Heparins and Coagulation

Learning Objectives

- ***What is the structure of heparin?***
- ***How is heparin isolated?***
- ***What are the biological activities of heparin?***
- ***What are low molecular weight heparins?***
- ***What are the similarities and differences between different heparins***
- ***What is biosimilarity?***
- ***What are the current rules for biosimilar biologics?***

Heparins and Coagulation

- ***Discovery***
- ***Structure of Heparin***
- ***Preparation of Heparin***
- ***Biological Activities of Heparin***
- ***Anticoagulant Heparin***
  - ✓ *Heparin regulation of coagulation*
  - ✓ *Heparin activation of antithrombin*
  - ✓ *Cyrstal structure of complex with antithrombin*
  - ✓ *Bridging mechanism*
  - ✓ *Differential regulation of coagulation enzymes*
  - ✓ *Binding to other coagulation serpins*
  - ✓ *Adverse Effects*
- ***Low Molecular Weight Heparins***
  - ✓ *Structure*
  - ✓ *Preparation*
  - ✓ *Composition*
  - ✓ *Specific case of a LMWH*
- ***Similarities and Differences Between UFH and LMWH***
- ***Concepts on Biosimilarity***

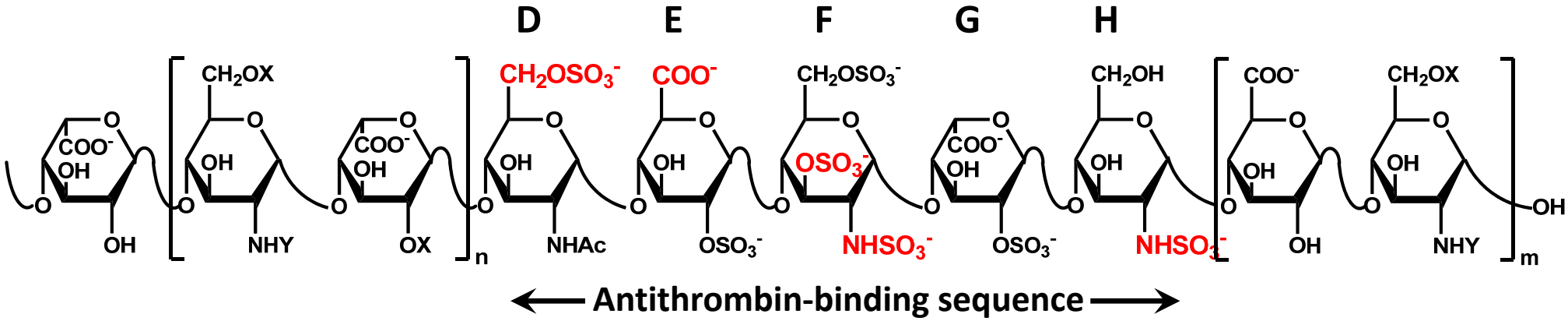
Heparin

- ***Discovery***
  - ✓ *Discovered in 1916 by Jay McClean, a second year MD student, in the laboratory of William Howell (JHU)*
  - ✓ *In 1928, Howell identified uronic acid as a constituent of heparin*
  - ✓ *In 1935-1936, Jorpes and Bergstrom identified the 2nd constituent as glucosamine*
  - ✓ *In 1940, heparin is sulfated was found, followed by ….*
  - ✓ *First clinical trials were performed in 1935s and subsequently it entered the clinic*
  - ✓ *In 1973, the mechanism of heparin action … binding to antithrombin … was discovered by Rosenberg and Damus*
  - ✓ *Structure of the key antithrombin binding pentasaccharide was identified by the Rosenberg and Lindahl laboratories*

Angew. Chem. Int. Ed. **2000**, *41*, 390 – 412.

Structure of Heparin

- ***Heparin is a mixture of millions of species!***
  - ✓ *M_R = ~13,000*
  - ✓ *Polydisperse = M_W/M_N = 1.3 → 1.4*
  - ✓ *Average chain length = 40 – 50 monomers*
  - ✓ *High proportion of trisulfated disaccharide (GlcNp2S6S – IdoAp2S)*
  - ✓ *High affinity pentasaccharide sequence between 25 – 30 % of chains*



Unfractionated Heparin

X = -SO_3Na or -H

Y = -SO_3Na or -$COCH_3$

Thromb. Haemost. **2008**, *99,* 807–818.

Preparation of Unfractionated Heparin

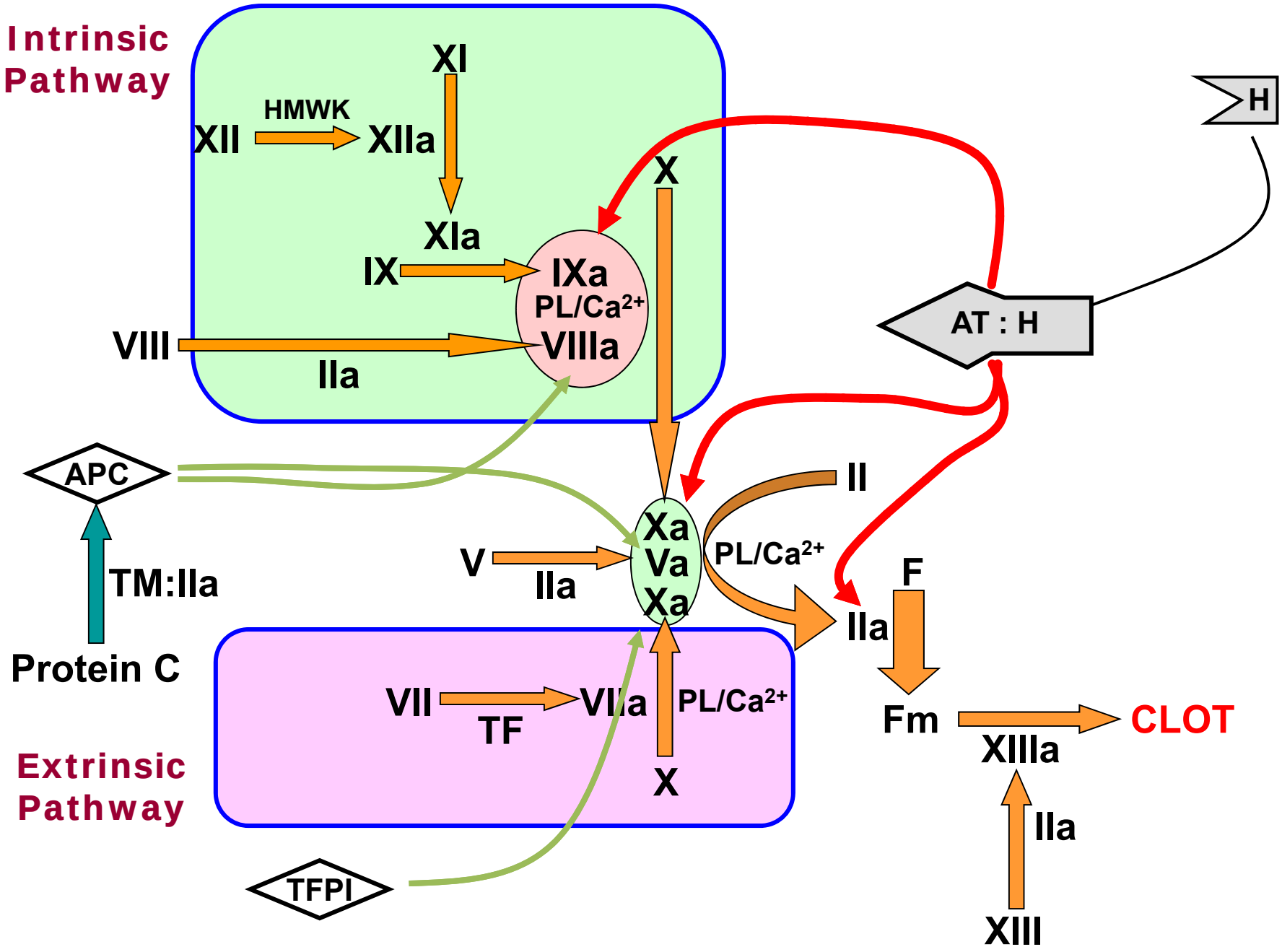
- ***Source of Commercial Heparin***
  - ✓ *Dog liver → Cow lung → Pig intestine*
  - ✓ *Different heparins in different species and tissues!*
- ***Basic Steps***
  - ✓ *Tissue preparation*
  - ✓ *Heparin extraction*
  - ✓ *Recovery of stage 12 heparin*
  - ✓ *Purification of heparin*
- ***Tissue Preparation***
  - ✓ *Collection of animal organ from the slaughterhouse*
  - ✓ *Tissue chopping / grinding to increase surface area*
- ***Heparin Extraction***
  - ✓ *Mild alkaline hydrolysis at elevated temp / pressure*
  - ✓ *Proteolytic enzymes to aid digestion*
  - ✓ *Filtration to remove particles*
  - ✓ *Filtrate contains GAGs, peptides and nucleic acids*
  - ✓ *Enzyme inactivation at 90 ℃ / 15 min; also sanitizes*
- ***Recovery of Stage 12 Heparin***
  - ✓ *Adsorption onto anion-exchange resin*
  - ✓ *Washing to remove unbound material*
  - ✓ *Elution with high salt and filtration*
  - ✓ *Precipitation with ethanol*
  - ✓ *Vacuum drying to yield stage 12 heparin*
  - ✓ *May be sold at this stage to other manufacturers of UFH and/or LMWH*
- ***Purification of heparin***
  - ✓ *Dissolution of stage 12 heparin in mildly acidic water and filtration*
  - ✓ *Oxidation at alkaline pH to sanitize, de-color and de-pyrogenate*
  - ✓ *Cation exchange resin to remove cations*
  - ✓ *Ethanol precipitation and re-precipitation*
  - ✓ *Freeze drying*

Biological Activities of Heparin

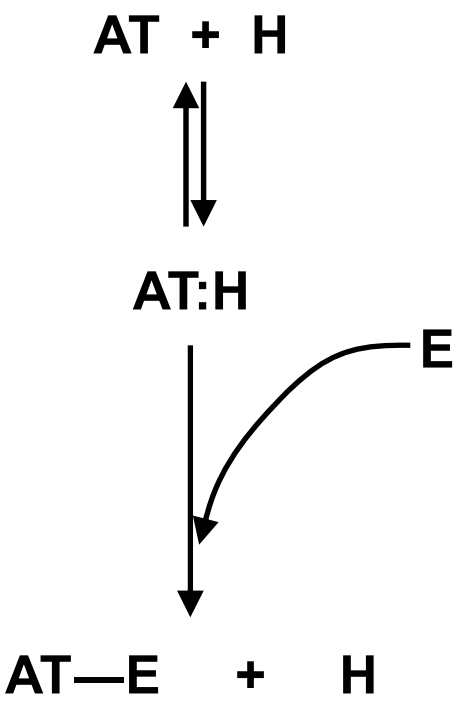
- ***Myriad Activities***
  - ✓ *Anticoagulant* … activates antithrombin to inhibit clot formation … major clinical use
  - ✓ *Proliferation* … activates growth factors to induce cell growth, differentiation, morphogenesis, …
  - ✓ *Wound healing* … binds to chemokines to modulate inflammation and wound healing
  - ✓ *Viral Invasion* … binds to enveloped virus glycoproteins to modulate infection of target cells
  - ✓ *Many other effects*

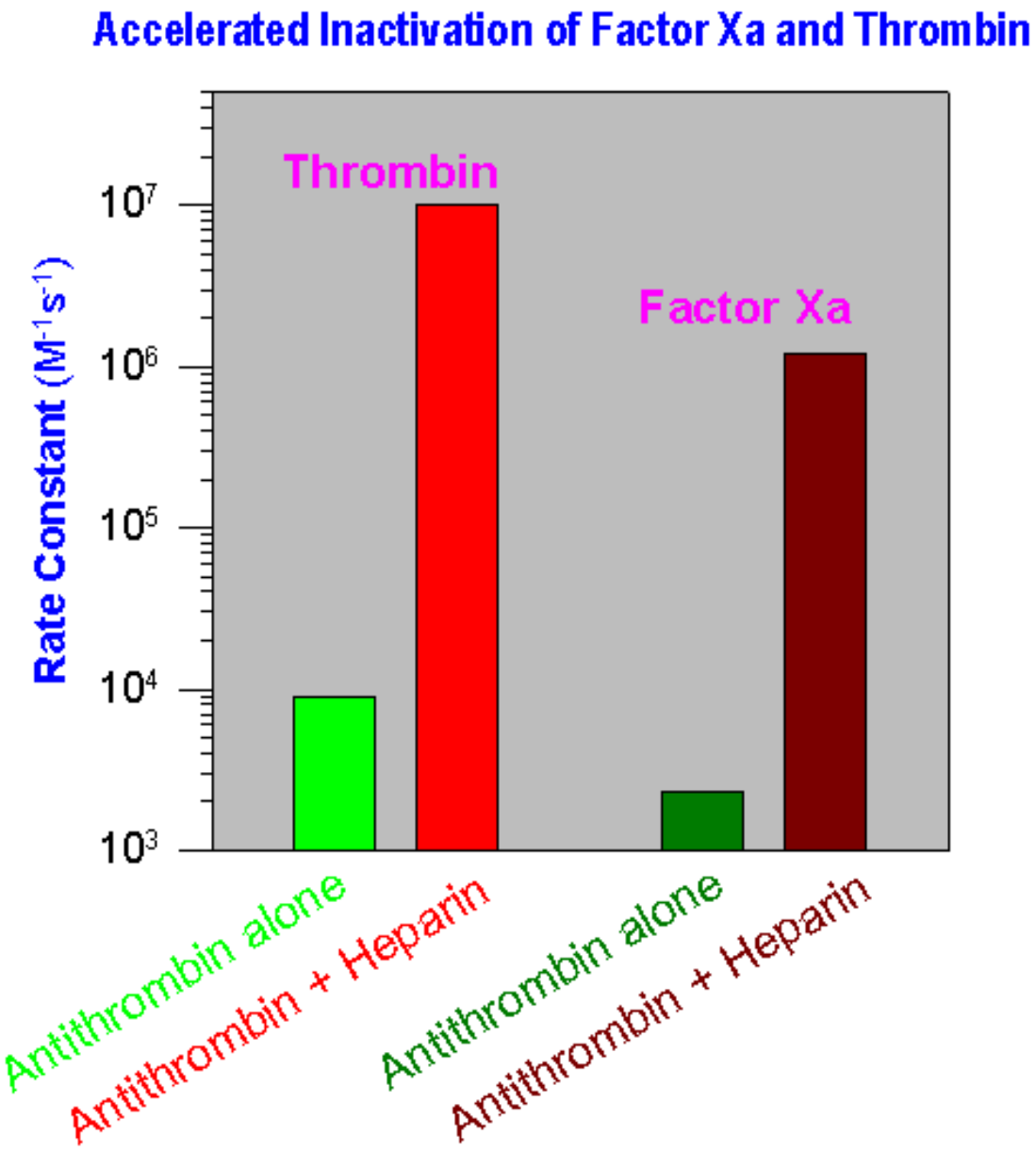
Angew. Chem. Int. Ed. **2000**, *41*, 390 – 412.

Heparin Regulation of Coagulation



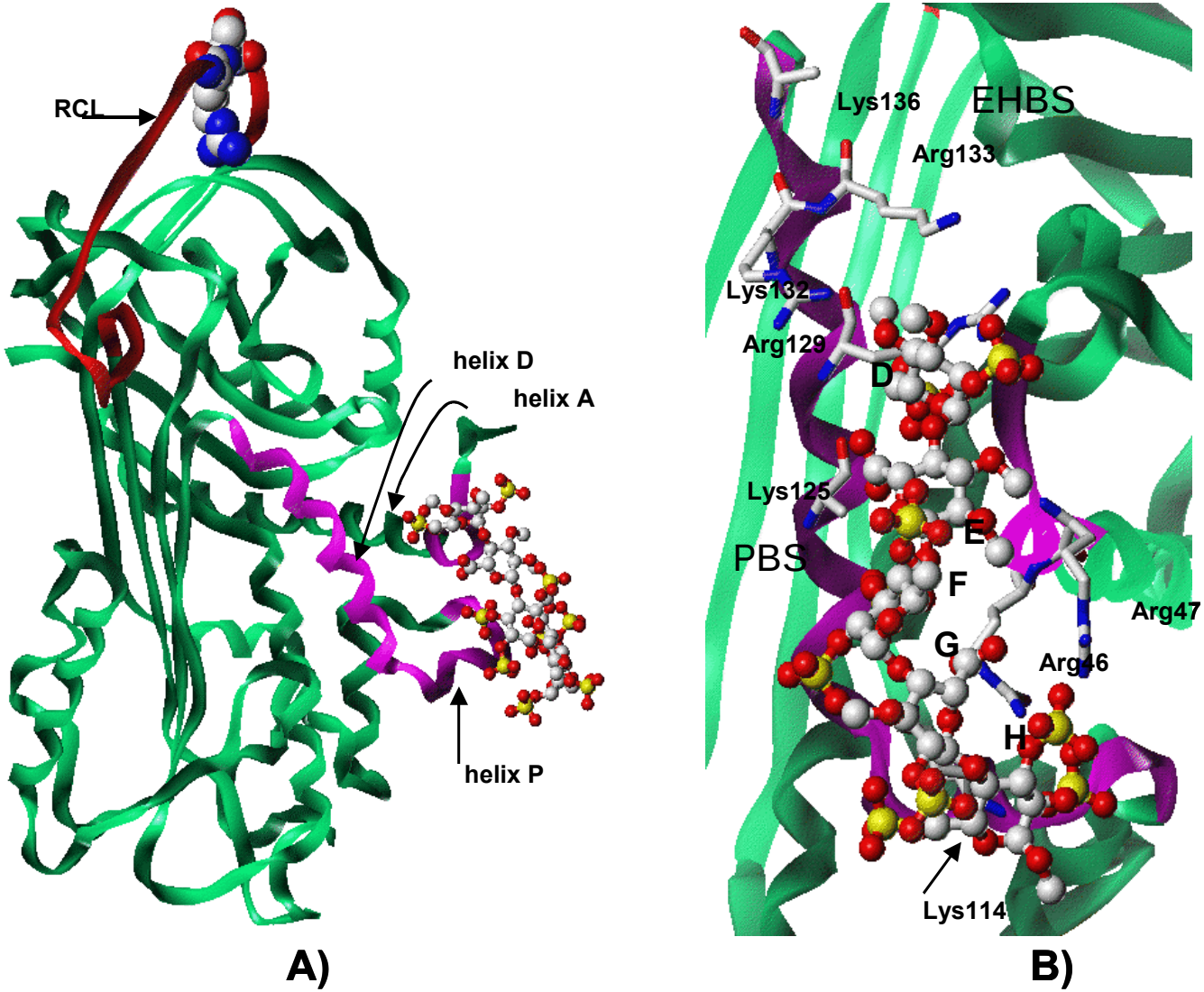
Heparin Activation of Antithrombin





Crystal Structure of Heparin – Pentasaccharide Complex

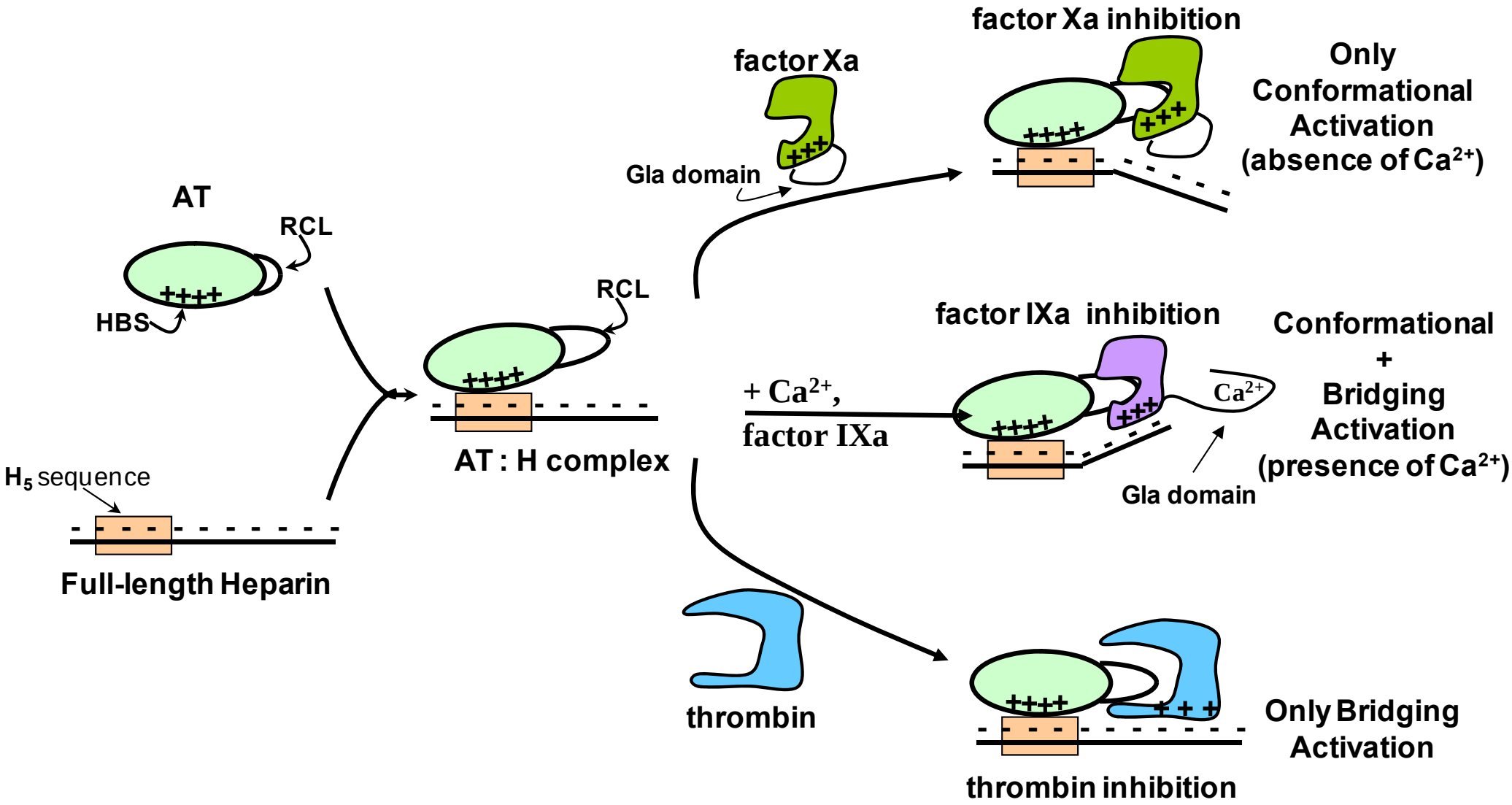
- ***Heparin Binding Site on Antithrombin***
  - ✓ *A trisaccharide and a disaccharide may function as allosteric effectors*



Proc. Natl. Acad. Sci. **1997**, *94, 14683-14688*

Molecular Mechanism of Heparin Action

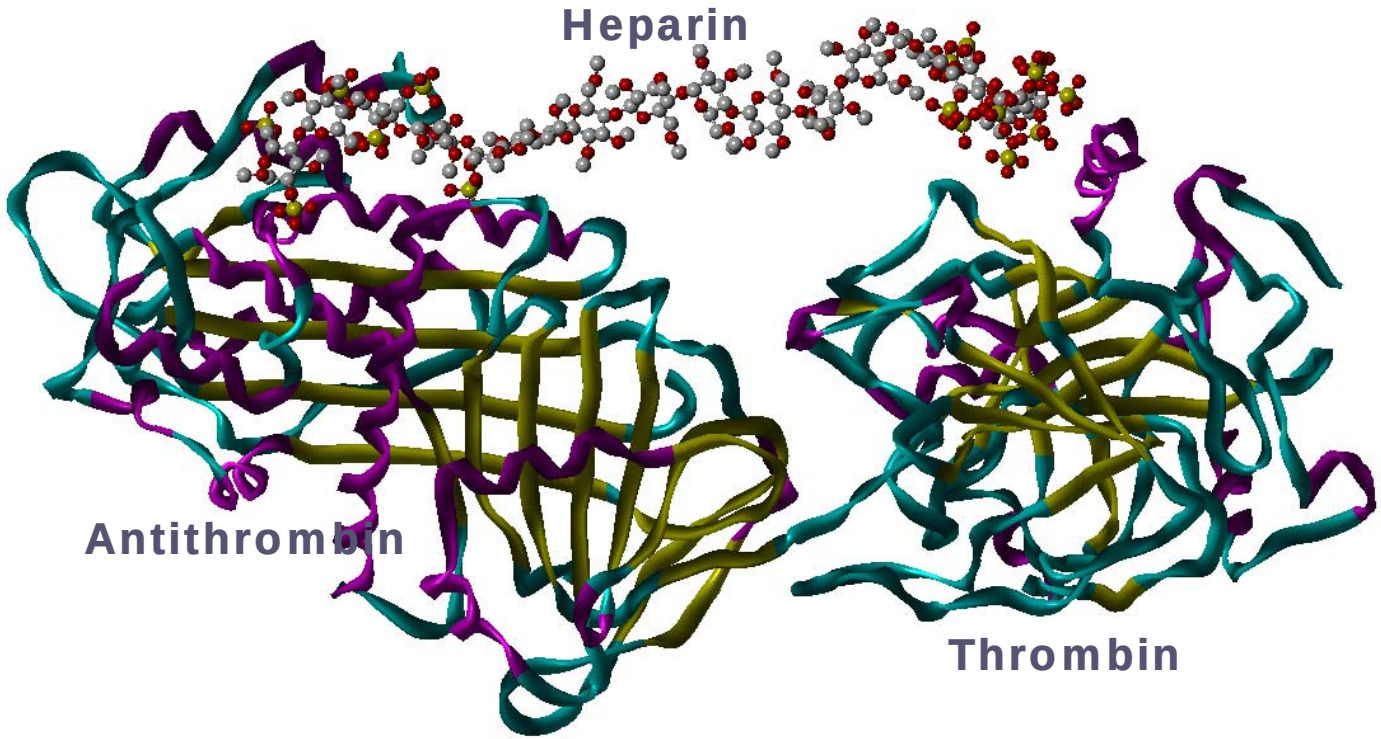
- ***Mechanism is more complex than just allosteric activation***



Med. Res. Rev. (2004) *24*:151-181

Molecular Mechanism of Heparin Action

- ***Proof of the Bridging Mechanism***
  - ✓ *A long heparin chain may engage more than one protein or protein monomers*



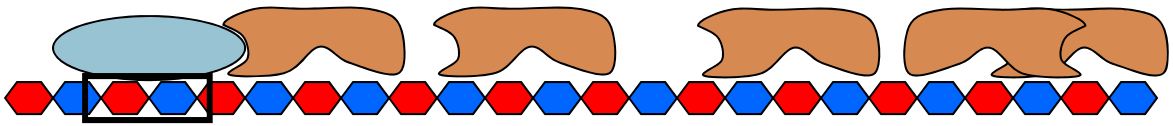
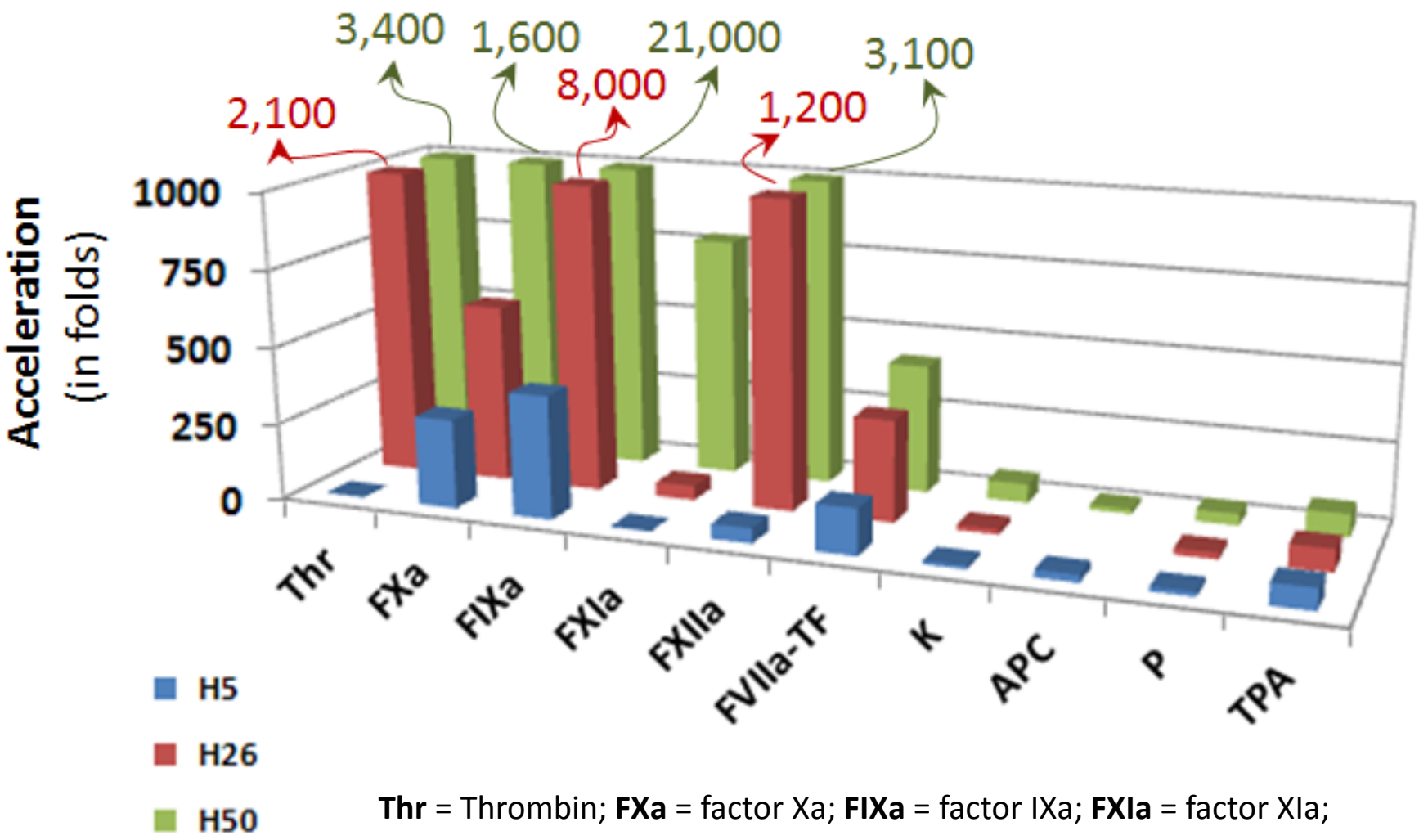


Image taken from Li et al. *Nature Struct. Mol. Biol.* ***2004****, 11*: *857-860*.

Differential Regulation of Coagulation Enzymes

- ***Chain length and heparin-binding site dependence***



Thr = Thrombin; **FXa** = factor Xa; **FIXa** = factor IXa; **FXIa** = factor XIa;

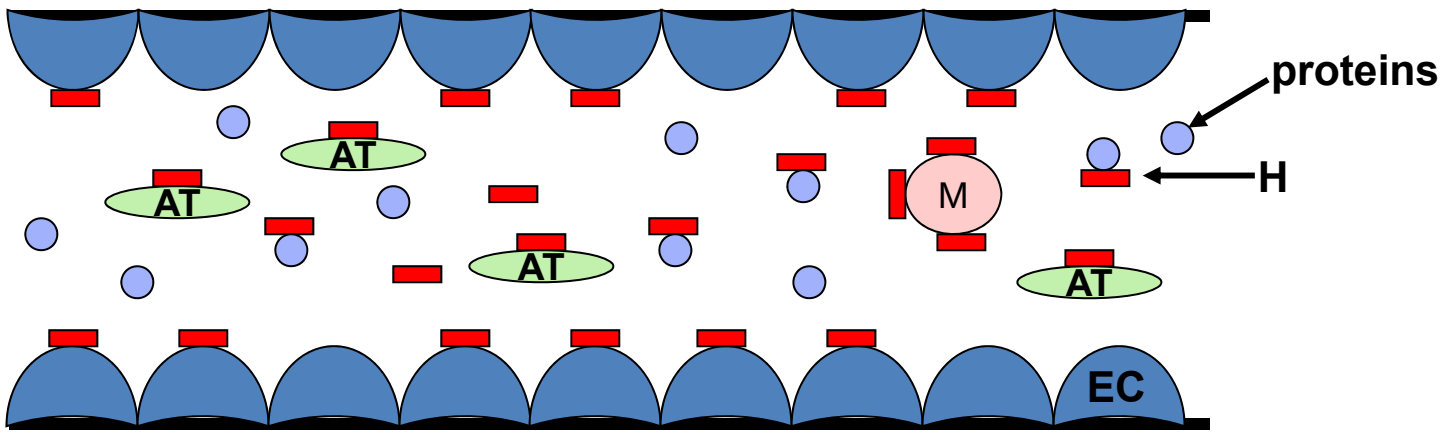
FXIIa = factor XIIa; **FVIIa-TF** = factor VIIa – tissue factor; **K** = kallikrein;

APC = activated protein C; **P** = plasmin; **TPA** = tissue-type plasminogen activator

Thromb. Hemost. (2004) *92*:929-939

Heparin's Adverse Effects

- ***Myriad Effects***



*Adapted from Hirsh et al. (2001) Chest **119**, 64S-94S*

- ✓ Heparin-induced thrombocytopenia …. 3.5% of treated patients
- ✓ Heparin resistance …. 25% of VTE patients
- ✓ Unable to inhibit factor Xa in prothrombinase complex
- ✓ Unable to inhibit thrombin bound to fibrin
- ✓ Binds to von Willebrand factor …. Inhibition of platelet function
- ✓ Binds to platelets …. inhibit platelet aggregation
- ✓ Binds to endothelial cells …. Heparin-induced bleeding
- ✓ Inhibits proliferation of vascular SMC
- ✓ Activates osteoclasts to promote bone loss
- ✓ Causes release of TFPI …. Added anticoagulant activity

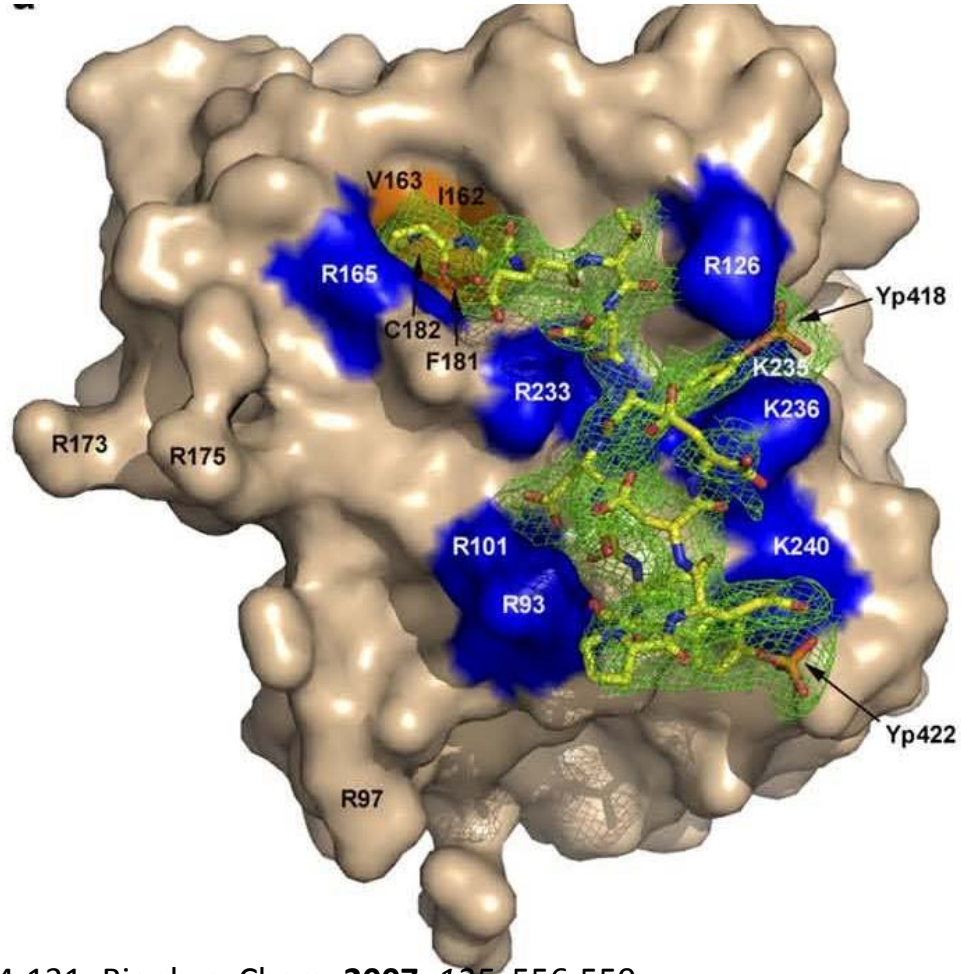
Heparin's Adverse Effects

- ***Heparin-Induced Thrombocytopenia***
  - ✓ *Significant drop in platelet count between 4 and 14 days after the start of heparin therapy*
  - ✓ *Type I HIT is not clinically relevant … drop in platelet count within 48 h … rebound without discontinuation … no immune reaction*
  - ✓ *Type II HIT is clinically relevant … ~3% patients develop clots!*
  - ✓ *UFH binds to platelet factor 4 (PF4) to form a tetrameric complex, which is antigenic … produces IgG and IgM antibodies … the immune complex further activates PF4 … setting up a cycle*
  - ✓ *Activated platelets aggregate and form a clot … effectively platelets are taken off circulation … thrombocytopenia*
  - ✓ *Most observed with UFH … less with LMWHs*
  - ✓ *Heparin pentasaccharide, fondaparinux, binds to PF4 but does not induce immune reaction*

Burger's Medicinal Chemistry, Drug Discovery, and Development, VIIth Edition (Ed. Abraham and Rotella) John Wiley and Sons, **2010**, 365-408.

Heparin's Adverse Effects

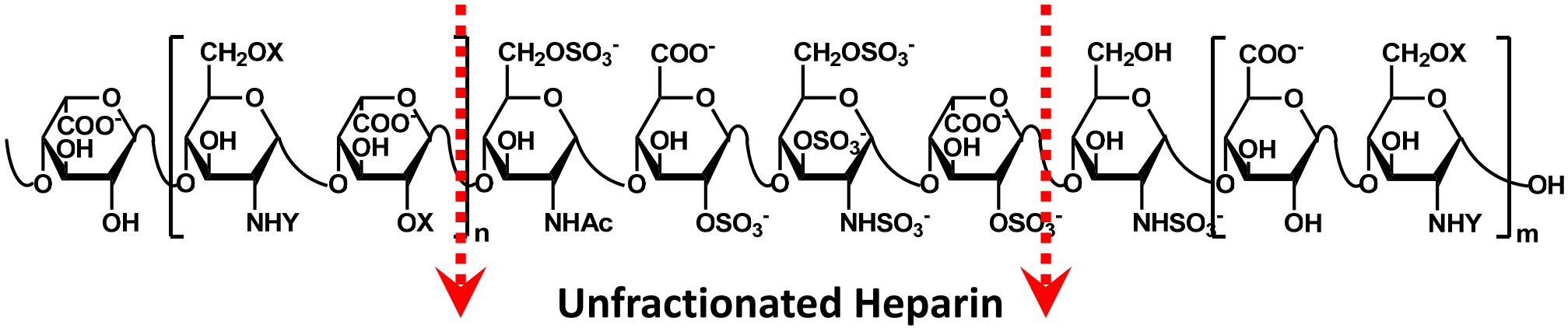
- ***Mechanism for Inability to Inhibit Thrombin in the Clot Bound Form***
  - ✓ *Thrombin binds to the γ chain of fibrinogen (7% of total) through its exosite 2*
  - ✓ *Competes with heparin binding to exosite 2 … prevents binding to the AT – H complex …. no inhibition of clot bound thrombin*



J. Thromb. Haemost. **2003**, ***1,*** 124-131; Biophys. Chem. **2007**, *125*, 556-559

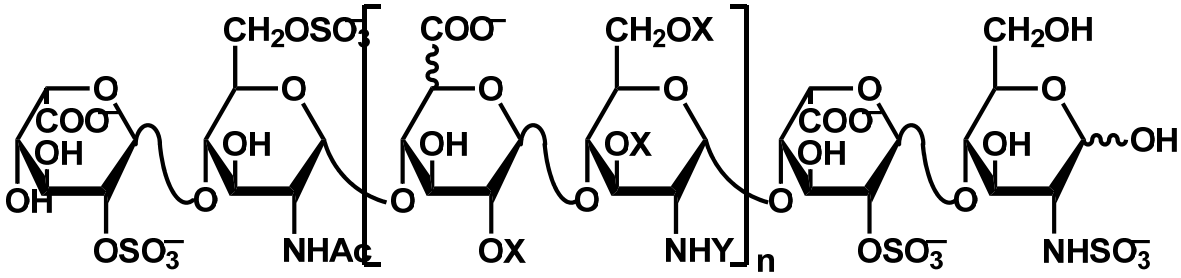
Low Molecular Weight Heparins (LMWHs)

- ***LMWHs are essentially similar to UFH***
  - ✓ *1/3rd the size*



Unfractionated Heparin

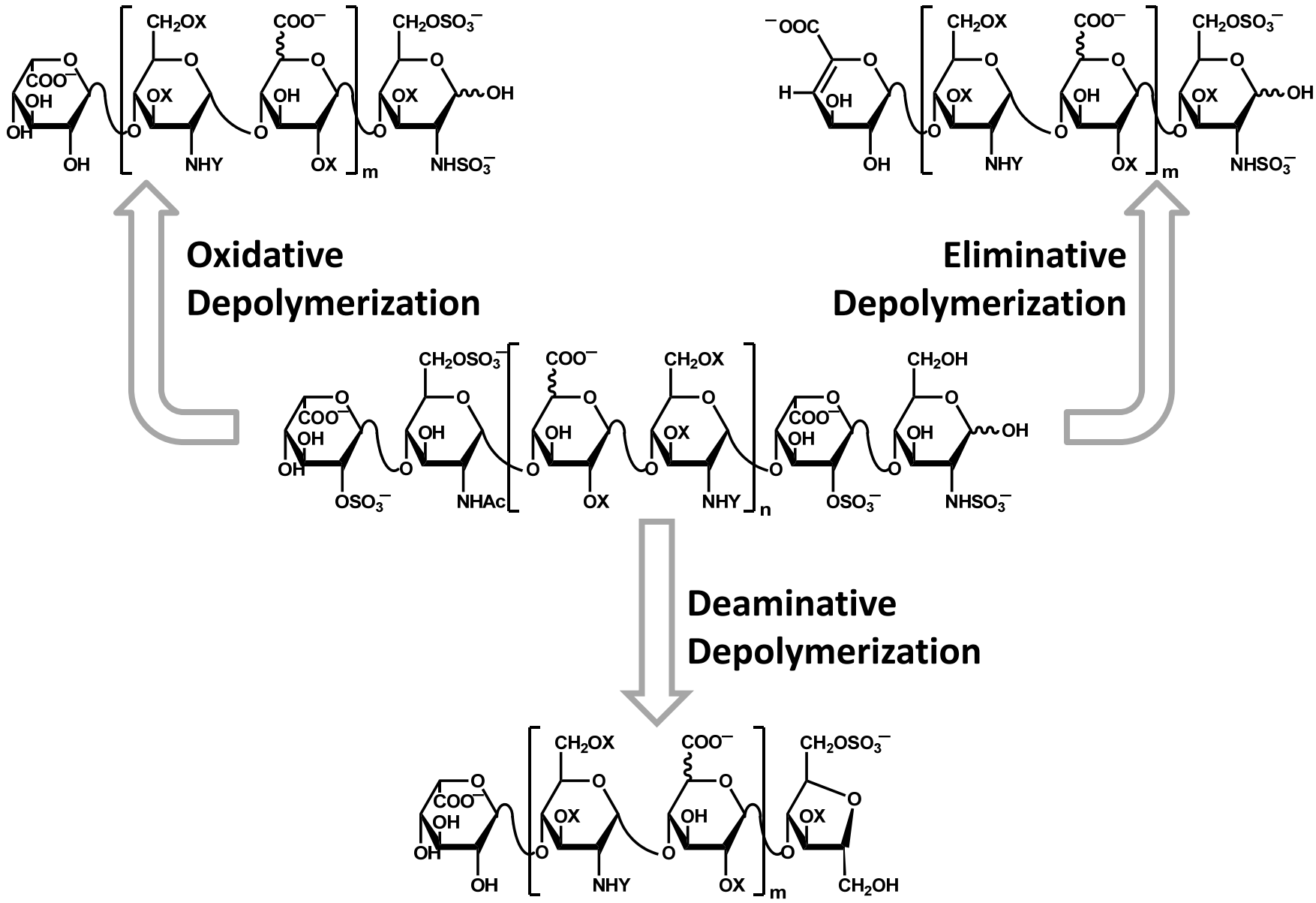
M_R = ~15,000



LMWH

M_R = ~5,000

Preparation of Low Molecular Weight Heparins



Sem. Thromb. Hemost. **1999**, *25 Suppl. 3,* 5-16

Preparation of Low Molecular Weight Heparins

- ***Source of UFH***
  - ✓ *Pig intestine*
  - ✓ *Purified UFH*
- ***Basic Group of Methods***
  - ✓ *Oxidative depolymerization*
  - ✓ *Deaminative depolymerization*
  - ✓ *β-Eliminative depolymerization*
- ***Oxidative Depolymerization***
  - ✓ *Using H_2O_2 w/ (parnaparin, Fluxum) or w/o (ardeparin, Normiflo) Cu^+*
  - ✓ *UFH is more susceptible to oxidants than to temp/acid/base*
  - ✓ *H_2O_2 generates OH radicals*
  - ✓ *Releases small mono-, di-, or tri- mers, especially unsubstituted at positions 2 and 3*
  - ✓ *Unsulfated uronic acids are more selectively oxidized*
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  - ✓ *N-nitrosation using nitrous acid (dalteparin, Fragmin; nadroparin, Fraxiparin; reviparin, Clivarin) or isoamyl nitrite (certoparin, Sandoparin) at the GlcNp2S*
  - ✓ *N-nitrososulfamide is unstable and gives C-2 carbocation*
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  - ✓ *Enzymatic with heparinase I (tinzaparin, Innohep) or chemical through alkaline hydrolysis (enoxaparin, Lovenox or Clexane)*
  - ✓ *Unsaturated uronate can be followed by UV at 232 nm*
  - ✓ *Enzymatic β-elimination at either GlcAp2S or IdoAp2S*
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  - ✓ *Enoxaparin from base hydrolysis of the benzyl ester of the benzethonium salt of heparin*
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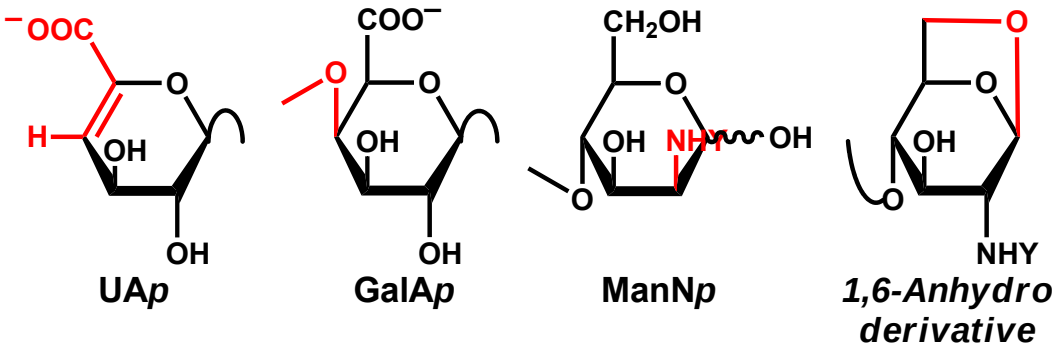
Specific Composition of a LMWH for Clinical Approval



- ***An Example of Enoxaparin***
  - ✓ *M_R = ~4,500*
  - ✓ *$P = M_W/M_N = 1.1 \rightarrow 1.5$*
  - ✓ *Chain length = 2 → 32 monomers*
  - ✓ *MW < 2,000 (<20%), 2,000 – 8,000 (68%), > 8,000 (<18%)*
  - ✓ *Fingerprints of 2 →12-mer (70–80%) …. M_R = 3,600*
  - ✓ *Contains odd + even chains*
  - ✓ *Remaining chains uncharacterized*
  - ✓ *Specific proportion of 8 known disaccharides*
  - ✓ *High affinity pentasaccharide sequence between 15 – 25 %*
  - ✓ *Presence of GalAp (% unknown)*
  - ✓ *Reducing end 70% GlcNp + 30% ManNp*
  - ✓ *1,6-Anhydro ring structures (15 – 25% chains)*

Similarities and Differences Between UFH and LMWH

- ***UFH***
  - ✓ *Animal product*
  - ✓ *Longer polysaccharide*
  - ✓ *Anti-IIa activity = 150 – 200 U/mg*
  - ✓ *Anti-Xa activity = 150 – 200 U/mg*
  - ✓ *Primarily intravenous*
  - ✓ *Half-life short … 1 – 2 h*
  - ✓ *Monitoring required … aPTT*
  - ✓ *Effectively reversed by protamine sulfate*
  - ✓ *Antithrombotic …. arterial and venous thrombosis, surgical anticoagulation, unstable angina, adjunct to cancer chemotherapy, …..*
  - ✓ *Several adverse effects, especially major and minor bleeds and HIT*

- ***LMWH***
  - ✓ *Derivatized animal product*
  - ✓ *Shorter polysaccharide*
  - ✓ *Anti-IIa activity = 20 – 45 U/mg*
  - ✓ *Anti-Xa activity = 80 – 125 U/mg*
  - ✓ *Sub-cutaneous*
  - ✓ *Half life longer … 4 – 6 h*
  - ✓ *Monitoring not strict*
  - ✓ *Reversibility with protamine not effective*
  - ✓ *Antithrombotic …. arterial and venous thrombosis, surgical anticoagulation, unstable angina, adjunct to cancer chemotherapy, …..*
  - ✓ *Lesser adverse effects … reduced bleeds and HIT*

Conceptual Understanding on Biosimilarity

- *What are biosimilars*
- *Why biosimilars?*
- *What are the rules on biosimilarity?*
- *What aspects dictate biosimilar low molecular weight heparins?*

Terminologies

- *Difference between biogeneric and biosimilar*
  - ✓ *Generic … inherently relates to 'identity of structure' … borrowed from the small molecular world*
  - ✓ *Similar … inherently relates to 'homology of structure' … borrowed from the protein world*
- *Example*
  - ✓ *An unglycosylated, unmodified native protein made up of standard amino acid residues when expressed recombinantly can have identical primary sequence, which gives identical three dimensional structure … **generic***
  - ✓ *But a glycosylated plasma protein when made recombinantly may not have the same saccharide composition … yet may be functionally (safety and efficacy) equivalent … **similar**!*

Definition

- *Biosimilars … biological medicinal products …*
  - ✓ *… that are similar in terms of quality, safety and efficacy to an already licensed reference medicinal product*
  - ✓ *… that follow expiry of market exclusivity period of the reference product*
  - ✓ *… that are authorized based on proof of similarity required by the regulatory bodies*
- *Biosimilarity …*
  - ✓ *… has not been applied as yet to cell therapies and other complex mixture of products, e.g., plasma concentrates*
  - ✓ *… applies typically to purified components, e.g., purified proteins or purified heparins*

Why Biosimilar?

- *Protein products are highly complex …*
  - ✓ *Size … MW 10,000 – may be 200,000 or higher*
  - ✓ *Structure … 1º, 2º, 3º, and 4º structure … not just one structure*
  - ✓ *SAR … usually less well defined in comparison to small molecules*
  - ✓ *Stability …generally low … depends more on temperature, storage conditions, excipients, pH, and a range of other factors*
  - ✓ *Microheterogeneity … PTMs and other modifications (oxidation, deamidation, denaturation, aggregation, etc) … also pegylation (one or more sites) … even highly purified proteins never consist of one single molecular entity …*

Why Biosimilar?

- *Manufacturing process … is critical*
  - ✓ *Living cells are the manufacturing facility …*
  - ✓ *Living cells are dynamic entities … running under some control, but that may not be identical between parent and alternate manufacturer*
  - ✓ *An alternate manufacturer … may not have access to original cells and exact operating procedures*
  - ✓ *Variations may generate heterogeneity … although not all heterogeneity may generate clinical variability*

Why Biosimilar?

- *Analytical characterization … may be insufficient*
  - ✓ *Highly orthogonal physicochemical methods of characterization …may not be able to identify all the different constituents of a biomolecule*
  - ✓ *Analytical methods … show comparability …. not identity*
  - ✓ *For example,*
    - ❖ *… glycosylation sequence … its location, its percent modification … become more difficult when protein is glycosylated at various positions*
    - ❖ *… some O-GlcNAc is time-dependent (dynamic)! … depends on the state of the cell*

Biosimilarity … US Regulation

- *Biologics Price Competition and Innovation Act of 2009*
  - ✓ *Abbreviated regulatory approval for biosimilars*
    - ❖ *… side-by-side comparison*
    - ❖ *… may require fewer patients and reduced efficacy / safety data*
    - ❖ *… may be approved for fewer indications*
    - ❖ *… may possess immunogenicity differences of no clinically significant value*

Kresse, G.-B. (2009) Eur. J. Pharmaceut. Biopharmaceut. 72:479-486.

Are Low Molecular Weight Heparins Biosimilar?

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  - ✓ *Pig intestine*
  - ✓ *Purified UFH*
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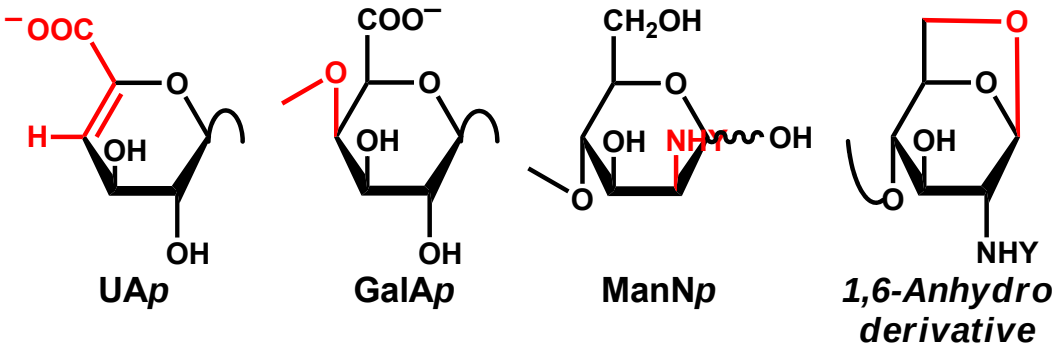
Can a Biosimilar LMWH be Approved for Clinical Use?

- ***Each LMWH is unique!***
  - ✓ *FDA … not inter-changeable*
  - ✓ *Significant structural differences*
  - ✓ *Significant pharmacological differences*
  - ✓ *Significant toxicological differences*
- ***Five Criteria***
  - ✓ *Used to evaluate similarity OR differences*
  - ✓ *Physicochemical characteristics*
  - ✓ *Source of UFH*
  - ✓ *Structural composition*
  - ✓ *In vitro biological activity*
  - ✓ *In vivo pharmacodynamic profile*



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