and 5.3. Therefore, it is important to compare the potency of each LMWH with its Reference Listed Drug (RLD). The LMWH and anticoagulant potency of ovine and bovine intestinal heparin-derived LMWHs were systematically evaluated and showed comparable profiles to branded enoxaparin (Brouwers, Roeters van Lennep, & Beinema, 2019; Jeske et al., 2018; Mourier et al., 2016).

2.3. Molecular weight distribution

Chain size is one of the factors affecting the biological activity of LMWH, and an accurate method to determine MW distribution and average molecular weight is essential for quality control. In the USP monograph, molecular weight distribution and weight average molecular weight are determined by size exclusion LC equipped with a differential refractive index (RI) detector in accordance with the monograph criteria. Two 7.8 mm × 300 mm columns in series and 0.5 M lithium nitrate mobile phase solution are used. The acceptance criteria for enoxaparin are Mw between 3800 and 5000 Da, the percentage of molecular weight lower than 2000 Da (M_{2000}) is between 12.0 % and 20.0 %, molecular weights in the range 2000–8000 Da ($M_{2000–8000}$) is between 68.0 % and 82.0 %, and molecular weights greater than 8000 Da (M_{8000}) is no more than 18.0 %. However, the accuracy of this method is ultimately dependent on calibration with high purity standards. In addition, the use of RI detection as specified in the monograph limits the analysis. Therefore, chemoenzymatically synthesized pure oligosaccharides with varied and determined molecular weights are options used as standards in combination with the Support Vector Machine (SVM) computational technique (Arnold et al., 2017). Furthermore, methods not requiring calibrants have been developed for the determination of molecular weight distributions. Size exclusion chromatography (SEC) relying on multiple angle laser scattering (MALS)/RI detectors has been developed for accurate analysis of HP and LMWH in the absence of standards (Beirne et al., 2011; Ouyang et al., 2017).

2.4. Disaccharide analysis

Repeated disaccharide units are characteristic structures of GAGs. LMWHs have different positions and numbers of sulfate groups in the repeating disaccharide units, and the proportion of these disaccharides with different positions and numbers of sulfate groups may vary depending on the animal source, preparation process, manufacturer and even batch. Therefore, the analysis of disaccharide composition after thorough enzymatic digestion is an essential element of quality control. The non-reducing end of the sugar chain of disaccharides or oligosaccharides released by heparin lyase degradation has an unsaturated uronic acid residue with a Δ4, 5-position double bond, which conjugates with the carboxyl, and exhibits a characteristic absorption at 232 nm. Deaminative cleavage using nitrous acid (HONO) is an alternative degradation method for disaccharides or oligosaccharide, which retains uronic acid epimerization and further determined by LC-MS (Gill, Wang, Shi, & Zaia, 2012; Kariya, Herrmann, Suzuki, Isomura, & Ishihara, 1998; Zhang et al., 2020). Qualitative and quantitative analysis can be achieved by combining different isolation mechanisms and appropriate detection methods, providing an important characterization of the basic composition for quality control.

Strong anion exchange (SAX) is a chromatographic method for the separation of disaccharides based on ionic strength using a high concentration of non-volatile salts as the mobile phase. SAX is characterized by high resolution, stability and quantitative accuracy in combination with a UV detector (Sadowski et al., 2017). However, it is not possible to use online coupled mass spectrometry due to the use of non-volatile salts as the mobile phase. A multiple heart cut (MHC) two-dimensional LC-MS/MS was developed to overcome this incompatibility. SAX-UV was used as first dimensional chromatography for the effective separation of LMWH products, and then designated chromatographic peaks were automatically entered into the second dimension of SEC for on-line desalting, followed by MS qualitative analysis (Chen et al., 2021; Chen et al., 2022). This method exploited the stability, accuracy and resolution of traditional SAX and enabled on-line qualitative analysis by MS.

Ion-pair reverse-phase MS (IPRP-MS) is an alternative method for disaccharide analysis due to its extremely hydrophilic nature, which is difficult to retain in conventional reversed-phase chromatographic columns. The addition of the ion-pairing reagents to the mobile phase results in the formation of neutral molecules with a certain lipophilicity, which increases the retention of the disaccharide in the chromatography and improves the separation. Commonly used ion-pairing reagents include tributylamine (TBA) and ammonium acetate, butylamine (BTA) and ammonium acetate, and pentylamine (PTA) and hexafluoroisopropanol (HFIP) (Galeotti & Volpi, 2013; Yang et al., 2011; Zhang et al., 2009). IPRP provides a high degree of chromatographic resolution and good MS compatibility, making it an efficient and convenient method for disaccharide analysis. However, the addition of ion-pairing reagents causes them to readily and irreversibly combine with the column packing material, leading to a reduction in column efficiency over time and a shortening of column lifetime. In addition, the ion-pairing reagent may impact MS performance.

Pre-column derivatization can alter chromatographic behavior and the introduction of a luminescent moiety diversifies the detection means and further improves detection sensitivity. Heparin disaccharides can be completely separated on RP, HILIC and capillary electrophoresis (CE) after derivatization with 2-aminoacridone (AMAC). Moreover, Wang

et al. used 6-amino-*N*-(2-diethylamino) ethyl-quinoline-(2-carboxamide) (AMQC) derivatization to quantify heparin disaccharide in MRM mode, with sensitivity increased by 20–320-fold compared to AMAC (Wang, Dhurandhare, et al., 2021). This high sensitivity and specificity make these methods ideal for the quantitative analysis of biological samples, such as cells, tissues, and body fluids.

HILIC, also known as "aqueous normal-phase chromatography", is a hybrid chromatography combining normal-phase and ion-exchange separation mechanisms, is suitable for the separation of highly polar compounds. HILIC has poor resolution, high and unstable UV background, but good compatibility with MS, and its combination with MS greatly improves the applicability of the method. Sun, et al. used HILIC for separation in combination with multiple reaction monitoring (MRM) for qualitative and quantitative analysis of thoroughly digested LMWH (Sun, Sheng, et al., 2016).

CE has the unique advantages of high resolution and high sensitivity for highly charged substances such as heparin disaccharides. Conventional CE is often used with UV or fluorescence detectors due to non-volatile salts such as phosphate buffer. Zhang et al. used the CE-UV method to analyze disaccharides in LMWHs with different AT III binding activity fractions (Zhang, Li, Zhang, & Kang, 2017). The interface of CE-MS is the key to establishing a successful method. With the development of CE and MS coupling technology, CE-MS has been used to qualitatively and quantitatively analyze heparin disaccharides with different degrees of sulfation (Ouyang et al., 2019; Sun, Lin, et al., 2016). Lin, et al. reported a volatile methanolic ammonium acetate electrolyte and sheath fluid linked to negative ion ESI-MS (Lin et al., 2017).

2.5. Terminal groups and minor modifications of LMWHs

Different cleavage methods create LMWHs containing distinctive terminal groups, which could be markers of manufacturing process. Therefore, it is required to be monitored by LC-UV, LC-MS and NMR methods for quality control. Enoxaparin is the most commonly used LMWH have unsaturated 4-enopyranosuronate residues at the non-reducing ends (NRE) and about 20 % of the materials contain a 1,6-anhydro derivative at the reducing end of the chain, the range being between 15 % and 25 % which is recorded in the United States Pharmacopoeia (USP) (United States Pharmacopeia, 2023). In other instances of LMWH, dalteparin and nadroparin cleaved by nitrous acid resulted in 6-*O*-sulfo-2,5-anhydro-D-mannitol at the reducing ends. In case of tinzaparin depolymerized by heparin lyase have an unsaturated uronic acid residue at its NRE. Although these unusual structures are in very low abundance, they are important indicators for the process conditions.

Minor modifications during different process procedures are also important quality attributes for LMWHs and need further investigation. For instance, linkage region oxidation, 2,3-epoxide and galacturonic acid GalA can be generated within the internal enoxaparin sequences (Gardini et al., 2021; Mourier & Viskov, 2004; Ozug et al., 2012; Rej & Perlin, 1990). The quantitative analysis of the linkage region tetrasaccharides in enoxaparin was also provided (Chen, Lin, et al., 2017). Ring contraction in dalteparin internal aminosugar residues was obtained using 2D NMR and LC-MS analysis (Alekseeva et al., 2014; Bisio et al., 2017). Extensive efforts, such as combined 2D NMR and LC-MS analysis have been devoted to the development of analytical techniques in terminal groups and minor modifications of LMWHs.

2.6. Oligosaccharide mapping

The oligosaccharides in LMWHs differ in degree of polymerization, glycosyl composition, and number of sulfate groups or positions at each degree of polymerization. The precise structure remains unknown. It is not possible to characterize every molecular species in LMWH preparations. Therefore, oligosaccharide mapping is an important supporting data for structure confirmation, conformational relationship, and quality control. However, due to the structural characteristics and micro-uniformity of the relative molecular mass distribution, the difficulty of analyzing these substances is much higher than that of disaccharides. Single chromatographic analysis is often unable to meet the requirements of oligosaccharide analysis. Liquid chromatography-mass spectrometry has become the main tools for LMWHs, and the commonly used chromatographic methods include IPRP, HILIC, SEC, and CE.

Top-down & bottom-up approaches have been developed for the quality control of enoxaparin and generic LMWH. The method uses a virtual data library generated by theoretical fractions generated by GlycoReSoft, consisting of enoxaparin to complete the retrieval and attribution of MS data. The compositions of oligosaccharides with different degrees of polymerization of enoxaparin from different manufacturers were compared (Li et al., 2012; Li, Yang, et al., 2014; Sadowski et al., 2020). The tremendous improvement in chromatographic resolution of LMWH by ultra-high-resolution SEC, online MS has also made great progress in the characterization of these oligosaccharides. However, the multiple adducts of volatile ammonium salts in the mobile phase and electrospray ionization have led to a large number of false positives. An online cation suppressor has been introduced to remove the cations, and an isotope MS database for heparin analogues was established to reduce the number of false positives (Zaia et al., 2016). Glycommaping software has also been introduced through chromatographic fitting and MS assignment correction, which greatly improves the performance of SEC separation. (Yan et al., 2022).

2.7. Bioinformatics-assisted parsing of mass spectrometry data

While mass spectrometry has played an important role in the analysis of polysaccharide composition, the large amount of data generated by MS makes assignment very tedious and challenging. In particular, LMWHs are composed of polydisperse and structurally heterogeneous oligosaccharides, which further complicates the analysis. The different oligosaccharides with close molecular weights may exceed the resolution limit of the instrument and the isotopic peaks may overlap. The strategies for computational algorithms and software-assisted MS annotation can be divided into three categories: structural library-based (Lohmann & von der Lieth, 2004), spectral library-based (Xue, Laine, & Matta, 2015) and *de novo* approaches (Hu et al., 2014). GlycReSoft is one of the most popular and successful tools that automates glycan recognition based on isotope peaks and charge (Maxwell et al., 2012). It has been applied successfully to the top-down analysis of LMWHs. In addition, the GlycCompSoft algorithm has been reported to support top-down analysis, significantly improving the speed and reliability of data interpretation (Wang et al., 2016). HS-SEQ and GAG-ID are two other algorithms for *de novo* sequencing of GAGs (Chiu, Huang, Orlando, & Sharp, 2015; Duan & Amster, 2018). In addition, HepParser has been developed to decipher the major components of LMWHs with significantly improved accuracy through perk merging strategy (Wang, Wang, et al., 2021). Furthermore, Glycomapping relies on an innovative chromatography fitting and a MS correction strategy to analyze and compare the enoxaparin produced by different manufacturers and animal sources (Yan et al., 2022). However, both of these algorithms or software do not provide a database, and thus, users need to generate on their own a hypothetical structural library. MsPHep provides various LMWH databases using a theoretical database-based strategy, which is a useful tool for rapid LMWH analysis and comparison (Xie et al., 2023).

2.8. NMR spectroscopy

NMR spectroscopy has been used extensively to characterize heparin and its derivatives. The successful detection of the potential adulterant, oversulfated chondroitin sulfate (OSCS), and other impurities in heparin and LMWHs led to the introduction and validation of proton NMR

(Guerrini et al., 2008). The U.S. Pharmacopoeia (USP) uses ^{1}H NMR and ^{13}C NMR to identify dalteparin and enoxaparin by the presence or absence of characteristic peak signals at defined chemical shifts. The advantage of NMR is that the sample does not require any further modification or chromatographic fractionation prior to the measurement. In addition, NMR provides a high-resolution qualitative and quantitative fingerprint that allows structural features to be assigned and compared. Generic and commercial branded LMWHs have been easily differentiated by coupling NMR and chemometric analysis (Guerrini et al., 2015; Jiang et al., 2022). Furthermore, LMWHs from different animal sources were investigated by NMR combined with multivariate analysis including principal component analysis (PCA), factor discriminant analysis (FDA), partial least squares-discriminant analysis (PLS-DA) and linear discriminant analysis (LDA) (Monakhova et al., 2018). Quantitative HSQC was applied to calculate the percentage of monosaccharide and disaccharides by normalizing the same monosaccharide and carbon-proton pair type of reference and target samples (Mauri et al., 2017). Therefore, 2D NMR is suitable for pharmaceutical quality control of LMWHs.

Although proton and carbon NMR are routinely applied for the quality assurance of pharmaceutical LMWHs, ^{15}N NMR has showed its own advantage for LMWH characterization (Langeslay, Beni, & Larive, 2011). Measurement of the [^{1}H, ^{15}N] HSQC spectra of enoxaparin determined the chemical exchange characteristics of the sulfamate protons, and [^{1}H, ^{15}N] HSQC-TOCSY spectrum was used to assign specific NH correlations through TOCSY cross peaks to sugar ring protons with chemical shifts characteristic of specific GlcNS microenvironments (Beecher et al., 2016; Langeslay et al., 2013). This method could distinguish enoxaparin from tinzaparin, which had a more diverse mixture of oligosaccharides, and dalteparin, which suffered from the loss of sulfamate groups in the formation of 2,5-anhydromannitol residue. Furthermore, ^{15}N NMR was proved to have the ability to detect the amine groups of GlcN and GlcN3S in aqueous solution, could be used for further study of the amine groups in LMWHs (Beecher & Larive, 2015; Pomin, 2016).

2.9. Antithrombin binding region

Heparin-AT binding is one of the most well studied interactions, which depends on a specific type of pentasaccharide sequence contains a 3-*O*-sulfo group in the central GlcN residue (Shi et al., 2022). LMWHs are derived through controlled cleavage of UFH, results in differences in anti-Xa and anti-IIa activity ratio. The structure features of different LMWHs regard to the distribution of chain length and sulfation patterns were investigated. LMWHs were fractionated into high affinity and no affinity pools with their ability to bind antithrombin and the fractions were characterized (Bisio et al., 2009; Chen et al., 2018). An alternative approach to determine 3-*O*-sulfation conditions is through 3-*O*-sulfated tetrasaccharides composition generated by exhaustive treatment with heparin lyase II (Chen, Ange, et al., 2017; Li et al., 2014). In addition, [^{1}H, ^{15}N] HSQC NMR has been reported to characterize the 3-*O*-sulfated tetrasaccharides (Beecher et al., 2016).

2.10. Risk of HIT

HIT is an adverse immunological disorder caused by antibodies to platelet factor 4 (PF4)-HP/LMWH complexes (Martel, Lee, & Wells, 2005). The analysis of HIT potential for LMWH and its generic products is important for quality control prior to clinical application. The methods addressing biomolecular interaction of HP/LMWH and PF4 include: (1) binding kinetics determined by surface plasmon resonance (SPR) (Pattnaik, 2005; Zhang, Datta, et al., 2020); (2) complex size determined by electron microscopy (Nevzorova et al., 2019); (3) complex charge determined by zeta potential method (Bertini et al., 2017); and (4) binding complex determined by size-exclusion chromatography or mass spectrometry (Shi et al., 2023; Wu et al., 2019). Even with a demonstration of active ingredient sameness for generic LMWH products, the HIT risk may still vary, since impurities may affect, such as DNA, protein and leachables from the container closure.

3. Preparation of LMWH by degradation

Enoxaparin, dalteparin, and tinzaparin have been manufactured by depolymerizing UFH using chemical or enzymatic methods. After expiration of the first LMWH patent, dalteparin by Lindahl et al. (Lindahl et al., 1979) in 2000, generic LMWHs have been developed with further innovation and new patents. To overcome the single-source nationality and regionality on the current supply chains. Apart from the LMWHs available on the market, numerous researchers have tested various techniques like chemical, physical, and enzymatic degradation to produce LMWHs. New version LWMHs derived from whether from other animal sources or chemoenzymatic synthesis or total synthesized LMWH are requested. Table 5 provides an overview of the principles, advantages, and disadvantages of different breakdown methods for producing LMWHs.

3.1. Chemical degradation

The chemical degradation mechanisms of UFH include three approaches: (1) hydrogen peroxide, (2) β-elimination, and (3) nitrite degradation (Fig. 2). All routes rely on breaking the glycosidic bond of heparin to derive LMWH. The hydrogen peroxide method oxidizes heparin under specific conditions to cleave the glycosidic bond. The benefit of this method is the acquisition of free radicals from several methods. However, contamination is a concern during processing. LMWHs, Ardeparin and Parnaparin are commercially manufactured using this method. Photochemical depolymerization catalyzed by titanium dioxide (TiO_2) can produce reactive oxygen species capable of randomly oxidizing sugar residues. However, issues on scaling up to 250 mL discouraged its commercial application (Higashi et al., 2012). Moreover, a precise H_2O_2/ascorbic acid process coupled with ultrasound was employed to produce free radicals for depolymerization, resulting in exceptionally pure products (Shen et al., 2019). The β-elimination method degrades the UFH through esterification followed by alkaline depolymerization. Enoxaparin sodium, the most significant type of LMWH, is obtained by β-elimination. This method provides the advantage of producing a more uniform LMWH by controlling the reaction conditions. However, the process has its drawbacks such as being cumbersome, requiring high technical expertise and cost, and having slightly inferior stability performance. Zhao et al. utilized a controlled β-elimination method followed by gel chromatography separation to produce a LMWH with significantly low polydispersity and narrow molecular weight distribution (Zhao et al., 2016). Finally, the nitrous acid degradation method involves adding a depolymerizing agent to heparin under acidic conditions, which breaks down the glycosidic bond over time (Lormeau, Petitou, & Choay, 1991). The reaction can be terminated by adjusting the pH to neutral. This is currently commonly used process for preparing LMWH at large scale, as it can effectively decrease the molecular weight through the use of different reaction conditions. Dalteparin and nadroparin are commercially available LMWHs produced through nitrous acid degradation followed by borohydride reduction (Davis & Faulds, 1997; Pineo & Hull, 2004).

3.2. Enzymatic degradation

Controlled enzymatic degradation of heparin is an important method for producing LMWH. Tinzaparin, is a representative form of LMWH manufactured by controlled enzymatic degradation of heparin (Amerali & Politou, 2022) (Fig. 2). Presently, LMWH is produced enzymatically using heparin lyase I/II/III or a combination of these enzymes. Previous studies have shown that heparin lyase I acts on the highly sulfated domain, →4)-α-D-GlcNS6S-(1 → 4)-α-L-IdoA2S-1(1→. In contrast,

Table 5
Degradation approaches of generic LMWHs.

Types	Methods	Mechanism	Advantage	Disadvantage	Ref
Chemical methods	Titanium dioxide (TiO_2)-catalyzed photochemical depolymerization	Produce reactive oxygen species that are capable of randomly oxidizing the sugar residues	Require no harsh or toxic reagents, free of chemical artifacts	Limited scales	Higashi et al., 2012
	H_2O_2/ascorbic acid and ultrasonic power system	Produce free radicals for depolymerization	Efficient and environmentally friendly	Biological activities *in vivo* was not determined	Shen et al., 2019
	Alkaline β-elimination followed by gel chromatography	a controlled β-elimination method	Producing a uniform LMWH by controlling the reaction conditions	cumbersome, requiring high technical expertise and cost, and having slightly inferior stability performance	Zhao, Sang, & Cui, 2016
Physical methods	Dielectric-barrier discharge (DBD)	Generate active free radicals	Quick, energy-efficient, low cost and environment-friendly	Sulfate group break off	Yang et al., 2018
	Ultrasonic wave	Hydrogen peroxide-catalyzed radical hydrolysis produced by ultrasonic waves	No harsh or toxic reagents, free of chemical artifacts	Lower anti-Xa and anti-IIa activity	Achour et al., 2013
	Combined ultrasonic wave and Fenton system	Generate active free radicals to deconstruct heparin	Efficient, improved activities	Side reactions that reduce final product efficiency and yields	Zhi et al., 2019
	Radiation-induced destruction with controlled irradiation conditions	Ionization that breaks the main chain	Original structure had not been significant modified	Involves a slight decrease of undersulfated sequences	Jeske et al., 1995; Bisio et al., 2001; Tuaeva et al., 2018
Enzymatic methods	Heparin lyase III	Enzyme action on unsulfated domains	Comparable anticoagulant activity	Costly	Xiao et al., 2011
	Photoswitchable heparin lyase III	Precise control of enzymes	Recyclable enzyme, precisely control of degradation	Unmatched anti-II activity compared to enoxaparin	Gu, Wu, Liu, Pan, & Chen, 2018
	Chitin-affinity immobilized platform	Immobilized enzymes cleaved the chains	Immobilized enzyme with high tolerance to heat and pH	Limited scale	Xu et al., 2017
	HepBp	Enzyme action on unsulfated domains	Specified reducing end cleaved by enzymes	Limited scale and further investigation needed	Bohlmann et al., 2015; Yu et al., 2019

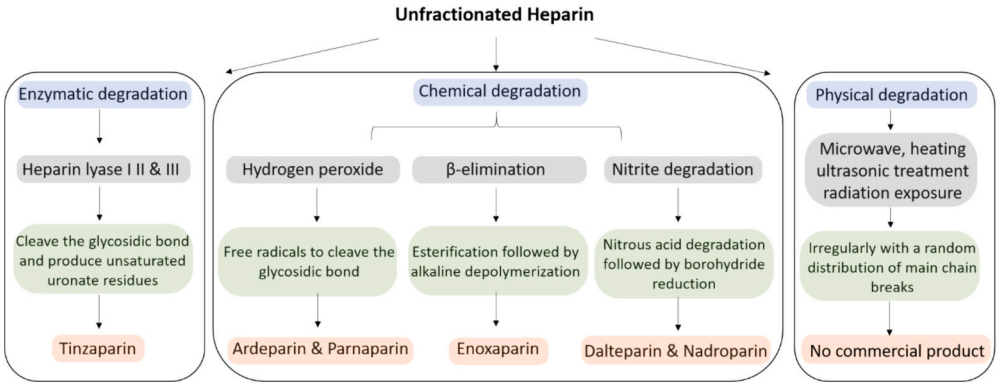

Fig. 2. Degradation mechanisms and the generated products.

heparin lyase II acts on lower sulfation heparin sulfate chains, →4)-α-D-GlcNAc/GlcNS/GlcNS6S-(1 → 4)-α-L-IdoA/β-D-GlcA-1(1→. Furthermore, heparin lyase II has a wider range of specificities, working on both HP and HS (Desai, Wang, & Linhardt, 1993; Lohse & Linhardt, 1992; Shaya et al., 2010). The challenge of controlling enzymatic conditions and producing large amounts of heparin lyase have limited its industrial implementation. The result of digesting heparin with heparin lyase III is a new low molecular weight heparin form due to the enzyme's action on unsulfated domains, which avoids cleavage of the linkages within the binding site for antithrombin III (Xiao et al., 2011). In addition, a photoswitchable heparin lyase III was developed for precise control over the preparation of LMWH (Gu et al., 2018). An enzymatic ultrafiltration reactor was developed to remove the high content of undesired disaccharides and tetrasaccharides during heparin lyase II treatment, and a novel LMWH was obtained (Fu et al., 2014). Xu and coworkers used chitin-affinity immobilized heparin lyase I platform to produce LMWHs (Xu, Zhang, Duan, & Chen, 2017). The combinations of heparin lyase I/II/III was used for synergistic degradation to obtain LMWHs (Wu, Zhang, Mei, Li, & Xing, 2014).

Heparin lyase cleavage produces unsaturated uronate residues at the reducing ends of the cut chains. Therefore, recombinant Δ4,5 unsaturated glycuronidase has been employed as it removes the unsaturated uronate residues present at the non-reducing ends of these lyase-depolymerized products (Su et al., 2015). A newly reported heparanase from the invasive pathogenic bacterium *Burkholderia pseudomallei* (HepBp) is an *endo*-acting glycoside hydrolase capable of degrading HS (Bohlmann et al., 2015). The specificity and action pattern of HepBp indicates that the enzyme (a hydrolase) cleaves the linkage between GlcA and GlcNAc, GlcNAc6S, or GlcNS in a low sulfated domain, suggesting that this enzyme may not be directly useful for the preparation of LMWHs (Yu et al., 2019). The advantage of this enzyme is that its cleavage site differs from that of heparin lyases. Furthermore, the exolytic heparinases which sequentially releasing unsaturated disaccharides from reducing ends could be a potential enzyme for preparation of LMWHs (Zhang et al., 2021).

3.3. Physical degradation

The degradation of polysaccharides can be achieved through various physical methods, such as microwave, heating, ultrasonic treatment, and radiation exposure (Fig. 2). Microwave and ultrasonic treatment can generate free radicals to aid in the heparin degradation process. Radiation-induced degradation relies on ionization to break the polysaccharide chain, resulting in reduction of heparin's molecular weight. This type of degradation occurs irregularly with a random distribution of main chain breaks. Each break in the main chain generates smaller non-uniform macromolecules, causing a reduction in the average molecular weight and altering the molecular weight distribution. Irradiation with γrays was applied for the depolymerization of UFH to obtain LMWHs, which exhibited very similar molecular weight profiles and comparable inhibitory activities (Bisio et al., 2001; Jeske et al., 1995). Tuaeva and colleagues (Tuaeva et al., 2018) applied radiation-induced destruction with controlled irradiation conditions to prepare LMWHs. This method led to the cleavage of the pyranose rings and the formation of compounds with carbonyls and carboxylic acids of heparin. Ultrasonic-assisted radical depolymerization of heparin leads to LMWHs that have comparable average molecular weight and anticoagulant properties to some commercial ones, but with lower degree of sulfation (Achour et al., 2013). Dielectric-barrier discharge (DBD) has been used to generate reactive free radicals and enable heparin depolymerization in aqueous solution (Yang et al., 2018). Additionally, a combined ultrasonic wave and Fenton system was utilized to deconstruct heparin into LMWHs. These LMWHs exhibit high anticoagulant activity, with HexN (GlcN and its derivatives) predominantly present at the end group and HexA (IdoA, GlcA and its derivatives) being broken down by hydroxyl radicals (Zhi et al., 2019).

4. Preparation of LMWH mimetics/heparin oligosaccharides by synthesis

Up to date, commercially available LMWHs in clinical use are exclusively prepared from natural heparin, except for fondaparinux, while the synthesized LMWH are still in its infant stage. Synthesis of LMWH mimetics can yield molecular species with a determinate structure. This ability is beneficial in examining the molecular underpinnings of interactions with proteins, ensuring quality control, and attaining regulatory compliance in the pharmaceutical industry. Moreover, it enables straightforward recognition of intellectual property. Chemical synthesis, bioengineering synthesis, and chemoenzymatic synthesis are three key methods for synthesizing LMWH mimetics.

4.1. Chemical synthesis

It remains difficult to synthesize long-chain oligosaccharide and polysaccharides using chemical approaches. Indeed, several challenges exist, such as low yields, multiple synthetic steps, protection and deprotection steps, and high costs. Chemical synthesis of a fondaparinux pentasaccharide, a chemically-defined ultra-LMWH structure, has been performed successfully; however, chemically synthesized LMWH products with defined structures are very expensive. Until now the completely chemically synthesized heparin oligosaccharide in current clinical use is fondaparinux (Keam & Goa, 2002). The first chemical synthesis of fondaparinux requires more than 50 steps with an overall yield of 0.1 % (Petitou et al., 1989). Recent efforts have been made to develop more efficient synthetic routes using traditional modular synthetic, one-pot, programmable methodologies (Chang et al., 2014; Dey, Lo, & Wong, 2019; Dey, Lo, & Wong, 2020; Jin et al., 2019; Li, Ye, et al., 2014). Heparan sulfate (HS) oligosaccharide synthesis including building block synthesis, oligosaccharides construction and chemical sulfation methods have been developed. Recently, a fluorous tagging strategy was used for synthesizing a universal tetrasaccharide building block for HS library, which will facilitate a detailed understanding of HS-protein interactions (Wang et al., 2023). The longest heparin-related oligosaccharide was chemically synthesized up to 40-mer using $[4]_n$ iterations strategy (Hansen, Miller, Cliff, Jayson, & Gardiner, 2015). The continued developments of synthetic strategies to HS synthesis, including automated glycan assembly and traditional solid phase synthesis will be needed to enable comprehensive HS libraries (Budhadev et al., 2019; Guedes et al., 2015). In addition, there are many excellent reviews addressing chemical approaches and methodology for synthesis of heparin oligosaccharides and the reader is directed to these literatures for a comprehensive survey of this field (Dulaney & Huang, 2021; Mende et al., 2016; Pongener, O'Shea, Wootton, Watkinson, & Miller, 2021).

4.2. Chemoenzymatic synthesis of LMWH mimetics

Chemoenzymatic synthesis presents a promising strategy for the production of glycosaminoglycans (GAGs) from non-animal sources, specifically for LMWH mimetics (Wang, Liu, & Voglmeir, 2020). This approach typically imitates the biosynthetic pathway of HP and HS, including backbone elongation and saccharide modifications. Initially, disaccharide polymerization was controllably performed using heparosan synthases and UDP (uridine diphosphate)-GlcNTFA (*N*-trifluoroacetyl glucosamine) and UDP-GlcA to generate oligosaccharides. The oligosaccharides produced were subsequently modified using *N*-sulfotransferase (NST), C5 *epi*merase, 2-*O*-sulfotransferase (2*O*ST), 6-*O*-sulfotransferase (6*O*ST) and 3-*O*-sulfotransferase (3*O*ST) in the presence of PAPS (3′-phosphoadenosine-5′-phosphosulfate) (Liu & Linhardt, 2014, Zhang et al., 2017).

Chemoenzymatic synthesis first reported by Liu and coworkers started with the acceptor GlcA-AnMan (anhydromannitol), yielding two well-defined anticoagulant ultra-low molecular weight heparins (Xu et al., 2011). Two glycosyltransferases—GlcN-acetylglucosaminyl transferase from *E. coli* K5 (KfiA) and heparosan synthase 2 from *Pasteurella multocida* (PmHS2)—were involved in the polymerization steps. A series of homogeneous active lead compound oligosaccharides were successfully prepared using this enzymatic synthesis approach (Xu et al., 2014; Xu, Pempe, & Liu, 2012). Homogeneous LMWH mimetics dodecasaccharides were chemoenzymatically synthesized from UDP monosaccharides using recombinant glycosyltransferase followed by C5-epimerase and various sulfotransferases. The resulting LMWH mimetics has promising anticoagulant activity and is neutralizable by protamine (Xu, Chandarajoti, et al., 2017). Cai and colleagues reported a fluorous-assisted chemoenzymatic approach to successfully synthesize a series of partial *N*-sulfated, 6-*O*-sulfated heparan sulfate oligosaccharides (Cai et al., 2014). Different functional groups and bioactivity heparin oligosaccharide analogs were synthesized following a chemoenzymatic scheme, for example, a nanomolar anti-FXa activity yet resistant to heparinase digestion HP oligosaccharides (Ham et al., 2023) and HS oligosaccharides composed of two or more epimerization and sulfation domains separated by unmodified domains of different length (Sun, Chopra, & Boons, 2022).

Other chemoenzymatic synthetic methodologies require pre-polymerized heparosan (Fig. 3). This heparosan starting material is available as a capsular polysaccharide from bacteria, such as *E. coli* K5 (Nehru, Tadi, Limaye, & Sivaprakasam, 2020). The molecular weight of the resulting heparosan was modified to produce low molecular weight heparosan (3 to 5 kDa) for the production of bioengineered LMWHs (Roy et al., 2021). In addition, a combined biocatalytic approach was developed for the size-controlled synthesis of longer heparosan oligosaccharides (Deng et al., 2022). Kuberan and coworkers synthesized antithrombin III-binding pentasaccharide using engineer cloned enzymes from nonsulfated polysaccharides treated with NDST and heparin lyase I (Kuberan, Lech, Beeler, Wu, & Rosenberg, 2003). A combination of heparin lyase III cleavage followed by chemoenzymatic modification resulted in oligosaccharides with comparable pharmacokinetic properties to Arixtra (Zhang, Yang, Wang, Cheng, & Liu, 2022). Yu and

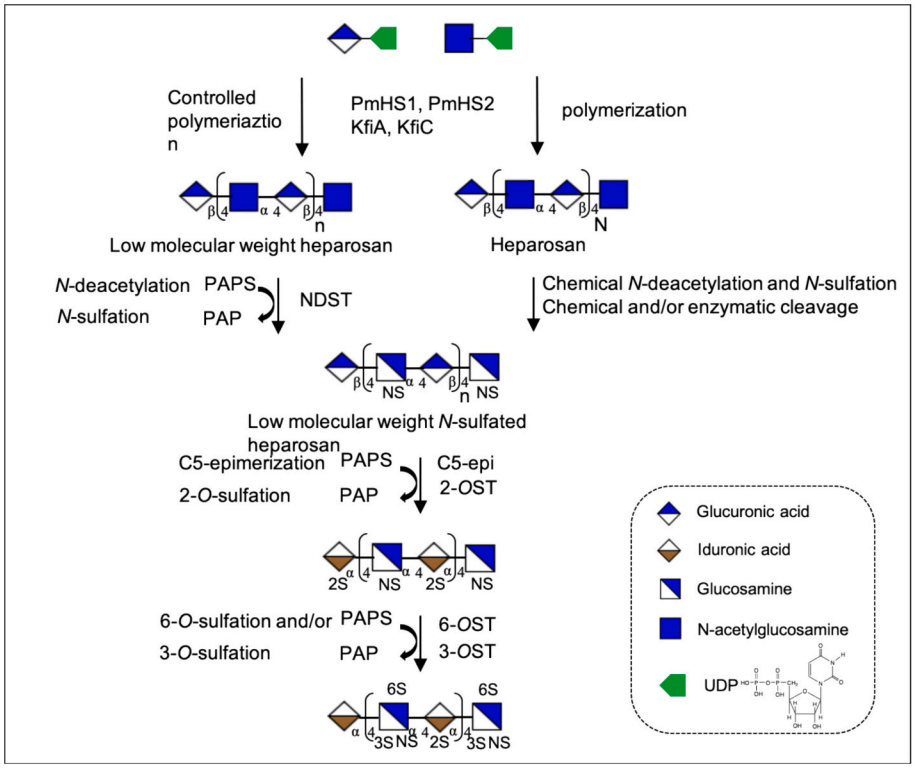

Fig. 3. Chemoenzymatic synthesis of LMWH through heparosan, PAPS: 3′-phosphoadenosine-5′-phosphosulfate, PAP: 3′-phosphoadenosine-5′ phosphate, NDST: *N*-Deacetylase/*N*-sulfotransferase, 2-*OS*T: 2-*O*-sulfotransferase, 3-*OS*T: 3-*O*-sulfotransferase, 6-*OS*T: 6-*O*-sulfotransferase.

coworkers start with heparin lyase III or heparinase Bp (Hep Bp) cleaved oligo-*N*-sulfated heparosan (NSH) followed by *O*-sulfation to produce generic enoxaparin LMWHs (Yu et al., 2022a; Yu et al., 2022b). Furthermore, it has been reported that the chemoenzymatic synthesized heparin with structural and functional properties similar to USP heparin has the ability to be converted into a LMWH similar to enoxaparin (Douaisi et al., 2024).

4.3. Bioengineered synthesis

Metabolic engineering synthesis of LMWHs is a process that re-engineers the heparosan pathway and recruits all related enzymes, including NDST, C5-epi, 2, 6, and 3*OS*Ts, in microorganisms (Fu et al., 2016; Jin, Zhang, & Linhardt, 2021). Bioengineered heparin derived from cells expressing high levels of recombinant heparin biosynthetic enzymes is a potential way to produce a more controllable heterogeneous product (Glass, 2018; Vaidyanathan, Williams, Dordick, Koffas, & Linhardt, 2017). In fact, the bioengineered synthesis methods mainly focus on synthesizing heparin, few reports has been directly targeted on LMWHs. It is reported that metabolically engineered CHO cells, which express enhanced levels of heparin biosynthetic enzymes under optimized bioprocessing conditions has been applied for producing heparin (Thacker et al., 2022). In addition, a microbial cell-free catalyst system has been established for the production of bioengineered heparin (Zhang et al., 2022).

5. Conclusion

Since the first approval of generic LMWH, FDA scientists established a scientific approach for demonstrating active ingredient sameness that takes into consideration the complexity of enoxaparin. Five criteria including (1) physical and chemical characteristics, (2) heparin material and chemical process, (3) arrangement of components, (4) anticoagulant activity, (5) drug's effect in humans ensure that a generic enoxaparin will have the same effects. Though, the technique has developed very fast, there still minor or undefined minor modifications or unpurified contaminate needs to be solved from different biological sources. Future effort would be directed towards developing and improving techniques and approaches towards the chemical or chemoenzymatic synthesis of LMWHs, as well as bioengineered synthesis approaches, which could be able to address the single-source and shortage of HP/LMWHs. However, new contaminate would also be introduced during the process. Therefore, quality control and safety assessment are critically important.

This review summarized the recent development for the quality control of new LMWH based on these criteria. The rapidly developing analytical techniques and LMWH-protein binding sites study will certainly improve the quality assurance during production. Furthermore, their minor modifications should also be carefully checked during the process and approval of new developed LMWHs. Currently, the high demand but limited source of LMWH require new development to diversify the supply of these critical life-saving drugs. Newer versions of LMWHs should be competitive and less expensive than the original versions. Therefore, different LMWH preparation methods including degradation and synthesis are presented. For those LMWHs degraded from animal sources UFH, the sameness should be carefully determined. As for chemoenzymatic and chemical synthesized or degradation from biological heparin, special attention should be paid to the minor modifications or contaminate.

CRediT authorship contribution statement

Yanlei Yu: Writing – original draft, Funding acquisition, Conceptualization. **Yue Song:** Writing – original draft. **Yunjie Zhao:** Writing – original draft. **Ningning Wang:** Writing – original draft. **Bin Wei:** Writing – review & editing. **Robert J. Linhardt:** Writing – review & editing, Funding acquisition. **Jonathan S. Dordick:** Writing – review & editing, Funding acquisition. **Fuming Zhang:** Writing – review & editing, Funding acquisition. **Hong Wang:** Writing – review & editing,

Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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