2021
8(2), 59-66

Chemical Biology
LETTERS

Isolation, Synthesis, and Medicinal applications of Heparin

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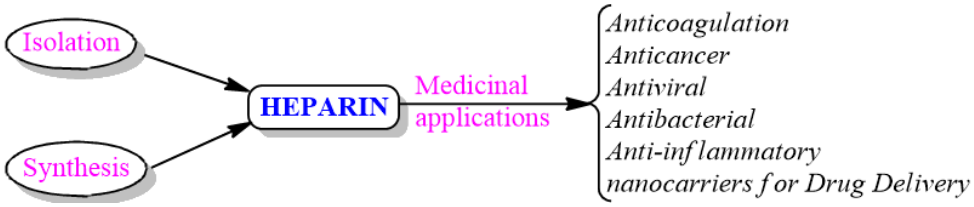
Submitted on: 25-Apr-2021, Accepted and Published on: 01-June-2021

Mini-Review Article

ABSTRACT



Heparin is a highly sulphated and most negatively charged natural biopolymer belonging to the glycosaminoglycan (GAG) family. This is about a 100-year-old anticoagulant drug. This is equally important for non-anticoagulant diseases also and is the reason for the recent burgeoning of interest in the molecule. Heparin has been isolated from both animal and non-animal sources; however, porcine mucosa remains the FDA-approved source for heparin. For the synthesis, chemical, chemoenzymatic, and biotechnological approaches have been studied. In recent times, the focus is more on synthesizing LMWH, ULMWH, and bioengineered heparins. This article is a compilation about the isolation, synthesis, and medicinal applications of heparin.

Keywords: Heparin, glycosaminoglycan, biopolymer, LMWH, ULMWH, anticoagulant

INTRODUCTION

Heparin (Figure 1) is a natural biopolymer belonging to the glycosaminoglycan (GAG) family.[1-3] This is a highly sulphated and most negatively charged polysaccharide. The heparin polysaccharide has disaccharide repeating units, where the disaccharide is 1,4-glycosidically linked and consists of uronic acid residues and D-glucosamine (GlcN). The uronic acid residue is either β-D-glucuronic acid or α-L-iduronic acid (IdoA).[4] The GlcN is further substituted with an N-acetyl (GlcNAc) or an N-sulpho (GlcNS) group, which can also be O-sulphated at the 6-position.[1] This molecule is heterogeneous in nature due to structural variations, which help to interact with different proteins leading to various biological activities. Some of the important properties of heparin are summarized in Figure 2.[5,6] Some of the important brands of heparin available with their generic name as enoxaparin, dalteparin, tinzaparin, heparin flush, danaparoid.

Many researchers have described the discovery and development of heparin. The three persons, William Henry Howell, Luther Emmett Holt (Jr), Jay McLean, names were involved in the discovery of this molecule in 1916-1918.[7,8] After about 14-15 years of work, this biopolymer was considered as an effective anticoagulant drug.[9] Further, medicinal investigation proved that heparin is equally potential as a pharmacological molecule for various non-anticoagulant diseases also.[6,10-12]

Being a biopolymer, heparin is biosynthesized by the concerted action of about 22 enzymes which reflects its complexity towards synthesis. This is biosynthesized in the endoplasmic reticulum (ER) and Golgi.[13-16] This molecule is isolated from porcine intestinal mucosa or bovine sources.[17,18] The isolation of this pharmaceutic is very tedious, with a low yield of about 180-260 mg per healthy animal. One of the major limitations of its production is that this molecule is mainly obtained from a single animal species. A lot of research has been done towards finding the other animal and non-animal sources with very little or no success. In non-animal source finding research, chemical and chemoenzymatic synthesis have been tried with very limited success. A most successful example of chemical synthesis is the chemical synthesis of Arixtra. The synthesis of this molecule takes place in about 50 steps with an overall yield of ~0.1%.[19,20] However, this is also not purely chemical synthesis.

"According to government data, the annual market for heparin is about Rs 50 crore for almost 63 lakh units and Rs 504 crores for enoxaparin, which is for about 150 lakh units. These estimates are of the product imported into India, which is about 2,500-3,000 kilos for Heparin from which about 1,000 Kgs of

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URN:NBN:sciencein.cbl.2021.v8.248 ISSN: 2347–9825

enoxaparin is processed in India. This doesn't account for the product that is imported directly as the finished product.[21-23]"

This article is a compilation about the isolation, synthesis and medicinal applications of this biomolecule by taking the representative examples.

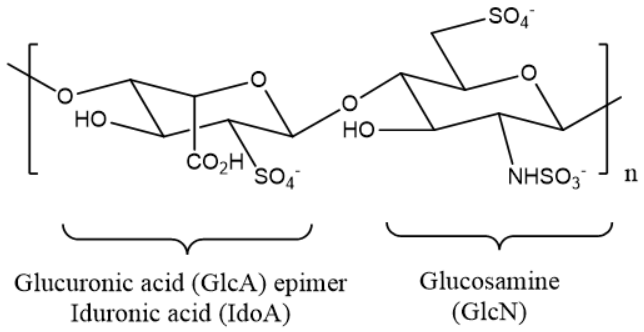

Figure 1. Maximum disaccharides in heparin

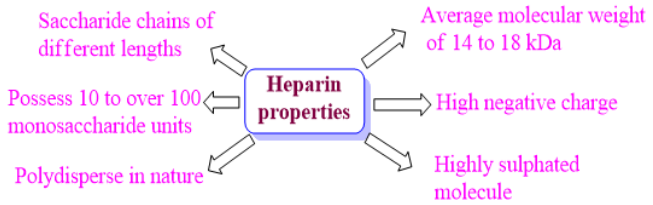

Figure 2. Important properties of heparin

ISOLATION AND SYNTHESIS OF HEPARIN

Isolation of heparin

Any animal used as the source for any drug isolation has to follow strict ethical guidelines. For heparin isolation also, the health and medication of slaughtered animals have to meet certain requirements.[24] There is full traceability from farm to pharma is maintained with proper regulations.[24-26] The earlier heparin isolation was done with canine or bovine livers; later, bovine lungs and mucosa were used (Figure 3).[18] However, due to the outbreak of mad cow disease (Bovine Spongiform Encephalopathy (BSE))[27], the use of bovine materials for heparin isolation decreased significantly.[28] The porcine mucosa remains the FDA-approved source for heparin; however, FDA encourages reintroduction of bovine-source also.[29] There is a strong requirement for enhanced knowledge about bovine disease and the heparin purification process to reduce the risk associated. Studies have shown that the bovine heparin is less active than the porcine heparin; this may be due to their structural differences between the two.[30]

The sheep (ovine) intestines have also been studied as a source to isolate pharmaceutical heparin.[28] Ovine heparin showed better disaccharide composition than the bovine heparin.[31,32] Further, the use of ovine is also free from any religious sentiments. However, the disease like scrapie in sheep found to have some concern, although this is not transmissible to humans.

Many mammalian sources have been studied to isolate heparin.[33,34] The human tissues were used as a natural source of heparin.[35,36] The frozen hemangioma tissue gave human heparin in the amount 649 µg/g of tissue.[35] Warda et al. isolated heparin from one-humped camel in 400 mg/kg amount from an adult camel.[37] This method and source gave better results in comparison to the source porcine intestine (250 mg/kg).

Among the non-mammalian sources, poultry, molluscs, fishes etc. have been explored to isolate heparin. Chicken intestines produced heparin with a lower degree of sulphation.[30] Heparin was also isolated from the turkey intestine but showed very poor activity.[38] Till today, to the best of my knowledge, there is no industry using poultry by-products to isolate heparin. In another study, tuna (saltwater fish) skins have been utilized to isolate heparin with the help of anion exchange resin.[39] Through steaming, the skin was obtained from the freshly caught tuna fish. The obtained heparin showed less potency than the commercial product.[39] Heparin was also isolated and purified from gills and intestines of salmon (*Salmo salar*) whose activity showed similarity with the activity of low-molecular-weight heparin (LMWH).[40] The heads of shrimp (*Penaeus brasiliensis*) produced LMWH with a yield of 32 mg/kg.[41] The isolation from clams (*Tapes phylippinarum*) gave heparin with a yield of ~2.1 g/kg dry tissue.[42] The commercial isolation/production from the discussed sources (Figure 3) is still a matter of research.

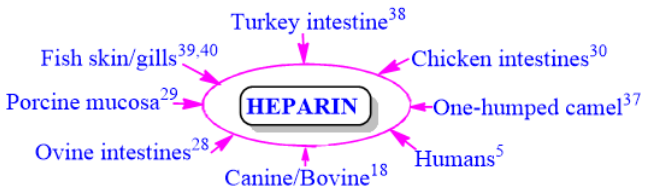

Figure 3. Natural sources of heparin

Synthesis of heparin

The synthesis of heparin is very challenging, and still a lot of work is going on towards this. Heparin of various polysaccharide chains has been synthesized/semi-synthesized.[43-46] As per the length and molecular weight, heparin is grouped as unfractionated heparin (UFH), low molecular weight heparin (LMWH), and ultra-low molecular weight heparin (ULMWH). Chemical synthesis and chemoenzymatic synthesis have been tried with limited success. In both the methods, oligosaccharide building method has been explored. The methods are multi-steps, taking place in more than 50-60 steps leading to very-very low yields. For example, a heparin, fondaparinux, was synthesized in about 60 steps with an overall yield of only 0.1%.[47,48] Fondaparinux is a pentasaccharide sequence having a similar binding region as heparin. The challenge associated with heparin synthesis are many like repetitive protection deprotection steps, couplings, functional group activation, intensive purification, and so on.[1,49]

The chemoenzymatic synthesis takes the help of polymerases which help in backbone building. The backbones have further been modified with enzymes like C5 epimerase and sulfotransferases.[50] The backbone of the chemoenzymatic method is the process involved in its biosynthesis; rather, one can say it is mimicking the biosynthesis of heparin.[51-53] Xu et al.

synthesized two ULMW heparins through a chemoenzymatic approach.[53] The focus of the synthesis is to make the pharmacophores of anticoagulant heparin.[54]

Another chemoenzymatic synthesis used the bacteria *E. coli* K5.[55] This gave an unsulphated precursor of the heparin, heparosan. The chemoenzymatic method came with a lot of limitations, like substrate specificities of the enzyme. The heparin-based oligosaccharide has also been synthesized using the polymer-supported method.[45,56] However, the glycosylation yield enhancement remained a challenge, and hence solution phase is still favoured.

The chemical synthesis of heparin is not like the total synthesis of heparin. Most of the researchers have synthesized the active portion of the chain like pentasaccharides or hexasaccharides as heparin-based oligosaccharide and further evaluated for the corresponding biological activity. This is an excellent approach to get the potential pharmaceutical active molecule with comparatively lesser effort.

MEDICINAL APPLICATIONS OF HEPARIN

Heparin for the first time used as postoperative anticoagulation in 1935, and since then, their applications have increased many folds.[57] The utility of heparin, directly or indirectly, in other therapy like vein thrombosis, heart-lung oxygenation, kidney dialysis, and coating of medical devices like stents make this molecule vital for medicinal applications.

Anticoagulation effect of heparin

A blood clot known as a thrombus is consists of fibrin and blood cells, which can form anywhere in the cardiovascular system.[58,59] The clot can be in the microcirculation, the heart, arteries, and veins. Many diseases are associated with thrombosis including, cardiac-related disease.[60]

Heparin is used for the treatment or prevention of thrombosis, re-thrombosis after thrombolysis, and other thrombosis-related diseases.[10] The blood-clotting cascade is inhibited by a serine proteinase inhibitor, anti-thrombin III (AT).[61] AT is a plasma protein and named by Abildgaard in 1968 as anti-thrombin III, which later referred to as anti-thrombin (AT)[62] after the study of Brinkhous et al. that heparin requires a plasma cofactor for its activity.[63] A pentasaccharide sequence (Figure 4) present in heparin is responsible for the anticoagulation effect.[64,65] The sulfo- and carboxyl groups possessing the negative charge bind tightly with AT.[66,67] AT itself is a weak protease inhibitor, but in combination with heparin pentasaccharide, increase its activity by about 300 times due to favourable conformational change.[68] The formation of insoluble fibrin clots from soluble fibrinogen is facilitated by thrombin. This step is blocked due to the formation of a complex AT-heparin-thrombin, and hence the formation of an insoluble fibrin clot is avoided.[69,70]

As per the mechanism of action, a number of coagulation factors such as XIIa, XIa, IXa, Xa, and IIa (thrombin) are inactivated by the heparin-AT complex.[6,71-73] The unfractionated heparin (UFH) possesses low bioavailability (30%) in comparison to LMWHs (90%) and a longer half-life (17–21 h) when compared to UFH. The LMWHs are given for thromboprophylaxis in fixed doses.[73,74] Due to low bioavailability, the doses need to be high, like 30,000 U/day, when administered via subcutaneous injection for therapeutic anticoagulation.[73]

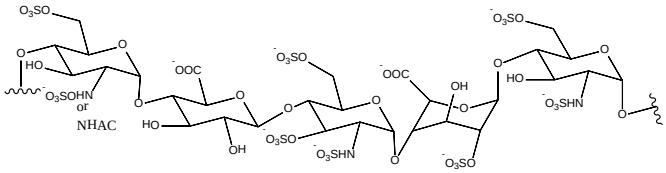

Figure 4. Heparin pentasaccharide that bind to AT

In an ongoing pandemic, COVID-19 also, heparin has been widely explored for its utility, which includes both its anticoagulant effect and antiviral effect.[10] The study showed that anticoagulant therapy with heparin has a positive effect and lowered mortality of COVID-19 patients. The COVID-19 patients with higher D-dimer levels or met the criteria of sepsis-induced coagulopathy (SIC) better impacted with heparin therapy.[75] Patients with 4-times higher D-dimer concentrations than normal were recommended for LMWH.[76] Some studies also confirmed the effectiveness of heparin in COVID patients with venous thromboembolism prophylaxis.[77,78]

Anticancer effect of heparin

The detailed studies of unfractionated and different molecular weight heparin showed improved effects on various types of cancer.[11,79-81] The cancerous tumors and thrombosis showed some link between them; this led to the use of antithrombotic molecules towards the treatment of cancer. The mechanism of action is still a matter of investigation; a noncoagulation pathway is a possible suggestion.[82] The study with heparin on animal models showed a reduction in metastasis of carcinoma cells.[83-85] This may be due to the non-deposition of fibrin around tumur cells.[83] The inhibition of biological processes that supports cell growth is an important approach to target cancer.[86] In some studies, heparin possessing non-anticoagulant property also inhibited metastasis.[84,85]

Several studies confirmed that heparin has effects on angiogenesis and also affects the formation and progression steps of the tumor.[87] They regulate the immune system due to their ability to bind with different proteins. Their role in cell adhesion, a barrier to leukocyte migration, and inflammation have been studied.[11,88] Based on various studies, several heparin-like molecules or heparin mimetics such as pixatimod, muparfostat, necuparanib, and roneparstat are currently in clinical trial for use in cancer treatment.[11] Heparin was used to study its impact on the treatment of angiogenic tumours in mice.[89] The result showed inhibition in downstream signalling and decreased angiogenesis. The heparin, by competition, interrupts the heparin sulphate proteoglycans interaction with fibroblast growth factors (FGF).[89]

The heparin sulphate is present in the extracellular matrix plays several characters towards cell division and proliferation, such as a positive or negative modulator.[90] This has been observed that proliferation of many cells is inhibited by heparin, and this behaviour is utilized for the treatment of cancer.[91] Due to the positive results obtained after many studies for cancer

treatment by heparin or its derivatives, more than 50 clinical trials are at different stages. However, the overall beneficial nature of these drugs is still a matter of investigation.

Antiviral effect of heparin

The GAGs are present on the cell surfaces, which allow them to serve as a non-specific receptor for virus binding.[92] Various studies have been done with heparin against a crowd of distinct viruses.[10,93-95] The human immunodeficiency virus (HIV) showed a reduction with heparin towards cytopathogenicity to MT-4 cells by preventing the adhesion of HIV to MT-4 cells.[96] The heparin-sulphate made the dengue virus ineffective by inhibiting the virus-cell attachment.[97] This has been observed that the heparin blocks the virus adhesion by competitive inhibition.[98] A study by Lin et al. showed the inhibitory action of heparin towards replication of Japanese encephalitis viruses in BHK-21 cells and dengue-2 in hepatoma.[99] The study was done with heparin at various dosages like 0.1, 1, 10, and 100 mg/ml). The result showed that the dengue-2 virus invasion can be stopped by heparin in different liver cells. The influenza virus strain H5N1 infection was also prevented by heparin and its derivatives.[100] The N-sulphation of HS showed its potential towards the infectivity of the Chikungunya virus.[101]

The heparin also showed its effectiveness towards SARS-CoV-2.[10] The angiotensin-converting enzyme 2 (ACE-2) is the major receptor, whereas heparan sulphate proteoglycan (HSPG) is the co-receptor for the virus, SARS-CoV-2, which this virus utilizes to infect the cells. Heparin inhibits the binding of SARS-CoV-2 to the cell surface by competing with co-receptor HSPG, and hence, stops the virus entry to the cell.[10,102] Conzelmann et al. studied SARS-CoV-2 infection inhibition using heparin as a repurposed drug.[103] The study was based on three important therapeutic actions of heparin, anti-inflammatory, anticoagulant, and antiviral. The results showed that the viral replication was completely inhibited with 500–1,000 μg/ml heparin and get suppressed to 60% at 125–250 μg/ml.[103]

Antibacterial activity of heparin

For the first time, Stoker reported the antibacterial effect of heparin against *Staphylococcus aureus*.[104] One year later, another group also reported the antibacterial effect of heparin against *S. aureus* and *Erwinia stewartia*.[105] Hanno et al. in 1978 studied the effect of heparin as an antibacterial agent on rabbit bladder.[106] The mucoprotein-deficient bladder was applied with heparin directly gave a very good result towards bacterial infection. Rosett and Hodges studied the effect of heparin on eight species of microorganisms in brain heart infusion broth.[107] It was observed in all the studies that the microorganism growth was inhibited by heparin. The gram-positive bacteria got inhibited more than the gram-negative bacteria. The unfractionated heparin showed inhibition in a dose-dependent manner to three of seven *S. pneumoniae* isolates and one of five *H. influenzae* isolates.[108] The heparin coating also showed antibacterial properties. The heparin-coated stents prevented the formation of bacterial biofilm and microbe attachment.[109] The biodegradable ureteral stents coated with heparin were also found to inhibit the early bacteriuria development.[110] The above examples confirm the utility of heparin as antibacterial agents. Systematic research is required to bring this as a broad-spectrum antibacterial agent.

Anti-inflammatory activity of heparin

Several studies have been done on heparin as an anti-inflammatory agent.[111-113] Different studies have discussed the mechanism of action; still, exact benefits and safety are yet to be established about heparin being an anti-inflammatory agent. Some of the early studies have suggested the positive use of intravenous heparin in asthma, a chronic inflammatory disorder of the airways.[114,115] The inhalation of heparin also gave positive effect towards bronchial hyperreactivity.[116] The heparin inhalation may be showing the suppressive type of activity on mast cell degranulation which is due to the absence of bronchodilation in airways.[116] In the burn's patients also, heparin was able to reduce the inflammation and also promoted tissue repair.[117]

Heparin and its derivatives showed anti-inflammatory property and benefits to the patients during cataract surgery, cardiopulmonary bypass, and asthma; however, other inflammatory diseases gave mixed and inconsistent results.[113] The adverse effect of heparin as anti-inflammatory agent has not been reported in maximum studies. Still, more studies are required to make this molecule as a versatile and robust anti-inflammatory agent.

HEPARIN-BASED NANOCARRIERS FOR DRUG DELIVERY

Heparin is biocompatible, and hence, heparin-based nanocarriers showed a lot of potential in drug delivery.[118,119] Different types of nanocarriers have been studied with heparin like self-assemblies, coated nanoparticles, nanogels, and polyelectrolyte complex nanoparticles.[119] The size and shape of heparin-based nanoparticles can easily be controlled by optimizing the heparin amount. The physical or chemical interaction using the cross-linked method allows the formation of heparin-based nanogels possessing 3-dimentional (3-D) porous network, which helps in the prevention of environmental degradation of drugs along with drug reservoirs. Chang et al. developed a heparin/berberine conjugate to deliver berberine at the infected site that reduced cytotoxic effects in infected cells.[120] This process reduced the unwanted side-effects caused due to antibiotic treatment for *Helicobacter pylori* bacteria. This was an excellent example of a nanoparticle berberine carrier with a heparin shell.[120] Choi et al. studied the controlled release of growth factors using heparin nanosponge.[121] The growth factors are useful to many diseases therapy and tissue engineering, but due to low bioavailability, non-specific biodistribution, and fast degradation, their efficacy is a limitation. These limitations are reduced with the use of heparin-based nanocarriers.

Heparin-based nanocarriers have been studied for cancer management either as a backbone, coating material, physical encapsulation, and conjugates.[119] An elevated antitumor effect was observed with doxorubicin encapsulation by a conjugate made from heparin and deoxycholic acid that was planned for SCC (squamous cell carcinoma).[122] This was amphiphilic

conjugate nanoparticles and gave a better result than without encapsulation. Zhang et al. studied conjugation and physical loading for two anticancer drugs, D (doxorubicin) and A (all trans retinoic acid) where D was loaded, and A was conjugated with LMWH forming D-loaded LMWH–A system.[123] The study reported that the anticancer effect with the prepared system was higher as compared to the free drugs in solution. Yang et al. studied the applicability of drug sorafenib in gastric cancers using an immobilized chitosan/heparin pluronic-coated system as a nano delivery system.[124,125] This was observed that the prepared nano delivery system enhanced the inhibition of cancer cells.

There have been numerous studies done to use heparin as a nano system for drug delivery, but still, no system has reached to the stage of successful clinical trials. Heparin being the hydrophilic molecule possessing a lot of modifiable groups, can provide an excellent potential in the drug delivery systems. Drug delivery is an essential aspect to provide treatment of various ailments, and a lot of research on varied topics are going on.[126,127]

SUMMARY AND WAY FORWARD

Heparin is a highly sulphated and most negatively charged natural biopolymer belonging to the glycosaminoglycan (GAG) family. This is about a 100-year-old anticoagulant drug, and still, the scientific community unable to find its substitute with similar efficacy. Various bottlenecks towards its isolation from animal sources kept the scientific community at their toes to search for similar alternatives. From publications point of view, many researchers have published its isolation from different sources, chemoenzymatic synthesis, chemical synthesis, biotechnological approach, but still, their commercial applicability is far away. In recent times, the focus is more towards the synthesis of LMWH, ULMWH, and bioengineered heparins.

Heparin is equally important for non-anticoagulant diseases and nanocarriers for drug delivery systems also. The effect of heparin and its oligosaccharides showed a better therapeutic effect towards cell proliferation, inflammation, microbial effect, thrombogenesis, and related diseases. More in-vitro, in-vivo, and clinical studies are required to understand the efficacy and treatment effect of heparin-based oligosaccharides, which are easy to synthesize in comparison to the total synthesis of heparin.

ACKNOWLEDGEMENTS

The authors are grateful to Delhi Technological University (DTU) for providing necessary facilities to carry out this work. The author SJ is also thankful for financial support from DTU.

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