

## Analytical Glycobiology

# Bottom-up analysis using liquid chromatography–Fourier transform mass spectrometry to characterize fucosylated chondroitin sulfates from sea cucumbers

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### Abstract

Fucosylated chondroitin sulfates (FCSs) from sea cucumbers have repetitive structures that exhibit minor structural differences based on the organism from which they are recovered. A detailed characterization of FCSs and their derivatives is important to establish their structure–activity relationship in the development of new anticoagulant drugs. In the current study, online hydrophilic interaction chromatography–Fourier transform mass spectrometry (FTMS) was applied to analyze the FCS oligosaccharides generated by selective degradation from four species of sea cucumbers, *Isostichopus badionotus*, *Pearsonothuria graeffei*, *Holothuria mexicana* and *Acaudina molpadioides*. These depolymerized FCS fragments were quantified and compared using the glycomics software package, GlycReSoft. The quantified fragments mainly had trisaccharide-repeating compositions and showed significant differences in fucosylation (including its sulfation) among different species of sea cucumbers. Detailed analysis of FTMS ion peaks and top-down nuclear magnetic resonance spectroscopy of native FCS polysaccharides verified the accuracy of this method. Thus, a new structural model for FCS chains from these different sea cucumbers was defined. This bottom-up approach provides rich detailed structural analysis and provides quantitative information with high accuracy and reproducibility and should be suitable for the quality control in FCSs as well as their oligosaccharides.

**Key words:** Fucosylated chondroitin sulfates, GlycReSoft, hydrophilic interaction chromatography–Fourier transform mass spectrometry, partial *N*-deacetylation–deaminative cleavage, fucosylation and sulfation

### Introduction

Fucosylated chondroitin sulfates (FCSs) found in the body wall of sea cucumbers have medicinal benefits in hemodialysis (Minamiguchi et al. 1997, 2003), atherosclerosis (Tovar and Mourao 1996; Igarashi et al. 1997), angiogenesis (Tapon-Brethaudiere et al. 2002), fibrosis

(Melo et al. 2010) and tumor growth and inflammation (Borsig et al. 2007; Zhao et al. 2013a; Panagos et al. 2014). FCSs have potent anti-coagulant and antithrombotic activities essentially driven through serine protease inhibitor-dependent and independent mechanisms (Nagase et al. 1995, 1997; Sheehan et al. 2006; Glauser et al. 2008).

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Furthermore, researchers have now focused on FCS oligosaccharides because of their selective inhibition on the intrinsic factor Xase complex in the coagulation pathway and the absence of adverse effects commonly observed in native (intact) FCSs, such as factor XII activation and platelet aggregation (Chen et al. 2013; Zhao et al. 2015; Yan et al. 2017). Thus, oligomeric derivatives have great potential as clinical anticoagulants.

The FCS glycosaminoglycan (GAG) consists of a chondroitin sulfate (CS) backbone composed of alternating  $\beta$ -D-glucuronic acid (GlcA) and *N*-acetyl- $\beta$ -D-galactosamine (GalNAc) disaccharide building blocks, with branches of sulfated  $\alpha$ -L-fucose (Fuc) linked to the 3-position of the GlcA residues of the CS backbone (Vieira and Mourao 1988; Chen et al., 2011). The sulfated Fuc branches are the key moieties responsible for the anticoagulant activity (Mourao et al. 1996; Wu et al. 2012). FCSs of several species of sea cucumbers have already been identified as having similar CS backbones but structurally different fucosylation and sulfation (SO<sub>3</sub>) patterns, such as 2,4-disulfated Fuc (Fuc2,4diS), 4-sulfated Fuc (Fuc4S), nonsulfated Fuc (Fuc0S) or no Fuc branch, depending on the species from which they are obtained (Myron et al. 2014). However, most of these structural analyses of FCS were carried out based on extensive structural studies involving 1D and 2D nuclear magnetic resonance (NMR) spectroscopy and Fourier transform infrared spectroscopy of the intact FCS polysaccharides. This “top-down” approach examines both fucosylation and SO<sub>3</sub> patterns are analyzed (Chen et al. 2013; Liu et al. 2016). However, it can be quite difficult to obtain precise structural information on intact chains and to differentiate the number of Fuc branches and their SO<sub>3</sub> pattern. While monosaccharide compositional analysis can provide the proportion of Fuc branches on the CS backbone, this method is limited by the acid hydrolysis conditions and can be variable due to incomplete hydrolysis or product degradation taking place on hydrolysis (Lv et al. 2009, 2014; Song et al. 2010). Thus, the FCS structures described by different research groups have differed somewhat (Myron et al. 2014; Santos et al. 2017; Soares et al. 2018).

The precise structures of intact polysaccharides or their oligosaccharide derivatives are required to develop the detailed structure–activity relationship needed in new drug development. This has motivated the search for specific degradation methods yielding intact FCS oligosaccharides of well-defined structure and to combine such methods with a flexible system of oligomer analysis, facilitating the determination of the structure of FCS polysaccharides and for the quality control of FCS oligosaccharides.

Enzymatic cleavage of FCS is not feasible for the preparation of well-defined oligosaccharides since the presence of Fuc branches prevents the action of CS lyases or hydrolases on FCS polysaccharides (Mourao et al. 1996). Chemical or physical degradation, relying on acid-catalyzed hydrolysis (Gao et al. 2014), free-radicals (Wu et al. 2010; Yang et al. 2015) and <sup>60</sup>Co irradiation (Wu et al. 2013) can be used to degrade FCS, but in most cases these methods result in the loss Fuc branches and desulfation as they randomly break the polysaccharide chains. Thus, the fragments obtained often do not reflect the original structure of the FCS polysaccharide. In contrast, partial *N*-deacetylation–deaminative cleavage exhibits a high selectivity and can successfully depolymerize FCS resulting in (2,5)-anhydro-D-talose (anTal-ol) residues at newly formed reducing ends without the loss of Fuc branches or desulfation (Wu et al. 2012; Zhao et al. 2015; Yan et al. 2017). Therefore, the resulting oligosaccharide fragments closely reflect the structure of native FCS polysaccharides.

Mass spectrometry (MS) is a powerful technique for structural characterization of complex anionic oligosaccharides, such as low molecular weight heparins (LMWHs) (Li et al. 2014) as well as FCS oligosaccharides (Chen et al. 2013) and provides both high sensitivity and high resolution. Santos et al. used a “bottom-up” analytical strategy to avoid complex NMR analysis, in which the Fuc branches and CS disaccharides obtained by acid hydrolysis and enzymatic cleavage were separately analyzed using disaccharide compositional analysis, NMR and electrospray ionization-high resolution mass spectrometry (ESI-HRMS). While this afforded structural information on the native FCS, desulfation resulting from acid hydrolysis hindered the quantitation of these units and this method was quite cumbersome because of the large number of complicated steps required for hydrolysis, enzymolysis, separation and purification (Santos et al. 2017).

Combining MS with powerful separation techniques can enhance the structural identification of polydisperse sulfated oligosaccharides. Online hydrophilic interaction chromatography (HILIC)–Fourier transform mass spectrometry (FTMS) has been widely applied in the analysis of oligosaccharide fragments of LMWHs generated through digestion with heparin lyases (Li et al. 2014). Bottom-up method profiles all these oligosaccharides and is suitable for the quality control and quality assurance in LMWH production. Li et al. analyzed oligosaccharides of defucosylated FCS from the sea cucumber *Holothuria mexicana* (*Hm*), which had been generated by free radical depolymerization using HILIC-FTMS and interpreted with GlycReSoft 1.0 software. However, the resulting total ion chromatogram (TIC) and the relative quantification results of the major oligosaccharides did not show a typical FCS structure because they had been defucosylation and randomly degraded by free radical depolymerization (Li et al. 2018).

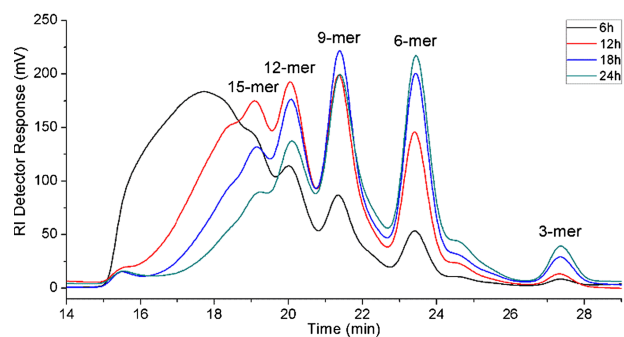
In the present study, we report a bottom-up analysis for the characterization of FCSs from four species of sea cucumbers, *Isotichopus badionotus* (*Ib*), *Pearsonothuria graeffei* (*Pg*), *Hm* and *Acaudina molpadioides* (*Am*). The FCSs from these species were selected because they have been preliminarily described as having well-defined structures, which suggests they consist of similar CS backbones and monosaccharide Fuc branches using NMR analysis, but the number of Fuc branches and their SO<sub>3</sub> patterns still need to be characterized. The method described herein relies on partial *N*-deacetylation–deaminative cleavage combined with HILIC-FTMS analysis. Data analysis was performed using GlycReSoft 1.0 software. The FCS oligosaccharides generated from these four sea cucumber species were quantified and compared to deduce the structures of their intact FCS chains. The bottom-up approach employed here represents an important analytical tool for quality control in the production of FCS and FCS oligosaccharides.

## Results and Discussion

### Depolymerization of FCS by partial *N*-deacetylation–deaminative cleavage

The GalNAc residues in FCS are *N*-acetylated, and treatment of hydrazine hydrate exposes a fraction of the amino groups in these hexosamines making these residues sensitive to selective oxidative cleavage using nitrous acid. After the reduction of the resulting products, a series of trisaccharide-repeating oligosaccharides, L-Fuc- $\alpha$ 1,3-D-GlcA- $\beta$ 1,3-(D-GalNAc- $\beta$ 1,4-[L-Fuc- $\alpha$ 1,3-]D-GlcA- $\beta$ 1,3-)<sub>*n*</sub>-D-anTal-ol (*n* = 0–6) with SO<sub>3</sub> on Fuc, GalNAc and anTal-ol, are produced. This degradation pathway had already been confirmed





**Fig. 1.** HPGPC profiles of depolymerized FCS from sea cucumber *Ib* obtained by different *N*-deacetylation time.

with FCS obtained from several different species of sea cucumbers (Zhao et al. 2013a; Yan et al. 2017). The oligosaccharides obtained by this depolymerization method are well defined and are believed to reflect the structure present in the native FCS chains.

The degree of *N*-deacetylation can be easily adjusted by controlling the reaction times and this decides the molecular weight (Mw) distribution of depolymerized oligosaccharides. The subsequent deaminative cleavage step is rapidly accomplished affording repeatable distributions of oligosaccharides (Zhao et al. 2013a). High performance gel permeation chromatography (HPGPC) profiles of depolymerized FCS from sea cucumber *Ib* obtained by different *N*-deacetylation times were used to investigate the appropriate degradation condition for further analysis of HILIC-FTMS (Figure 1). A 6 h of *N*-deacetylation time was insufficient for the optimal depolymerization of FCS. However, too longer treatment with hydrazine hydrate could result in obvious  $\beta$ -elimination cleavage of FCS as well as heparan sulfate (Guo and Conrad 1989; Yan et al. 2017), which caused significant reduction of GlcA content in saccharide chains under alkaline condition. Since the 12 h of *N*-deacetylation time has resulted in obvious oligomers from 3-mer to 15-mer; this incubation time was optimum for deaminative cleavage and further analysis of HILIC-FTMS.

#### HILIC-FTMS analysis of the depolymerized FCS

HILIC affords good separation of these samples based on the different polarity of their constituent FCS oligosaccharides just as had been previously reported for the analysis of oligosaccharides from LMWHs (Li et al. 2012, 2014). Combined with high resolution and mass accuracy of ESI-FTMS, different FCS trisaccharide-repeating oligosaccharides with nearby fragment peaks from four species of sea cucumbers were well separated and could be identified in their TIC (Figure 2). A short column was only sufficient for separating oligosaccharides of a degree of polymerization (dp) of 2–6 (Li et al. 2014), while a longer column was required to separate oligosaccharides of  $dp \leq 18$ , although these longer chains were detected with much lower sensitivity. These HILIC separations demonstrate favorable run-to-run reproducibility and the TIC of the four species of sea cucumbers analyzed showed very similar oligosaccharide distributions with much better separation (resolution) than obtained using HPGPC. However, there were some obvious differences in the four TICs. The TICs of FCS-*Ib* and FCS-*Hm* showed dominant trisaccharide-repeating oligosaccharides with a small percentage of nearby fragments separated based on their different polarities. The TICs of FCS-*Pg* and FCS-*Am* were less regular, since there were remarkable ion peaks close to those of the trisaccharide-repeating oligosaccharides.

These abundant fragments afford information on a large portion of other compositions; those are different from the trisaccharide-repeating oligosaccharides of the depolymerized products.

The TIC of every species of sea cucumber FCS oligosaccharides contained many ion peaks. Moreover, the mass spectrum of each peak, such as the 6-mer, contained a mixture of multiple components with various compositions and degrees of SO<sub>3</sub> and were too complex to be completely interpreted. For further analysis, the raw data from the HILIC-FTMS were deconvoluted using Decon Tools, and then, the output from Decon Tools was processed by GlycResoft with a hypothesis that each component contained a different number of five moieties, [GlcA, Fuc/anTal-ol, GalNAc, acetyl (AC), SO<sub>3</sub>] that could be used to generate matching structures and to provide quantitative information (Karamanos et al., 2012). The result of the semi-quantitative comparison of the depolymerized FCS oligosaccharides from the four species of sea cucumbers was provided in Figure 3. Typical compositions in depolymerized FCS oligosaccharides were matched using GlycResoft. The majority of these oligosaccharides were no more than 9-mer, although some longer oligosaccharides were also identified. As expected, the sulfated trisaccharide, hexasaccharide, nonasaccharide and other trisaccharide-repeating oligosaccharides were the major components in each sample, as suggested by the results of HPGPC profiles and TICs.

However, there were many differences among the GlycResoft matched results from the four examined species. Depolymerized FCS-*Ib* (Figure 3a) was mainly composed of trisaccharide-repeating oligosaccharides, including trisaccharides, hexasaccharides and nonasaccharides, each having different degrees of sulfation. For example, according to the hypothesis, the most significant composition [1, 2, 0, 0, 4] includes a GlcA residue, two Fuc/anTal-ol residues (actually one Fuc residue and one anTal-ol residue), a GalNAc residue, no additional AC groups and four sulfate groups in this fragment. Thus, this composition could be interpreted as a chain of L-Fuc2,4diS- $\alpha$ 1,3-D-GlcA- $\beta$ 1,3-D-anTal-ol4,6diS (Chen et al. 2013; Zhao et al. 2015; Yan et al. 2017). Similarly, composition [2, 3, 1, 0, 8] including two GlcA residues, three Fuc/anTal-ol residues (actually two Fuc residues and one anTal-ol residue), two GalNAc residues, no additional AC groups and eight sulfate groups, corresponds to L-Fuc2,4diS- $\alpha$ 1,3-D-GlcA- $\beta$ 1,3-D-GalNAc4,6diS- $\beta$ 1,4-[D-Fuc2,4diS- $\alpha$ 1,3]-D-GlcA- $\beta$ 1,3-D-anTal-ol4,6diS. Similarly, compositions such as [2, 3, 1, 0, 4] and [2, 3, 1, 0, 7] corresponded to hexasaccharides with partial monosulfated or unsulfated Fuc and GalNAc/anTal-ol residues. Since the compositions of [2, 3, 1, 0, 8] and [3, 4, 2, 0, 12] accounted for most of the hexasaccharides and nonasaccharides, respectively, there was only a small portion of partial monosulfated or nonsulfated oligosaccharides in the FCS-*Ib* oligosaccharides.

Fragments of depolymerized FCS-*Pg* (Figure 3b) showed significant difference from those of depolymerized FCS-*Ib*. The most obvious difference was that depolymerized FCS-*Pg* contained a large portion of nonfucosylated or partial nonfucosylated oligosaccharides. For instance, the composition of [1, 1, 0, 0, 2] lacks the disulfated Fuc branch presence in the composition of [1, 2, 0, 0, 4], and similarly the difference between compositions of [2, 2, 1, 0, 6] and [2, 3, 1, 0, 8], compositions of [3, 3, 2, 0, 10] and [3, 4, 2, 0, 12] and compositions of [4, 4, 3, 0, 14] and [4, 5, 3, 0, 16], respectively. These data clearly demonstrate that the polysaccharide chains of FCS-*Pg* contain some nonfucosylated positions. In addition, the oligosaccharides of depolymerized FCS-*Pg* had a higher proportion of monosulfated Fuc branches than those of depolymerized FCS-*Ib* as seen by comparing the relative intensity of their compositions of [1, 2, 0, 0, 3] and [1, 2, 0, 0, 4], compositions of [2, 3, 1, 0, 7] and [2, 3, 1, 0, 8].

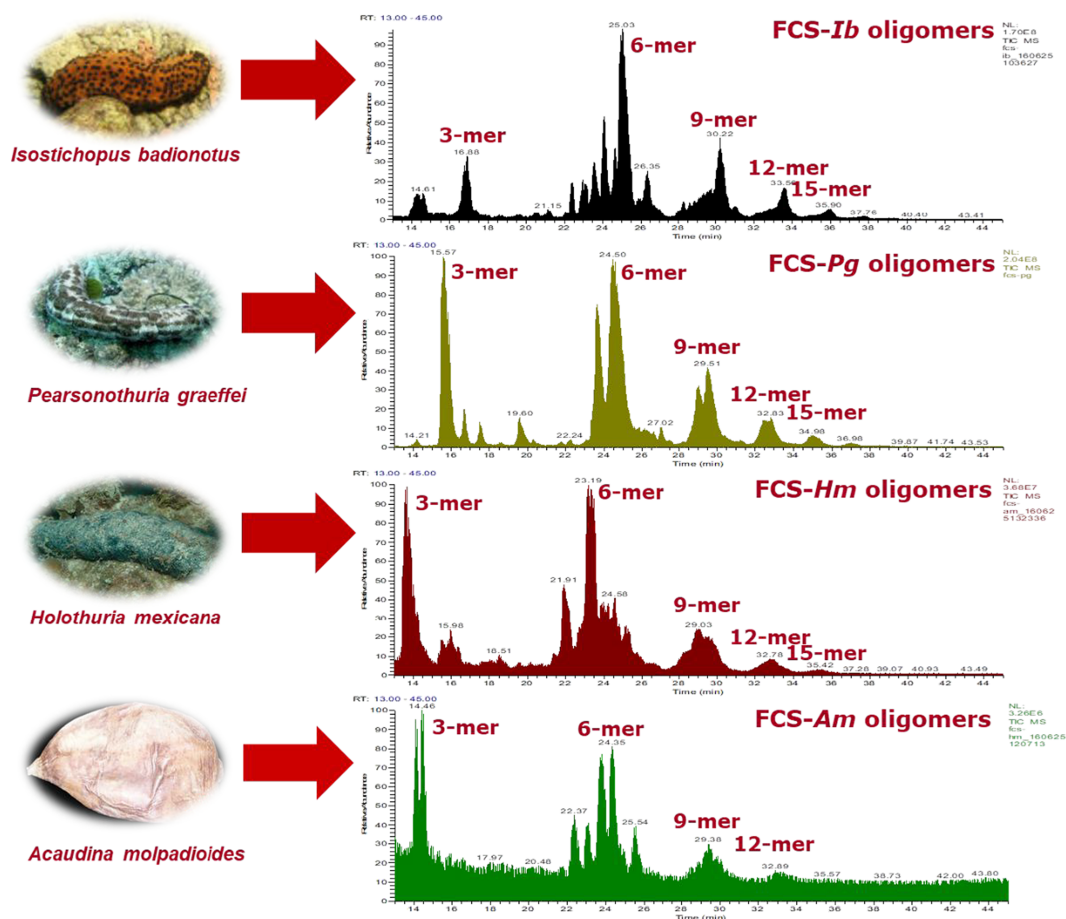


Fig. 2. TICs of depolymerized FCS by partial *N*-deacetylation–deaminative cleavage from four species of sea cucumbers profiled by HILIC-FTMS.

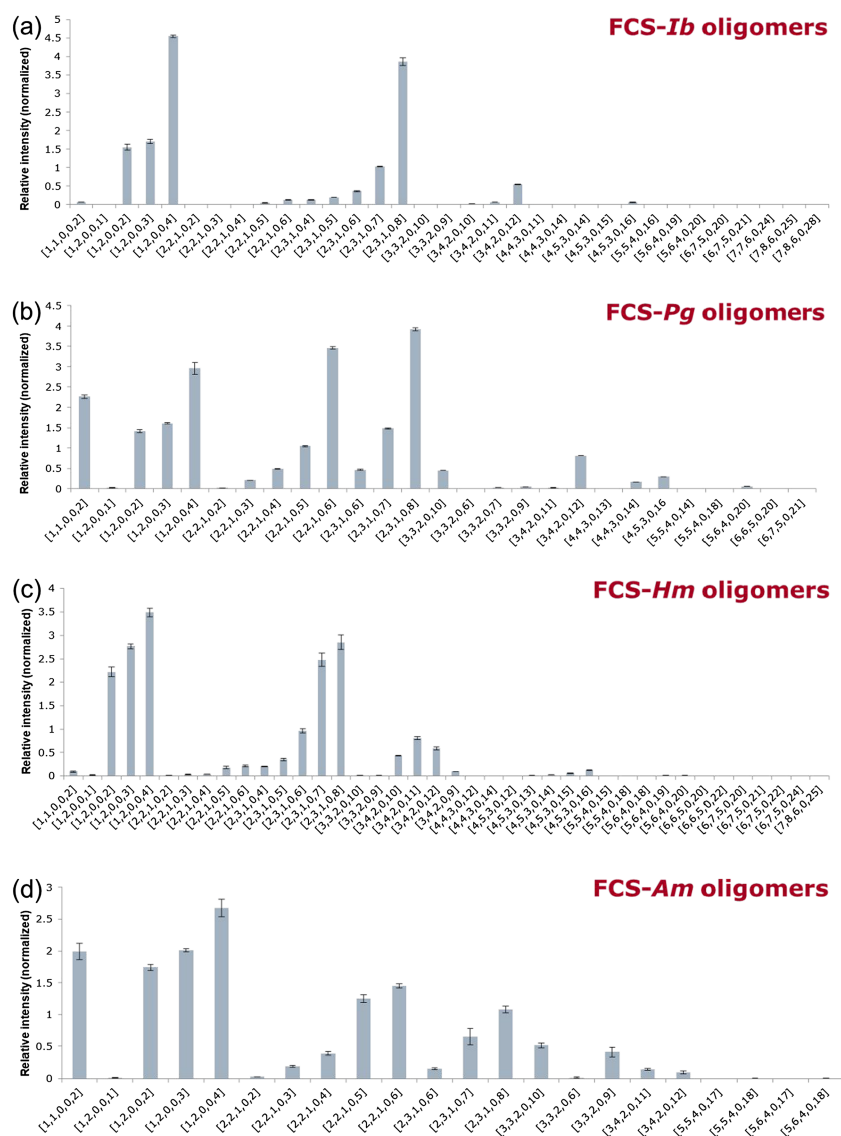
These data are consistent with the previous conclusion supported by NMR analysis that there is approximately 8% higher proportion of monosulfated Fuc branches in FCS-Pg than FCS-Ib polysaccharides (Chen et al. 2013; Soares et al. 2018).

Oligosaccharides in depolymerized FCS-Hm and depolymerized FCS-Ib were rarely nonfucosylated or partially nonfucosylated structures, since there were almost always compositions of [1, 2, 0, 0, X] (X = 1–4), compositions of [2, 3, 1, 0, Y] (Y = 4–8) and compositions of [3, 4, 2, 0, Z] (Z = 9–12), corresponding to trisaccharides, hexasaccharides and nonasaccharides, respectively. However, oligosaccharides in depolymerized FCS-Hm had more partial monosulfated or nonsulfated Fuc (or GalNAc/anTal-ol) on oligosaccharides such as the compositions of [1, 2, 0, 0, 3], [2, 3, 1, 0, 7] and [3, 4, 2, 0, 11] than those found in depolymerized FCS-Ib. These results are again consistent with previous studies by NMR showing that FCS-Hm polysaccharides contain considerable amounts of monosulfated or nonsulfated Fuc branches (Mou et al. 2017).

The matched compositions in depolymerized FCS-Am were similar to those in depolymerized FCS-Pg. First, there were obvious nonfucosylated or partially nonfucosylated compositions such as [1, 1, 0, 0, 2], [2, 2, 1, 0, 6], [3, 3, 2, 0, 10] in the oligosaccharides of depolymerized FCS-Am. Meanwhile, partial monosulfated or nonsulfated portions were quite common in the depolymerized FCS products from this species of sea cucumber because there were noticeable partially monosulfated or nonsulfated Fuc branches in the polysaccharide chains (Dong et al. 2014).

The major compositions in the TICs, including 3–12-mer, of depolymerized FCS from the four sea cucumber species were analyzed to investigate the accuracy of the GlycResoft matching. The high-resolution FTMS spectra with matched compositions of the 6-mer group (hexasaccharide with nearby saccharides) were shown in Figure 4. This group of ion peaks afforded abundant information with remarkably strong signals in each TIC (Figure 2). It has been reported previously that trisaccharide-repeating residue, L-Fuc2/3,4diS- $\alpha$ 1,3-D-GlcA- $\beta$ 1,3-D-GalNAc4,6diS, is the most abundant residue in the FCS polysaccharides of these four sea cucumber species (Chen et al. 2013; Dong et al. 2014). Furthermore, it is known that D-GalNAc4,6diS is transformed into D-anTal-ol4,6diS at new reducing ends under partial *N*-deacetylation–deaminative cleavage (Zhao et al. 2013a). Meanwhile, prior tandem MS and NMR measurements have respectively well explained the pattern of sulfation in the Fuc residues for these four species of FCS from oligosaccharides or polysaccharides (Chen et al. 2011; Dong et al. 2014; Mou et al. 2017; Agyekum et al. 2018). Based on these understandings, the ions information of the most abundant 6-mer group from four species of depolymerized FCS were matched for composition while using symbol “M” as a intact hexasaccharide, L-Fuc2/3,4diS- $\alpha$ 1,3-D-GlcA- $\beta$ 1,3-D-GalNAc4,6diS- $\beta$ 1,4-[D-Fuc2/3,4diS- $\alpha$ 1,3-D-GlcA- $\beta$ 1,3-D-anTal-ol4,6diS.

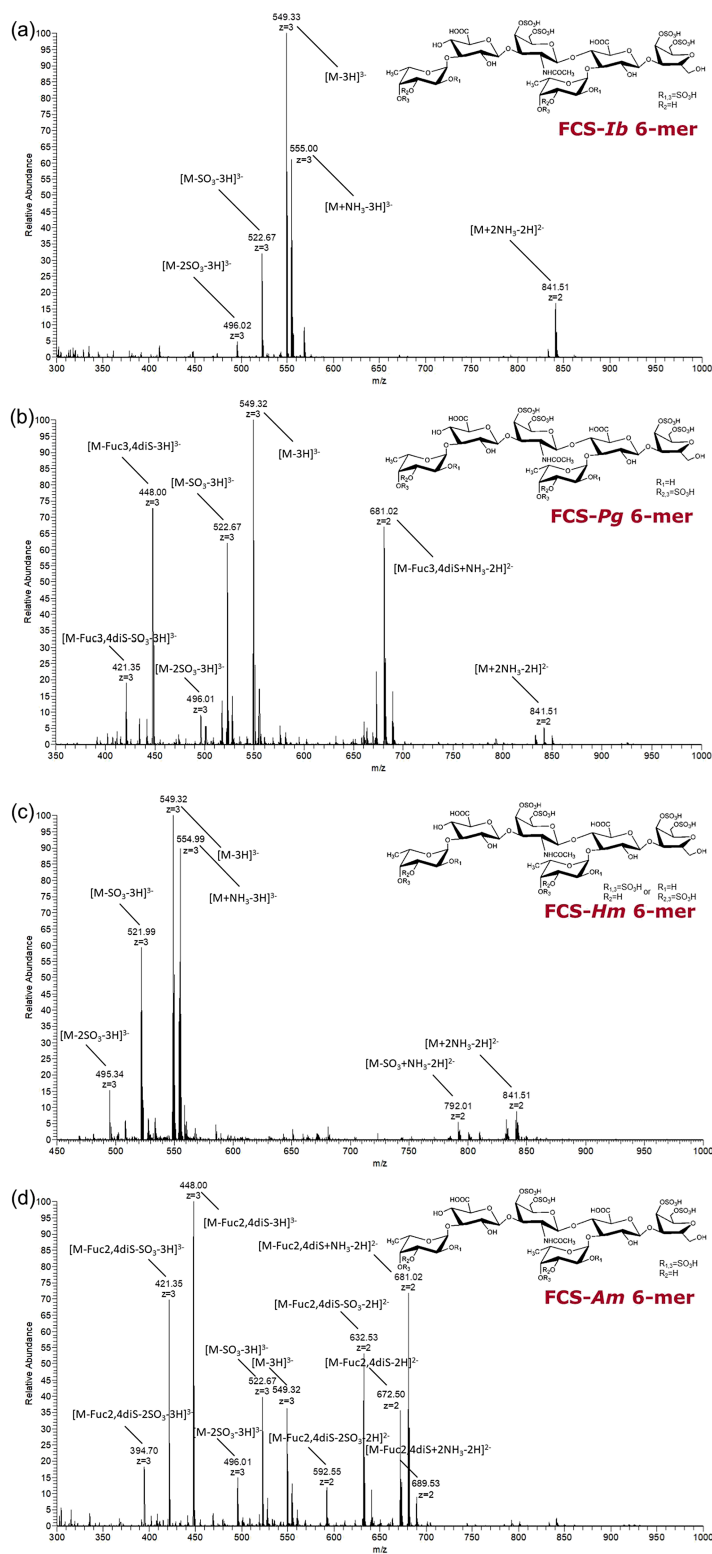
MS and tandem MS data acquired for the same original dp 6 of both FCS-Ib and FCS-Pg have been reported (Agyekum et al. 2018), where fully deprotonated ion as well as extensive cross-



**Fig. 3.** Typical oligosaccharide composition quantification of depolymerized FCS from four species of sea cucumbers calculated by GlycResoft. Oligomer composition plotted on the x-axis was given as [GlcA, Fuc/anTal-ol, GalNAc, Ac (acetyl), SO<sub>3</sub> (sulfation)] (e.g. [1,2,0,0,4] means 1 GlcA, 2 Fuc/anTal-ol, 0 GalNAc, 0 Ac, 4 SO<sub>3</sub>).

ring fragmentations were observed to confirm the presence of the GlcA units, Fuc branches with sulfate groups and di-sulfated monosaccharide GalNAc residues. So, it could be regarded as a standard for matching objective ions in the current TICs. Consequently, the most abundant oligosaccharides, partially nonfucosylated oligosaccharides and partially nonsulfated-Fuc oligosaccharides from these 6-mer groups, matched exactly. While these results vary somewhat among the depolymerized FCS from different sea cucumber species, they were matched those from GlycResoft. Ions of  $m/z$  549.33 [M-3H]<sup>3-</sup>,  $m/z$  555.00 [M+NH<sub>3</sub>-3H]<sup>3-</sup> and  $m/z$  841.51 [M+2NH<sub>3</sub>-2H]<sup>2-</sup> corresponded exactly to the charged abundant hexasaccharides with several ionized protons and probable ammonium adducts. These ions corresponded to all of the major FCS-Ib 6-mers as shown in Figure 4a, also reflecting that FCS-Ib polysaccharides contain major disulfated Fuc branches. The ions of the FCS-Hm 6-mer group shown in Figure 4c compared well with those that of FCS-Ib 6-mer group. There were significantly

more ions of  $m/z$  521.99 [M-SO<sub>3</sub>-3H]<sup>3-</sup> and  $m/z$  495.34 [M-2SO<sub>3</sub>-3H]<sup>3-</sup> corresponding to charged partial monosulfated or nonsulfated Fuc in the hexasaccharides, which is in accordance with the result of matched compositions provided using GlycResoft. The ions of FCS-Pg and FCS-Am 6-mer groups were much more complex. As shown in Figure 4b and d, there were obvious ions of  $m/z$  421.35 [M-Fuc2/3,4diS-SO<sub>3</sub>-3H]<sup>3-</sup>,  $m/z$  448.00 [M-Fuc2/3,4diS-3H]<sup>3-</sup>,  $m/z$  672.50 [M-Fuc2,4diS-2H]<sup>2-</sup>,  $m/z$  681.02 [M-Fuc2/3,4diS+NH<sub>3</sub>-2H]<sup>2-</sup> and  $m/z$  689.53 [M-Fuc2,4diS+2NH<sub>3</sub>-2H]<sup>2-</sup>, which exactly matched charged partially nonfucosylated oligosaccharides. Meanwhile, ions matching nonfucosylated oligosaccharides in the FCS-Am 6-mer group occupied much higher proportion of the total mixture than those in FCS-Pg 6-mer group. These results are consistent with those matched by GlycResoft in both quality and quantity. Additionally, in previous structural elucidation of the same original FCS-Ib and FCS-Pg 6-mer by using tandem MS, revealed cross-ring products <sup>0,2</sup>X<sub>2αf</sub> was diagnostic for 2,4-disulfated Fuc while



**Fig. 4.** High-resolution FTMS spectrograms of the 6-mer group (hexasaccharide with nearby saccharides) with matched structures of depolymerized FCS by partial *N*-deacetylation–deaminative cleavage from four species of sea cucumbers. The patterns of sulfation in the fucose residues are based on literature (Chen et al. 2011; Dong et al. 2014; Mou et al. 2017; Agyekum et al. 2018) and combined with <sup>1</sup>H NMR spectra analysis of native FCS in Figure 5.

revealed cross-ring products  ${}^{0,2}A_{\alpha f}$  and  ${}^{2,4}X_{2\alpha f}$  were diagnostic for 3,4-disulfated Fuc (Agyekum et al. 2018). Meanwhile, NMR measurements of FCS-*Hm* and FCS-*Am* polysaccharides also guided

the pattern of sulfation in the Fuc residues of their oligosaccharides (Dong et al. 2014; Mou et al. 2017). Thus, we also marked the pattern of sulfation in the Fuc residues in Figure 4.

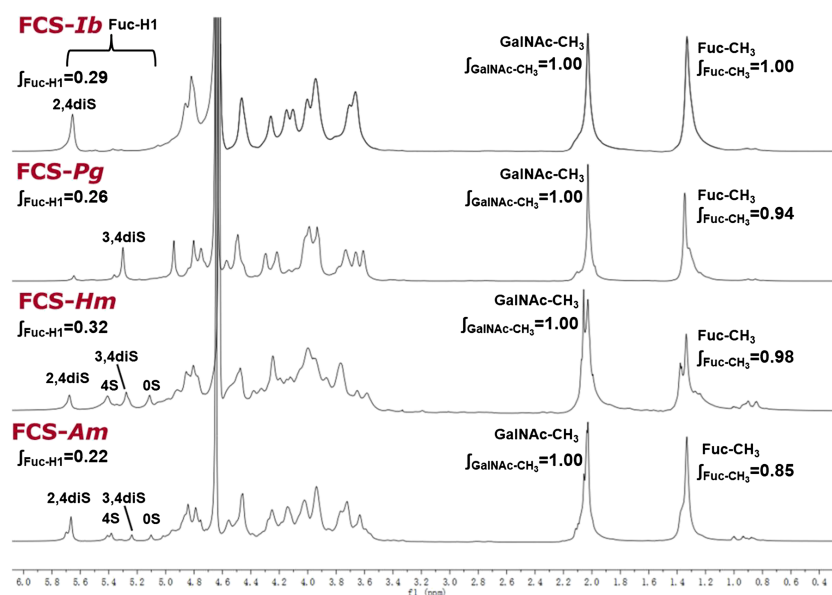


Fig. 5.  $^1\text{H}$  NMR spectra analysis of native FCS from four species of sea cucumbers.

FCS	3-mer	5-mer	6-mer	8-mer	9-mer	11-mer	12-mer
FCS-Ib	✓		✓		✓		✓
FCS-Pg	✓	✓	✓	✓	✓	✓	✓
FCS-Hm	✓		✓		✓		✓
FCS-Am	✓	✓	✓	✓	✓	✓	✓
Definition of FCS structure	Unreasonable one		Reasonable one				

Fig. 6. New model structure definition of FCS polysaccharide chains from four species of sea cucumbers.

High-resolution FTMS spectra of the 3-mer, 9-mer and 12-mer groups with matched compositions of depolymerized FCS coming from four species of sea cucumbers (Supplementary Figure S1) also showed that FCS-Ib oligosaccharides mainly contained disulfated Fuc branches, FCS-Pg oligosaccharides contained a remarkable portion of partial nonfucosylation and partially monosulfated or nonsulfated Fuc branches, FCS-Hm oligosaccharides contained partially disulfated Fuc branches and partially monosulfated or nonsulfated Fuc branches and FCS-Am oligosaccharides contained obvious amount of partial nonfucosylation and partially monosulfated or nonsulfated Fuc branches. These results demonstrate the accuracy of analysis by GlycResoft.

### NMR spectral (top-down) analysis of native (intact) FCSs

The  $^1\text{H}$  NMR spectra of intact FCS from four species of sea cucumbers were obtained to further verify the accuracy of the results obtained using GlycResoft with this hypothesis and the key signals were identified (Figure 5). First, the sulfation patterns of Fuc branches of each native FCS could be easily identified according to signals for the anomeric protons at 5.05–5.80 ppm (as labeled) according to previous report (Chen et al. 2011; Dong et al. 2014; Mou et al. 2017). These were consistent with those identified as having partial monosulfated and nonsulfated Fuc on oligosaccharides by GlycResoft. Signals of Fuc-H1 as well as GalNAc-CH<sub>3</sub> and Fuc-CH<sub>3</sub> were



integrated in each  $^1\text{H}$  spectrum with the GalNAc-CH<sub>3</sub> peak area set as 1.00 to determine the degree of nonfucosylation for each native FCS. In the NMR spectra of FCS-*Ib* and FCS-*Hm*, the ratio of signal intensity for Fuc-H1, GalNAc-CH<sub>3</sub> and Fuc-CH<sub>3</sub> were 0.29: 1.00: 1.00 and 0.32: 1.00: 0.98, respectively, exactly reflecting the trisaccharide-repeating unit of these polysaccharides. However, the signal intensities of Fuc-H1 and Fuc-CH<sub>3</sub> in FCS-*Pg* and FCS-*Am* were markedly weaker, reflecting the partial nonfucosylation in polysaccharide chains. Thus, strengths of these signals fit the partial nonfucosylation of depolymerized FCS oligosaccharides matched by GlycResoft.

### New model structure definition of FCS polysaccharide chains

The main fragments based on the selective depolymerization of FCS polysaccharides with accurate matching of their oligosaccharides by GlycResoft for each species of sea cucumber are presented in Figure 6. The building blocks of these FCS fragments from these four sea cucumber species provide a new structural model for these polysaccharides. FCS-*Ib* polysaccharides consist of a CS backbone consisting of alternating  $\beta$ -D-GlcA and 4,6-disulfated *N*-acetyl- $\beta$ -D-GalNAc disaccharide building blocks with almost all branches corresponding to 2,4-disulfated  $\alpha$ -L-Fuc linked to the 3-position of GlcA residues of the CS core. The other three species of FCS chains had almost the same backbones. However, FCS-*Pg* polysaccharide chains lacked some Fuc branches on the CS core, and their Fuc branches mostly consisted of 3,4-disulfated patterns. FCS-*Hm* polysaccharide chains contained almost full Fuc branches with 2,4-disulfated, 4-sulfated and nonsulfated patterns on the CS backbone. FCS-*Am* polysaccharide chains showed the most complex Fuc branches including a lack of some Fuc branches on the CS backbone and various sulfation patterns on these Fuc branches. Thus, we conclude that the repeating trisaccharide unit with a possible deletion of Fuc branches corresponds to the structure motif of all of these FCS polysaccharides. A model structure of disaccharide building blocks with Fuc or Fuc disaccharide branches is, therefore, unreasonable for these FCSs. Thus, this new structural definition for the FCS polysaccharide chains provides a more precise structure and represents a significant advance in developing structure–activity relationships required for new drug development.

### Conclusion

In the present study, we establish a bottom-up analysis for complex FCS polysaccharides relying on selective depolymerization and using HILIC-FTMS with matching by GlycResoft. The accuracy of this method was verified by detailed analysis of FTMS ion peaks and through the top-down NMR spectroscopy of native (intact) FCS polysaccharides. Based on our bottom-up analysis of FCS oligosaccharides, many structural details, such as partial nonfucosylation and partially monosulfated and nonsulfated Fuc branch among the FCS oligosaccharides of different sea cucumber species, were examined. This method represents a fast and accurate way to analyze the precise structure of FCS polysaccharide chains from different sea cucumber species and provides the foundation for structure–activity research and new drug development. In addition, our method is suitable for quality control of FCS oligosaccharides being developed as potential anticoagulant and antithrombotic agents. This strategy for structure analysis will also be meaningful for other marine polysaccharides.

## Materials and methods

### Materials

Native FCS-*Ib*, FCS-*Pg*, FCS-*Hm* and FCS-*Am* were isolated and purified from the sea cucumbers *Ib*, *Pg*, *Hm* and *Am*, respectively. Isolation and purification of these polysaccharides were performed as previously described (Li et al. 2016). Hydrazine hydrate (containing about 64 wt.% hydrazine in water) and hydrazine sulfate were obtained from Aladdin Reagent (Shanghai, China). All other chemicals and reagents were of analytical grade.

### Partial *N*-deacetylation of FCS

Partial *N*-deacetylation of FCS was performed using our previously described method (Yan et al. 2017). Briefly, dried FCS (100 mg) and 1.50 mL hydrazine hydrate containing 1% hydrazine sulfate were added in a reaction tube. The tube was sealed and incubated at 90°C for 12 h on a magnetic stirrer at 250 rpm. After the reaction, the solution was added to ethanol (quadruple the solution volume). When several drops of saturated sodium chloride were added, a white precipitate formed. The precipitate was collected by centrifugation and dissolved in distilled water. This precipitation and dissolution procedure was repeated four times to remove the hydrazine and hydrazine sulfate. The resulting solution was dialyzed against flowing tap water for 2 days and distilled water for 1 day with a 3500 Da Mw cut-off membrane and subsequently lyophilized. Experiments with different reaction time were performed to optimize the reaction conditions.

### Deaminative cleavage of partial *N*-deacetylated FCS

The deaminative cleavage was based on our previously described method (Yan et al. 2017). The nitrous acid reagent was prepared by mixture of 0.5 M H<sub>2</sub>SO<sub>4</sub> and 5.5 M NaNO<sub>2</sub> at volume ratio of 3:5. In brief, 1 mL ice cold 20 mg/mL partially *N*-deacetylated FCS solution was added to 2 mL of pre-cooled nitrous acid reagent in a reaction tube. The reaction was performed for 10 min in an ice bath, and the excess nitrous acid was neutralized to pH 7 by addition of 0.5 M NaOH. Immediately, 150  $\mu$ L 300 mg/mL NaBH<sub>4</sub> (dissolved in 0.05 M NaOH) was added and reduced at 50°C for 2 h. Finally, the sample was acidified to pH 7.0 and dialyzed with a 500 Da Mw cut-off and lyophilized as described above.

### Profiles of the depolymerized FCS by HPGPC and HILIC-FTMS

HPGPC profiles of depolymerized FCS samples dissolved at 3 mg/mL in pure water were obtained using a Superdex Peptide 10/300 GL column (10  $\times$  300 mm) eluted by 0.2 M NaCl at the flow rate 0.5 mL/min monitored with a refractive index detector.

Depolymerized FCS samples were dissolved in 50% acetonitrile at 1 mg/mL for HILIC-FTMS analysis, which was performed on an Agilent 1290 LC UPLC system (Agilent Technologies, Wilmington, DE) equipped with a LTQ ORBITRAP XL mass spectrometer (Thermo, SCIENTIFIC). The FCS oligosaccharides were separated by a Luna HILIC column (150  $\times$  2.00 mm, 3  $\mu$ m, Phenomenex) at 25°C. The mobile phase was a mixture of 5 mM NH<sub>4</sub>OAc/98% acetonitrile (solvent A) and 5 mM NH<sub>4</sub>OAc/H<sub>2</sub>O (solvent B) at a flow rate of 150  $\mu$ L/min. The gradient was programmed as 92% A in the beginning, linearly changed to 60% A in 58 min. The analysis was performed in the negative-ion mode using a capillary temperature of 275°C. The spray voltage was 4.2 kV and nitrogen dry gas flowed

at 40 L/min. Data acquisition and analysis were performed using Xcalibur 2.0 software and GlycReSoft 1.0 software.

### NMR spectral analysis

For NMR spectrum analysis, samples (30 mg) were dissolved in 500  $\mu$ L of D<sub>2</sub>O (99.9%) and lyophilized three times to substitute the exchangeable protons and then dissolved in 500  $\mu$ L D<sub>2</sub>O, and finally transferred to NMR microtubes and performed on a Hudson-Bruker SB 600 MHz Spectrometer (Madison, WI) at room temperature.

### Supplementary data

Supplementary data are available at *Glycobiology* online.

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### Conflict of interest statement

None declared.

### Abbreviations

Ac, acetyl; Am, *Acaudina molpadioides*; anTal-ol, (2,5)-anhydrotalose; CS, chondroitin sulfate; dp, degree of polymerization; ESI-HRMS, electrospray ionization-high resolution mass spectrometry; FCS, fucosylated chondroitin sulfate; FT-IR, Fourier transform infrared; FTMS, Fourier transform mass spectrometry; Fuc, fucose; GAG, glycosaminoglycan; GalNAc, galactosamine; GlcA, glucuronic acid; HILIC, hydrophilic interaction chromatography; Hm, *Holothuria Mexicana*; HPGPC, high performance gel permeation chromatography; Ib, *Isostichopus badiionotus*; LMWHs, low molecular weight heparins; mer, oligomer; MS, mass spectrometry; Mw, molecular weight; NMR, nuclear magnetic resonance; oligomer composition was given as [GlcA, Fuc/anTal-ol, GalNAc, Ac, SO<sub>3</sub>] (e.g. [1,2,0,0,4] means 1 GlcA, 2 Fuc/anTal-ol, 0 GalNAc, 0 Ac, 4 SO<sub>3</sub>); Pg, *Pearsonothuria graeffei*; sulfate, S; SO<sub>3</sub>, sulfation; TIC, total ion chromatogram

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