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Perspective

Semi-synthetic heparin derivatives: chemical modifications of heparin beyond chain length, sulfate substitution pattern and *N*-sulfo/*N*-acetyl groups

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Abstract—The glycosaminoglycan heparin is a polyanionic polysaccharide most recognized for its anticoagulant activity. Heparin binds to cationic regions in hundreds of prokaryotic and eukaryotic proteins, termed heparin-binding proteins. The endogenous ligand for many of these heparin-binding proteins is a structurally similar glycosaminoglycan, heparan sulfate (HS). Chemical and biosynthetic modifications of heparin and HS have been employed to discern specific sequences and charge-substitution patterns required for these polysaccharides to bind specific proteins, with the goal of understanding structural requirements for protein binding well enough to elucidate the function of the saccharide–protein interactions and/or to develop new or improved heparin-based pharmaceuticals. The most common modifications to heparin structure have been alteration of sulfate substitution patterns, carboxyl reduction, replacement *N*-sulfo groups with *N*-acetyl groups, and chain fragmentation. However, an accumulation of reports over the past 50 years describe semi-synthetic heparin derivatives obtained by incorporating aliphatic, aryl, and heteroaryl moieties into the heparin structure. A primary goal in many of these reports has been to identify heparin-derived structures as new or improved heparin-based therapeutics. Presented here is a perspective on the introduction of non-anionic structural motifs into heparin structure, with a focus on such modifications as a strategy to generate novel reduced-charge heparin-based bind-and-block antagonists of HS–protein interactions. The chemical methods employed to synthesize such derivatives, as well as other unique heparin conjugates, are reviewed.

Keywords: Heparin; Heparinoids; Heparin analogs; Semi-synthesis; Glycosaminoglycans

1. Introduction

1.1. Heparin structure and activities

Heparin‡ is a linear, polydisperse polyanionic polysaccharide.[1] Heparin is composed of repeating β-D-glucopyranosiduronic acid or α-L-idopyranosiduronic acid (1→4) linked to *N*-acetyl or *N*-sulfo D-glucosamine (Fig. 1). The glucosamine and uronic acid residues are variably substituted with anionic *O*-sulfo (sulfate) and *N*-sulfo (sulfoamino) groups. Heparin and low molecular weight heparins (LMWHs) derived from the depolymerization of heparin are primarily recognized for their clinical use as anticoagulants.[2] Heparin has been ascribed an increasing number of biological activities and potential therapeutic applications as a consequence of binding to hundreds of prokaryotic and eukaryotic proteins.[1,3] The endogenous ligand for many of these proteins is heparan sulfate‡ (HS). HS is found on the surface of virtually all mammalian cells and in the extracellular matrix where it plays a profound role in many physiological processes ranging from intercellular communication to host–pathogen interactions.[1,3] Heparin

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‡ In this manuscript, the terms heparin and heparan sulfate refer to the polysaccharide (glycosaminoglycan) component of these proteoglycans.

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and derivatives of heparin have thus served as lead structures toward identifying structures that bind HS-binding proteins and block HS–protein interactions.[4,5]

Heparin and HS, being microheterogeneous polysaccharides, consist of variably substituted sequences along each polysaccharide chain.[1,6] The different sequences often have varied affinities for different heparin-binding proteins.[7] Over the past few decades, the specific sequence(s) within heparin and HS having highest affinity for many heparin/HS-binding proteins have been elucidated. Identifying the sequences in heparin that are responsible for heparin binding to specific proteins has been invaluable toward understanding the biological activities of exogenous heparin and the endogenous functions of HS. This information has yet, however, to lead to fulfillment of the vast potential for new therapeutic agents as inhibitors of HS–proteins interactions, in particular because many HS-binding proteins bind to the same highly charged sequences in heparin and HS with similar affinity.

1.2. Agonist versus antagonist functions of heparin-based therapeutics

Much of the effort to identify heparin derivatives and heparin-mimicking molecules (heparinoids) to exploit the numerous potential therapeutic applications ascribed to these molecules has focused on modifying heparin or preparing highly charged sulfated saccharides.[4,5,8,9] The design platform for these agents has often relied on optimizing the degree and/or spatial display of anionic substituents on heparin-based, oligosaccharide-based or non-carbohydrate-based scaffolds. A trend with the chemical modification of heparin has been to maintain anionic substituents and sulfate substitution patterns identified to be required for heparin to bind target protein with highest affinity. Maintaining a requisite spatial display of anionic groups or a specific sulfate substitution pattern along a saccharide chain is certainly critical to achieve agonist-like actions of heparin, such as the binding and activation of antithrombin by a unique heparin pentasaccharide (Fig. 1C). However, there are few compelling reasons to limit heparinoid design to this constraint when the therapeutic goal of a heparinoid-based agent is to bind the HS-binding site of a protein and block HS–protein interactions. There are clear differences in the absolute structural requirements for heparinoid 'agonists', where critical contacts between protein and saccharide must occur to allosterically modulate or otherwise mediate protein function, versus heparinoid-based bind-and-block 'antagonists' of HS–protein interactions, where affinity and selectivity of a heparinoid for the HS-binding site of a protein are the critical factors regardless of the ultimate binding orientation, sugar conformation, and/or saccharide–protein contacts.

Because early work toward the design and preparation of heparin derivatives focused on identifying novel anticoagulants (e.g., agonists of serine protease inhibitors such as antithrombin), it was required that chemical modifications to heparin, including the replacement of anionic groups with non-anionic moieties, employ only partial substitution of anionic groups or impart only modest structural alterations such that key saccharide–protein contacts required for agonist-like function would not be lost. Persulfated and other polyanionic oligosaccharide and polysaccharide derivatives prevailed as potential heparin mimics in efforts to capitalize on critical charge–charge interactions for agonist function. As the number of proposed therapeutic applications for bind-and-block antagonists of HS–protein interactions has increased, an emphasis on evaluating highly charged polyanions as heparin mimics to bind these proteins has persisted, although more extensive removal of charge, more dramatic alteration of heparin structure, and the

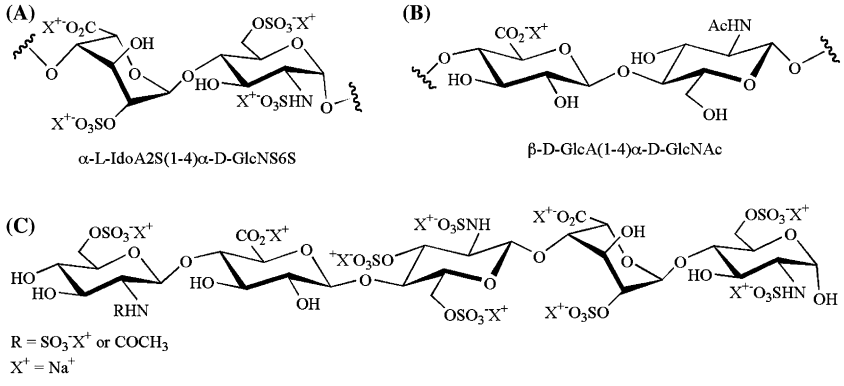

Figure 1. Representative heparin and HS structures. (A) A tetra-anionic disaccharide unit of heparin comprised of α-L-idopyranosiduronic acid (1→4) linked to *N*-sulfo-D-glucosamine. This disaccharide predominates in the most highly charged domains of heparin and HS. (B) A disaccharide unit of heparin comprised of β-D-glucopyranosiduronic acid (1→4) linked to *N*-acetyl-D-glucosamine. This disaccharide is the lowest charge repeating sequence found in heparin and HS. (C) The antithrombin-binding pentasaccharide sequence found in heparin and HS. This pentasaccharide sequence binds and promotes proteolytic activity of antithrombin, and is thus representative of unique sequences in heparin and HS that modulate the function of specific proteins in an agonist-like manner.

introduction of non-anionic moieties into heparinoid-based structures is increasingly being reported in the literature.

Limited protein-binding selectivity and undesired physical properties are significant problems thwarting therapeutic application of many current heparinoids as antagonists of HS–protein interactions.[10] As described in Sections 2–6 below, a number of laboratories have contributed to the development of chemical methods to introduce non-anionic structures into heparin toward developing novel heparinoids with improved physical properties and/or more selective binding to target proteins. However, the vast majority of these efforts have looked to retain key aspects of heparin structure because of the aforementioned agonist model for the structural requirements of heparinoids. We recently detailed a rationale for replacing anionic groups on heparin, even anionic groups required for heparin to bind target proteins, with certain types of non-anionic moieties.[11,12] The goal of such an approach is to minimize charge and optimize spatially more stringent saccharide–protein-binding contacts to generate heparinoids with improved selectivity for binding specific proteins, thus affording novel lead structures for reduced-charge bind-and-block antagonists of HS–protein interactions. In this work we demonstrated that the *N*-sulfo groups on heparin, which were known to be required for high affinity binding of heparin to the target proteins, could be replaced with structurally diverse non-anionic moieties to yield reduced-charge heparin derivatives that more selectively bound certain heparin-binding proteins over other heparin-binding proteins without loss of affinity. Similar results have also been obtained upon preparing and evaluating a panel of novel heparin amides.[12]

Replacing anionic moieties in heparin with structurally diverse non-anionic groups can be likened to generating a structurally diverse library of reduced-charge heparinoid sequences, even if these sequences exist as mixtures along the polysaccharide chains. Identification of the uniquely modified heparin derivatives that bind a target protein, followed by elucidating the individual sequences responsible for protein binding is expected to afford novel lead structures upon which to guide the preparation of smaller charge-reduced bind-and-block antagonists of HS–protein interactions. While oligosaccharides prepared by this approach may not epitomize drug-like compounds, they are certain to have improved physical properties over the more highly charged heparinoids currently under evaluation as inhibitors of HS–protein interactions.

As a process for identifying lead structures, replacing anionic groups on heparin with structurally diverse non-anionic moieties is anticipated to ultimately yield short, low-charge heparinoids that are more drug-like than current polyanions. Perhaps of greater significance, identifying novel reduced-charge heparin sequences that bind specific proteins will pave the way for new generations of heparinoids derived from the de novo synthesis of short oligosaccharides based on these lead heparin-derived sequences. Advances in the synthesis of heparin oligosaccharides have already resulted in synthetic oligosaccharide therapeutics based on lead sequences from unmodified heparin. For example, synthetic versions of the native and O-methylated antithrombin-binding pentasaccharide are now in the clinic as anticoagulants (fondaparinux and idraparinux).[13] The synthesis of heparinoid-based uronic acid conjugates bearing structurally diverse groups has recently been reported.[14] Indeed, the synthesis of short heparinoid-based oligosaccharides functionalized with non-anionic moieties holds great promise as selective antagonists of HS–protein interactions. The design and synthesis of such agents can be envisioned from de novo strategies based on the design and synthesis of target oligosaccharide structures, or from the synthesis of target oligosaccharide structures guided by lead structures derived from the diversity-oriented chemical modification of heparin.

Many early efforts to introduce non-anionic moieties into heparin structure were performed with the intent of not altering the agonistic actions of heparin as an anticoagulant.[§] To this end, partial substitution of anionic groups with non-anionic groups toward increasing the hydrophobicity of heparin or otherwise altering heparin properties without effecting anticoagulant activity was the goal. More recently, we and others have more aggressively altered heparin structure with destruction of anticoagulant activity being one goal, and selective binding to target protein being the other goal. A number of chemical modifications to heparin structure have also been undertaken toward the preparation of chemical probes for pharmacological, metabolic or protein-interaction studies. In Sections 2–6 below we attempt to provide a comprehensive review of the chemical modifications of heparin that have been reported where the introduction of structural moieties into heparin go beyond altering chain-length, chain integrity, or anionic group substitution patterns.

1.3. Solubility of heparin salt forms[¶]

The polyanionic nature of heparin limits solvent and reagent options for derivatization/modification chemistry.

[§] A number of the early methods employed to modify heparin have been discussed in some detail (see Ref. 81) and presented in a review on polysaccharide modifications (see Ref. 176).

[¶] The reports for chemical modification of heparin discussed in Sections 2–6 provide numerous examples of manipulating heparin salt forms to achieve desired solubility for chemical reactions on heparin. Additional references that pertain specifically to alkali metal salts and ionic liquids are provided.[177,178]

Alkali metal salts of heparin are soluble only in water and aqueous buffer, water/buffer combined with water miscible organic co-solvent, formamide, and room temperature ionic liquids. Many of the chemical modifications to heparin outlined in the sections below employ the sodium salt form of heparin dissolved in water-based solvent systems. Salt forms of heparin that improve solubility in polar organic solvents are known. Examples of such salt forms found throughout the sections below include the pyridinium salt form of heparin (soluble in pyridine, DMSO, DMF, and other polar organic solvents) and *n*-alkylammonium salt forms of heparin (soluble in many protic and aprotic polar organic solvents including methylene chloride).

2. Chemical modification of glucosamine C-2-amino groups in heparin

2.1. Amine substitution after N-desulfonation

Substitution of the glucosamine C-2 amino groups in heparin has been primarily employed to prepare *N*-acyl heparin derivatives (see Fig. 2). Early accounts describing the N-desulfonation/N-acylation of heparin seemed to primarily modulate anticoagulant activity. In the late 1950s, Velluz and co-workers reported the preparation and anticoagulant evaluation of N-desulfonated/*N*-acyl heparin derivatives substituted with aliphatic and aryl moieties.[15] The aliphatic derivatives consisted of acetyl, methylsulfonyl, ethylacetyl, and 2-phenylbutanyl *N*-acyl groups. The aromatic derivatives were benzoyl, 4-methylbenzoyl, 3,5-dimethylbenzoyl, 4-nitrobenzoyl, 3,5-dinitrobenzoyl, 2,4,6-trinitrobenzoyl, 2-bromobenzoyl, 3,4,5-trimethoxybenzoyl, 2-ethoxybenzoyl, 4-hydroxybenzoyl, 2-carboxybenzoyl, 4-methylbenzoyl, and 2-naphthoyl.[15,16] In the early 1960s, Foster and co-workers published a series of N-desulfonated/N-acylated heparin derivatives, which were evaluated for antimicrobial activity.[17] In this work, as well as in an earlier work by Velluz and co-workers, N-desulfonation with concomitant loss of some *O*-sulfo groups was achieved using acid hydrolysis. The amino groups of N-desulfonated heparin were reacted with acid chlorides in water, acetone, or aqueous ethanol to afford *N*-acyl derivatives including benzoyl, benzyloxycarbonyl, *m*-trifluoromethylbenzoyl, nicotinoyl, isonicotinoyl, toluene-*p*-sulfonyl, *p*-acetamidobenzenesulfonyl, 2,4-dinitrophenyl, and di-*O*-phenyl-phosphoryl.[17] These early methods for N-desulfonation/N-acylation of heparin toward improving anticoagulant and antilipemic activities are also documented in the patent literature. For example, nearly 20 N-desulfonated/N-acylated heparins were prepared using acid chlorides in aqueous sodium bicarbonate.[18] Similar methods were reported in the preparation of *N*-succinyl-, *N*-(2-sulfobenzoyl)-, *N*-(2,4-disulfobenzoyl)-, *N*-(3,5-disulfobenzoyl)-, and *N*-(3-sulfobenzoyl)-heparin derivatives.[19,20]

In the 1970s, Hirano and Ohashi reported the synthesis of N-desulfonated/*N*-acyl heparin derivatives using hydrochloric acid-mediated N-desulfonation followed by coupling of the amines with acid anhydrides in formamide solvent.[21,22] Coupling was reported without additives or with variations of the procedure using base/catalyst to provide *N*-acylated heparins that included *N*-acetyl, *N*-propionyl, *N*-butanoyl, *N*-hexanoyl, *N*-octanoyl, *N*-decanoyl, *N*-lauroyl, *N*-myristoyl, *N*-palmitoyl, *N*-stearoyl, *N*-(2-carboxybenzoyl), *N*-benzoyl, and *N*-nicotinoyl.[21,22]

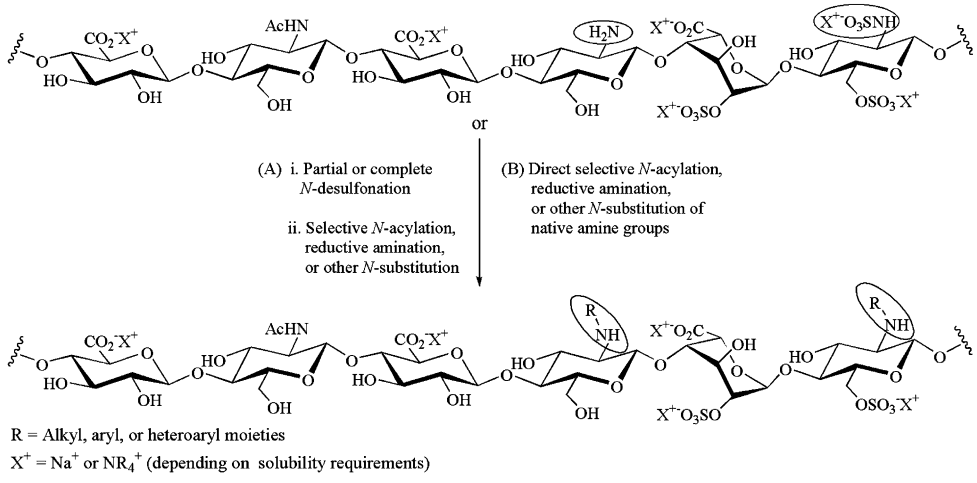

Figure 2. Chemical substitution of the glucosamine C-2 amino groups in heparin can be achieved through substitution of the limited number of native amino groups present in heparin or through amino groups introduced to varying degrees via N-desulfonation procedures. Substitution of amino groups in heparin has predominately been achieved through selective N-acylation employing activated carboxylic acids such as anhydrides and NHS esters. Coupling of amino groups in heparin with isocyanates and other reagents capable of selective substitution of amines over hydroxyl groups is also common.

The preparation and evaluation of *N*-acyl heparin derivatives has more recently been directed toward exploiting heparin's non-anticoagulant activities, which parallels the increasing number of putative therapeutic applications for bind-and-block antagonists of HS-binding proteins. In addition, these later procedures typically employ newer methods for the selective, solvolytic N-desulfonation of pyridinium heparin rather than acid-mediated desulfonation. In 1991 Moczar and Hornebeck described the partial N-desulfonation of heparin followed by N-acylation with oleoyl chloride and triethylamine in DMSO.[23] Similarly, heparin with different degrees of *N*-oleoyl and *N*-palmitoyl groups were prepared.[24,25] These partially N-desulfonated/N-acylated heparins have been evaluated for modulation of plasmin, urokinase, elastase, and cathepsin G activity and for inhibition of HIV infection.[23–26] The anti-HIV and anti-metastasis activities of N-succinylated heparin and LMWH have been evaluated.[27,28] The inhibition of tumor metastasis by heparanase inhibiting *N*-hexanoyl heparins has been studied.[29] Preparation and enzymatic degradation of *N*-propionyl heparin derivatives was reported by Moffat and co-workers.[30] The NHS esters of stearic acid and cholic acid were reacted with partially N-desulfonated heparin to afford *N*-stearoyl and *N*-cholate heparins that were evaluated for anticoagulant activity and physical properties.[31] In 2006, we reported the parallel coupling of 19 different NHS activated carboxylic acids with each of the seven different heparin fractions having degrees of N-desulfonation ranging from 10% to 100%.[11] The resulting library of *N*-acyl heparin derivatives was evaluated for binding to growth factors and thrombin. This work demonstrated that select *N*-acyl groups substituted into heparin in place of *N*-sulfo groups known to be required for high affinity binding to certain proteins afforded heparin derivatives that maintained affinity for protein while other *N*-acyl substitutions did not. Members of this library are under further investigation for selective binding to a number of protein targets and for anticoagulant as well as other biological activities.

A number of reports describe approaches for the N-substitution of glucosamine residues in heparin other than N-acylation. The N-arylation of heparin was reported in the literature almost 40 years ago; N-desulfonated heparin was reacted with 1-fluoro-2,4-dinitrobenzene to yield *N*-(2,4-dinitrophenyl)-heparin. These derivatives were synthesized for structural studies and evaluated for anticoagulant activity.[32,33] Two *N*-carboxymethyl heparin derivatives were prepared by alkylation of N-desulfonated heparin with bromoacetic acid in aqueous sodium bicarbonate, and evaluated for interactions with paramagnetic cations.[34] A 1994 report describes the synthesis of glutaraldehyde adducts of heparin.[35] Heparin was partially N-desulfonated using mild acid hydrolysis followed by treatment with glutaraldehyde, in phosphate buffer to form the imine. The resulting high MW adducts and hydrogels were evaluated for anticoagulant properties. Increasing the hydrophobicity of partially N-desulfonated heparin via an imine adduct was similarly achieved using dodecanal.[31]

2.2. Direct N-acylation of heparin via native amino groups

A number of synthetic procedures for modifying heparin describe N-acylation of heparin without preliminary N-desulfonation (Fig. 2, Path B). Two scenarios account for the presence of amino groups in heparin or LMWH available for N-acylation when no preliminary modification or otherwise purposeful N-desulfonation of the glucosamine residues is performed. First, heparin and LMWH inherently contain low levels of free amine (unsubstituted amine of glucosamine), and the reducing terminus of a portion of the chains, depending on the heparin employed, may be linked to residual serine residues remaining from the proteoglycan form. Second, random N-desulfonation/N-acylation of heparin likely occurs during certain uncontrolled acylation reactions. For example, highly reactive acylating agents such as acyl chlorides are known to afford increased levels of N-acylation, as well as O-acylation, under conditions where the corresponding anhydrides and many NHS esters afford selective N-acylation of pre-existing amino groups without additional N-desulfonation/N-acylation.[11,36,37]

In 2002, Zamora and co-workers reported N-linked benzyl-bis(dimethylsilylmethyl)oxycarbamoyl-heparin derivatives, which were prepared by coupling the corresponding NHS ester to heparin over varied reaction time. These amphipathic heparins have been employed for surface coating applications relating to peptide/protein adsorption and delivery.[38,39] Lee and co-workers reported the synthesis and evaluation of a series of hydrophobic heparin derivatives, which were prepared by coupling heparin and/or LMWHs with the NHS esters of deoxycholic acid, cholesterol (carboxymethyl substituted), palmitic acid, and lauric acid.[40–42] NHS and sulfo-NHS esters of biotin derivatives have been used to achieve direct coupling of biotin to amino groups in heparin.[43–59] or amine rich heparin obtained after reacting heparin with 3-bromopropylamine hydrobromide.[44]

Modifications to amino groups in heparin, native or introduced via N-desulfonation have been employed to prepare a variety of *N*-acyl heparin derivatives as chemical probes. To this end, heparin and/or partially N-desulfonated heparin and LMWHs have been reacted with a variety of reactive N-acylating species for the introduction of fluorescent tags including fluorescein,[60–64] coumarin,[65] dansyl,[66–69] and Rhodamine B.[69] Partially N-desulfonated heparin has been coupled with sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD) and the NHS ester of 4-azidosalicylic acid to allow radioiodination and subsequent

photo-conjugation of heparin to complement factor H.[70] Heparin has been similarly coupled directly with activated carboxylic acids on the surface of solid supports.[71] Additional types of unique *N*-acyl probes and radioiodinated ligands are discussed in Section 6. An increasing number of these labeled heparin derivatives are becoming commercially available.

3. Derivatization of the carboxylate groups in heparin

3.1. Synthesis of heparin esters via carboxylate substitution

Ester derivatives of heparin are most commonly used as the starting point for chemical beta-eliminative depolymerization, and reports in this area are found throughout the patent literature. For example, reaction of the benzethonium salt form of heparin with benzyl chloride affords the benzyl ester of heparin, which is treated with base to afford beta-eliminative cleavage followed by ester hydrolysis to afford the LMWH.[72–74] Methyl esters have been similarly employed, where the initial ester is formed by reacting a tetrabutylammonium salt of heparin with diazomethane.[75] Esterification of heparin serves to lower the pK_a of the hydrogen atom at the C-5 position of uronic acid residues, thus facilitating proton abstraction and subsequent beta-elimination under basic conditions.[74] Reports describing the selective formation of heparin esters without otherwise altering heparin structure in the published literature other than en route to LMWHs are more limited. Treatment of the free acid form of heparin with ethereal diazomethane affords heparin methyl ester (Fig. 3).[76] Esterification of a heparin-derived disaccharide was reported during attempts to benzyl protect unsubstituted hydroxyl groups, where the tetramethylammonium form of the saccharide was reacted with NaH and benzyl bromide in DMF.[37] Benzyl and butyl esters of LMWH were also prepared by alkylation of the tetrabutylammonium salt form of heparin with alkyl halide in DMF.[77] This method was subsequently employed in the synthesis of a series of LMWH esters for evaluation as anti-HIV agents.[78]

3.2. Synthesis of heparin amides via carboxylate substitution

The earliest preparation and characterization of heparin amides appears in the patent literature, where the formation of alkylamides is exemplified by aminolysis of heparin methyl ester with *N*-methylamine (Fig. 4, Path A).[75] In the 1970s, Danishefsky and co-workers reported the formation of heparin amides via activation of the carboxylate groups in heparin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) followed by coupling to amino groups in glycine methyl ester, L-phenylalanine methyl ester, or glycyl-L-phenylalanine amide (Fig. 4, Path B).[79] Heparin amides from aminomethanesulfonic acid, taurine, and hydroxylamine were similarly prepared by Danishefsky and co-workers.[76,80] A number of these heparin amides were evaluated for anticoagulant properties as well as antimetastatic activity.[76,80–82] It is notable that this carbodiimide-mediated coupling of heparin carboxyl groups with amines is the chemistry of choice today for linking small molecules, chemical probes or macromolecules to the carboxyl groups in heparin via the amide bonds.[‖]

In 1981, Lasker and co-workers coupled heparin to 4-amino-TEMPO, creating a spin-labeled amide of heparin that was used to study the heparin–antithrombin complex.[84] EDC-mediated coupling of heparin with biotin hydrazide has been employed to prepare carboxy-biotinylated heparins.[59,85,86] Similarly, a variety of fluorescent amines and hydrazides have been linked to heparin through the carboxyl groups including Alexa Fluor 488 hydrazide[59,87] and 5-fluoresceinamine.[88–90] A number of reports describe EDC-mediated coupling of heparin carboxylate groups either directly to proteins (zero-length cross-linking) or indirectly via diamine linkers, as well as compare carboxyl-mediated cross-linking to other cross-linking methods.[91–93] An example of carboxyl-mediated coupling of heparin with solid supports compares this method to other immobilization techniques.[71]

A 2004 report describes the preparation of a heparin amide with deoxycholic acid amine (DOCA-NH_2) in the preparation of amphiphilic conjugates/nanoparticles.[94] A 2005 report describes the preparation of hydrophobic heparin–deoxycholic acid conjugates where LMWH is coupled with differing ratios of deoxycholylethylamine to achieve the corresponding heparin amides having varied degrees of carboxyl groups substituted.[95] More recently, we have been employing EDC-mediated coupling of heparin to amines to prepare structurally diverse heparin amides and LMWH amides toward identifying antagonists of HS–protein interactions.[12]

4. Derivatization of heparin through O-acylation and O-alkylation

4.1. Acylation of unsubstituted hydroxyl groups in heparin

Sulfate substitution within heparin is variable, and thus heparin contains varied levels of unsubstituted hydroxyl groups at positions 2 and/or 3 of uronic acid residues

[‖] Although not a modification where unique structures are introduced into heparin, the activation of heparin carboxylates with EDC to form the *O*-acylisoureas followed by reduction with borohydride species is commonly employed to prepare carboxyl-reduced heparin, see Ref. 85 and citations within for original procedures.[83]

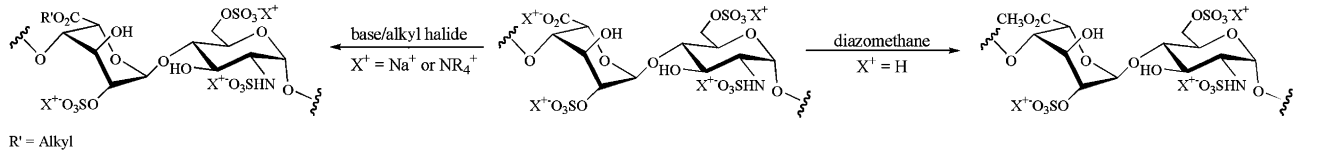

Figure 3. General procedures for preparing esters of the carboxylate groups in heparin.

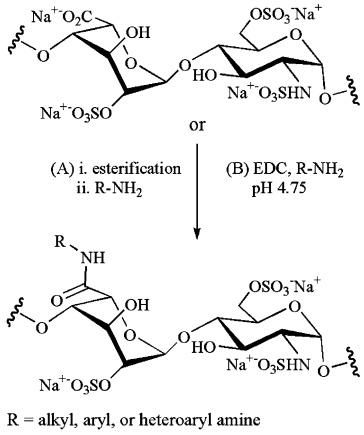

Figure 4. Carboxamide derivatives of heparin. Heparin amides are most often prepared using EDC-mediated coupling of heparin with amines (B). Esterification followed by aminolysis of the ester has also been reported (A).

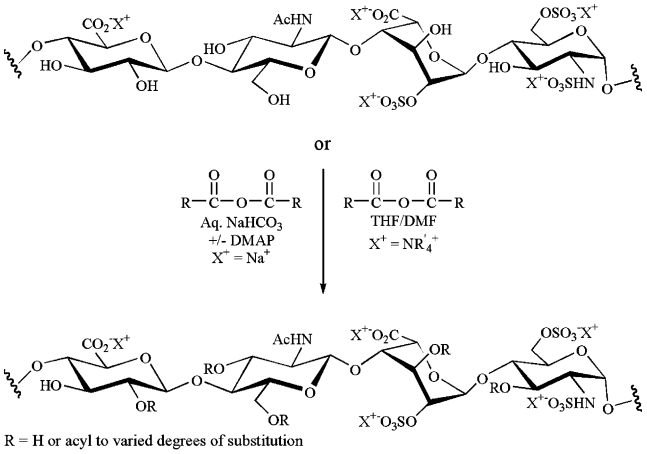

Figure 5. The most successful methods for selective O-acylation of heparin have relied on the coupling of acid anhydrides with heparin in aqueous or organic solvent. Such coupling reactions in organic solvent typically require heparin to first be converted to a quaternary ammonium salt form to achieve sufficient solubility.

and positions 3 and/or 6 of glucosamine units. Early efforts to selectively *O*-acylate heparin employed a variety of methods.[96–99] None of these methods afforded high yield selective O-acylation, providing low levels of substitution, concomitant N-acylation to varied degrees, and/or were subsequently shown to yield derivatives other than the putative *O*-acyl products.[36] In 1992 Petitou and co-workers undertook the selective O-acylation of heparin to ascertain the effects of heparin O-acylation on anticoagulant activity, antithrombotic properties, pharmacokinetics, and other pharmacological activities.[36] To this end, the selective O-acylation of unfractionated heparin and LMWH was achieved by conversion of heparin or LMWH to a tributyl- or tetrabutylammonium salt to improve solubility in organic solvents. Subsequent treatment with carboxylic acid anhydride in the presence of DMAP afforded selective O-acylated heparins where reaction time, temperature and stoichiometry of the acid anhydride modulated the degree of O-acylation (Fig. 5). Mixed N,O-acylated products were obtained employing acid chlorides. Some formation of mixed anhydrides between the carboxylate group of the polysaccharide and an acyl group of the acylating agent was observed. In these cases, treatment with aqueous sodium bicarbonate reversed the side reaction.[36] This method was subsequently used to prepare various *O*-butanoyl, *O*-acetyl, *O*-hexanoyl, *O*-benzoyl, *O*-octanoyl, *O*-succinyl, and *O*-decanoyl heparin and/or LMWH derivatives and these various derivatives have been evaluated for a number of pharmacological activities including anticoagulant, antithrombotic, antiproliferative, anti-HIV, and inhibition of proteases.[77,78,100–103]

In 1994 Malsch et al. reported the preparation of *O*-acetyl-, *O*-benzoyl-, *O*-butanoyl-, *O*-caproyl-, and *O*-octadecyl heparin and LMWH derivatives by coupling the tetrabutylammonium salt form of the heparin with acid anhydrides in THF/DMF solvent.[104] In 1995 Kerns et al. described the introduction of hydroxyl protecting groups on heparin-derived disaccharides, including selective pivaloylation of the hydroxyl group at C-3 of the uronic acid residue.[37] The preparation of methacrylate esters is described in a patent by Nakaya.[105] Heparin O-acylation is recurrently encountered in patent literature where the precise degree of O-acylation, position(s) of O-acylation, and effect on net sulfate content are varied and often not discernable. A general trend observed in these investigations is that using acid chlorides is likely to afford concomitant N-acylation, presumably because of N-desulfonation followed by N-substitution, while reaction with acid anhydrides can be controlled to afford variable degrees of selectively O-acylated products.

4.2. Alkylation of unsubstituted hydroxyl groups in heparin

Reports of selective O-alkylation of heparin in the literature appear to be limited to selective O-methylation

reactions, with a primary aim of structural analysis. A 1969 report describes the results for a number of methods to achieve selective O-methylation of N-desulfonated/N-acetylated heparin.[106] Quantitative methylation was reportedly achieved by reaction of the pyridinium salt of carboxyl-reduced heparin with dimethylsulfinyl anion in DMSO.[107] Similarly, Kovensky et al. reported a method for the methylation of sulfated polysaccharides, including heparin, for structural analysis. In general, pyridinium salts of sulfated polysaccharides can be methylated by sonicating in DMSO, followed by addition of butyllithium and methyl iodide.[108]

5. Heparin derivatives obtained through chemical modification at the reducing end

Reductive amination and similar amine-carbonyl coupling chemistry is the most common method for derivatizing the reducing end of heparin (Fig. 6). The reductive amination procedure for heparin typically requires extended reaction time with excess amine to form the initial imine, which is reduced in situ with sodium cyanoborohydride ($NaCNBH_3$) or sodium triacetoxyborohydride ($NaBH(OAc)_3$) (see Fig. 6, Path A). Coupling with a hydrazide instead of amine affords a more stable initial adduct than the imine, a hydrazone, which undergoes rearrangement and thus reduction is not required. This approach for heparin modification has been used mainly to attach chemical probes (chromophores, etc.) onto heparin for detection purposes. The use of NaB^3H_4 to reduce and thus radiolabel the reducing end aldehyde of nitrous acid fragmented heparin, although not derivatization per se, is a useful strategy to prepare radiolabeled heparins (see Fig. 6, insert for reducing-end structure of these fragments).[74,109] An increasing number of heparin derivatives bearing reporter groups at the reducing end are commercially available. Most of the methods reported to prepare these derivatives are also applicable to the preparation of semi-synthetic heparin oligosaccharides bearing structurally diverse moieties at the reducing end, and as outlined below this approach for modifying heparin has been employed to introduce a variety of structures onto the reducing end of heparin and LMWHs.

Hydrophobic heparin derivatives prepared by coupling aliphatic chains to the reducing end of heparin were reported by Liu et al.[110] In this work, a molar excess of acyl hydrazides were coupled to the reducing end of heparin in formamide (solvent for octyl (C_8), lauryl (C_{10}), and capryl (C_{12}) hydrazides coupling with sodium heparin) or dichloromethane (solvent for stearyl (C_{18}) hydrazide coupling with tetrabutylammonium heparin) to form hydrazones, which are stabilized by tautomerization (see Fig. 6, Path B). Hydrogenation of the azo bond in the laurate derivative was performed to couple a second hydrophobic group via N-acylation (Fig. 6, Path A).[110] Employing an alternative strategy reducing end alkyl heparins, Matsuda et al. converted the reducing end lactol of heparin to the lactone,

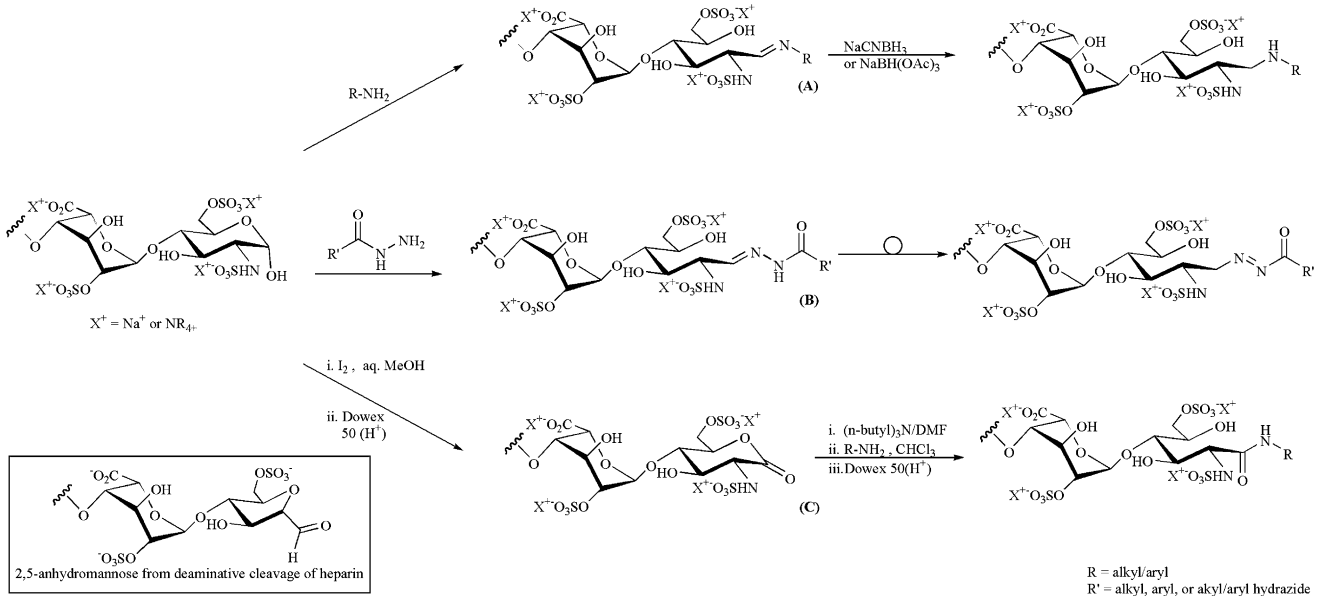

Figure 6. Predominant approaches employed for introducing structures at the reducing-end of heparin. (Path A, upper) Imine formation followed by reduction to form the stable amine-linked conjugate. (Path B, Middle) Coupling of reducing end lactol with acyl hydrazides affords stable conjugates upon tautomerization. (Path C, lower) Lactonization of reducing-end residues followed by aminolysis affords the amide linked conjugates. The inset structure, lower left, depicts the reducing terminus of heparin oligosaccharides after nitrous acid-mediated chain fragmentation of heparin. The resulting aldehydes undergo Path A and B transformations analogous to the ring-open form of reducing-end lactol.

followed by ring opening with alkylamines to introduce butyl, octyl, lauryl and stearyl groups (Fig. 6, Path C).[111] Similarly, L-α-dipalmitoylphosphatidylethanolamine has been coupled to the reducing end of polysaccharides including heparin.[112]

Heparin disaccharides have been reductively aminated to introduce dihexadecyl phosphatidylethanolamine, an aminolipid.[113] Rong and co-workers developed a general procedure to link together heparin or HS oligosaccharides to form neo-glycosaminoglycan conjugates. In this work, heparin oligosaccharides having a reducing terminal 2,5-anhydromannose residue were substituted by reductive amination with cystamine in the presence of NaB^3H_3CN to afford concomitant introduction of ^3H. After further manipulation, the resulting reducing end thiol was conjugated with an iodoacetylated fragment of heparin to obtain neo-glycosaminoglycans.[114] Reducing end chemistry has also been employed to introduce fluorescent probes into heparin. Examples include 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS),[115,116] 2-aminoacridone (AMAC),[117] 2-aminopyridine,[115] 2-aminobenzamide and 2-anthranilic acid,[118] 2-aminobenzoic acid,[119] 3-aminotyramine,[120] 7-amino-1,3-naphthalenedisulfonate,[121] and 4-amino-1-naphthalene sulfonic acid.[122] An informative summary of fluorophores employed in the reductive amination of reducing sugars has recently been reported.[123]

The coupling of tyramine to the reducing end of heparin has received much attention. LMWH–tyramine has been prepared by reductive amination of LMWH with tyramine in the presence of $NaCNBH_3$ in water at pH 7.[104,124] The introduction of tyramine has been followed by fluorescent labeling with FITC,[124–126] or radioiodination of the tyramine residue.[124,127–132] Fluorescent labeling by tyramination has been performed on solid support.[120] A FITC–tyramine–LMWH conjugate has also been coupled to protamine microbeads.[133] A number of different biotin derivatives have been linked to the reducing end of heparin for a variety of purposes.[50,115,134,49,59] For example, coupling of LMWH with biotinamidocaproylhydrazide (BACH) has been used to immobilize heparin on sensor surfaces for surface plasmon resonance.[135] Reductive amination of heparin with various structures, such as bis-amine moieties, has also been used to facilitate the immobilization of heparin in a defined orientation,[136] or to directly immobilize heparin and LMWH to amine-functionalized surfaces.[71,137–140]

6. Synthesis of uniquely labeled heparin probes or probes via unique chemistry

Many of the methods for the semi-synthetic modification of heparin discussed in Sections 2–5 above have been employed to introduce fluorophores or other common labeling-type moieties into heparin and/or LMWH. Some of these methods have been used to prepare unique heparin derivatives labeled with specific reporter groups for specific application, while a number of other chemical methods employed to tag heparin with labeling groups are somewhat unique. Cyanogen bromide (CNBr) activation of heparin followed by aminoalkylation with diaminohexane and coupling with biotin-Σ-aminocaproic acid NHS ester (Biotin-LC-NHS) has been used to biotinylate heparin.[141–144] Similarly, CNBr-activated heparin has been reacted with fluoresceinamine,[145–147] and to link heparin to dextran, ficoll, and albumin.[148] Oxidation of vicinal diols in heparin affords carbonyl groups, which have been coupled with Biotin hydrazides.[58,149–151] More recently this approach has been employed to prepare non-anticoagulant glycol-split heparins.[152,153,5f,154] Heparin has also been photo-labeled with photo-activated biotin derivatives for immobilization procedures,[155,156] and a photo-activatable hetero-bifunctional cross-linking agent for covalent conjugation with proteins.[157]

A number of unique heparin derivatives have been prepared for imaging applications and metabolic/pharmacodynamic studies. Metal chelating agents have been covalently linked to heparin. The dianhydride of diethylenetriaminepentaacetic acid (DTPA anhydride) was coupled to heparin to afford heparin–DTPA, which has been complexed with Indium-111.[158] Heparin-coupled and LMWH-coupled chelating agents complexed with a variety of paramagnetic metal cations and radionuclides are also described in the patent literature. Heparin has been directly labeled with ^{51}Cr by reaction with $^{51}CrCl_3$ for metabolism studies.[159,160] Technetium-99m has also been complexed with heparin and LMWH in the presence of $SnCl_2$.[161–163] As a general rule, ion pair-based complexes of heparin would not necessarily be considered derivatives. One interesting case however; heparin-stabilized silver nanoparticles of varied sizes have been prepared from heparin and $AgNO_3$.[164] Radioiodination of heparin has been achieved through coupling of heparin to structures that are susceptible to iodination. Acylation of amino groups present in unmodified heparin has been reported using the NHS ester of 3-(4-hydroxyphenyl)propionic acid followed by labeling with ^{125}I or ^{131}I.[165–171] Introduction of tyramine at the reducing end of heparin and LMWH has similarly been followed by radioiodination.[124,128,129,132] A number of fluorescein-labeled heparins and LMWHs have also been radioiodinated.[44,172–175]

7. Conclusions

In conclusion, heparin is a complex structure endowed with a plethora of biological activities as a consequence of bind-and-block antagonism of HS–protein interactions.

While a goal of developing novel therapeutics based on known heparin–protein interactions includes reducing charge and simplifying structure of potential drug candidates, the most rapid route to achieving both of these outcomes may actually require increasing the structural diversity of heparinoids to elucidate lead structures that will ultimately allow this goal to be met. As outlined above, decades of work by many research groups has yielded a surprisingly diverse array of chemical methods to selectively modify functional groups in heparin to generate semi-synthetic heparinoids. Employing these methods to more aggressively modify heparin and heparin-based structures with structurally diverse non-anionic moieties holds great potential for ultimately identifying novel, selective antagonists of specific HS-mediated biological processes.

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