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Structure and anticoagulant activity of fucosylated glycosaminoglycan degraded by deaminative cleavage

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ABSTRACT

Fucosylated glycosaminoglycans (FGs) are complex glycosaminoglycans that exhibit potent anticoagulant activity. To study the relationship between molecular size and biological activity, oligosaccharides with (2,5)-anhydro-D-talose units at new reducing ends were prepared by hydrazine deacetylation and nitrous acid depolymerization. The product chemical structures were analyzed by one- and two-dimensional NMR methods. Additionally, anticoagulant activities were evaluated by clotting assay and chromogenic substrate cleavage. The results demonstrated that under mild deacetylation and deaminative cleavage conditions, both products were relatively homogeneous and sulfated fucose branch types and sulfate substituents remained stable. These depolymerized FGs with different molecular sizes had potent intrinsic anticoagulant activities, which were similar to those that were obtained by free-radical depolymerization with similar molecular weights. Decreasing molecular weight may weaken activity but not significantly affect factor Xase and heparin cofactor II (HCII)-mediated thrombin inhibition.

1. Introduction

Fucosylated glycosaminoglycans (FGs) extracted from sea cucumbers have a chondroitin sulfate E-like structure that contains large numbers of sulfated α-L-fucopyranose branches linked to β-D-glucuronic acid residue position 3 (Mourão et al., 1996; Wu, Xu, Zhao, Kang, & Ding, 2010a, 2010b; Wu et al., 2012; Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992), while the FG fucose branches may have distinguishable sulfate substitution patterns and proportions (Wu et al., 2012). Chondroitin sulfate derivatives have high anticoagulant and antithrombotic properties (Buyue & Sheehan, 2009; Sheehan & Walke, 2006). However, FGs also exhibit undesirable effects such as inducing factor XII activation and platelet aggregation (Fonseca et al., 2010; Li & Lian, 1988). To minimize side effects, its low molecular weight derivative depolymerized FG was prepared, which retained antithrombotic and anticoagulant activities without causing platelet aggregation in human platelet-rich plasma (Suzuki, Kitazato, Takamatsu, & Saito, 1991; Yoshida et al., 1992). Furthermore, pharmacological research has indicated that the depolymerized product and its native FG share common mechanisms for factor tenase inhibition in the intrinsic pathway and thrombin inhibition by activating heparin cofactor II (HCII) (Buyue & Sheehan, 2009; Sheehan & Walke, 2006). The literature and our preliminary studies also confirmed that low-molecular-weight fragments exhibit a better antithrombotic–hemorrhagic ratio than unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) (Kitazato, Kitazato, Sasaki, Minamiguchi, & Nagase, 2003; Sheehan & Walke, 2006; Wu et al., 2010b). Therefore, to further study the relationship between molecular size and FG biological activity, depolymerized derivatives with different structural characteristics should be prepared.

Based on these facts, searching for a proper protocol to efficiently prepare depolymerized FGs may be necessary. However, a limited number of techniques for molecular reduction have

Abbreviations: GAG, glycosaminoglycan; FG, fucosylated glycosaminoglycan; DFG, depolymerized fucosylated glycosaminoglycan; UFH, unfractionated heparin; LMWH, low-molecular-weight heparin; DD, deacetylation degree; GalNAc4S6S, 4,6-O-disulfated N-acetylgalactosamine; GlcUA, glucuronic acid; GalN, galactosamine; anTal, anhydro-D-talose; Fuc3S, 3-O-sulfated fucose; Fuc4S, 4-O-sulfated fucose; Fuc2S4S, 2,4-O-disulfated fucose; APTT, activated partial thromboplastin time; HCII, heparin cofactor II; EC_{50}, half maximal effective concentration.

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been described. Unlike heparin and chondroitin sulfate, FG cannot be directly digested by polysaccharide degrading enzymes such as heparin lyases or chondroitin ABC lyase, possibly because their sulfated fucose branches may be linked to position 3 of GlcA residues (Luo et al., 2013; Mourão et al., 1996). Free radical depolymerization is the first commonly used method for degraded sample preparation (Kitazato, Kitazato, Nagase, & Minamiguchi, 1996; Suzuki et al., 1991). Recently, the ^{60}Co irradiation method was reportedly used to prepare depolymerized FG with a M_w between 2.5 and 7.6 kDa (Wu et al., 2013). In our previous study, we developed hydrogen peroxide-mediated free radical FG depolymerization in the presence of cupric ion (Wu et al., 2010a), which produced FG fragments with no obvious sulfate or fucose branch loss. These preparation processes may produce different products with different structures, molecular weights, anticoagulant activities, and pharmacological properties. Thus, a need still remains to develop efficient and different methods to prepare low-molecular-weight fragments for chemical structure analysis and structure–activity relationship research. Here, we investigated the application of deaminative cleavage for preparation of depolymerized FG (DFG).

Deaminative cleavage with nitrous acid is a successful chemical method for heparin and heparan sulfate depolymerization because of its advantages such as high selectivity, mild depolymerization conditions and no preferential side chain cleavage (Petitou, Casu, & Lindahl, 2003). This method has been used for LMWH production such as dalteparin, nadroparin and reviparin (Linhardt & Gunay, 1999). However, because the hexosamines in FG are all N-acetylated hexosamine residues, FG cannot be directly depolymerized by nitrous acid. To apply the deaminative cleavage method to prepare depolymerized FG, it may be necessary to partially remove the FG acetyl group. Hydrazinolysis was a commonly used method for glycosaminoglycan (GAG) deacetylation such as chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) (Guo & Conrad, 1989; Shaklee & Conrad, 1984). Because of these fucose branches, the FG structure is more complex than CS, DS and HS structures. Therefore, the current work sought to investigate the deacetylation and deaminative cleavage for FG depolymerization and to characterize the product structures and anticoagulant activities.

2. Experimental design

2.1. Materials

FG (purity: 99.9%, average molecular weight: 69.930 kDa, sulfate/carboxyl groups: 3.54) was isolated and purified from the sea cucumber *Thelenota ananas*. Isolation, purification and characterization of this glycosaminoglycan were performed as previously described (Wu et al., 2010a, 2010b). Hydrazine hydrate (containing about 64 wt.% hydrazine in water) was obtained from Aladdin Reagent (Shanghai, China). Anhydrous hydrazine was obtained from hydrazine hydrate according to the method of Takasaki, Mizuochi, and Kobata (1982). Hydrazine sulfate and sodium nitrite were purchased from DamaoChem., Ltd. (Tianjin, China). Deuterium oxide (D_2O) containing 0.05 wt.% trimethylsilyl-propionic acid (TSP) sodium salt was obtained from Sigma (St. Louis, MO, USA). Activated partial thromboplastin time (APTT) assay kits (Lot No. 10917752) and Tris–HCl (Lot No. 20110726) were obtained from TECO Medical Instruments Co., Germany and Amresco Co., USA, respectively. A chromogenic assay kit for measuring factor VIII: C in concentrates (Lot No. 12101-PK:11) and HCII assay reagents including HCII (Lot No. 09041F), human thrombin IIa (Lot No. 100628A) and thrombin chromogenic substrate (Lot No. 11204-1-PK:1) were purchased from Hyphen Biomed (France). Human coagulation factor VIII was obtained from Shanghai RAAS Blood Products, Ltd., China. All of the other chemicals and reagents used were of analytical grade.

2.2. FG deacetylation

FG deacetylation was performed using Fukuda's modified method that has been described previously (Fukuda, Kondo, & Osawa, 1976; Shaklee & Conrad, 1984). Briefly, dried FG (60 mg) and 1.45 ml hydrazine hydrate containing 1% hydrazine sulfate were added in a reaction tube. The tube was flushed with nitrogen, 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 was formed. The precipitate was collected by centrifugation and dissolved in distilled water. This precipitation and dissolution procedure was repeated 4 times to remove the hydrazine and hydrazine sulfate. The resulting solution was dialyzed against flowing tap water for 2 d and distilled water for 1 d with a 3500 Da molecular weight cut-off and subsequently lyophilized.

To optimize the reaction conditions, a series of experiments were performed based on the above representative protocol with varied reaction parameters (time, catalyst type, temperature and concentration of hydrazine), and each experiment was performed at least in duplicate. In the protocol, the sample deacetylation degree (DD) was calculated using the two methyl peak area ratios that were approximately 1.36 and 2.06 ppm in the ^{1}H NMR spectrum, which were from the N-acetylgalactosamine (GalNAc) methyl group and from the fucose sulfate methyl group, respectively. The N-deacetylated FG yield was calculated using the N-deacetylated and native FG sample weights.

2.3. Deaminative FG cleavage

The deaminative cleavage protocol was designed according to the literature (Bienkowski & Conrad, 1985; Shively & Conrad, 1976). The pH 4 nitrous acid reagent was prepared by addition of 0.5 M H_2SO_4 to 5.5 M $NaNO_2$ until pH 4 was reached. In total, 1 ml ice-cold 20 mg/ml N-deacetylated FG solution was added to 2 ml pre-cooling nitrous acid reagent in a reaction tube. The reaction was performed for different times in an ice bath, and the excess nitrous acid was destroyed by addition of 0.5 M NaOH until pH 8 was reached. Finally, the sample was dialyzed with a 1000 Da molecular weight cut-off and lyophilized as described above.

Different reaction times (4, 6, 8, 10, 15, 20, 25, 30 min) and reaction temperatures (room temperature and an ice bath) were investigated using 35% N-deacetylated FG according to the above representative protocol. The deaminative cleavage protocol was then investigated using native FG (M_w 69.930 kDa) and the depolymerized sample (M_w 13.710 kDa) by free radical method, and DFG samples of different M_w (5021–22240 Da) were prepared using deaminative cleavage for structure and activity measurements.

2.4. NMR analysis

NMR analyses were performed at 300 K with a 500 MHz Bruker Advance DRX 500 spectrometer (Bruker BioSpin GmbH) that had been equipped with a ^{13}C/^{1}H dual probe in the FT mode as described previously (Gao et al., 2012). The native, N-deacetylated and depolymerized FG samples were dissolved in deuterium oxide (99.9% D) at 10–20 mg/ml and lyophilized thrice to replace the exchangeable protons with deuterium.

Table 1
Effects of different reaction conditions on deacetylation degree and deacetylated product physicochemical properties.

Variable-level[a]		DD (%)	Molecular weight, M_w (kDa)	Polydispersity (M_w/M_n)	Yields (%)
Reaction time (h)	2	6.5	69.970	1.503	100.0
	4	12.3	66.040	1.545	100.1
	6	16.7	65.330	1.495	99.9
	8	23.7	65.130	1.578	100.2
	10	30.1	64.400	1.547	99.1
	12	31.0	63.380	1.553	99.8
	14	34.2	56.790	1.587	99.6
	24	51.9	55.130	1.580	98.0
	36	69.1	34.590	1.587	94.6
	48	78.4	26.840	1.488	85.0
Catalyst type	Absence of catalyst	11.9	65.343	1.403	99.2
	$N_2H_4{\cdot}H_2SO_4$	32.0	63.603	1.468	99.0
	HCl	31.4	63.063	1.467	98.7
	$N_2H_4{\cdot}2HCl$	33.8	63.413	1.476	99.3
Reaction temperature (°C)	60	1.0	70.000	1.393	100.0
	75	13.4	69.840	1.391	99.3
	90	31.9	63.420	1.481	99.6
	105	58.2	41.540	1.627	98.3
Concentration of hydrazine in water (wt.%)	32	8.4	69.370	1.406	99.0
	64	31.5	63.320	1.501	98.7
	100	70.4	45.120	1.552	97.0

[a] The 60 mg dried FG samples were hydrazinolyzed by 1.45 ml of hydrazine hydrate containing 1% hydrazine sulphate at 90 °C for 12 h, precipitated, dialyzed, lyophilized and determined by HPGPC and NMR. The conditions for hydrazinolysis were investigated as these model reactions while varying levels of the variables in this table.

2.5. Molecular weight and sulfate/carboxyl group measurement

To investigate the molecular weight changes during the deacetylation and deamination reaction process, the weight-average molecular mass (M_w), number-average molecular mass (M_n) and molecular weight distribution (M_w/M_n) of the native, N-deacetylated and depolymerized FG samples were determined by high-performance gel permeation chromatography (HPGPC) using an Agilent technologies 1200 series (Agilent Co., USA) apparatus that had been equipped with a Shodex OH-pak SB-804 HQ column (8 mm × 300 mm). Chromatographic procedures and conditions were performed according to a previous method (Tsukamoto, Hattori, Sakabe, & Haginaka, 2001; Wu et al., 2010a). A standard curve was calibrated for molecular weight estimation using standard D-series Dextrans (D-0, 2, 3, 4, 5, 6, 7 and 8); native and free radical-depolymerized FG samples from the sea cucumber *T. ananas* with known M_w of 65.820, 11.580 and 4.961 kDa were used for sample standardization. Molecular weight calculations were performed using GPC software version B01.01 (Agilent Co., USA).

Native, N-deacetylated and depolymerized FG sample sulfate/carboxyl groups were determined by a conductometric method according to the literature (Casu & Gennaro, 1975).

2.6. DFG anticoagulant activity

The DFG APTT was determined at ten gradient concentrations (2.56, 1.28, 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01 and 0.005 mg/ml) using an APTT assay kit on a coagulometer (TECO MC-4000, Germany) as described previously (Gao et al., 2012). Antithrombin activity in the presence of HCII and factor Xa generation by the factor IXa-factor VIIIa complex were determined by the methods described previously; similar M_w were prepared by deaminative cleavage and free-radical depolymerization (Wu et al., 2012).

3. Results and discussion

3.1. FG deacetylation

In this study, hydrazinolysis conditions were investigated by varying different parameters. As demonstrated in Table 1, the reaction may be pseudo-first-order ($k = 0.0319\ min^{-1}$) in the presence of hydrazine sulfate at 90 °C from 2 to 48 h. Thus, controlling reaction time may be an optimal way to obtain samples with different DD for deaminative cleavage. However, compared with data from the literature that was obtained under similar conditions (Guo & Conrad, 1989), reaching the same DD for FG may require more reaction time than what is required for other GAGs such as heparin.

The data in Table 1 also demonstrate that the FG DD was obviously increased in the presence of different catalysts (hydrazine sulfate, hydrochloric acid and hydrazine dihydrochloride) at 90 °C for 12 h compared with no catalyst. Although a common catalyst used in glycoprotein and GAG hydrazinolysis is hydrazine sulfate (Bradbury, 1956; Fukuda et al., 1976), no significant differences were found among the FG sample DDs that were deacylated with different catalysts, indicating that an acid catalyst that provides the same amount of protons may accelerate the hydrazinolysis reaction rate with a similar deacetylation rate.

Hydrazine temperature and concentration in water may also be critical parameters that affect the deacylated FG reaction rate and yield. As demonstrated in Table 1, with increasing reaction temperature and hydrazine concentration in water, the deacylated FG DD obviously increased. However, the N-deacetylated FG M_w was obviously decreased after reaction with 64% hydrazine in water at 105 °C for 12 h or with anhydrous hydrazine at 90 °C for 12 h. The reagent containing 70% hydrazine in water can also reportedly eliminate the heparan sulfate polymer chain β-eliminative cleavage (Guo & Conrad, 1989), which coincides with our observations that less depolymerization occurred with lower hydrazine concentrations in water. Thus, this M_w reduction of the products may be because of β-elimination resulting in FG cleavage under alkaline conditions.

The hydrazinolysis method has been commonly used for partial glycoprotein and GAG such as chondroitin sulfate and heparan sulfate deacylation for structural modification and analysis (Bradbury, 1956; Fukuda et al., 1976). Our results revealed that the hydrazinolysis method might be successfully applied to FG, although FG has unique sulfated fucopyranose branches.

Table 2
Relationship of DD and DFG M_w obtained from raw materials of two different M_w.

M_w of raw materials[a] (kDa)	DD (%)	Theoretical, M_w (kDa)	Measured, M_w (kDa)	Polydispersity
13.710	11.0	8.245	8.777	1.401
13.710	13.8	6.577	6.751	1.396
69.930	16.0	5.683	6.083	1.162
69.930	8.3	10.984	9.702	1.292

[a] The raw material with a M_w 13.710 was prepared by free-radical depolymerization method according to Wu et al. (2010b).

3.2. FG deaminative cleavage

When N-deacetylated FG with 35% DD was treated with nitrous acid (pH 4) for 4–30 min in an ice bath or at room temperature, high-performance gel permeation chromatography (HPGPC) product profiles were very similar (similar retention time and elution curve shape, not shown). These results revealed that the N-deacetylated FG deaminative cleavage with nitrous acid occurred fast, and that these reactions could complete in very short time (<4 min) at a low temperature, which is consistent with previous reports on heparin depolymerization with nitrous acid (Shively & Conrad, 1976). In addition, these products had similar yields (approximately 80%) and similar sulfate/carboxyl groups (approximately 3.1–3.4).

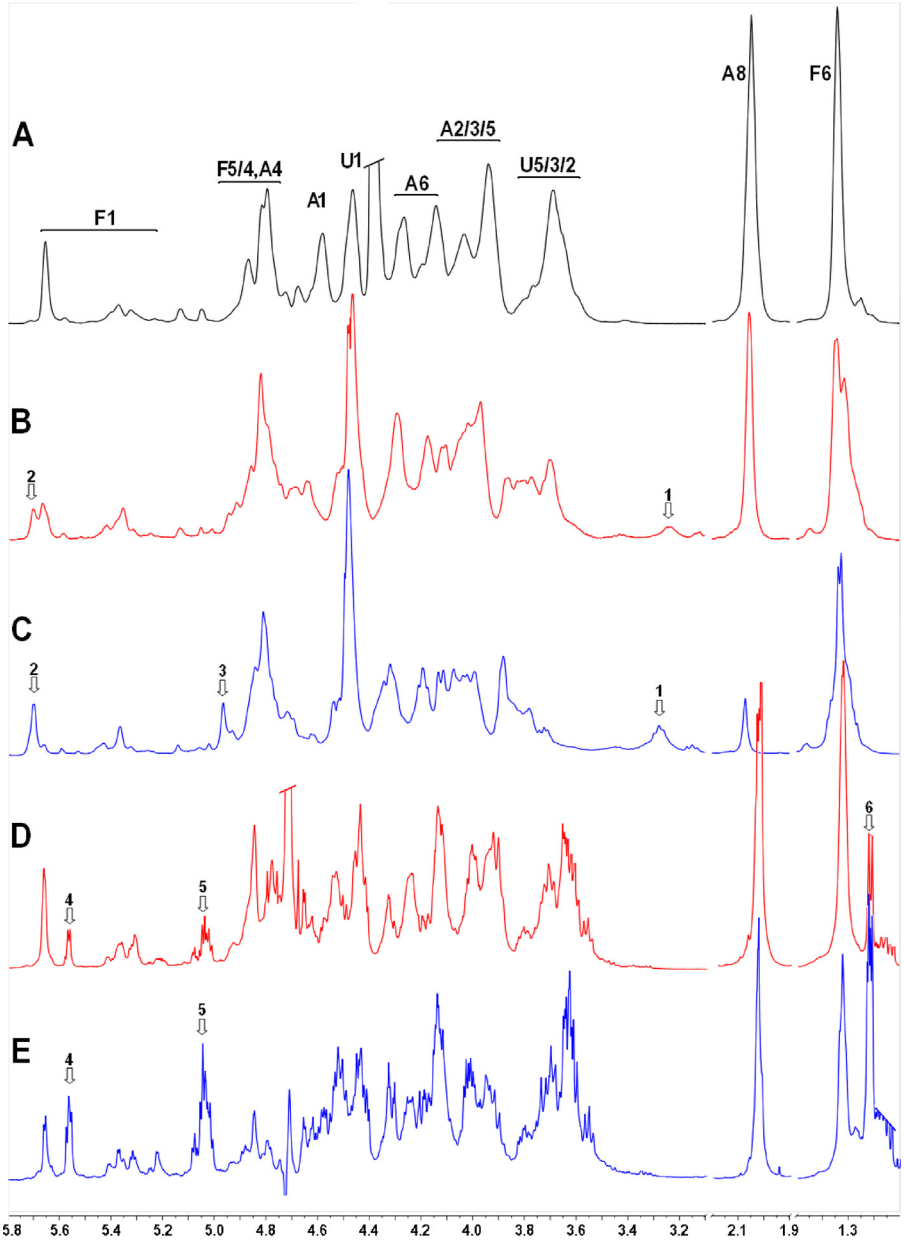

Fig. 1. ^{1}H NMR spectra of native (A), N-deacetylated (B and C) and deaminative depolymerized FGs (D48 and D88) (D and E). N-deacetylated FG DDs are 48% and 88%. Symbols "1", "3" are GalN H-2 and H-4 signals; "2" is a new Fuc2S4S H-1 signal found in N-deacetylated FGs; "4" is another Fuc2S4S H-1 signal found in depolymerized FGs; "5" is a set of the H-1 and H-4 anTal (diol) signals; "6" is terminal fucose H-6 signals.

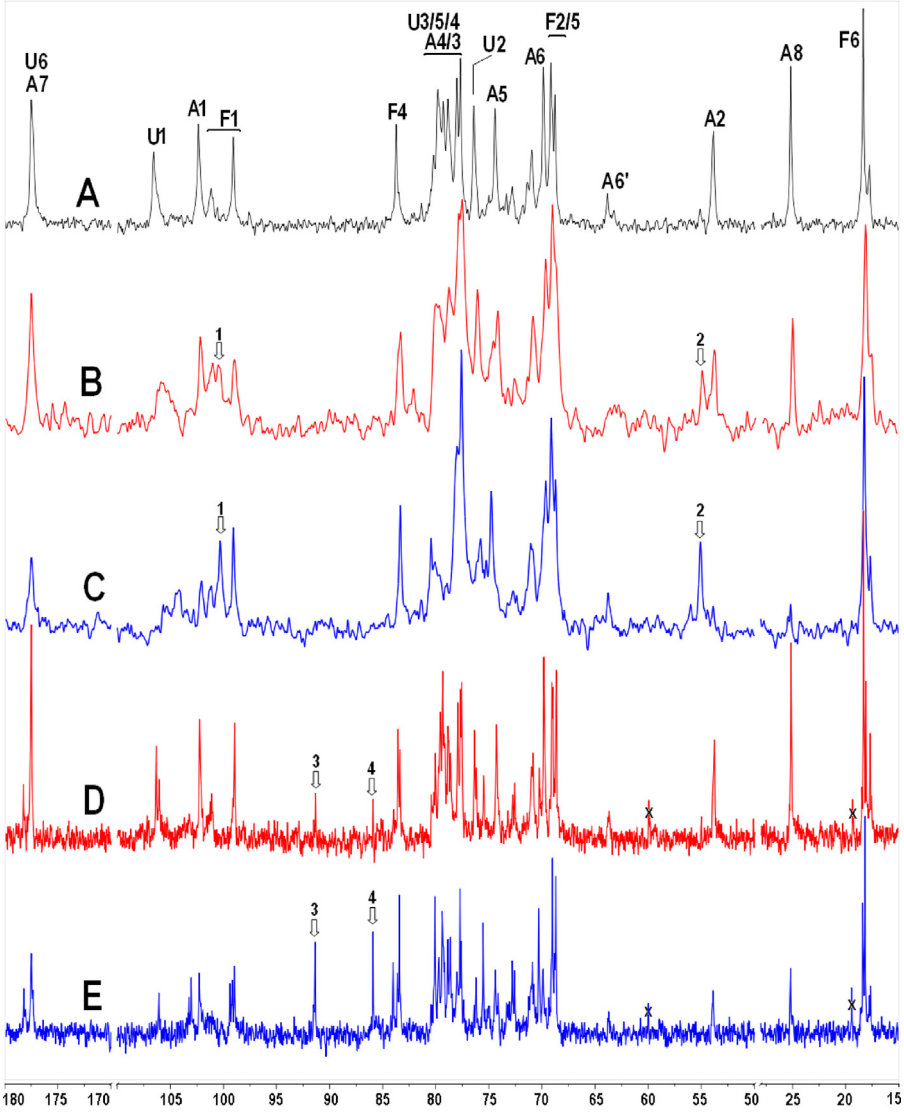

Fig. 2. Native (A), N-deacetylated (B and C) and deaminative depolymerized FG (D48 and D88) ^{13}C NMR spectra (D and E). N-deacetylated FG DDs are 48% and 88%. Symbols "1" and "2" are GalN C-1 and C-2 signals, respectively; "3" and "4" are anTal (diol) C-1 and C-2 signals.

To investigate the relationship between the deacetylated FG DD and the M_w of its deaminative cleavage products, N-deacetylated FG samples with four different DDs were prepared by varying the reaction times, using native FG (M_w 69.930 kDa) and depolymerized samples (M_w 13.710 kDa) with free radicals as raw materials. The four N-deacetylated FG samples were then depolymerized by nitrous acid treatment (pH 4) for 10 min at room temperature. The results indicated that the practical M_w as measured by HPGPC coincided with the theoretical DFG M_w, which is calculated using the formula: $M_w = 1/DD \times 907$ (where 907 is the M_w of every constitutional FG unit), indicating that the DFG M_w may be determined by the FG DD and might not be related to the M_w of the raw materials. Therefore, we may obtain the DFG with different M_w by controlling the FG DD using raw materials of different M_w (Table 2).

According to the above depolymerization investigation, the DFG samples of different M_w (5021–22240 Da) were prepared by

Table 3
Deaminative cleavage-prepared DFG physicochemical properties, yields and anticoagulant activities.

Sample	M_w (kDa)	Polydispersity	Sulfate/carboxyl ratios	Yields (%)	Doubling APTT (μg/ml)	Anticoagulant activity (U/mg)
DFG1	22.240	1.35	3.33	93.0	3.40	79.29
DFG2	17.100	1.29	3.48	86.0	3.55	76.05
DFG3	12.567	1.11	3.12	86.5	4.22	63.92
DFG4	11.332	1.54	3.27	86.5	4.85	55.68
DFG5	10.497	1.63	3.20	85.0	4.92	54.85
DFG6	9.476	1.48	3.35	81.0	6.14	43.94
DFG7	8.336	1.40	3.36	81.0	6.25	43.16
DFG8	7.000	1.36	3.40	79.0	6.78	39.79
DFG9	6.603	1.39	3.34	78.8	7.81	34.56
DFG10	5.021	1.20	3.36	72.5	12.38	21.79

cleaving N-deacetylated FG samples with nitrous acid at room temperature. The molecular weight distribution (polydispersity) was used to measure the molecular weight distribution width, which represents polymer homogeneity and dispersibility (Tsukamoto et al., 2001). As demonstrated in Table 3, the distribution data (1.1–1.6) indicated that the products are relatively homogeneous. In contrast to the native FG, the DFG physicochemical properties such as polydispersity and sulfate/carboxyl groups demonstrated no obvious changes, indicating some similarly in their chemical structures (see below for discussion).

3.3. Native FG, deacetylated FG and DFG structural analysis and comparison

To obtain the desirable DFG with a certain M_w, it may be required that the basic N-deacetylated and depolymerized FG structure should be similar to the native FG without loss of *O*-sulfate substituents and sulfated fucose branches. Thus, native FG, N-deacetylated FG of different DD (48% and 88%) and DFG (named D48 and D88) structures were obtained by cleaving 48% and 88% N-deacetylated FGs and studied by 1D and 2D NMR spectroscopy. Their ^{1}H–^{1}H COSY, TOCSY and ROESY spectra are demonstrated in Figs. 1–4. The chemical shifts in Table 4 are based on the interpretations of the COSY, TOCSY, ROESY, HMBC and HSQC spectra.

Native FG ^{1}H and ^{13}C NMR signals could be assigned as described previously (Mourão et al., 1996; Wu et al., 2012; Yoshida et al., 1992). Compared with these native FG signals, some new ^{1}H and ^{13}C signals appeared in the N-deacetylated FG with different DD NMR spectra, which could be assigned to protons signals and ^{13}C of GalN (i.e., the N-deacetylated products of GalNAc).

As demonstrated in Fig. 1B and C, the signals at 3.1–3.3 ppm assigned as '1' were from H-2 of GalN. We found two GalN spin systems by analyzing ^{1}H–^{1}H COSY, ROESY and TOCSY N-deacetylated FG spectra (Fig. 4A), which may belong to different types of sulfate substituted GalN, i.e., major 4,6-O-disulfated N-acetylgalactosamine (GalN4S6S) and minor GalN4S. Similarly, we also observed that some terminal 2,4-O-disulfated fucose (Fuc2S4S) and glucuronic acid (GlcUA) H signals were shifted downfield after deacetylation, and their shifted integrals were proportional to their DDs, which are consistent with the observations from chondroitin and dermatan sulfate deacetylation (Nadkarni et al., 1996). As demonstrated in Fig. 2B and C, compared with the corresponding GalNAc signals, the GalN C-1 and C-2 signals appeared at approximately 100.3–100.6 and 55.04–55.06 ppm, which were shifted upfield by approximately 2 ppm and downfield by approximately 1 ppm, respectively. Fucose H-1 signals were at 5.2–5.7 ppm, which were different spin systems of three sulfated fucose types, namely Fuc2S4S, 3-O-sulfated fucose (Fuc3S) and 4-O-sulfated fucose (Fuc4S) in the ROESY and TOCSY spectra (Fig. 4A). Although H-1 fucose signal splitting could be seen in these spectra, the sulfated fucose types and 1H signal integral ratios for the three fucose types were similar to those of the native FGs. Based on these analyses, N-deacetylated FG ^{1}H and ^{13}C NMR signals were assigned as demonstrated in Table 4, suggesting that the main FG structures may be essentially stable during FG deacetylation with hydrazinolysis, except occurrence of deacetylation and partial depolymerization.

Similar to the oligosaccharides that were produced by nitrous acid chitosan depolymerization, the existing form of the reactive (2,5)-anhydro-D-mannose-unit at new reducing end was almost hydrated aldehyde (Tommeraas, Varum, Christensen, & Smidsrod, 2001). In our work, the H-1 and C-1 resonances from the sugar unit at the new reducing end were found at 5.0–5.1 ppm and 91–92 ppm regions (Figs. 1 and 2), respectively, indicating that the H_2O molecule was added to the carbonyl group (also called gem diol). Identification of the resonances within the (2,5) anhydro-D-talose

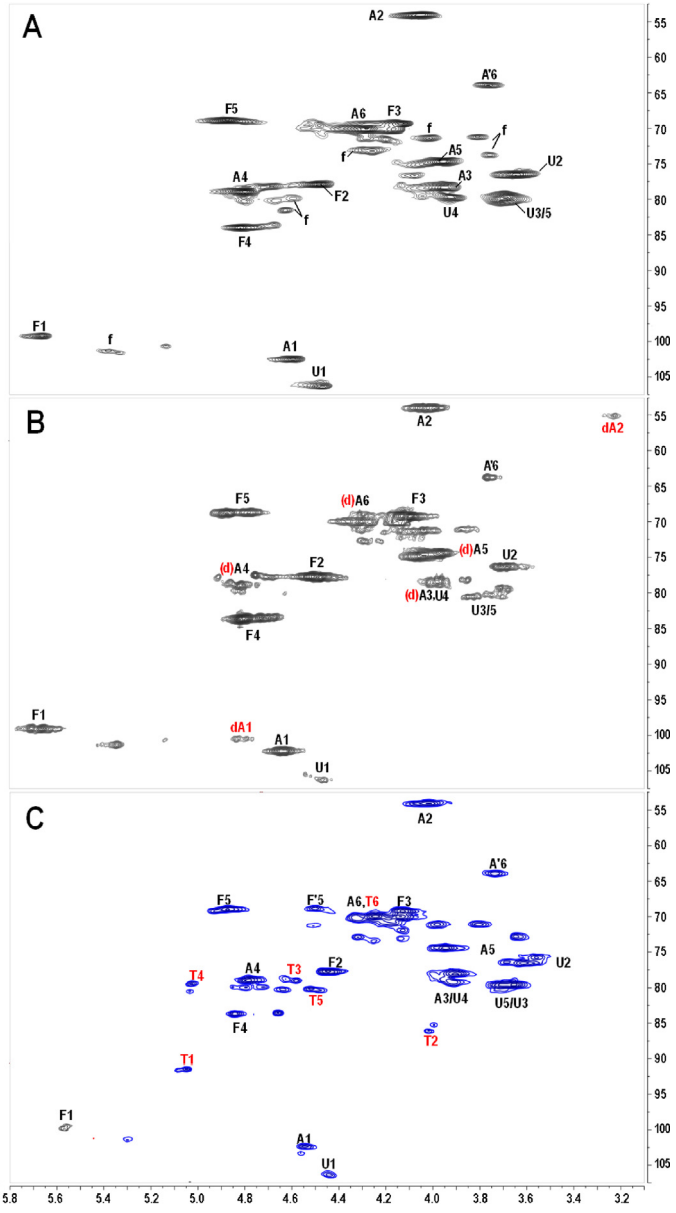

Fig. 3. Native (A), N-deacetylated (DD 48%) (B) and depolymerized FG (D88) ^{1}H–^{13}C HSQC spectra (C). Labels F, U, A, dA and T represent Fuc2S4S, GlcUA, GalNAc, GalN and anTal (diol), respectively. f signals are from Fuc3S or Fuc4S.

(anTal) (diol) system (Figs. 3 and 4) was relatively straightforward according to proton spectra assignments observed for (2,5) anhydro-D-talitol rings (Laudera, Huckerbya, Nieduszynskia, & Sadlerb, 2011). Confirmation that the chondroitin sulfate polymer chain cleavage using hydrazinolysis followed by nitrous acid does indeed produce anTal-ol residues from GalNAc precursors may be obtained from NMR spin–spin coupling data (Laudera et al., 2011). The cleavage process is proposed to involve diazotization followed by inversion at the C2 site in GalNAc with loss of elemental nitrogen, giving rise to the *talo* configuration in which H2 and H3 are on opposite sides of the furanose ring plane; however, H-3, H-4 and H-5 all reside on the same ring face (Laudera et al., 2011). $J_{2,3}$ of anTal is consistently of greater magnitude (approximately 8.0 Hz) compared with $J_{3,4}$ and $J_{4,5}$, which both have values of approximately 4.0 Hz, in accord with the relative coupling pathway geometries. Based on analysis of these signals from ROESY and TOCSY spectra, the spin systems of anTal were assigned as demonstrated in Fig. 4B and Table 4.

Table 4
Assignment of $^{1}H/^{13}C$ NMR signals of native FG and its N-deacetylated and depolymerized products.

	Chemical shift (ppm)														
	H1	H2	H3	H4	H5	H6	Ac	C1	C2	C3	C4	C5	C6	Ac	
							—CH_3							—CH_3	—C=O
Native FG															
β-GalNAc4S6S	4.58	4.05	3.92	4.79	3.94	4.16, 4.26	2.05	102.6	54.1	79.8	79.2	76.8	70.3	25.5	177.6
β-GlcUA	4.46	3.64	3.73	3.94	3.68	–	–	106.8	76.8	80.5	79.8	80.4	177.6	–	–
α-Fuc2S4S	5.66	4.48	4.14	4.83	4.88	1.34	–	99.4	78	69.6	84.1	69	18.6	–	–
α-Fuc4S	5.33	3.82	4.01	4.81	4.91	1.34	–	101.4	71.4	74.0	80.5	69	18.6	–	–
α-Fuc3S	5.37	4.15	4.69	4.03	4.52	1.25	–	101.2	71.8	83.9	73.3	69.3	18.6	–	–
48% N-deacetylated FG															
β-GalNAc4S6S	4.63	4.02	3.94	4.81	3.96	4.16, 4.29	2.06	102.1	53.8	77.9	79.7	74.8	69.8	25.2	177.5
β-GalN4S6S	4.78	3.24	4.06	4.75	4.07	4.16, 4.29	2.06	100.6	55	77.9	79.7	74.4	69.8	–	–
β-GlcUA	4.52	3.7	3.96	3.95	3.87	–	–	106.2	76.2	80.2	78.8	79.8	177.5	–	–
	4.47	3.67	3.83	3.97	3.86	–	–	105.4	76.2	80.5	78.8	79.8	177.5	–	–
α-Fuc2S4S	5.69	4.5	4.12	4.75	4.79	1.31	–	99	77.7	69.1	83.6	68.6	18.3	–	–
	5.66	4.52	4.11	4.82	4.79	1.31	–	99	77.9	69.1	83.4	68.6	18.3	–	–
α-Fuc4S	5.34	3.87	4.03	4.83	4.87	1.34	–	101.3	71.1	71.4	79.9	68.9	17.8	–	–
α-Fuc3S	5.36	4.16	4.68	4.03	4.53	1.26	–	101.2	72.1	83.5	73.3	68.9	17.8	–	–
88% N-deacetylated FG															
β-GalNAc4S6S	4.72	4.03	3.99	4.86	3.99	4.19, 4.31	2.07	102	53.8	77.9	79.6	74.8	69.7	25.1	177.7
β-GalN4S6S	4.81	3.33	4.07	4.78	4.08	4.20, 4.33	2.07	100.3	55.3	77.9	79.9	74.4	69.7	–	–
β-GlcUA	4.54	3.72	3.82	4	3.89	–	–	105.4	75.8	80.5	79	79.8	177.7	–	–
α-Fuc2S4S	5.7	4.53	4.12	4.76	4.82	1.33	–	98.9	77.7	70.8	83.3	68.7	18.3	–	–
α-Fuc4S	5.37	3.87	4.07	4.85	4.89	1.38	–	101.3	71.1	71.5	79.9	69.1	17.8	–	–
α-Fuc3S	5.44	4.2	4.7	4.28	4.55	1.27	–	101.2	72.3	83.1	73.3	69.1	17.8	–	–
Depolymerised DFG (named D48) by deaminative cleavage															
β-GalNAc4S6S	4.54	4.02	3.92	4.77	3.95	4.14, 4.24	2.02	102.3	51.8	78.1	79	74.4	67.7	23.2	177.6
anTal4S6S	5.05	4.02	4.58	5.02	4.5	4.19, 4.32	–	91.6	86.32	79.1	80.5	80.4	68.2	–	–
β-GlcUA	4.44	3.59	3.66	3.89	3.72	–	–	106.4	76.4	80.3	79.2	79.4	177.6	–	–
α-Fuc2S4S	5.66	4.45	4.13	4.85	4.89	1.33	–	99	77.8	70.1	83.6	68.8	18.5	–	–
α-Fuc4S	5.31	3.83	3.97	4.8	4.86	1.33	–	101.3	71.1	72.6	79.9	69	17.8	–	–
α-Fuc3S	5.38	4.13	4.63	4.02	4.5	1.22	–	101.2	72.8	83.5	71.3	68.8	18.2	–	–

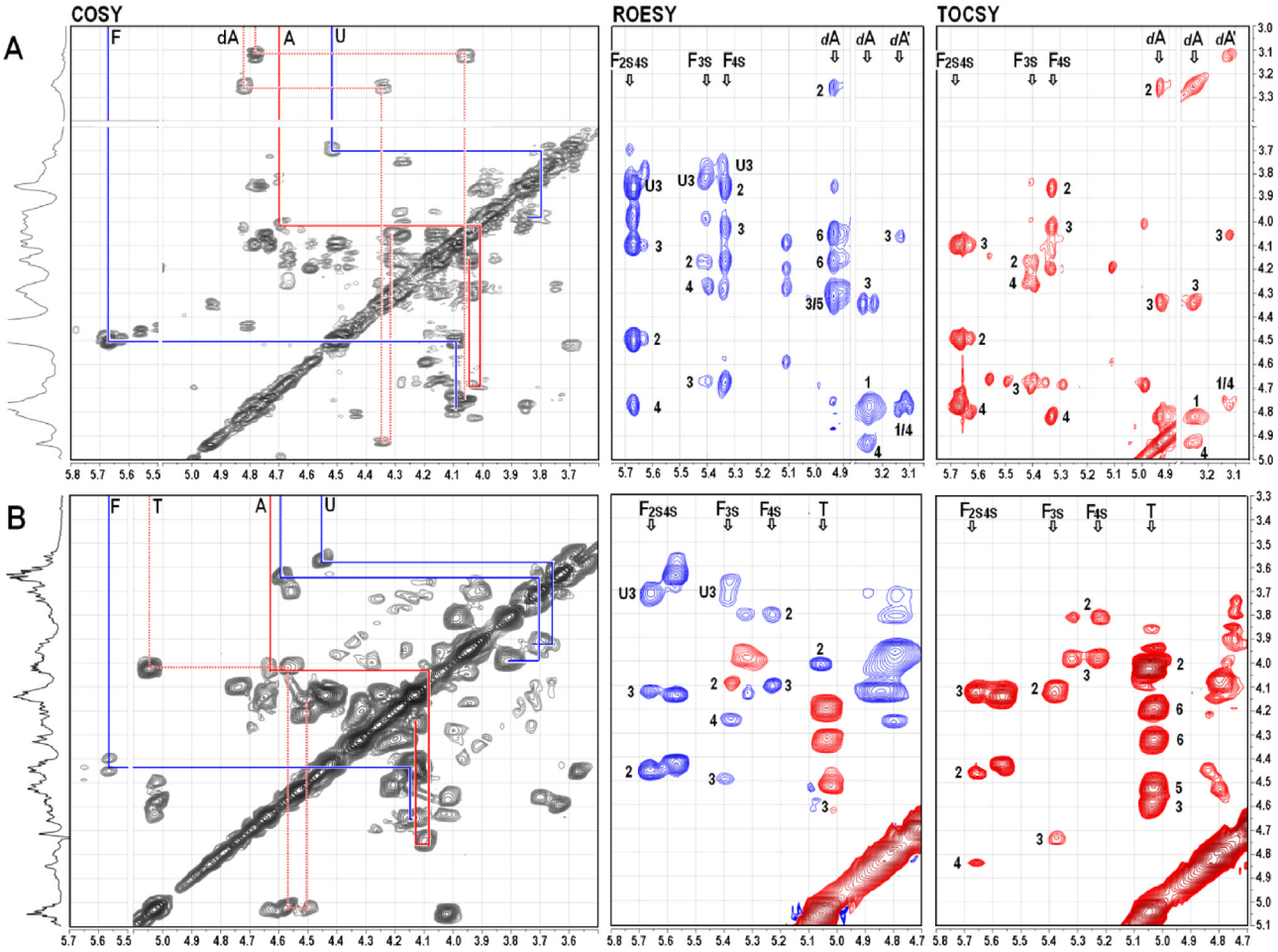

Fig. 4. Partial ^{1}H–^{1}H COSY, ROESY and TOCSY spectra of N-deacetylated (A) and depolymerized FG (B). Labels U, A, dA and T represent GlcUA, GalNAc, GalN and anTal (diol), respectively. F_{2S4S}, F_{3S} and F_{4S} represent three sulfated fucose types in FGs, 2,4-O-disulfated fucose, 3-O-sulfated fucose and 4-O-sulfated fucose, respectively.

It is notable that the signals at 3.1–3.5 ppm that were assigned to H-2 of GalN in ^{1}H NMR of N-deacetylated FG disappeared in the DFG ^{1}H NMR, indicating that D-β-GalN in the N-deacetylated FGs was almost deaminated by nitrous acid. Besides these, in contrast to the $^{1}H/^{13}C$ N-deacetylated FG NMR signals, the DFG signals were more similar to those of native FG (Figs. 1 and 2). Compared with the ^{1}H–^{13}C HSQC (Fig. 3), ROESY and TOCSY (Fig. 4) native FG spectra, signals from GalNAc and GlcUA in DFG could have been assigned as demonstrated in Table 4. As demonstrated in Fig. 4B and Table 4, although 1H signals from each type of fucose split into two positions (signed "4" in Fig. 1D and E), DFG also contained three types of sulfated fucoses, i.e., Fuc2S4S, Fuc3S and Fuc4S, which was similar to native and N-deacetylated FG. The results also implied that fucose side chains and their substituted sulfate might be stable during the deacetylation and deaminative cleavage reactions.

3.4. Anticoagulant properties

Previous studies demonstrated that the peroxidation-prepared depolymerized FG with a molecular weight from 10,000 to 15,000 has powerful anticoagulant activities and treats thromboembolism with less bleeding than UFH and LMWH (Minamiguchi et al., 2003; Nagase et al., 1995). In the present study, nine DFG samples with different molecular weights were prepared by deaminative cleavage and evaluated for their anticoagulant activities by APTT assay (Tables 3 and 5). The results demonstrated that all of the DFG samples strongly prolonged human plasma APTT, and the concentrations of DFG with higher than 6.0 kDa M_w that were required to double the APTT were less 12 μg/ml, indicating that these derivatives can inhibit intrinsic coagulation. At the 0.005–2.560 mg/ml concentration, different molecular weight DFGs prolonged APTT activities in a concentration-dependent manner (not shown). Additionally, as demonstrated in Table 3, the APTT prolonging activities of DFG with different M_w decreased logarithmically in proportion to the molecular sizes, which is in agreement with previous reports (Gao et al., 2012; Wu et al., 2010b). Because of hematological activity, if DFG is to be used as an anticoagulant drug, its molecular weight might not be below 6.0 kDa, which is in agreement with our previous study on free-radical depolymerization-prepared DFG (Wu et al., 2010b). Interestingly, deaminative cleavage-prepared DFG exhibited slightly stronger anticoagulant activity than free-radical degradation-obtained DFG with similar M_w and polydispersity (Table 5).

Previous pharmacological studies of depolymerized and native FG have demonstrated that the factor Xase-inhibiting activities and HCII-dependent antithrombin activities may be the main mechanisms for their anticoagulant activities (Glauser, Pereira, Monteiro, & Mourão, 2008; Nagase et al., 1995; Sheehan & Walke, 2006; Wu et al., 2012). To evaluate the DFG anticoagulant mechanisms, effects of different molecular size DFG on factor Xase inhibition and HCII-mediated thrombin inhibition were further investigated. The results demonstrated that DFGs effectively inhibited intrinsic tenase-mediated factor X activity (anti-f.Xase activity), and the half maximal effective concentration (EC_{50}) was 20–40 ng/ml (Table 5), demonstrating high potency for anti-f.Xase activity. In the presence of heparin cofactor II, the deaminative cleavage-prepared

Table 5
Physicochemical properties and anticoagulant activities of deaminative cleavage (DFG3, DFG7, and DFG10) and free-radical depolymerization (DTHG1–DTHG3)-prepared DFG samples with similar M_w.

Sample	M_w (kDa)	Polydispersity	Doubling APTT (μg/ml)	Anti-f.Xase (EC_{50}, ng/ml)	Anti-IIa (EC_{50}, ng/ml)
DTHG1	12.730	1.52	6.52	23.47	265.99
DFG3	12.567	1.11	4.22	21.34	285.51
DTHG2	8.549	1.33	9.86	38.07	156.59
DFG7	8.336	1.40	6.25	30.46	182.67
DTHG3	4.961	1.55	19.50	52.88	141.42
DFG9	5.021	1.20	12.38	38.18	227.18

DFG samples demonstrated potent thrombin inhibition (anti-IIa activity; EC_{50} < 286 ng/ml). Additionally, deaminative cleavage-prepared DFG samples exhibited similar anti-f.Xase and anti-IIa activity potency as free-radical depolymerization products. Furthermore, the deaminative cleavage-prepared DFG samples with M_w higher than 6.0 kDa may prolong the APTT and strongly inhibit thrombin via HCII and factor Xa generation via the intrinsic tenase complex in a manner that is similar to native glycosaminoglycan, indicating that the effects of their molecular sizes on factor Xase inhibition and HCII-mediated thrombin inhibition may be very slight.

4. Conclusion

In this work, partial N-deacetylation and deaminative cleavage of FG were investigated, and its products with a (2,5)-anhydro-D-talose unit at the new reducing end were prepared by hydrazinolysis and nitrous acid treatment depending on experimental conditions. The product chemical structures were studied by one- and two-dimensional ^1H and ^{13}C NMR methods. Under mild conditions, basic N-deacetylated FG and DFG structures are similar to those of native FG, indicating that fucose side chains and their substituted sulfate may remain stable during deacetylation and deaminative cleavage reactions. In contrast to DFGs with similar molecular mass by free radical degradation, these products also exhibit potent anticoagulant activity and inhibit thrombin via HCII and factor Xa generation via the intrinsic tenase complex. Therefore, oligosaccharides with the anhydro-D-talose unit at the new reducing end can be used to constitute a compound library that will be useful for elucidating the FG structure–activity relationship.

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