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Introduction

# Heparin centenary – an ever-young life-saving drug

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ABSTRACT

On the centenary of the discovery of heparin, the *International Journal of Cardiology* agreed to publish a collection of mini reviews that summarize the historical development of this ever-young life-saving drug. The present articles deal not only with the historical milestones, but also with current and future perspectives regarding the development of heparin in terms of its structure, as well as on-going biochemical, biological and clinical research. Attention is focused on recent applications of heparin derivatives to non-anticoagulant or antithrombotic therapies, providing particular emphasis on their inhibitory activities, including their potential as anti-cancer agents. In the Chapter, entitled '*Recent innovations in the structural analysis of heparin*', some recent technological advances are described for the problem of monitoring the purity and reproducibility of pharmaceutical heparin. These now permit sensitive detection of non-heparin impurities, as well as the detection of heparin from different animal sources, to be made in pharmaceutical heparin samples. In '*Past, present, and future perspectives of heparin in clinical settings and the role of impaired renal function*', the author traces the history of heparin and the development of low molecular weight heparin, highlighting the large number of clinical trials in which it has been involved, and reviewing its efficacy among patients with impaired renal function. In the final chapter, '*Old and new applications of non-anticoagulant heparin*', the authors survey some of the many non-anticoagulant activities of heparin and its derivatives, including glycol-split heparin, which has demonstrated promising activities in a wide-range of situations.


## 1. Introduction

We now stand at the centenary of heparin's discovery and can reflect upon a story of early difficulties, followed by many years of success and short periods of crisis but, throughout, heparin has remained available as a life-saving drug. The therapeutic use of heparin has been principally as an anticoagulant but, more recently other, non-anticoagulant activities and applications have begun to be explored. Heparin was discovered in 1916 [1] as "cephalin" and re-named "heparin" in 1918 [2], was commercialized in the early 1920s and subsequently became a well-established anticoagulant drug. From the mid-1970s the numbers of reports regarding its fractionation and depolymerization to generate low molecular weight heparins (LMWs) increased, accompanied by work on both unfractionated heparin and derivatives that targeted non-anticoagulant applications. Approaching the centenary, several publications appeared, including the review "Re-visiting the structure of heparin" [3] and the book "Heparin a century of progress" [4], that includes the well-documented "history of heparin" [5] and "non-coagulant effects of heparin: an overview" [6].

## 2. The sources of, and crisis with, pharmaceutical grade heparin

The first pharmaceutical preparations by Roche-Organon from bovine lung were commercialized in USA as "Liquemin" in 1939. In the 1950s bovine lung was replaced by porcine mucosal heparin (PMH) as the major source and partially by bovine mucosal heparin (BMH) in the 1960s [5], but BMH was withdrawn from Western countries during the 1990s because of potential prion contamination and the risk of infection with bovine spongiform encephalopathy (BSE), which was prevalent at that time in Europe. Concerns with other animal sources aroused the search for biotechnological heparins starting from the microbial polysaccharide K5, comprising a repeating disaccharide sequence [-4) D-GlcA β (1-4) GlcNAc α (1-], common to that of N-acetyl heparosan, the biosynthetic precursor of heparin. Through a combination of enzymatic and chemical steps, heparin anticoagulant mimetics were obtained [7,8] and their development has been reviewed recently [9]. A second crisis, in late 2007 and early 2008 mainly in the USA, was caused by adverse events, including fatalities, in patients treated with PMH contaminated arised from an "unnatural" fully O-sulfated chondroitin sulfate [10]. As a consequence, demand increased for pharmaceutical grade PMH and derived LMWHs, and provoked the development of more rigorous controls [11], but also raised concerns regarding the supply of the starting material, which is produced mainly in China. These events have induced EC authorities

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and the US FDA to consider the reintroduction of bovine heparin, which has been produced and clinically used continually in South American and other countries. Its potential re-introduction into Western markets was one of the topics of two recent meetings: "The 6th Workshop on Characterization of Heparin Products" held on August 6–7, 2015, in São Paulo, Brazil and the "23rd Symposium on Glycosaminoglycans", which addressed the "Heparin Centenary: Present and Future Perspectives", held in Milan and at Villa Vigoni, Loveno di Menaggio (Co), Italy on 17–19 September 2015.

## 3. Early clinical studies and discovery of anti-thrombin mechanism of action and binding sequences

The first parental preparations from canine liver, estimated to be around 14–15% pure, were tested in patients around 1924 with several side effects [12], but these were no longer present by the arrival of the first commercial heparin preparations in the early 1930s [13]. Later, it was discovered [14] that heparin required a plasma co-factor for its anticoagulant activity, which was identified as antithrombin III (AT) [15]. Heparin binding to AT, discovered in 1973 [16], was found to increase the rate of AT inhibition of thrombin by up to one hundred times [17,18] and to potentiate the inhibition of other serine proteases involved in the coagulation process, as recently reviewed [19]. The most active sequence in heparin that is involved in AT binding and activation was identified in oligosaccharides derived from both enzymatic and chemical depolymerisation processes that unexpectedly preserved the active sequence, a pentasaccharide core of the AT binding region (ATBR), characterized by a crucial internal trisulfated glucosamine (Fig. 1) [20–23]. Utilising the natural, high affinity components from oligosaccharides extracted from 50 kg of heparin, allowed the correct identification of an "anomalous" residue in the ATBR sequence by NMR spectrometry [24]. Despite this success, and the contribution that NMR made to the debate around GlcA and IdoA content [25] and conformational studies [26], these NMR results were initially rejected in favour of the more-doubtful measurement of sulfate content. 1981 could represent the year in which NMR spectrometry began to be used for the analysis and characterization of GAGs on a significant scale, a process that continues today, culminating with the present requirement for NMR identification of heparin-based drugs [27,28]. The discovery of this sequence, which is present in only one third of heparin chains paved the way for the search for LMWH. The ATBR sequence was the major one found in PMH [29[, but in a bovine lung heparin sample, a de-N-acetylated, re-N-sulfated variant was identified [30]. The possible re-introduction of BMH, and the dearth of recent compositional studies concerning its ATBR containing sequences, prompted our group to compare the sequence profiles of pharmaceutical grade BMH and PMH samples selected from same producer. NMR and LC/MS analyses of heparin samples of their AT affinity chromatographic fractions and comparison of their heparinase digestion products revealed the main differences, consisting of lower ATBR content of BMH and some of its components, mainly tri-sulfated glucosamine (A*), despite an anti-factor Xa activity comparable to that of PMH. This may be explained by the presence in some BMH chains of peculiar ATBR sequences, such as one bearing two trisulfated glucosamine residues [31].

## 4. Low molecular weight heparins (LMWs)

The first LMW tested in clinical trials was "Fraxiparine" (Choay Labs) [32], prepared by gel filtration, which resulted in the loss of two thirds of the heparin starting material. By the 1980s, four other LMWs, prepared using less costly processes were commercialized by European companies. LMWHs currently available in the EU and USA, produced by partial depolymerization of pharmaceutical grade

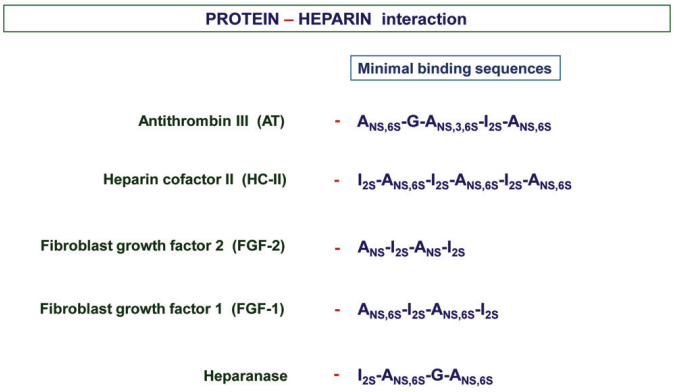


**Fig. 1.** Schematic representation of the minimal binding sequences of heparin chain specific for some of the target proteins. $A_{NS}$: (1-4) N-sulfated α-D glucosamine, $A_{NS,6S}$: (1-4) N-sulfated,6-O-sulfated α-D-glucosamine, $A_{NS,3,6S}$: (1-4) N-sulfated, 3-,6-O-sulfated α-D-glucosamine, G: (1-4) β-D-glucuronic acid, I: (1-4) α-L-Iduronic acid, I2S: (1-4) 2-O-sulfated α-L-iduronic acid.

heparin show mean $M_w$ between 3.5 and 6 kDa (corresponding to 12–20 disaccharide units), comprising at least 60% material with $M_w$ <8 kDa [33]. When compared to heparin, LMWs have a lower affinity towards plasma proteins, endothelial cells and macrophages, but better bioavailability and pharmacokinetics with more predictable dose responses [34]. The chemical-physical characterization of LMWs has been reviewed recently [35]. The names, brands, production processes and mean $M_w$ of historical and currently available LMWs in the EU and USA are shown in Table 1. Each depolymerization process gives rise to chain-bearing, specific marker residues at the reducing (RE) and non-reducing (NRE) ends as shown in Fig. 2.

Some generic LMW appeared recently on the US market. Owing to the structural heterogeneity of heparin, the approval of these generic drugs by the FDA has required very complex structural characterizations. This has had a positive side through development of sophisticated survey techniques for primary chemical structure determination.

## 5. Ultra-low molecular weight heparins (uLMWHs) as antithrombotic

Disclosure of the ATBR pentasaccharidic sequence [23], found in an affinity hexasaccharide obtained by heparin depolymerization [37] promoted intensive synthetic programs, the most successful leading to the synthesis of the α-methyl glycoside of the N-deacetylated-N-sulfated natural pentasaccharide [38]. The product, endowed with AT binding and antithrombotic activity reached the market in 2001 as Fondaparinux (Aristra-Aspen), representing the first synthetic heparin oligosaccharide ($M_r$ 1728 as the sodium salt) to be used as a drug. However, it requires an expensive synthetic process (about 50 steps and consequent low overall yield) and this has encouraged the search for less expensive leads through new heparin depolymerization or chemo-enzymatic processes. The development of a fully O-methylated O-sulfated Fondaparinux analogue, Idraparinux (Sanofi) was discontinued in phase III trials presumably due to intracranial bleeding. A biotinylated formulation, Idrabiotaparinux, was further clinically investigated [39]. Semuloparin (AV5026) is a ULMWH ($M_w$ 2.4 kDa, corresponding to ~8 disaccharide units) [40], obtained by heparin through a modified Enoxaparin depolymerization process protecting ATBRs, that has been evaluated in Phase III/IV trials, but its development was halted in 2012. Finally, active oligosaccharides from hexa- up to dodecasaccharides have been obtained by a chemo-enzymatic approach starting from the bacterial polysaccharide K5 using a recombinant version of the heparan biosynthesis enzymes [41].