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Variations of pH as an additional tool in the analysis of crowded NMR spectra of fucosylated chondroitin sulfates

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ABSTRACT

The influence of pH variation on chemical shift values in NMR spectra of fucosylated chondroitin sulfates was studied using polysaccharides isolated from three sea cucumber species *Apostichopus japonicus, Actinopyga mauritiana* and *Cucumaria japonica*. The signals of glucuronic acid residues were found to be the most sensitive to pH changes in comparison to the chemical shifts of the sulfated galactosamine and fucosyl units, most of which were altered insignificantly. It was shown that in the presence of imidazole-HCl buffer (pH 7.2) NMR spectra of the polysaccharides from *A. japonicus* and *A. mauritiana* were sufficiently resolved, whereas under acidic conditions their ^{1}H NMR spectra were complicated by overlapping of H-1 signals of GlcA and GalNAc. In the case of polysaccharide from *C. japonica* bearing 3-O-fucosylated and 3-O-sulfated glucuronic acid residues in the backbone, acidification of the medium led to separation of H-1 signals of GlcA3*S* and GalNAc. Therefore, the combination of data obtained at different pH values may be useful for interpretation of overcrowded spectra of fucosylated chondroitin sulfates.

1. Introduction

Fucosylated chondroitin sulfates (FCS) are components of the body walls of sea cucumbers possessing several types of biological activity, such as anti-inflammatory, antithrombotic, anticoagulant, antibacterial and others.[1–3] These biopolymers are built up of chondroitin core [→3)-β-D-GalNAc-(1→4)-β-D-GlcA-(1→]$_n$ bearing α-L-fucosyl residues as branches. Structural diversity of FCS isolated from different holothurian species depends on variations in number of branches and sites of their attachment, as well as on sulfation pattern of all monosaccharide components.[4–7] Characterization of FCS usually requires a combination of chemical and physicochemical methods, among which non-destructive NMR spectroscopy can be regarded as the most informative one. Partial depolymerization of FCS is often performed to obtain well resolved NMR spectra.[8–10] However depolymerization is usually accompanied by partial loss of some structural fragments, and hence, may lead to errors in reconstruction of the native structures.

Acidic polysaccharides are known to be sensitive to pH changes. Incomplete ion exchange of protons on metal cations (e.g. Na^+) in sulfate or carboxyl groups during isolation and purification lead to acidification of polysaccharides. This could result in difference of NMR spectra of polysaccharide samples isolated from the same source by different methods. In the worst case, acidification of polysaccharides may initiate their autohydrolysis, which leads to significant destruction of macromolecules.

In this communication the influence of pH on the signal positions in NMR spectra of the native FCS isolated from three sea cucumber species *Apostichopus japonicus, Actinopyga mauritiana* and *Cucumaria japonica* has been studied.

2. Experimental procedures

Isolation of FCS from sea cucumbers *Apostichopus japonicus*, *Actinopyga mauritiana* and *Cucumaria japonica* was described previously.[7,11] Briefly, polysaccharides were isolated from minced body walls with extraction in the presence of papain followed by Cetavlon precipitation and anion-exchange chromatography.

2.1. Method A

Sample of FCS (25 mg) was dissolved in 99.9% D_2O, freeze-dried, dissolved in 99.96% D_2O and placed into Shigemi tube. ^{1}H and ^{13}C spectra were recorded using a Bruker AV-600 spectrometer at 303 K with HOD suppression by pre-saturation. COSY, HSQC, and ROESY spectra were recorded using standard Bruker pulse sequences. Chemical shifts are measured relative to sodium 3-(trimethylsilyl)propionate-*2,2,3,3,-d*$_4$ at 0 ppm for ^{1}H and at –1.6 ppm for ^{13}C spectra.

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2.2. Method B

Sample of FCS (25 mg) was dissolved in 99.9% D_2O, freeze-dried, dissolved in 99.96% D_2O and pH adjusted to 2.0 by addition of 4% CF_3COOH in D_2O. The solution was placed into Shigemi tube and NMR spectra were recorded as described above.

2.3. Method C

Sample of FCS (25 mg) was dissolved in 0.3 mL of imidazole-HCl buffer (90 mM, pH 7.2), freeze-dried, then dissolved in 99.9% D_2O, freeze-dried again, dissolved in 99.96% D_2O and placed into Shigemi tube. NMR spectra were recorded as described above.

3. Results and discussion

The mixtures of water-soluble polysaccharides were isolated from the body walls of sea cucumbers *Apostichopus japonicus*, *Actinopyga mauritiana* and *Cucumaria japonica* by solubilization with papain[12] followed by precipitation of the sulfated components as cetyltrimethylammonium salts. Their transformation into a water-soluble sodium salts by stirring with NaI in ethanol and further purification by ion-exchange chromatography led to the samples of fucosylated chondroitin sulfates **AJ**, **AM** and **CJ**, respectively. Details of isolation and structural characterization of these polysaccharides were described previously.[7,11] It was shown that the backbones of **AJ** and **AM** were composed of repeating disaccharide units →3)-β-D-GalNAc-(1→4)-β-D-GlcA-(1→ of type **I** (Fig. 1), whereas for **CJ** two types (**I** and **II**) of repeating blocks were found in the chain. Branches Fuc2*S*4*S* (**G**) and Fuc3*S*4*S* (**H**) attached to O-3 of GlcA were present in different proportions in all studied FCS (Table 1). Besides,

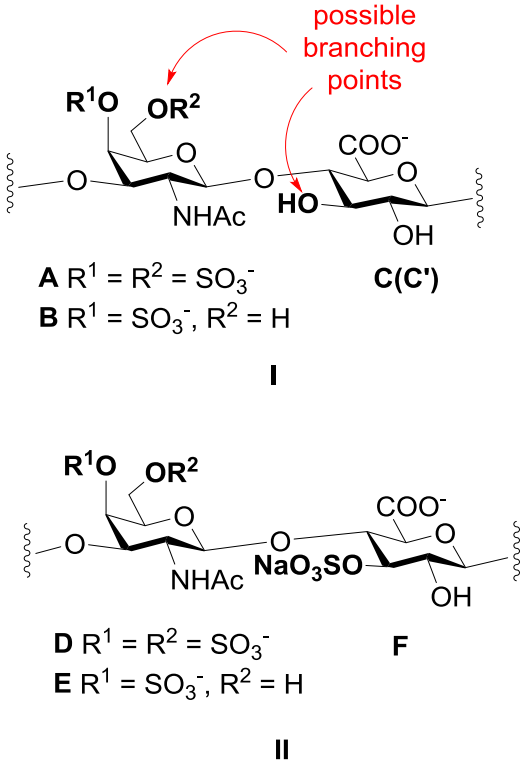

Fig. 1. Backbone repeating blocks of fucosylated chondroitin sulfates **AJ** (constituent units **A**-**C**,**C′**), **AM** (constituent units **A**-**C**,**C′**) and **CJ** (constituent units **A**-**F**,**C′**). Units **C** and **C′** bear Fuc2*S*4*S* and Fuc3*S*4*S* substituents, respectively, at O-3, as pointed in Table 1.

Table 1
Structural characteristics of fucosylated chondroitin sulfates **AJ**, **AM** and **CJ**

FCS	Sea cucumber	Backbone repeating blocks	Branches	
			At O-3 of GlcA	At O-6 of GalNAc
AJ	*A. japonicus*	Type I	Fuc2S4S (**G**)[a] Fuc3S4S (**H**)[b]	None
AM	*A. mauritiana*	Type I	Fuc2S4S (**G**) Fuc3S4S (**H**)	Fuc3S4S (**J**)
CJ	*C. japonica*	Types I and II	Fuc2S4S (**G**) Fuc3S4S (**H**)	None

[a] Attached to unit **C**.
[b] Attached to unit **C′**.

AM contained small amounts of unit Fuc3*S*4*S* (**J**) attached to an unusual O-6 position of GalNAc.

Usually samples of water-soluble acidic polysaccharides for NMR experiments are prepared in D_2O without any additional treatments. Therefore, the first sample of **AJ** was prepared by simple dissolution of the polysaccharide in D_2O (Method A). Unfortunately, partial overlapping of the H-1 signals of GlcA (**C**,**C′**) and GalNAc (**A**,**B**) in ^1H NMR spectrum was observed (see a fragment of HSQC spectrum, Fig. 2A). Acidification of the sample to pH 2.0 by addition of CF_3COOH (Method B) did not improve the spectrum (Fig. 2B), while the application of imidazole-HCl buffer with pH 7.2 as a medium (Method C) led to separation of H-1 signals of the backbone units (Fig. 2C). It was an important result because the main signal assignment in NMR spectra of the polysaccharides was performed with the help of 1D and 2D ^1H spectra. Application of buffers is very rare for the preparation of polysaccharide samples for NMR analysis. Panagos et al. used phosphate buffer (pH 7.2) for recording NMR spectra of FCS from *Holothuria forskali*.[8]

Complete assignment of the signals in the ^1H and ^{13}C NMR spectra of **AJ** at pH 7.2 was performed using a series of 2D NMR experiments (COSY, HSQC, ROESY).[11] Selected values of the chemical shifts (δ, ppm) are given in Table 2. These data were helpful for the assignment of the signals of **AJ** at pH 2.0. The 2D NMR spectra of **AJ** and **AM** recorded at pH 7.2 and 2.0 are shown in Figs. S1 and S2. One could notice, that several signals of glucuronic acid residues (**C**,**C′**) were sensitive to pH changes and moved downfield under acidic conditions, while the majority of signals belonging to the sulfated galactosamine and fucosyl units altered insignificantly (Table 2). For instance, the difference between δ of H-5 (GlcA,**C′**) in acidic and neutral medium was 0.20 ppm. Such alterations might be attributed to conformational distortions of the pyranose ring of GlcA caused by change of the effective radius of the carboxyl group upon its protonation/deprotonation (Fig. 3A and B). Experimental evidence for such changes was obtained from the ROESY spectra of polysaccharides (Fig. S3). It could be seen that in polysaccharide **AM** no intra-ring correlation H1-H3 of the GlcA residue was observed at pH 2.0, which suggested that its conformation deviated from classical $^4C_1$, as was the case for polysaccharide **AJ**. Analogous dependence of several signal positions on pH in NMR spectra of heparin fragments was observed previously.[13,14] Besides, Lemieux described the effect of protonation of amino groups in aminosugars on the chemical shifts in NMR spectra.[15] Alterations of the spectra recorded at different pH were explained by conformational transformations of the pyranose rings of the studied compounds.

Interestingly, the values of δ H-1 of GlcA residues **C** and **C′** substituted at O-3 by Fuc2*S*4*S* and Fuc3*S*4*S*, respectively, were the same at pH 7.2, while at pH 2.0 the difference between δ H-1 (**C**) and δ H-1 (**C′**) was 0.05 ppm (Fig. 3B). This effect could be connected with the presence of sulfate group at C-2 of fucosyl unit (Fig. 3C). The solvation of sulfate group is altered at different pH. These changes influence the effective volume of sulfate and consequently conformational properties in the neighboring fragment.