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Minireview

NMR structural determination of unique invertebrate glycosaminoglycans endowed with medical properties

Vitor H. Pomin*

Program of Glycobiology, Institute of Medical Biochemistry Leopoldo de Meis, University Hospital Clementino Fraga Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, 21941-913, Brazil

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ABSTRACT

Glycosaminoglycans (GAGs) are sulfated polysaccharides of complex structure endowed with numerous biomedical functions. Although ubiquitously distributed in vertebrates, GAGs can also occur in certain terrestrial or marine invertebrates. Solution nuclear magnetic resonance (NMR) spectroscopy has been the analytical technique mostly employed in structural characterization of GAGs from any source. This review aims at illustrating the application of NMR in structural determination of few representative invertebrate GAG examples of unique structures and endowed with therapeutic actions. They are the holothurian fucosylated chondroitin sulfate, the acharan sulfate isolated from the snail *Achatina fulica*, the dermatan sulfates with distinct sulfation patterns extracted from ascidian species, the sulfated glucuronic acid-containing heparan sulfate isolated from the gastropode *Nodipecten nodosum*, and the hybrid heparin/heparan sulfate molecule obtained from the shrimp *Litopenaeus vannamei*. These invertebrate GAGs exhibit distinct structures when compared to those extracted from mammalian GAGs. The distinct structures of the invertebrate GAGs lead also to different mechanisms of actions as compared to the mammalian GAG standards. Invertebrate GAGs comprise promising therapeutic candidates in fights against diseases. Solution NMR has been playing a pivotal role in this carbohydrate-based drug research, discovery and development.

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1. Introduction

Glycosaminoglycans (GAGs) are glycans of diverse functions and complex structures, although composed of disaccharide building blocks. These repeating blocks are in turn composed of alternating uronic acid and hexosamine monosaccharide units, which can be differently sulfated depending on the GAG families. Members of the same GAG family share common structural features. For example, heparan sulfate (HS) and heparin (Hp) are composed of α-D-glucosamine (GlcN) units as hexosamine type. While HS is less modified by N- and O-sulfation and uronic acid C5 epimerization, which converts the composing β-D-glucuronic acid (GlcA) into α-L-iduronic acid (IdoA); Hp is highly processed by these modifications.[1,2] The other two biologically active GAGs highly abundant in cell surface or extracellular matrix-associated proteoglycans are the chondroitin sulfate (CS) and dermatan sulfate (DS). Although CS and DS are both composed of N-acetyl-β-D-galactosamine (GalNAc) units, the former is made up primarily of GlcA whereas IdoA composes the latter.[3]

Due to this great structural variety and presence of sulfation, GAGs can bind to an incredible number of functional proteins, including, but not limited to, inflammatory cytokines and chemokines,[4] angiogenesis-related growth factors like fibroblast growth factors 1,[5] and 2 (FGF2),[6] and plasma cofactors involved in blood coagulation like thrombin (IIa),[7] and antithrombin (AT).[7,8] Because of these and other unmentioned roles, GAGs are ubiquitously distributed in all vertebrates usually linked to a protein core forming thus the well-known proteoglycans. Structural and functional analyses of GAGs from mammals are largely carried out worldwide given to the fact that these molecules play key roles in multiple pathophysiological processes such as cancer,[9,10] inflammation,[11] thrombosis,[12] and neovascularization.[13] The mechanisms of action of GAGs in these events are directly related to their structural features.

However, GAGs of medical utilities are not derived exclusively from mammals. Therapeutic GAGs from invertebrates, from terrestrial or marine habitat, are also available and have shown

* R. Prof. Rodolpho Paulo Rocco, 255, HUCFF 4A01, Ilha do Fundão, Rio de Janeiro, RJ, 21941-913, Brazil. Tel.: +55 21 3938 2939; fax: +55 21 3938 2090.
E-mail addresses: vhpomin@gmail.com, pominvh@bioqmed.ufrj.br.

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curiously different structures as compared to the mammalian standards. Among numerous invertebrate GAGs that have been studied so far in terms of both structure and function, representative examples are the holothurian fucosylated chondroitin sulfate (FucCS),[14,15] the acharan sulfate (AS) from the giant African snail *Achatina fulica*,[16–18] the ascidian DSs of unique sulfation patterns,[19–21] the novel HS from the bivalve *Nodipecten nodosus*,[22] and the hybrid HS/Hp found in the head of the shrimp *Litopenaeus vannamei*.[23] Like in structural analyses of mammalian GAGs, solution nuclear magnetic resonance (NMR) spectroscopy alone or combined with other techniques, has been the preferred method used in structural characterization of the invertebrate molecules. Here, this NMR-based analysis of GAGs of unique structures found exclusively in invertebrate animals is being overviewed in order to offer a straightforward technical guidance to future researches in the area. Discussion about the medicinal implications of these distinct invertebrate GAG structures is also provided in order to highlight their potential benefits in future treatments and prophylaxis of certain diseases.

2. Holothurian FucCS

The first evidence describing the existence that sea cucumbers (Echinodermata, Holothuroidea) could synthesize a different GAG in their body wall seems to come from reference 24. Through monosaccharide composition analyses, the new GAG extracted from the holothurian species *Ludwigothurea grisea* was reported to be composed of equimolar quantities of α-L-fucopyranose (Fuc*p*), β-D-GlcA and β-D-GalNAc units.[25] In a further work of the same group, the investigators have expanded the structural characterization of this holothurian GAG by using NMR spectroscopy combined with methylation analyses.[26] In this work, the authors have unequivocally confirmed that this novel GAG from *L. grisea* is composed of branching sulfated Fuc*p* units 3-linked to the GlcA units, which together with GalNAc units forms a CS-like backbone as commonly seen in mammalian CSs (Fig. 1). This new holothurian GAG was then named fucosylated chondroitin sulfate (FucCS). Evidence supporting the physiological occurrence of FucCS linked to a protein core as seen in GAG-composed proteoglycans was inclusively raised.[26] The structure of the *L. grisea* FucCS was observed to be very heterogeneous, and the definitive proposition for its structure

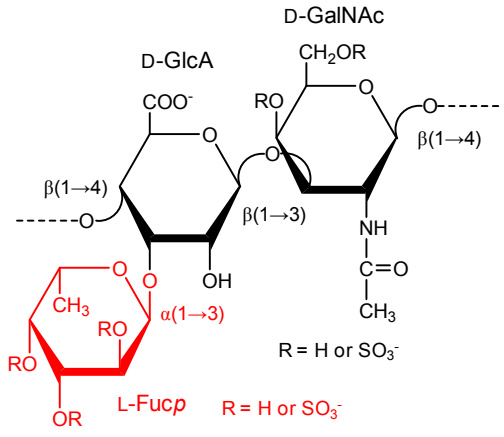

Fig. 1. Structural representation of the holothurian FucCS (black and red monosaccharides) and the regular CS (black monosaccharides) as seen in mammals. They are α-L-fucopyranose (L-Fuc*p*) in red, β-D-glucuronic acid (D-GlcA), and N-acetyl-β-D-galactosamine (D-GalNAc). Note that the holothurian GAG differs from the mammalian counterpart only by the addition of the sulfated fucosyl unit highlighted in red(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

was reached only at two further works of the same group.[14,27] A comprehensive review concerning the background and history of this sea cucumber GAG has been published recently.[28] The composition and amounts of units currently accepted for FucCS of *L. grisea* as well as to FucCSs obtained from other sea cucumber species studied so far are displayed in Table 1. These FucCS structures were determined primarily by liquid-state NMR spectroscopy. Below, I illustrate the NMR-based structural characterization of FucCS from three of these holothurian species, based on the outstanding reference 15. A brief discussion about medical activities and the structural requirements of FucCSs in some of these activities are reported after the NMR analysis.

Fig. 1 depicts the partially assigned one-dimensional (1D) ^{1}H (panels A–C) and ^{13}C (panels D–F) NMR spectra of FucCSs isolated from three sea cucumber species: *Isostichopus badionotus*, *Stichopus tremulus*, and *Holothuria vagabunda*. The signals at 1.21 and 1.89 ppm derive respectively from methyl protons of Fuc*p* (CH_3) and GalNAc acetyl groups ($COCH_3$). The signals between 5.1 and 5.6 ppm belong to the anomeric atoms of variously sulfated Fuc*p* units.[14,33]

The ^{1}H NMR spectrum of FucCS from *I. badionotus* shows anomeric signals at 5.23 and 5.56 ppm (Fig. 2A). This indicates the presence of 4-mono-sulfated (Fuc4S) and 2,4-di-sulfated (Fuc2,4S) Fuc*p* units (Table 2). Integral values of these peaks show sulfation content to be 4.1% and 95.9% for mono- and di-sulfated units, respectively (Table 1). The ^{1}H spectrum of FucCS from *S. tremulus* shows three clear signals in the anomeric region (Fig. 2B). The peaks at 5.23 and 5.56 ppm belong to Fuc4S and Fuc2,4S units, respectively (Table 2). The signal at 5.33 ppm was assigned to the 3,4-di-sulfated Fuc*p* (Fuc3,4S) based on the studies of FucCSs obtained from *Stichopus japonicus* and *L. grisea*.[14,33] FucCS from *S. tremulus* is then composed of 24.8%, 22.4% and 52.8% of Fuc4S, Fuc2,4S and Fuc3,4S, respectively (Table 1). In the anomeric ^{1}H region of FucCS from *H. vagabunda* (Fig. 2C) peaks at 5.17 and 5.23 ppm belong both to the Fuc4S unit, while those at 5.33 and 5.56 ppm belong to the Fuc3,4S and Fuc2,4S units, respectively. The signal at 5.09 ppm is assigned to a non-sulfated fucose unit (Fuc0S). Based on integral values of these peaks, the sulfation pattern of the side-chain Fuc*p* units in FucCS from *H. vagabunda* is 25.6%, 50.2%, 15.8%, and 8.4% for Fuc0S, Fuc4S, Fuc2,4S, and Fuc3,4S (Table 1).

Different sulfation patterns of Fuc*p* branches can affect the chemical shifts of methyl protons in this own unit but also in adjacent GalNAc units (top insert panels of Fig. 2). For instance, *I. badionotus* FucCS has a major methyl proton signal at 1.19 ppm (Fig. 2A), which can be readily assigned to a Fuc2,4S. FucCSs from *S. tremulus* and *H. vagabunda* exhibit both a complicated Fuc*p* methyl signal profile (Fig. 2B and C) similar to their anomeric proton signals, indicating thus complex sulfation patterns of their Fuc*p* units. The methyl proton signals of GalNAc acetyls (1.8–2.0 ppm) are also different among the three FucCSs. A major peak at 1.89–1.93 ppm was found in the spectra of FucCSs from *S. tremulus* and *I. badionotus*, indicating that they share a similar sulfation pattern on the GalNAc units within their disaccharide-composed backbones. However, *H. vagabunda* FucCS gives a more complex GalNAc methyl signals due to the complex sulfation pattern of its composing GalNAc units, as discussed below.

1D ^{13}C NMR spectra of FucCSs (Fig. 2D–F) do not show any apparent difference in the sulfation patterns of the Fuc*p* branches. However, the major signals were useful to assign the CS backbone sequence as these carbon signals are very similar to those present in the spectrum of a standard chondroitin sulfate-E (CS-E),[33] and in the FucCS from *S. japonicus*[37], which contains the CS-E as its backbone (Table 1). In CS-E, the GalNAc is sulfated at both 4- and 6-O-positions. A signal at 67.5 ppm, present in all three spectra, is from

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