INVITED REVIEW

Development of glycosaminoglycan mimetics using glycopolymers

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Glycosaminoglycans (GAGs) are polysaccharides found in living systems that have key biological roles and function as polyelectrolytes owing to their large number of sulfate groups. There have been many reports describing the syntheses of GAGs and the development of GAG mimetics and analogs. The preparation of such GAG mimics has utilized versatile methods ranging from total syntheses to synthetic polymer chemistry approaches. The core of GAG mimetic production is the fusion of complex chemical structures with polymeric properties. Multivalent interactions of the saccharides with specific biological targets, such as proteins, are an essential function of GAGs and other multivalent saccharides. In this review, methods for generating GAGs from glycopolymers are presented and research reports describing the functional characterization of the synthesized GAGs are outlined.

INTRODUCTION

Saccharides on cell surfaces have important roles in living systems[1] and exist as glycolipids, glycoproteins and polysaccharides. Glycans have various functions that include serving as an energy source, increasing the water solubility of proteins and chemicals, forming components of cell walls and acting as biological ligands for molecular recognition. In particular, their role as biological ligands has received significant attention in the fields of biochemistry and biomaterials. Saccharides on the cell surface mediate biological signals through molecular recognition. Among the numerous types of saccharides, glycosaminoglycans (GAGs) and proteoglycans are important biological ligands, because they mediate an abundance of biological phenomena and control living systems. GAGs and proteoglycans exist universally on the cell surface. Proteoglycans are glycoproteins that have GAGs and core proteins, making their functions dependent on GAGs (Figure 1).

GAGs have an alternating copolymer structure composed of amino-sugars (*N*-acetyl glucosamine (GlcNAc) and *N*-acetyl galactosamine (GalNAc)) and uronic acids (iduronic acid (IdoA) and glucuronic acid (GlcA)).[2] Most GAGs are highly sulfated, with the exception of hyaluronic acid, and their sulfation patterns and saccharide combinations are used to distinguish the different types of GAGs. GAGs are natural polymers and polyelectrolytes with molecular weights reaching 50 kDa, in the case of heparan sulfate. GAGs are divided into different types of polysaccharides based on their unique saccharide structures, sulfation patterns and molecular weights (Figure 2). For example, hyaluronic acid is an unsulfated alternating copolymer of GlcNAc and GlcA. Heparin and heparan sulfate are highly sulfated alternating copolymers of GlcNAc and GlcA or IdoA, respectively. Similarly, chondroitin sulfate is a sulfated alternating copolymer of GalNAc and GlcA or IdoA. The complex sulfation patterns of the saccharides, saccharide structures, chiral (epimeric) centers and stereo-selective glycoside bonds result in rich structural diversity.

Because GAGs cover the cell surface as an extracellular matrix, they have numerous roles in living systems, including cell growth, cell differentiation and defense against pathogen infection. Some GAGs have been used in practical settings. The antithrombogenic activity of heparin has received significant attention for use as a therapeutic and biomaterial.[3] Heparin, which is used clinically, binds antithrombin III (ATIII) to control the activity of thrombin and inhibits thrombus formation. The application of heparin-immobilized materials for antithrombogenic applications has been previously investigated, for example, for use in artificial vessels.[4,5] The cell growth factor of b-fibroblast growth factor (FGF) has been reported to bind heparin; such heparin binding facilitates dimerization, activation and cell proliferation.[6] Other growth factors, such as vascular endothelial growth factor and epidermal growth factor, have been reported to bind GAGs for activation and stabilization of these proteins.[7,8] GAGs also protect cells from pathogen infection by bacteria, viruses and other toxic proteins. Although GAGs protect cells from infectious pathogens,[2] some viruses specifically target GAGs for cell invasion.[9–11] By contrast, the infection of some viruses is inhibited by the presence of GAGs.[12,13] GAGs also regulate inflammation by interacting with selectins.[14–16]

Studies of GAGs hold a prominent position in the fields of glycoscience, biochemistry and biomaterials. GAGs are challenging to study because of difficulties associated with their availability, whereby their synthesis or production is a limiting factor. Research

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Correspondence: Professor Y Miura, Department of Chemical Engineering, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan.
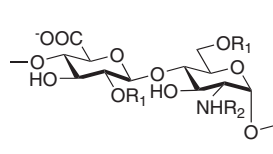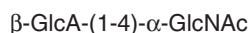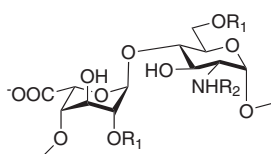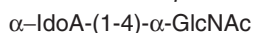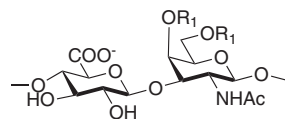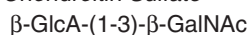E-mail: miuray@chem-eng.kyushu-u.ac.jp

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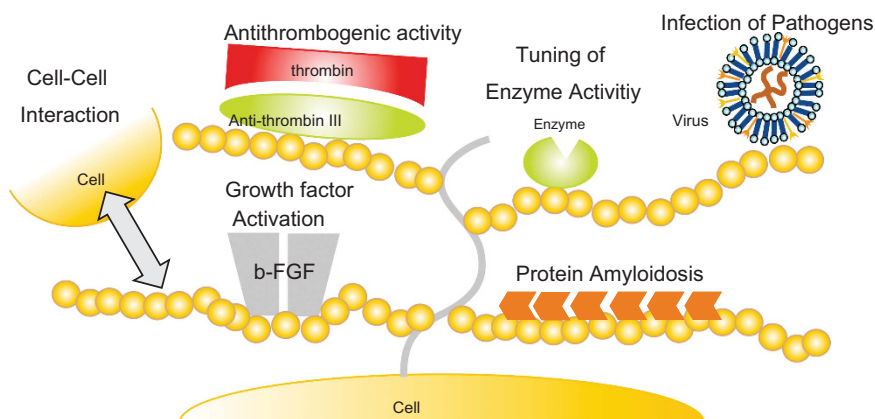
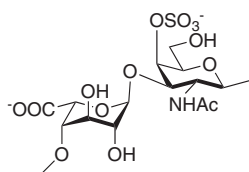**Figure 1** Schematic illustration of the functions of GAGs on the cell surface.
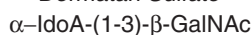
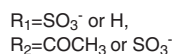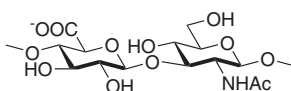## Disaccharide Structure of Glycosaminoglycans

Heparin and Heparan Sulfate

α–IdoA-(1-4)-α-GlcNAc   β-GlcA-(1-4)-α-GlcNAc

Chondroitin Sulfate
β-GlcA-(1-3)-β-GalNAc

Dermatan Sulfate
α–IdoA-(1-3)-β-GalNAc

Hyaluronic Acid
β-GlcA-(1-3)-β-GlcNAc

$R_1 = SO_3^-$ or H,
$R_2 = COCH_3$ or SO_3^-
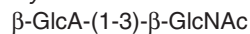
Figure 2 Chemical structure of GAG components.
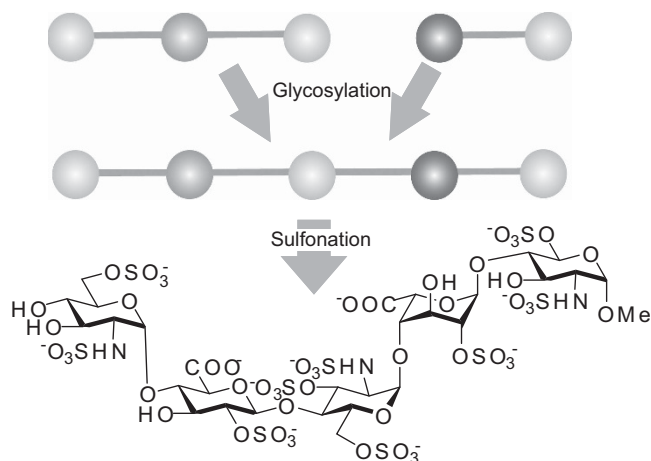
Figure 3 Total syntheses of GAG pentasaccharides. The syntheses were attained by conjugating functional disaccharides and trisaccharides and were designed retro-synthetically. A full color version of this figure is available at the *Polymer Journal* online.

has focused on preparing GAGs using chemical and biological syntheses, with a particular emphasis on oligosaccharide production.[17] However, GAGs are natural 'polymers' and function as polyelectrolytes; thus the production of new GAG mimetics using polymer chemistry has considerable potential. In this review, we describe the preparation of glycopolymers that act as GAG mimetics.

## SYNTHESIS OF GAGS USING SYNTHETIC AND BIOLOGICAL METHODS

GAGs have been prepared using both chemical and biological methods. Because of their complex saccharide structures, they have been a target of total synthesis within the field of organic chemistry. The total synthesis of GAGs enables the detailed investigation of the various functions of GAGs; however, complicated glycosylation and regioselective sulfonation procedures are required (Figure 3). The syntheses are frequently designed by retro-syntheses. An efficient synthetic method that includes the use of protective groups and glycosylation is necessary to successfully synthesize GAG oligosaccharides. There have been many reports describing the synthesis of oligosaccharides of GAGs,[17,18] with numerous research groups reporting novel methods for synthesizing GAGs, including efforts to prepare longer saccharides using facile synthetic approaches.

The synthesis and biological activity of heparin and heparan sulfate oligosaccharides have been previously studied. The biological functions of these saccharides have been investigated using various oligosaccharides. IdoA(2S)(α1-4)GlcNS(6S) is a major structure[17] that has been reported to bind to ATIII,[18] blood platelets,[19,20] basic FGF (b-FGF)[21] and the Alzheimer amyloid precursor protein.[22] The Petitou group reported the synthesis of the disaccharide IdoA(2S) (α1-4)GlcNS(6S),[23] whereas the Russo group reported the synthesis of a different GAG disaccharide.[24]

The Kusumoto group reported the syntheses of heparin and heparan sulfate oligosaccharides.[20,25,26] This group reported the synthesis of IdoA(2S)(α1-4)GlcNS(6S) and found that the disaccharide moiety inhibited heparin from binding to blood platelets.[20] They also reported the synthesis of GlcNS(6S)(α1-4)GlcA(β1-4)GlcNS(3S, 6S) (α1-4)IdoA(2S)(α1-4)GlcNS(6S), which efficiently inhibited heparin binding to platelets. The Suda group reported the syntheses of a glycoconjugate and multivalent compounds using a heparin oligosaccharide, which is described in the next section.[27] The Sinaÿ group reported the binding of an oligosaccharide to ATIII.[28] By modifying a heparin pentasaccharide, the authors found that this compound had a reduced affinity toward ATIII compared with heparin. This group also
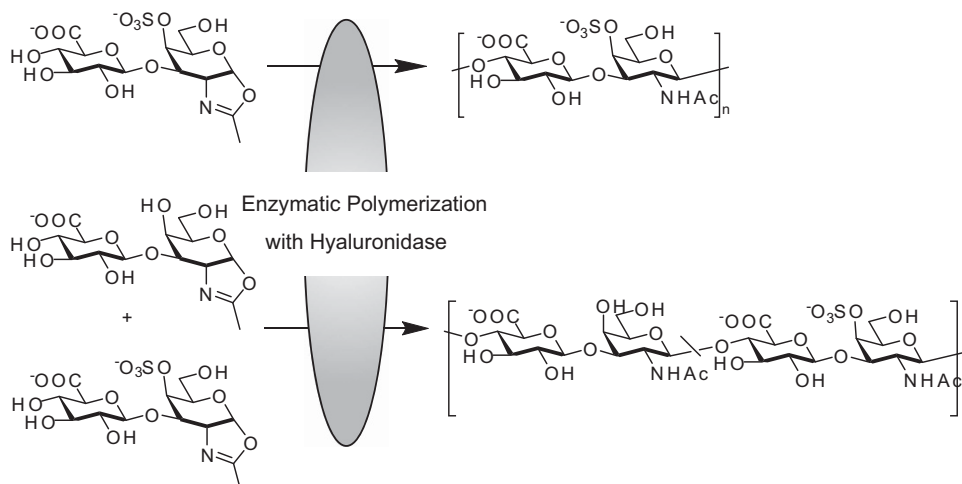
Figure 4 Preparation of chondroitin sulfate using chemoenzymatic synthesis based on the enzymatic polymerization of hyaluronidase.
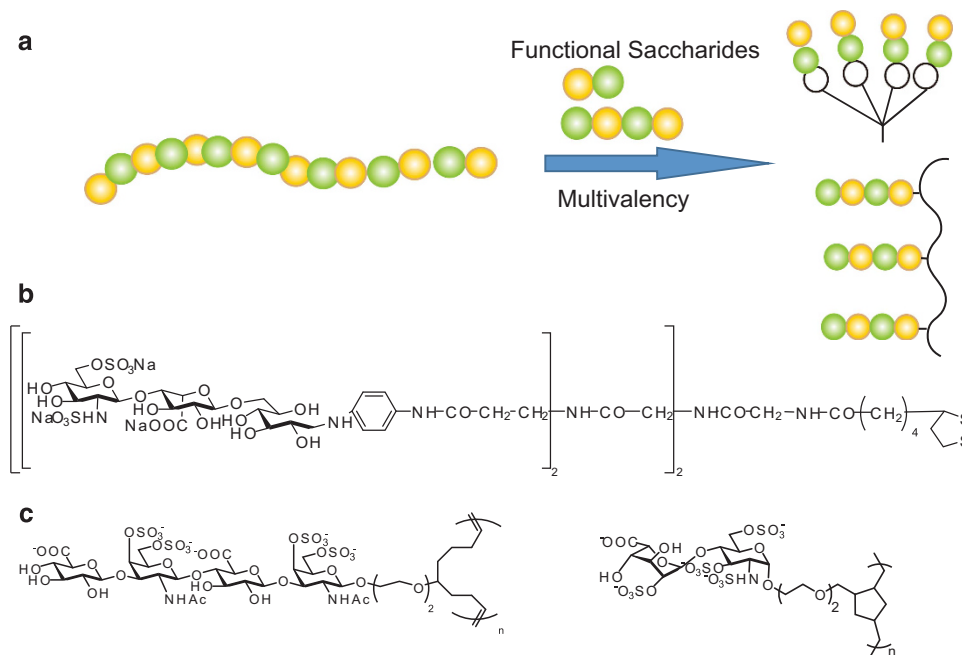
Figure 5 Preparation of GAG mimetics with multivalent saccharides. (**a**) The concept of multivalent GAG mimetics. The functional oligosaccharides were modified to be multivalent compounds. (**b**) Chemical structure of heparin disaccharides reported by the Suda group. (**c**) Chemical structures of glycopolymers with chondroitin sulfate and heparin oligosaccharides reported by the Hsieh-Wilson group.

synthesized heparan sulfate oligosaccharides, which were shown to bind to b-FGF.[29]

The Hung group synthesized a library of oligosaccharides from heparin and heparan sulfate, including various oligosaccharides of 3-*O*-sulfated heparan sulfate with α-selective glycosylation using trichloroacetimidate and selective protecting group strategies. They reported the total syntheses of tetrasaccharide to octasaccharide GAGs and investigated their interactions with various proteins, viruses and bacteria.[30–32] Cole *et al.* reported the syntheses of hepta- to dodecaoligosaccharide GAGs and studied their endothelial cell functions.[33,34] The Seeberger group also reported the syntheses of GAG oligosaccharide libraries[35,36] and developed microarrays for high-throughput analyses with proteins, such as growth factors. The authors also attempted to fabricate an automated procedure for

solid-phase syntheses of GAG oligosaccharides based on synthetic procedures developed for chondroitin sulfate oligosaccharides.[37]

The biosynthesis of GAGs has also been investigated.[38] The majority of commercially available GAGs are currently extracted from animals, making it difficult to produce them in large quantities. GAGs isolated from animal tissue also have a risk of contamination. Therefore, biosynthesis has received significant attention as a mass production approach. However, biosynthesis faces the same challenges as organic syntheses, which includes the preparation of complex saccharide structures and regioselective sulfonation procedures. Saccharide structures have been prepared using enzymes, such as saccharide transferase, epimerase and sulfatase. Gene technology for cloning enzymes for the GAG synthesis and gene analyses that include GAG synthesis have also been studied thoroughly. However, biosynthesis

remains too costly for commercial production owing to the high cost of necessary enzymes and sugar nucleotides.

The combination of organic synthesis and biosynthesis represents an alternative method for the efficient preparation of GAGs. The Kobayashi group reported the preparation of GAGs using a reverse reaction with glycosidase (sugar hydrolase) (Figure 4).[39] Kobayashi used sugar oxazoline derivatives as transition-state analogs for the enzyme, which were then polymerized into oligosaccharides, such as cellulose, chitinase and hyaluronidase.[40] The authors also synthesized hyaluronic acid by polymerizing transition state analogs of GlcA(β1-3) GlcNAc oxazoline derivatives (Figure 4)[41] and succeeded in synthesizing chondroitin sulfate by polymerizing disaccharide oxazoline derivatives with hyaluronidase.[42,43]

GLYCOPOLYMERS CARRYING GAG SACCHARIDES

Although the properties of GAGs are partially based on their oligosaccharide structure, they also function by acting as natural polyelectrolytes. Here the molecular weights of GAGs have important roles in defining their biological activities, which can be demonstrated by the difference in biological activities between high and low molecular weight heparin.[44]

Saccharide interactions have been investigated in detail with regards to multivalent effects.[1,45] For example, glycolipids form densely packed 'raft'-like saccharide clusters, whereas glycoproteins display branched multivalent saccharides. These saccharide clusters induce multivalent binding, which enhances the saccharide–protein interactions. Synthetic multivalent compounds such as glycopolymers, glycodendrimers and other polyvalent saccharide structures have been shown to amplify these interactions.[46] Controlled polymerization has been applied to bioconjugation methods. Glycopolymers are frequently prepared using radical, living radical and ring-opening metathesis polymerization (ROMP) chemistry owing to their orthogonality to hydroxyl and sulfate groups.

The chemical structures of polymer backbones also affect the physical properties of GAGs. The Kiessling group reported the synthesis of various multivalent glycoconjugates with dendrimers using ROMP and radical polymerization chemistry.[47] The Kiessling group also reported that these glycopolymers interacted strongly with proteins, where the glycopolymers prepared using radical polymerization had stronger interactions than those prepared using ROMP. Because the affinities of the polymers depend on various factors, such as multivalent effects, polymer mobility and target proteins, the comprehensive design of glycopolymers requires further investigation.

GAGs are polymers with linear structures that undergo multivalent binding with target proteins (Figure 5a),[2] usually in the form of proteoglycans. These multivalent interactions are considered to be essential for the biological function of GAGs. Suda and Kusumoto investigated the synthesis of heparin oligosaccharides and their interactions with platelets.[25–27] Trisaccharide fragments of GAGs could bind platelets but had weaker binding affinities than natural

saccharides, suggesting a lack of a multivalent amplification effect. To overcome these weak interactions, Suda et al.[27] investigated methods to amplify saccharide–protein interactions using dendritic multivalent compounds (Figure 5b). The authors synthesized GlcNS (6S)(α1-4)IdoA(β1-4)Glc as well as monomeric, trimeric and tetrameric variants of GlcNS(6S)(α1-4)IdoA(β1-4)Glc. The saccharides were then immobilized onto a Au-substrate to quantitatively evaluate hemostatic protein binding of the von Willebrand factor using surface plasmon resonance. The affinity of tetrameric saccharides for von Willebrand factor was amplified strongly by the multivalency effect.

The Hsieh-Wilson group reported the syntheses of glycopolymers with GAG oligosaccharides using ROMP in the form of polymer chains with pendant saccharides (Figure 5c).[48–50] This group synthesized disaccharide and tetrasaccharide forms of chondroitin sulfate (that is, (GlcA(β1-3)GalNAc(3S)(6S) and GlcA(β1-3)GalNAc (3S)(6S)(β1-4)GlcA(β1-3)GalNAc(3S)(6S)) and investigated their biological activity. Specifically, their ability to stimulate neuronal growth was investigated.[48] The monomeric disaccharide and tetrasaccharide showed no clear activity toward neurons, but the polymer and natural chondroitin sulfate inhibited the outgrowth of the hippocampal neurons. Their activity against neurons was dependent on their polymer chain length, with longer chains offering sufficient multivalent interaction sites. Glycopolymers with a heparin disaccharide of IdoA(2S)(α1-4)GlcNS(6S) were also prepared using ROMP, which exhibited anticoagulant activity. ATIII binding and thrombin activity were amplified by the glycopolymers in a molecular weight-dependent manner.[49] The Hsieh-Wilson group also reported glycopolymer immobilization via a biotin end-functionalized polymer and demonstrated that GAG recognition proteins could tolerate modifications to the polymer backbone, highlighting the significance of the multivalent effect.[50]

GAG MIMETICS USING POLYMERS

It remains difficult to obtain GAGs with high molecular weights using total synthesis methods. The functions of GAGs are based on saccharide structures, but GAGs also function as polyelectrolytes. In some cases, anionic polyelectrolytes represent an alternative to GAGs.

The Maynard group investigated the GAG mimetic function of a polystyrene sulfonic acid-based polymer and its activity as a polyelectrolyte (Figure 6).[51,52] They found that poly(styrenesulfonic acid-co-poly(ethylene glycol) methacrylate) could bind FGF and vascular endothelial growth factor fragment peptides. Growth factor binding occurs as a result of the strong acidity of GAGs, an effect that was successfully replicated by polystyrene sulfonic acid. Polymethacrylate did not interact with cell growth factors because of its modest acidity. The polymers were immobilized onto a substrate in a micropattern using hydrophobic interactions and end-functionalization. The authors also investigated the effects of protein modification using the copolymer poly(styrene sulfonic acid-co-poly (ethylene glycol) methacrylate) by conjugating the polymer to b-FGF through disulfide bonds.[52] Because b-FGF has a positive net charge, b-FGF was stabilized by conjugation with the polymer. The modified b-FGF showed much higher stability under high temperature (55 °C), low temperature, acidic conditions and in the presence of a protease. The polymer-modified b-FGF also enhanced cell growth. Heparin has been reported to increase stability and induce b-FGF activation[53] through electrostatic interactions; polystyrene sulfonic acid altered this GAG function.

The Chaikof group has also reported the preparation of GAG mimetics with glycopolymers (Figure 7a). They prepared sulfated glycopolymers using sulfated GlcNAc[54,55] and lactose.[56,57] The sulfated
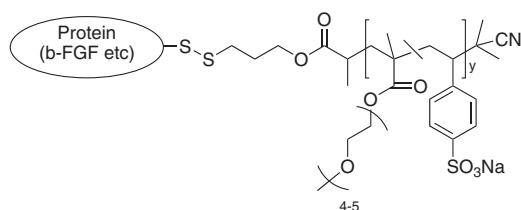
Figure 6 A polystyrene sulfonate-based glycosaminoglycan mimetic conjugated to a protein.

glycomonomers were polymerized with cyanoxyl-persistent radicals and were found to exhibit anticoagulant activity and interact with b-FGF.[55] The anticoagulant activity responded to changes to the molecular weight of the polymers and the sugar ratios. Additionally, the Chaiikof group prepared dendrimer-like sulfated saccharides with a polyethylene oxide linker.[57] These GAG mimetic compounds had stronger interactions with L-selectin than heparin and multivalent sialyl Lewis[X] and significantly reduced inflammation *in vivo*.[58]

The Akashi group investigated a glycopolymer with sulfonated Glc (Figure 7b)[59] by polymerizing methacrylate with per-sulfonated Glc. The sulfated glycopolymer exhibited anticoagulant activity, and its activity was compared with those of heparin and sulfated dextran. The activities were found to be related to the degree of sulfation. This demonstrated that the highly sulfated glycopolymer exhibited sufficient activity demonstrated some of the functions of GAGs.

BIOINSPIRED GAG MIMETIC POLYMERS TO INHIBIT PROTEIN AMYLOIDS

As multivalent interactions and polyelectrolyte properties are important features of GAGs, their structures can be modified to a

Figure 7 Glycosaminoglycan mimetic glycopolymers from the Chaikof group (**a**) and Akashi group (**b**).

certain extent. GAG functions that rely solely on saccharide structures are usually not observed.

We attempted to recreate GAG functions using glycopolymers with regioselectively sulfated saccharides. Despite the many functions of GAGs,[2] we focused on protein amyloidosis because most of the proteins involved in amyloidosis interact with GAGs.[60–62] The Goto group investigated amyloidosis in detail and found that the charge at the cell surface had an important role in protein aggregation.[63] Vinyl saccharides with regioselective sulfonation were subject to polymerization.[64] An advantage of this method is the facile preparation of GAG mimetic polymers. It was easy to verify the structure of the monosaccharide, the sugar ratio of the polymer, the molecular weight and the structure of the polymer backbone.

GAG mimetic polymers with sulfated GlcNAc acrylamide phenyl were prepared (Figure 8) using protecting group chemistry to generate 6-sulfo-, 4-sulfo-, 3-sulfo- and 3,4,5-sulfo-GlcNAc variants. An acrylamide phenyl derivative of GlcA was also prepared using a uronic acid monomer. These saccharide monomers were copolymerized with acrylamide using a radical initiator. To investigate the activity of these GAG mimetics, we focused on the 6-sulfo GlcNAc monomer, as it has been shown to have an important role in various living systems.[62]

The effects of amyloid β (Aβ) peptides on Alzheimer amyloidosis have been investigated using poly(AAm-*r*-6-sulfo-GlcNAc).[65–67] Glycopolymers with various sugar contents (10 − 100%) and molecular weights were synthesized using 6-sulfo-GlcNAc and subsequently incubated with Aβ (1 − 42) and (1 − 40) peptides. Aβ (1 − 42) spontaneously aggregated to form nanofibrils, but the addition of sulfated glycopolymers inhibited aggregation. The aggregation of Aβ was inhibited by strong interactions with the glycopolymers, which resulted from electrostatic interactions between the sulfated glycopolymers with basic residues of Aβ.[65] Inhibition of Aβ aggregation was dependent on the sugar content of the polymers. Poly(AAm-*r*-GlcNAc(6S)) demonstrated stronger inhibitory effects against Aβ aggregation than poly(AAm-*r*-GlcA), and a terpolymer of poly(AAm-*r*-GlcNAc(6S)-*r*-GlcA) showed the strongest activity.[66] The morphology changes of Aβ aggregates upon incubation with glycopolymers were investigated using atomic force microscopy. The original nanofibrils of Aβ adopted round micrometer-scale shapes. The glycopolymers also neutralized cell cytotoxicity.

The GAG mimetic polymers were synthesized using controlled polymerization methods. The molecular weights of GAGs correlate
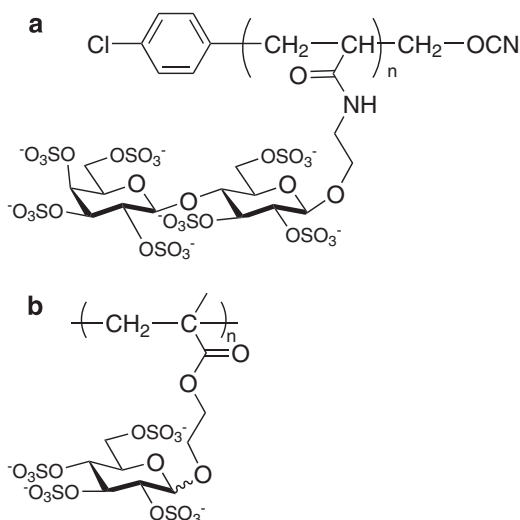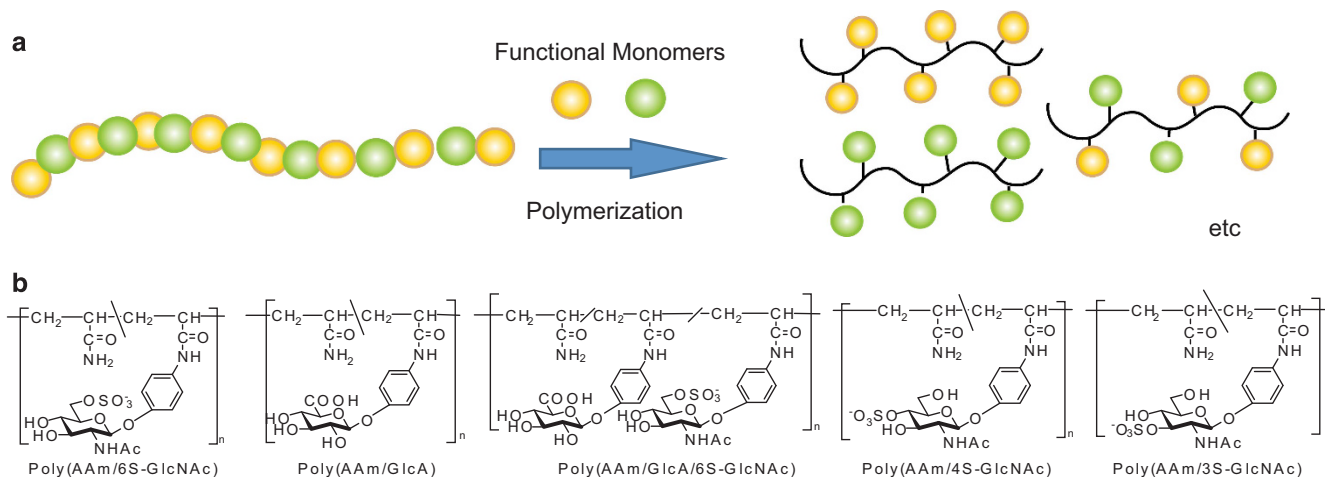
Figure 8 Preparation of GAG mimetic glycopolymers. (**a**) The concept of GAG mimetics using glycopolymers. (**b**) Chemical structures of GAG mimetics using glycopolymers with GAG monosaccharides.

a

b Protein   Interaction with protein and cell

GAGs mimic sugar arrays
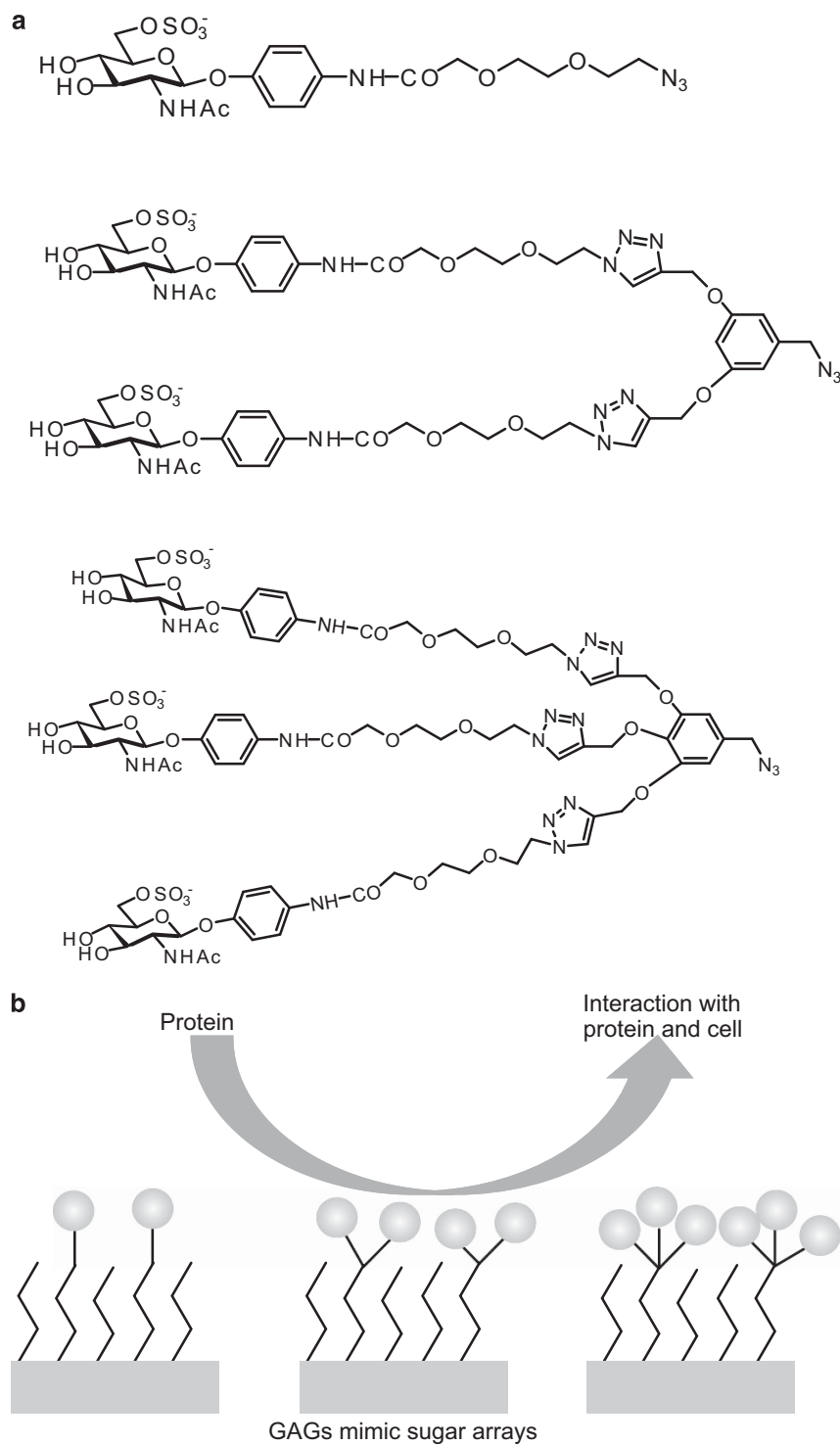
Figure 9 GAG mimetic multivalent arrays. (**a**) Chemical structure of dendritic 6-sulfo-GlcNAc. (**b**) Saccharide microarray for protein analysis. A full color version of this figure is available at the *Polymer Journal* online.

with their biological activity,[68] and this effect was studied using living radical polymerization. The molecular weights of glycopolymers carrying 6-sulfo-GlcNAc and GlcA were controlled by reversible addition fragmentation chain transfer (RAFT) polymerization, and their inhibitory effects on Aβ aggregation were investigated.[66] Interestingly, their inhibitory effects on Aβ aggregation were dependent on their molecular weights: glycopolymers with lower molecular

weights exhibited stronger inhibition effects owing to their greater mobility.

The enzymatic activity of Alzheimer disease is also tuned by GAG interactions. β-Secretase (BACE-1) is an enzyme that produces Aβ peptides, and its activity is fine-tuned through GAG interactions.[69,70] The activity of BACE-1 was examined in the presence of GAG mimetic glycopolymers. The enzymatic activity was dependent

on the saccharide structure of the GAG mimetic polymers. The polymers with 6-sulfo-GlcNAc and 3,4,6-sulfo GlcNAc, along with heparin, exhibited inhibitory activity against BACE-1.[71] Polymers carrying 3-sulfo-GlcNAc and 4-sulfo-GlcNAc did not have an effect on BACE-1 activity, suggesting that the sulfate groups of the saccharides are necessary for enzymatic inhibition. Thus these polymers have the potential to clarify the function of each of the saccharide variants.

Addition polymerization is a facile and useful method to prepare various GAG mimetics, but it provides poor structural control. Dendritic 6-sulfo-GlcNAc compounds were prepared to control the multivalency of GAG mimetic structures. Monomeric, dimeric and trimeric 6-sulfo-GlcNAc variants were prepared to investigate their interactions with Aβ (1–42) (Figure 9).[72,73] The dendritic 6-sulfo-GlcNAc was immobilized on a gold substrate using click chemistry. The interaction of Aβ with the monomeric saccharide was weak, but its interaction with bivalent and trivalent 6-sulfo-GlcNAc was stronger, as determined by surface plasmon resonance. The morphologies of Aβ (1–42) on sugar layers were dependent on the sugar valency; Aβ formed nanofibrils on monomeric 6-sulfo-GlcNAc and spherical objects on the trimeric saccharide. Aβ was not toxic on monomeric 6S-GlcNAc but was cytotoxic on the trimeric saccharide. These GAG mimetic studies demonstrate that the multivalency of GAGs is significant for their biological function and that such protein functions can be controlled by modifying their interactions with GAGs. Additionally, the 6-sulfo-GlcNAc-containing dendrimers inhibited the aggregation of Aβ.[71]

CONCLUSIONS

There are numerous reports that describe the synthesis of GAG mimetics. However, no methods have prevailed as preferred synthetic routes. A survey of GAG-related molecules shows that multivalency is a key factor that affects their biological activity and function. The saccharide scaffold provides an important structural template for GAG function, and polymers with pendant monosaccharides and oligosaccharides are able to efficiently amplify the biological activity of GAGs. In contrast to the challenges associated with total syntheses of GAGs using organic chemistry or biosynthesis, oligosaccharide preparation and the amplification of multivalency using polymerization strategies are facile and practical. We proposed the synthesis of novel GAG mimetics using glycopolymers with regioselectively sulfated saccharides and investigated their abilities to inhibit the progression of Alzheimer disease. Other groups have succeeded in activating antithrombotic activity and stimulating growth factor activation. Considering the strong demand for natural GAGs and their scarcity, the preparation and functionalization of GAG mimetics represents a suitable alternative.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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