ACIDIC POLYSACCHARIDES: THEIR MODIFICATION AND POTENTIAL USES

Robert J. Linhardt, Ali Al-Hakim and Jian Liu

Division of Medicinal and Natural Products Chemistry
College of Pharmacy
University of Iowa
Iowa City, IA 52242

Acidic polysaccharides are known for their wide range of biological activities. These natural products are obtained from animal sources (i.e., glycosaminoglycans) or are prepared by the chemical sulfation of naturally occurring microbial and plant polysaccharides. For medical applications, heparin is the most commonly used acidic polysaccharide.

Heparin is a polydisperse, highly sulfated, linear polysaccharide of repeating uronic acid and glucosamine residues. Although heparin has been used clinically as an anticoagulant for the past 50 years, its precise structure remains unknown. Heparin's primary application is as an anticoagulant, however, it is more appropriate to consider heparin as a polyelectrolytic drug having a multiplicity of biological activities. Virtually any cationic protein and many anionic proteins are capable of binding to heparin under physiological conditions. Within the past decade, a growing number of biological activities have been demonstrated to be regulated by heparin, ranging from its effect on angiogenesis to the regulation of the immune response. Low molecular weight heparins have been undergoing clinical trials as antithrombotic agents for use in a wide variety of diseases ranging from deep vein thrombosis to non-hemorrhagic stroke. This chapter discusses new agents including natural products chemically or enzymatically derived from heparin, synthetic acidic oligosaccharides and polysaccharides, and heparin covalently immobilized to polymers.

INTRODUCTION

Glycosaminoglycans are a group of highly sulfated acidic polysaccharides.[1] One or more polysaccharide chains are typically found attached to a protein core rich in serine-glycine repeating sequences. Unlike the highly branched oligosaccharides found in glycoproteins, these glycosaminoglycans are linear (Figure 1). Although these linear polysaccharide are typically O-linked to a serine residue in their core protein, they

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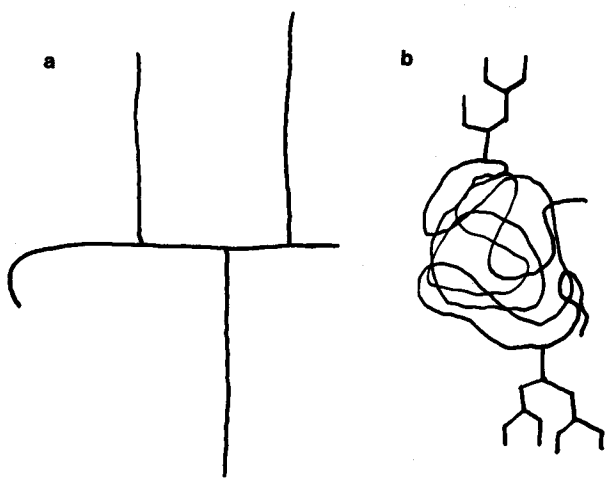

Figure 1. Proteoglycan and glycoprotein structures. Representations are shown of linear glycosaminoglycan chains extending from the protein core of a proteoglycan (a) and the branched oligosaccharides of a glycoprotein (b).

are also found N-linked similar to glycoprotein oligosaccharides.[2]

There are several major classes of glycosaminoglycans.[1] These include hyaluronic acid, chondroitin sulfates A and C, dermatan sulfate, heparan sulfate, heparin and keratan sulfate. The structure of the major repeating disaccharide unit in each glycosaminoglycan is shown in Figure 2. Hyaluronic acid contains no sulfate and is not found linked to a core protein.[2] Its structure is (→3)-β-D-N-acetylglucosamine-(1→4)β-D-glucuronic acid (1→). Chondroitin sulfates are another class of PGs which can be broken down into two major classes: chondroitin sulfate A, (→3)-β-D-N-acetylgalactosamine-4 sulfate-(1→4)-β-D-glucuronic acid (1→); chondroitin sulfate C, (→3)-β-D-N-acetylgalactosamine-6 sulfate (1→4)-β-D-glucuronic acid (1→); Dermatan sulfate B is also a galactosaminoglycan and is primarily a polymer of (→3)-β-D-N-acetylgalactosamine-4 sulfate (1→4)-α-L-

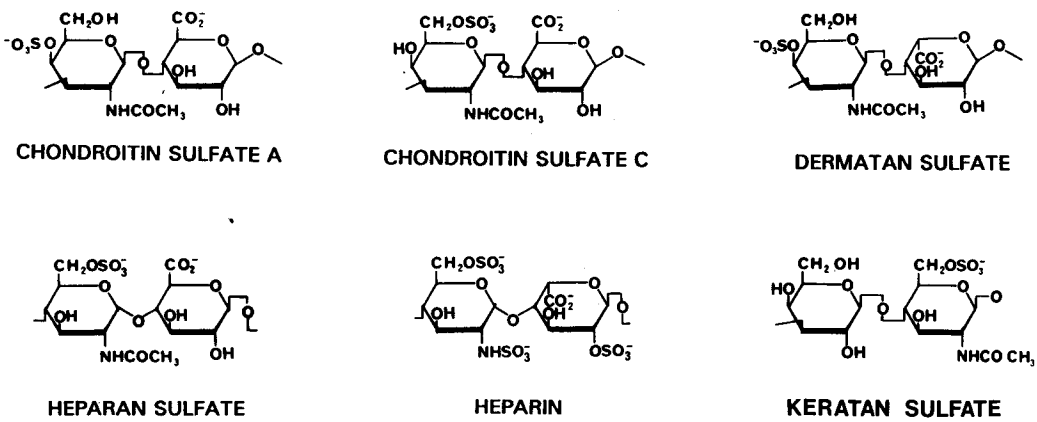

Figure 2. Major repeating disaccharide units found in various glycosaminoglycans.

iduronic acid (1→).[2] Heparin and heparan sulfate are structurally similar glucosaminoglycans[3] comprised of(→4)-β-D-N-acetyl or N-sulfated glucosamine (6-O-sulfation is possible), (1→4)-α-L-iduronic acid or β-D-glucuronic acid (with possible 2-sulfation) (1→). Keratan sulfate, the final class of glycosaminoglycans, has a major disaccharide repeating unit: (→3) β-D-galactose (1→4)-β-D-N-acetylglucosamine-6 sulfate (1→).[2]

Although heparin's primary application is as an anticoagulant, it is more appropriately considered a polyelectrolytic drug having a multiplicity of biological activities.[4,5] Most cationic proteins (pI > 7), and many anionic proteins, are capable of binding to heparin under physiological conditions. In the past decade, a number of new biological activities have been demonstrated to be regulated by heparin.[1,6] These range from its effect on angiogenesis to the regulation of the immune response.[7,8] Low molecular weight heparins, heparin oligosaccharides and other glycosaminoglycans have been undergoing clinical trials as anticoagulant/antithrombotic agents for use in a wide variety of disease states ranging from deep vein thrombosis to non-hemorrhagic stroke.[1,9,10-12] These agents include both natural products, chemically or enzymatically derived from heparin, as well as fully synthetic acidic oligosaccharides and polysaccharides. This chapter focuses on the enzymatic preparation of oligosaccharides from heparin and other glycosaminoglycans. Although they have been primarily studied in their soluble form, methods are being developed to incorporate these oligosaccharides into synthetic polymers.

EXPERIMENTAL

1. Materials

Heparin, sodium salt, from porcine intestinal mucosa (approximately 150 U/mg) was obtained from Hepar (Franklin, OH). Other glycosaminoglycans including heparan sulfate, chondroitin sulfates A and C, dermatan sulfate and hyaluronic acid (also sodium salts) were from Sigma (St. Louis, MO). Raw (unbleached) heparin, in which approximately 10% of the polysaccharide chains contain peptide bonded covalently at their reducing end, was either prepared in our laboratory from porcine intestinal mucosa or purchased from Sigma. Lyase enzymes including heparinase, heparinase II, heparitinase, and chondroitinases A, AC and B were prepared in our laboratory.[12-14] These enzymes, as well as chondroitinase ABC and hyaluronidase, are also commercially available from Sigma and Seikagaku Kogyo America (Rockville, MD).[15] Reactigel (HW-65F), a polyvinyl alcohol based beaded resin pre-activated with 1,1'-carbonyliimidizole was from Pierce (Rockford, IL). Sulfopropyl Sephadex C-50 was from Sigma. Controlled pore dialysis bags were from Spectrum Medical (Los Angeles, CA).

2. Methods

2A. Lyase Catalyzed Depolymerization of Glycosaminoglycan

Heparin was prepared in 5 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride at 10 mg/mL. To 80 µl of heparin, 20 µl of heparin lyase (0.03 IU in the same buffer) was added. The reaction mixture was incubated overnight at 30°C. After the reaction was complete, protein was removed from the reaction mixture by adjusting its pH to 3.0 and passing this solution through a 1 mL column of sulfopropyl-Sephadex adjusted with 30 mM hydrochloric acid to the same pH. The material fail-

ing to bind to this support was collected and its pH adjusted to 7.0 with sodium hydroxide solution. The oligosaccharide products could be desalted by exhaustive dialysis using 500 or 1000 molecular weight cut-off bags. The resulting salt-free solution of heparin oligosaccharides were concentrated by freeze-drying and reconstituted in distilled water. Oligosaccharides derived from other glycosaminoglycans were similarly prepared by depolymerization using the appropriate enzyme, reaction time and temperatures.[14,15]

2B. Fractionation of Acidic Oligosaccharides

Two methods were used to fractionate oligosaccharide mixtures, preparative strong anion-exchange high pressure liquid chromatography[16] and preparative gradient polyacrylamide gel electrophoresis.[17] These methods were used to prepare multimilligram quantities of homogeneous oligosaccharides for structural characterization, further chemical or enzymatic modification and ultimately for chemical coupling to polymer surfaces.[18-21]

2C. Covalent Binding of Peptide-Containing Glycosaminoglycans

Raw, unbleached, heparin was prepared at 100 mg/mL in 0.1 M sodium borate buffer at pH 8.5. To this heparin, 2 mL of activated beaded resin (activation level: >50 µmoles/mL gel) was added and the 8 mL of suspension was shaken at 25°C for 24 h. After the reaction was complete, the beads were washed two times with 3 volumes of 6 M urea followed by two 3 volume washes with distilled water, two 3 volume washes with 2M sodium chloride and finally by two 3 volume washes with distilled water. After resuspension in distilled water the content of immobilized heparin was measured by dye binding assay.[22]

RESULTS AND DISCUSSION

1. Heparin's Biological Activities

The biological activities of heparin and other glycosaminoglycans are primarily mediated through their binding to proteins and subsequent regulation of their activities. To be effective, substitute polyanions must bind to these proteins and regulate these same activities. Heparin's binding capability is primarily through electrostatic interactions and depends on its high charge density. Most heparin substitutes that have been proposed are highly sulfated polyanions, prepared enzymatically or chemically, and can take the place of heparin by the positioning of these charged groups. Ideally, these heparin substitutes should exhibit tighter binding to these proteins to have higher potency. Only the antithrombin III (ATIII) binding site (Figure 3) in heparin has been sufficiently studied to develop a well defined structure activity relationship. A synthetic heparin pentasaccharide containing this binding site has an ATIII binding constant that is comparable to heparin's.[23]

In addition to its anticoagulant activity, heparin exhibits a number of side-effects. Not all of heparin's side-effects, however, are undesirable. Some of these side-effects might be exploited and new pharmacologically active agents prepared. For example, heparin releases and activates lipoprotein lipase (LPL) but does so only at concentrations that are fully anticoagulating.[24] If a heparin were prepared devoid of this anti-

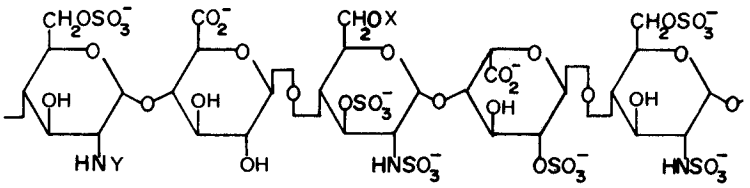

Figure 3. Heparin's antithrombin III-binding site. A pentasaccharide sequence at which antithrombin III can tightly bind is shown where X = H or SO_3^- and Y = SO_3^- or $COCH_3$.

coagulant activity, but with high LPL releasing activity, it might represent a useful agent in the treatment of atherosclerosis. Heparin inhibits complement activation but only at concentrations much greater than those required for full anticoagulation.[8] Recent results in our laboratory demonstrate that it is possible to prepare oligosaccharides from heparin that are equipotent with heparin (on a weight basis) in inhibiting complement activation but without anticoagulant activity.[8,21] Such a drug might be very useful in preventing complement activation in extracorporeal therapy. There are scores of other heparin side-effects that might be usefully exploited resulting in the preparation of new classes of therapeutic agents.

Hemostasis is the "spontaneous arrest of bleeding from ruptured blood vessels".[25] This broad physiological process includes the blood coagulation system and involves plasma coagulation factors, platelets, monocytes, and endothelial cells that line the blood vessels. The coagulation cascade consists of a sequence of reactions in which protease precursors are converted from enzymatically inactive to enzymatically active forms. In the final stages of the coagulation cascade, fibrinogen is converted by thrombin (factor IIa) into the spontaneously polymerizable fibrin monomer. Polymerization and subsequent crosslinking of fibrin monomers produces gelatinous fibers which enmesh platelets forming a primary hemostatic plug.

Heparin catalyzed anticoagulation is primarily attributable to its binding to antithrombin III (ATIII), a serine protease inhibitor. On binding to heparin, ATIII undergoes a conformational change that enhances its activity as serine protease inhibitor. Thrombin, a serine protease then binds to the heparin-ATIII complex, acts on ATIII, and is irreversibly inactivated. Thus the conversion of fibrinogen to fibrin clot is blocked and the blood is anticoagulated. Heparin also inhibits coagulation and thrombosis through a number of other mechanisms the details of which are beyond the scope of this chapter.[1]

2. Polymers As Heparin Substitutes

Polymers that are structurally related to heparin and that possess certain of its biological properties, such as anticoagulant activity, are commonly called heparinoids. Non-heparin glycosaminoglycans having anticoagulant/antithrombotic activities are sometimes classified as heparinoids. Heparinoids are also prepared by modification of naturally occurring polysaccharides, by the total synthesis of heparin-like polymers, and most recently by the synthesis of small sulfated heparin-like oligomers.

Dermatan sulfate and heparan sulfate have been used in animal studies and in clinical studies in Europe as antithrombotic agents.[10,26,27] Other glycosaminoglycans, such as hyaluronic acid and chondroitin sulfates, may also have low antithrombotic activity.[10]

Chitin, a major organic component of the exoskeleton of insects,[2] can be de-N-acetylated to prepare chitosan. On chemical sulfation and/or carboxymethylation, chitosan affords a polyanion with certain structural and activity similarities to heparin.[28]

Pentosan, extracted from the bark of the beech tree, can be sulfated by chemical methods resulting in an anticoagulant with one-tenth of heparin's activity on a weight basis.[29,30]

Dextran, a branched glycan polymer, can be chemically sulfated to prepare dextran sulfate,[31] having low anticoagulant activity.[2] Dextran sulfate has been used as a heparin replacement in anticoagulation and has recently been immobilized on plastic tubes to prepare non-thrombogenic surfaces.[32]

Synthetic polymers such as poly(vinyl sulfate) and poly (anethole sulfonate) are highly charged heparin-like polyanions that also exhibit anticoagulant activity.[33] However, these agents are not used *in vivo* as they are resistant to metabolism and thus remain in the body for extended periods resulting in toxic side effects.[1,33]

3. Defined Oligosaccharides as Heparin Substitutes

A synthetic 3-O-sulfated pentasaccharide, representing heparin's ATIII binding site (Figure 3) was first prepared by Choay, et.al., in a multi-step synthesis.[34] Clinical studies on this pentasaccharide, as an antithrombotic agent, demonstrated that it was not as effective as heparin itself and its cost probably precludes its use as a therapeutic agent.[35]

Heparin-oligosaccharides of defined structure have been prepared by our laboratory using lyases (Figure 4) as described in the experimental section. These heparin oligosaccharides have important biological activities including complement inhibitory activity with an *in vitro* potency nearly equal to heparin on a weight basis.[21] Methods are being developed

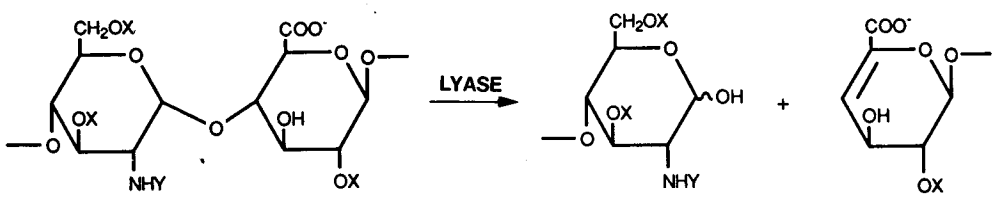

Figure 4. Depolymerization of heparin using polysaccharide lyase. The glycosidic linkage between a substituted glucosamine (X = H or SO_3^-, Y = SO_3^- or CH_3CO) and uronic acid (iduronic or glucuronic, X = SO_3^- or H) is cleaved eliminatively affording oligosaccharide products. The non-reducing end of each product formed contains a Δ-4,5 unsaturated uronic acid residue.

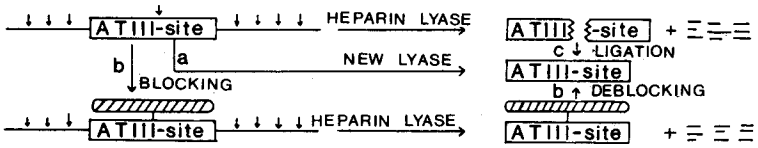

Figure 5. Strategies to prepare homogeneous oligosaccharides having an intact antithrombin III-binding site. (a) Heparin is depolymerized using a new highly specific lyase that cuts only glycosidic linkages outside the ATIII-binding site. (b) The ATIII-binding site in heparin is blocked while heparinase depolymerizes the surrounding heparin chain. The blocking agent is then removed. (c) Two partial ATIII-binding sites are ligated by reverse enzymic catalysis using heparinases to form an intact ATIII-binding site.

for the large-scale and inexpensive preparation of these heparin oligosaccharides.[16,17]

The ATIII-binding site contains an unique arrangement of five different saccharide units. Because this binding site contains such a wide variety of saccharide types; no method of chemical depolymerization has yet been devised that is capable of excising this ATIII-binding site from a heparin chain without breaking it. Enzymatic methods using lyases also cleave heparin's ATIII-binding site.[14,16] Multistep, low-yield chemical synthesis remains the only reliable method to prepare heparin oligosaccharides containing intact ATIII-binding sites. Herein lies the problem: to prepare in a few steps, and in high yield and high purity, oligosaccharides having intact ATIII-binding sites. We are examining three methods to prepare these ATIII-binding sites (Figure 5).

The first method (Figure 5a) uses a lyase to cleave selectively at (→4)-β-D-N-sulfated glucosamine 6-sulfate (1→4)-α-L-iduronic acid 2-sulfate (1→) linkages occurring only outside the ATIII-binding site. An ATIII-binding octasaccharide from porcine heparin (or hexasaccharide from bovine heparin) could be prepared using this lyase in a single step in high yield. Of the lyases that we have characterized, heparin lyase comes closest to meeting this specificity requirement; but its specificity also permits it to cut the (→4)-β-D-N-sulfated glucosamine 3,6-disulfate-(1→4)-α-L-iduronic acid 2-sulfate (1→) linkage, located in the center of the ATIII-binding site. This linkage, containing an additional 3-sulfate group, seems to be cut preferentially, probably because of a reduced Km of the enzyme for this linkage.[16] Reaction conditions are being examined that decrease the action of heparin lyase at this linkage within the ATIII-binding site. The specificity of new lyases are being examined to see if these can cleave heparin while leaving the ATIII-binding site intact.

The second method (Figure 5b) approaches this problem by selectively blocking the action of heparin lyase at the ATIII-binding site. ATIII (Kd ~10^{-7} M) has been used successfully to compete with heparin lyase (Km ~10^{-8} M) for heparin's ATIII-binding site. ATIII non-covalently binds to heparin, blocking lyase action at this site, and affording oligosaccharides with intact ATIII-binding sites.[36]

The third approach (Figure 5c) involves the enzymatic or chemical ligation of a broken ATIII-binding site. There is no reported reverse

enzymic catalysis utilizing polysaccharide lyases. Such an approach, however, if successful, might be extremely useful in re-assembling oligosaccharides into acidic polysaccharides of defined structure and with a variety of desirable biological properties. Our laboratory prepared a hexasaccharide, using heparin lyase, in a single step (Figure 4) from porcine mucosal heparin.[18,37] Approximately 95% of this hexasaccharide is recovered and it represents about 5-10% of heparin's mass. Based on the principle of microscopic reversibility, heparin lyase should catalytically re-form an appropriate glycosidic linkage between two hexasaccharides (each containing a portion of heparin's ATIII binding site). The result would be a single intact ATIII-binding site.

4. Preparing Stable Blood Compatible Surfaces

The major problem associated with the design and preparation of blood compatible surfaces is the lack of a complete understanding of coagulation and thrombosis. The ideal blood compatible polymer should mimic blood's natural container, the vessel lined with endothelial cells, as closely as possible. The surface should be stable and survive enzymatic and chemical attack from the components present in the circulation. The vascular endothelium is lined with heparan sulfate, a heparin-like molecule containing ATIII-binding sites.[1] The production of stable heparinized, antithrombotic surfaces, however, is very difficult. Simple adsorption of heparin onto a polymer produces a blood compatible surface that only lasts a short period of time until the heparin leaches from the surface.[38,39] Covalent immobilization should offer an alternative, chemically stable linkage. Many of these surfaces, however, also have a short lifetime possibly due to the enzymatic stripping of heparin from the surface. Enzymes that act on heparin, including both exoglycuronidases and endoglycuronidases are present in the circulation.[40-42] The precise

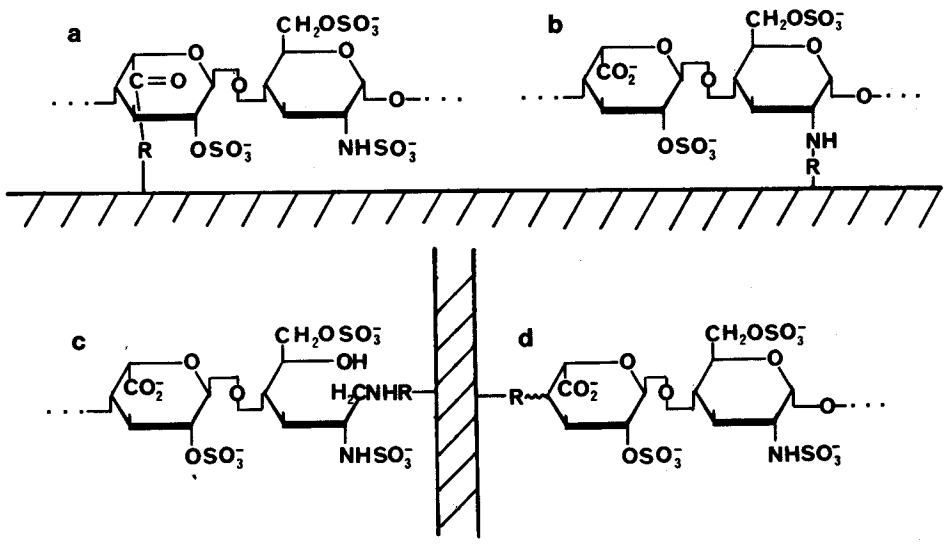

Figure 6. Orientations of immobilized heparin chains on the surface of a polymer. Possible orientations include coupling through: the center of a heparin chain utilizing (a) carboxyl or (b) amino functionality; (c) heparin's reducing-end by reductive amination orthrough a peptide linkage; and (d) heparin's non-reducing end.

nature of the surface-heparin linkage as well as its orientation (Figure 6) may control enzyme access to immobilized heparin chains resulting in stable heparinized surfaces.

Our laboratory is examining the question of how the orientation of a heparin chain that is immobilized to a surface (i.e., coupled through either its reducing-end or its non-reducing end), affects its linkage stability when exposed to enzymes in the circulation (Figure 6). The first of these defined heparinized surfaces have been prepared by coupling heparin at its reducing end to a synthetic polymer. The coupling chemistry relies on residual peptide present in raw (unbleached) heparin. This heparin can be immobilized with a high loading (1 mg heparin/mL of swelled polymer bead). A peptide linkage is used to assure the chemical stability of this heparinized surface. Studies of the *in vitro* stability in blood as well as the *in vivo* stability of this heparinized surface are currently underway. Surfaces with heparin (or oligosaccharides prepared from heparin using lyases) bound in other orientations (Figure 6) are also being prepared for testing.

Finally, it may be possible to incorporate heparin directly into the backbone of a synthetic polymer. The approach makes use of heparin oligosaccharides prepared using a lyase (Figure 4). These oligosaccharides contain an unusual unsaturated uronic acid at their non-reducing end.[15] This unsaturated uronic acid residue represents an activated site through which the oligosaccharide might be incorporated into a synthetic polymer. Although this unsaturated uronic acid residue resembles an acrylic acid type Michael acceptor, it is extremely unreactive towards nucleophiles. This low reactivity may rule out ionic-based polymerization methods for incorporation into synthetic polymers. An alternative method of incorporating these oligosaccharides into synthetic polymers relies on free-

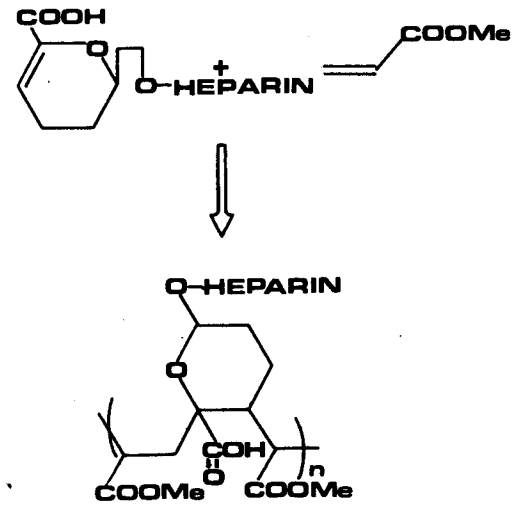

Figure 7. Scheme for the free-radical copolymerization of unsaturated heparin oligosaccharide and an acrylic acid based monomer. A heparin oligosaccharide (R = 1,3,5... sugar residues) prepared using a lyase is copolymerized with methyl acrylate in the presence of a peroxide catalyst and initiator. The polymer prepared contains the non-reducing terminal sugar of the oligosaccharide in the backbone of the polymer.

radical polymerization methods. Here a monomer such as methyl acrylate might be copolymerized with a heparin oligosaccharide to form a heparin-containing synthetic polymer (Figure 7). Such an approach has recently resulted in the successful incorporation of lyase prepared heparin oligosaccharides into polyacrylamides.[43]

5. Future Prospects

Heparin has been in widespread clinical use for over a half a century. Despite heparin's structural complexity, multiple activities and numerous side-effects, its use continues and has even expanded during the past decade. The major factor behind heparin's success is that it remains the most effective anticoagulant/antithrombotic agent available. Thus, the prospects for new and improved heparins or heparinized surfaces are very good. The twenty-first century should bring an array of monodisperse, homogenous, potent heparinoid drugs with high therapeutic indices and increased specificities. In addition, heparinized biomaterials will probably be in great demand for use in both extracorporeal devices and implanted artificial organs.

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