Effects of Native Fucosylated Glycosaminoglycan, Its Depolymerized Derivatives on Intrinsic Factor Xase, Coagulation, Thrombosis, and Hemorrhagic Risk

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Abstract

A native fucosylated glycosaminoglycan from sea cucumber *Holothuria fuscopunctata* (nHG), mainly branched with Fuc_{3S4S}, exhibited potent anticoagulant activity by intrinsic tenase iXase (FIXa-FVIIIa complex) and antithrombin-dependent factor IIa (FIIa) inhibition, but also had the effects of FXII activation and platelet aggregation. For screening a selective iXase inhibitor, depolymerized nHG (dHG-1 ~ –6) and a pure octasaccharide (oHG-8) were prepared. Like nHG, dHG-1 ~ –6 and oHG-8 could potently inhibit iXase, and competitive binding assay indicated that dHG-5 and oHG-8 could bind to FIXa. Nevertheless, dHG-5 and oHG-8 had no effects on FXII and platelet activation. nHG, dHG-5, and oHG-8 could significantly prolong the activated partial thromboplastin time of human, rat, and rabbit plasma. In the rat deep venous thrombosis model, dHG-5 and oHG-8 showed potent antithrombotic effects in a dose-dependent manner, while the thrombus inhibition rate of nHG at high dose was markedly reduced. Additionally, dHG-5 and oHG-8 did not increase bleeding at the doses up to 10-fold of the effectively antithrombotic doses compared with nHG and low molecular weight heparin in the mice tail-cut model. Considering that dHG-5 possesses strong anti-iXase and antithrombotic activities, and its preparation process is simpler and its yield is higher compared with oHG-8, it might be a promising antithrombotic candidate.

Keywords
- dHG-5
- iXase
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- antithrombotic
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Introduction

Venous thromboembolism (VTE) is a common underlying pathology of cardiovascular disease, which is a global burden associated with high morbidity and mortality.[1,2] Unfractionated heparin (UFH) and low molecular weight heparin (LMWH) are effective in prevention and treatment of thrombosis.[3,4] UFH and LMWH target both factor Xa (FXa) and thrombin (FIIa) which are located at the common coagulation pathway and essential for hemostatic function. Thrombin and FXa are most sensitive to inhibition by UFH in the presence of antithrombin (AT), and thrombin is approximately 10-fold more sensitive to inhibition than FXa. Unlike UFH has high bleeding risk, LMWH have reduced anti-FIIa activity relative to anti-FXa activity and showed more favorable pharmacokinetic profiles without monitoring requirement.[5–7] Compared with UFH and LMWH, oral direct anticoagulants have more predictable pharmacokinetic and pharmacodynamic profiles, without the requirement of routine monitoring. However, these drugs also affect the common coagulation pathway and have increased risk of bleeding.[8,9]

Previously, it was found that intrinsic coagulation pathway plays an important role in the pathological process of thrombosis, while it is not essential for physiological hemostasis.[10–13] The inhibitors of intrinsic coagulation pathway may prevent thrombosis with negligible bleeding risks, which has attracted more attention of researchers to develop new anticoagulants.[11–20] Hereditary deficiency of FXII or FXI impaired thrombus formation while having a minimal impact on hemostasis.[15,16] Several strategies of targeting FIX, including monoclonal antibodies, synthetic active site-blocked competitive inhibitors, and ribonucleic acid aptamers, have been explored. FIX inhibitors showed effective anticoagulation and reduced risk of bleeding compared with UFH.[11,12] The intrinsic factor Xase (iXase) complex consisting of FIXa–FVIIIa is the final and rate-limiting enzyme of the intrinsic coagulation pathway. Thus, the iXase may represent an important therapeutic target for effective and safe antithrombosis.[2,21–24]

Fucosylated glycosaminoglycan (FG), extracted from sea cucumber, is a unique glycosaminoglycan with fucose branches. FG has shown potent anticoagulant and antithrombotic effects. The mechanisms might be attributed to its anti-iXase activity, heparin cofactor II (HCII)-dependent anti-FIIa activity, and AT-dependent FXa or FIIa inhibition activity.[25] FG also has the undesired effects of inducing platelet aggregation and activating FXII.[26–28]

Depolymerization is an effective method to reduce the side effects of FG.[29,30] Some depolymerized FG (dFG) had potent anti-iXase activity while reduced AT-dependent anti-FIIa and anti-FXa activities, indicating that dFGs have higher selectivity than the native FG, and that their anticoagulant mechanisms are different from heparins.[31–35] Previously, we prepared a nonasaccharide from the FG extracted from the sea cucumber *Stichopus variegatus*, and found that it could inhibit venous thrombosis in rats with negligible risk of bleeding.[33] Subsequently, an octasaccharide derivative from the FG originated from *Bohadschia argus* was found to be the minimum structural fragment as a potent iXase inhibitor.[36]

Previous study has showed that natural FG branched with Fuc_{3S4S} has strong anticoagulant activity with relatively weaker FXII activation and platelet aggregation activities.[34] Herein, a native FG mainly bearing Fuc_{3S4S} branches was extracted from sea cucumber *Holothuria fuscopunctata* (nHG). Its depolymerized fractions, dHG-1∼6 (►**Fig. 1A**) and a pure octasaccharide oHG-8 (►**Fig. 1B**) were obtained from its β-eliminative depolymerized products. Anti-iXase activities and the effects on FXa and FIIa in the presence or absence of AT or HCII of nHG, dHGs, and oHG-8 were evaluated. Then, the pharmacological and pharmacodynamic activities of nHG, dHG-5, and oHG-8 in vitro and in vivo were systematically compared. The results may provide valuable experimental data for the development of dHG-5 as a new antithrombotic candidate with low hemorrhagic risk.

Methods

Materials

Sprague-Dawley rats (male, body weight 180 ∼ 220 g, approved no. SCXK 2016–0002), Kunming mice (male, body weight 18 ∼ 22 g, approved no. SCXK 2016–0002), and New Zealand white rabbits (male, body weight 1.5 ∼ 2.0 kg) were all purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). Animals were housed at 24 ± 2°C, 40 to 60% relative humidity, air change 10 times each hour. Animal experiments were reviewed and approved by the Animal Ethics Committee of Kunming Institute of Botany, Chinese Academy of Sciences. LMWH (enoxaparin, 0.4 mL × 4000 AXaIU, molecular weight [Mw] ∼4,500 Da) was from Sanofi-Aventis (Paris, France). Dermatan sulfate (DS, Mw ∼15,000–45,000 Da) and adenosine diphosphate (ADP) were from Sigma-Aldrich (Saint Louis, Missouri, United States). Oversulfated chondroitin sulfate (OSCS) was from National Institutes for Food and Drug Control (Beijing, China). The activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) reagents, and human coagulation control plasma were all purchased from Teco Medical (Munich, Germany). BIOPHEN FVIII:C kit, BIOPHEN AT-IIa kit, BIOPHEN AT-Xa kit, HCII, human thrombin (FIIa), human FXII, thrombin chromogenic substrate CS-01(38), and kallikrein chromogenic substrate CS-31(02) were all from HYPHEN Biomed (Neuville Sur Oise, France). Recombinant coagulation FVIII was from Bayer HealthCare LLC (Berkeley, California, United States). Human FIXa was purchased from Haematologic Technologies Inc (Essex Junction, Vermont, United States). Amine-PEG_3-Biotin was from MedChemExpress LLC (Monmouth Junction, New Jersey, United States). Streptavidin (SA) biosensors were from Fortébio (Fremont, California, United States). All other chemical reagents were of analytical grade and obtained commercially.

Preparation of nHG, dHGs, and oHG-8

nHG was extracted and purified from the body wall of the sea cucumber *H. fuscopunctata* as previously described.[37] The low molecular weight depolymerized products of nHG (dHGs) were prepared using a β-eliminative depolymerization method, which is highly selective to cleave the β1,4 glycosidic bonds between GalNAc and GlcA residues.[32,36,38] Six fractions (dHG-1∼6) with different molecular weight

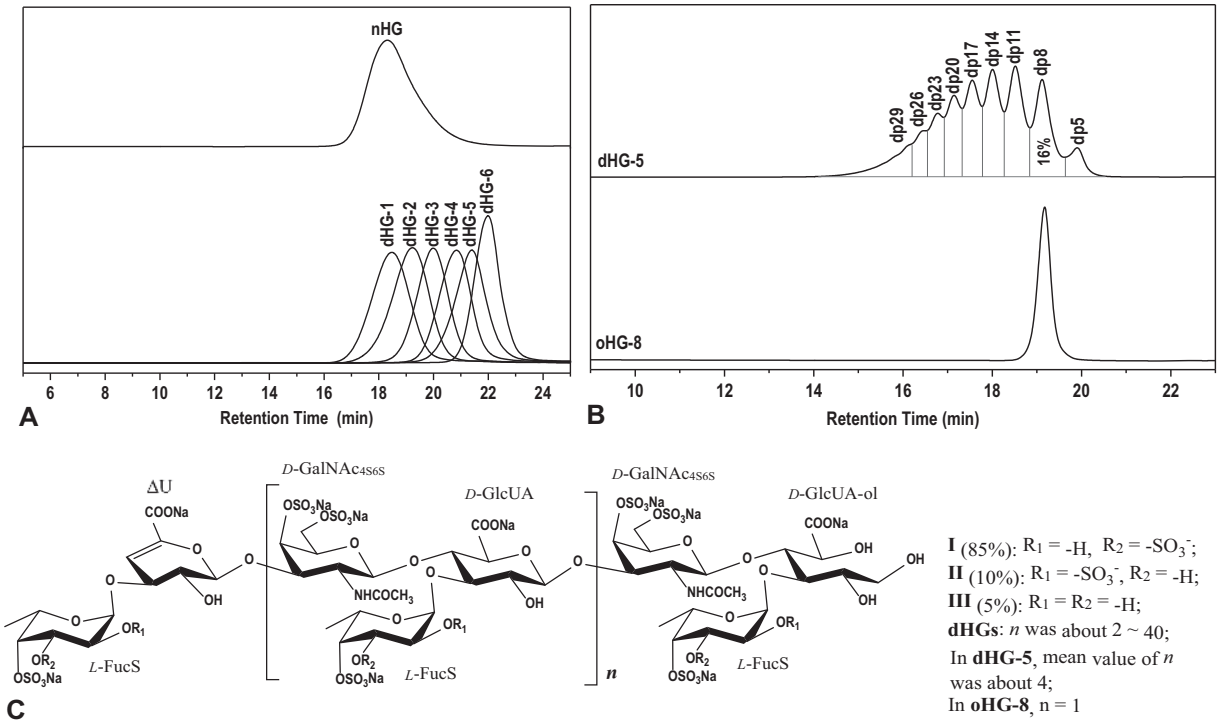

Fig. 1 (**A**) High-performance liquid chromatography (HPLC) profiles of native FG (nHG) and depolymerized nHG (dHG)-1∼6 which were determined on Shodex OHpak SB-804 HQ column eluted with 0.1 M NaCl solution at a flow rate of 0.5 mL/min under the differential refractive index detector. (**B**) HPLC profiles of dHG-5 and octasaccharide (oHG-8) which were determined on TSK G2000SW XL GL column eluted with 0.1 M NaCl solution at a flow rate of 0.5 mL/min under the differential refractive index detector. And oHG-8 accounts for ∼16% component of dHG-5. (**C**) The structures of dHGs and oHG-8.

were prepared by controlling the esterification degree and purified by gel permeation chromatography. Furthermore, a pure octasaccharide fragment (oHG-8) was obtained from the dHG-5 by gel permeation chromatography.[36,38,39] The structures of nHG, dHGs, and oHG-8 were determined by one-dimensional/two-dimensional nuclear magnetic resonance (NMR) spectra (800 MHz, at 298 K, in 99.9% D_2O). The molecular weight of nHG and dHG-1∼6 were measured by a size-exclusion chromatography with multiple angle laser light scattering. The molecular weight of oHG-8 was determined by electrospray ionization quadrupole time-of-flight mass spectrometry.

Anti-iXase Activities and Effects on FXa and FIIa in the Presence or Absence of AT or HCII

The iXase inhibition activity was detected using the BIOPHEN FVIII:C kits (containing R1, R2, and R3 solutions) and recombinant human FVIII using previous method.[33,34] Briefly, 30 µL each compound solution at different concentrations (or Tris-HCl solution as control), 30 µL FVIII (2 IU/mL), and 30 µL R2 solution (containing 60 nM FIXa, human thrombin, phosphatidylcholine/phosphatidylserine, and Ca^{2+}) were mixed and incubated at 37°C for 2 minutes. Then, 30 µL R1 solution (containing 50 nM FX and thrombin inhibitor) was added. After incubation for 1 minute at 37°C, the residual FXa activity was measured by the addition of 30 µL R3 solution (FXa chromogenic substrate SXa-11). Anti-FIIa and anti-FXa activities in presence (or absence) of AT were measured with Biophen heparin anti-FIIa kits and Biophen AT kits. Inhibition activity of thrombin by human HCII was measured with thrombin chromogenic substrate CS-01(38). A mixture of 30 µL HCII (1 mM) and 30 µL gradient concentration of each compound solution (or Tris-HCl solution as control) was incubated at 37°C for 2 minutes. Then, 30 µL aliquot of 20 NIH/mL thrombin was added. After incubation at 37°C for 1 minute, 30 µL of 4.5 mM thrombin chromogenic substrate CS-01(38) solution was added and the thrombin activity was measured. The absorbance of the reaction mixture was all detected at 405 nm using a BioTek Microplate Reader (ELx 808, United States).

Binding of FIXa to Immobilized nHG and Competitive Bindings of Soluble nHG, dHG-5, and oHG-8

nHG with reducing terminals was biotinylated by Amine-PEG_3-Biotin and the reaction mixture was desalted using Sephadex G-25 column. Then the biotinylated nHG was immobilized onto the surface of SA biosensors. Gradient concentrations of FIXa were interacted with immobilized nHG. A competition binding assay was performed to determine their respective EC_{50} values as previously described.[40] All the experiments were conducted at 30°C in HEPES buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, and 0.1% bovine serum albumin) by biolayer interferometry (BLI) using an Octet Red 96 instrument (FortéBIO, United States).

Preparation of Rat and Rabbit Plasma

Blood was drawn from the abdominal aorta of rats after anesthetizing with chloral hydrate or the central aural artery

of rabbits by disposable needles and was collected into a glass tube containing 3.8% sodium citrate (9:1, v/v). Then after the blood was centrifuged at $1,000 \times g$ for 15 minutes at 15°C, the rat and rabbit plasma were obtained, respectively.

FXII Activation and Platelet Aggregation

The activation of human FXII in the presence of samples was assessed using previous method with modifications.[41,42] Note that 40 µL diluted plasma and 30 µL different concentrations of each compound were mixed and incubated at 37°C for 1 minute. Then, 30 µL of 0.3 mM kallikrein chromogenic substrate CS-31(02) was added and the absorbance at 405 nm was recorded for 5 minutes. The method to determine the activity is based on the difference in absorbance between the p-nitroanilide formed and the original substrate. Venous blood was drawn from young healthy volunteers anticoagulated with 3.8% sodium citrate (9:1, v/v). The volunteers participated in this study voluntarily and had not taken any antiplatelet drugs prior to drawing blood. Platelet-rich plasma (PRP) was obtained from blood after centrifugation at $180 \times g$ for 10 minutes at 15°C. And the platelet-poor plasma (PPP) was obtained from the residual blood centrifugation at $1,200 \times g$ for 20 minutes at 15°C. Washed platelets were prepared according to the method of Walsh et al[26,43] with modification. After centrifugation at $1,200 \times g$ for 10 minutes, the supernatant was discarded. The platelets were washed in calcium-free Tyrode's buffer and centrifuged at $1,200 \times g$ for 10 minutes. Then the supernatant was discarded. The washing process was repeated twice. Finally, platelets were suspended in Tyrode's buffer with 0.5 mM Ca^{2+} and adjusted to approximately $300 \sim 500 \times 10^9$/L. Note that 250 µL of PRP or washed plasma was incubated in a cuvette at 1,000 revolutions per minute at 37°C for 5 minutes and then 2.5 µL of each compound solution (30 µg/mL) or ADP solution (10 µM) was added. The change of optical density as the result of platelet aggregation were recorded by a Chrono-log 700 aggregometer (Havertown, Pennsylvania, United States) based on Born's method.[44]

Anticoagulation Assays

The APTT, PT, and TT of human, rat, and rabbit plasma were detected using a coagulometer (TECO, MC-4000, Germany).[34,41] Note that 5 µL samples solution or Tris-HCl buffer (as control) and 45 µL plasma was mixed and incubated at 37°C for 2 minutes, then 50 µL APTT solution was added. After incubating for 3 minutes, timing was started after the addition of 50 µL $CaCl_2$ (0.02 M). Note that 5 µL samples solution or Tris-HCl buffer (as control) and 45 µL plasma was mixed and incubated at 37°C for 2 minutes, then 100 µL PT solution was added and timing was started. Note that 10 µL samples solution or Tris-HCl buffer (as control) and 90 µL plasma was mixed and incubated at 37°C for 2 minutes, then 50 µL TT solution was added and timing was started.

Effect of nHG, dHG-5, and oHG-8 on Tissue Thromboplastin-Induced Venous Thrombosis

Antithrombotic activity of the tested compounds was evaluated by the tissue thromboplastin-induced venous thrombosis in male Sprague-Dawley rats.[33,34,42] Briefly, vehicle, tested compounds, or LMWH were administered dorsally and subcutaneously. After 1 hour, rats were anesthetized by 10% chloral hydrate (0.3 mL/kg). The inferior vena cava were ligated and maintained for 20 minutes at a time of 20 seconds after tissue thromboplastin was injected intravenously. The cavity was then reopened, and the thrombus formed was removed and dried for 24 hours at 50°C, then weighted. For each group, the mean thrombus weight was determined and expressed as mean ± standard deviation (SD). The absence of any inhibition of thrombus formation (compared with the mean thrombus mass in control group administered with vehicle) was represented as 100%.

Bleeding Assay

Different volumes (10, 20, 40, 80, 160 µL) of blood were collected by capillary pipette and then diluted in 40 mL of purified water. The absorbance of solutions was determined by measuring the concentration of hemoglobin at 540 nm using an ultraviolet spectrophotometer (UV-2450, SHIMADZU, Japan).[33] Standard curve was drawn based on the correlation between blood volume and average absorbance ($n = 3$). Then, different doses of the tested compounds were subcutaneously injected into the dorsum of Kunming mice. After 60 minutes, the tails of the mice were cut 5 mm from the tip and immersed in 40 mL distilled water for 90 minutes at 37°C with stirring. Blood loss was determined by the same method described above and the volume of blood loss was calculated by the standard curve.

Data Analysis

The IC_{50} was determined by fitting the data (mean ± SD) to the following equation using the Origin 2017 software (OriginLab, United States): $A = {IC_{50}}^n/({IC_{50}}^n + [I]^n)$. Where A represents the enzyme activity, $[I]$ represents the concentration of compound used as an inhibitor, n represents Hill coefficient, and IC_{50} represents the concentration of compounds that causes 50% inhibition of the enzyme and expressed as means ± standard error (SE). BLI kinetic data were analyzed using the Octet software version 7.0 and the binding curves were globally fitted using a 2:1 model. The EC_{50} was determined by fitting the data to the following equation using the Origin 2017 software (OriginLab): $A = {EC_{50}}^n/({EC_{50}}^n + [I]^n)$ and results are expressed as $EC_{50} \pm SE$.

The data of thrombus formation and blood loss were analyzed using one-way analysis of variance followed by Duncan's multiple-range test using IBM SPSS. All values for each group were given as the means ± SD. Note that p-values less than 0.05 was considered to be statistically significant (i.e., $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$).

Results

Chemical Characteristics of nHG, dHGs, and oHG-8

The anticoagulant and antithrombotic activity of FG derived from various sea cucumber species has been reported.[34] Herein, a native FG consisting of a chondroitin sulfate E (CS-E) backbone composed of alternating β-*D*-glucuronic acid and N-acetyl-β-*D*-galactosamine disaccharide and

Table 1 Effects of nHG, dHGs, and oHG-8 on coagulation factors[a]

Compounds	Mw (kDa)	iXase (IC_{50}, ng/mL)	FXa (AT) (IC_{50}, ng/mL)	FIIa (AT) (IC_{50}, ng/mL)	FIIa (HCII) (IC_{50}, ng/mL)
nHG	42.6	41.9 ± 2.76	> 10,000	448 ± 23.6	589 ± 62.4
dHG-1	39.9	35.8 ± 1.90	> 10,000	732 ± 56.8	543 ± 54.1
dHG-2	27.8	32.4 ± 3.11	> 10,000	1,080 ± 133	456 ± 36.1
dHG-3	14.9	31.2 ± 2.12	> 10,000	1,800 ± 285	316 ± 14.4
dHG-4	8.24	31.5 ± 1.14	> 10,000	3,850 ± 524	221 ± 20.3
dHG-5	5.30	69.7 ± 6.89	> 10,000	> 5,000	152 ± 16.1
dHG-6	3.12	159 ± 15.0	> 10,000	> 5,000	286 ± 25.7
oHG-8	2.46	156 ± 23.3	> 10,000	> 10,000	576 ± 19.4
LMWH	4.50	164 ± 15.4	21.9 ± 4.68	43.0 ± 2.11	231 ± 9.30
DS		/[b]	/	/	99.9 ± 9.82

Abbreviations: AT, antithrombin; DS, dermatan sulfate; FIIa, factor IIa (thrombin); FXa, factor Xa; iXase, intrinsic factor Xase; LMWH, low molecular weight heparin; Mw, molecular weight; SE, standard error.
[a]IC_{50} value was expressed as mean ± standard error (SE).
[b]Not detected.

branches of sulfated α-*L*-fucose linked to position 3 of glucuronic acid residues was obtained from *H. fuscopunctata* (nHG) (►**Fig. 1A**) with yield of approximately 1.0%. The ^{1}H NMR spectrum (►**Supplementary Fig. S1**, available in the online version) showed that nHG contains *L*-Fuc_{3S4S} (~85%) as the main type of branches, and minor amounts of *L*-Fuc_{2S4S} and *L*-Fuc_{4S} branches (~15%).

The high-performance gel permeation chromatography (HPGPC) profiles of dHG-1 ~ –6 are shown in ►**Fig. 1A**, indicating that they are homogeneous products and the molecular weight decreases successively (39.9 ~ 3.12 kD, ►**Table 1**). dHG-1 ~ –6 were the mixture of oligosaccharides with different degree of polymerization based on HPGPC analysis, like dHG-5 (►**Fig. 1B**). The structural difference among these depolymerized oligosaccharides was the number of the repeating trisaccharide unit [-3-D-GalNAc4s6s-β1,4-(L-Fuc-α1,3)-D-GlcA-β1]. According to the ^{1}H/^{13}C NMR spectra (taking dHG-5 as an example, ►**Supplementary Fig. S2**, available in the online version), the nonreducing ends of dHGs were $\Delta^{4,5}$-unsaturated glucuronic acids (ΔU) and the reducing terminals were the alditol of glucuronic acids (*D*-GlcA-ol, *L*-gulonic acid) (►**Supplementary Fig. S2**, available in the online version).

Recently, we have found that octasaccharide from the β-eliminative dFG was the minimum fragment exhibiting strong and selective iXase inhibitory activity.[36] Thus, an octasaccharide (oHG-8) was purified from dHG-5 for comparative analysis, and its structure was characterized by HPGPC (►**Fig. 1B**) and ^{1}H/^{13}C NMR spectra (►**Supplementary Fig. S3**, available in the online version).

The Anti-iXase Effects of nHG, dHG-1~6, and oHG-8

Increasing concentrations of nHG and its depolymerized products (dHG-1~6) could essentially result in complete inhibition of FXa generation by anti-iXase (►**Fig. 2**). The results shown in ►**Table 1** indicated that nHG (Mw ~42.6 kD) and dHG-1~5 (Mw from 39.9 ~ 5.30 kD) had significant iXase inhibitory activities, with the IC_{50} values of 31.2 ~ 69.7 ng/mL. Anti-iXase activities of dHG-1~5 were approximately threefold higher than that of LMWH (IC_{50}, 164 ng/mL). The IC_{50} values of dHG-6 (Mw ~3.12 kD) and oHG-8 (Mw ~2.46 kD) were 159 and 156 ng/mL, respectively, suggesting the iXase inhibitory activity of them were reduced.

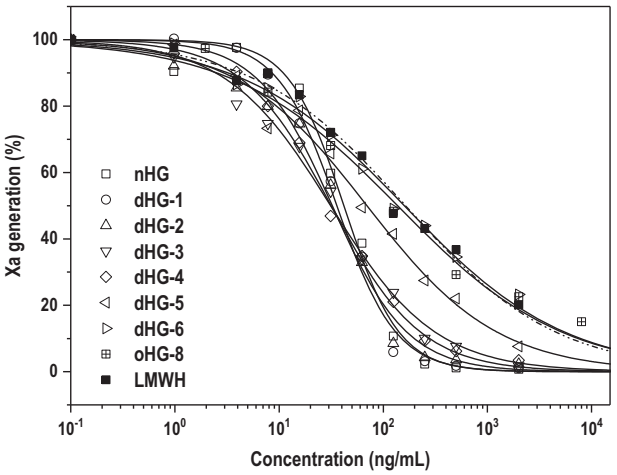

Fig. 2 Effects of native FG (nHG), depolymerized nHGs (dHGs), and octasaccharide (oHG-8) on intrinsic tenase (iXase). Different concentrations of each compound solution (30 µL), 30 µL factor VIII (FVIII), and 30 µL R2 solution (containing 60 nM FIXa, human thrombin, phosphatidylcholine/phosphatidylserine [PC/PS], and Ca^{2+}) were mixed and incubated at 37°C for 2 minutes. Then, 30 µL R1 solution (containing 50 nM FX and thrombin inhibitor) was added. After incubation for 1 minute at 37°C, the residual FXa activity was measured by the addition of 30 µL FXa chromogenic substrate SXa-11. Substrate hydrolysis was detected using a BioTek Microplate Reader. The data were nonlinear fitted by the Origin 2017 software and the IC_{50} values (mean ± standard error [SE]) are showed in ►**Table 1**.

Effects on FXa or FIIa in the Presence or Absence of AT or HCII

It has been reported that FGs possess no significant effect on FVII and FIXa in the presence or absence of AT. And FGs containing higher Fuc_{2S4S} branches exhibit stronger AT-dependent anti-IIa activities.[33,34] To study and compare the anticoagulant mechanism and pharmacological targets of nHG and its derivatives, their effects on FXa and FIIa were tested using pure coagulation factors by a chromogenic substrate method (►**Table 1**, ►**Fig. 3**).

nHG and its derivatives showed no significant AT-dependent anti-FXa activity ($IC_{50} > 10{,}000$ ng/mL) compared with LMWH (IC_{50}, 21.9 ng/mL). The AT-dependent anti-FIIa activities of these compounds were decreased with the reduction of their molecular weight. nHG (IC_{50}, 448 ng/mL) had certain AT-dependent anti-FIIa activity compared with LMWH (IC_{50}, 43.0 ng/mL), while dHG-5 ($IC_{50} > 5{,}000$ ng/mL) and oHG-8 ($IC_{50} > 10{,}000$ ng/mL) showed no such effect. Moreover, all tested compounds showed no significant anti-FXa and anti-FIIa activities in the absence of AT (►**Supplementary Fig. S4**, available in the online version).

The HCII-dependent anti-FIIa activity of dHG-5 (IC_{50}, 152 ng/mL) was weaker than the positive control DS (IC_{50}, 99.9 ng/mL) and slightly stronger than LMWH (IC_{50}, 231 ng/mL). nHG (IC_{50}, 589 ng/mL) and oHG-8 (IC_{50}, 576 ng/mL) was relatively weaker in HCII-dependent anti-FIIa activity.

Among the dHGs, dHG-5 with a relative low molecular weight showed potent iXase inhibitory activity and certain HCII-dependent anti-FIIa activity. Additionally, it had no anti-FXa and anti-FIIa activities in the presence or absence of AT. Therefore, dHG-5 was selected for further pharmacodynamics analysis compared with nHG and oHG-8.

Competitive Bindings of Soluble nHG, dHG-5, and oHG-8 to FIXa with Immobilized nHG

iXase is an enzyme complex formed by FIXa and cofactor FVIIIa on the membrane surface of activated platelets. It has been demonstrated that nonasaccharide inhibits iXase activity through binding to FIXa and disrupting the interaction between FIXa and FVIIIa A2 domain.[40] Thus, the interactions between FIXa and nHG, dHG-5, and oHG-8 were detected for confirming their mechanism of anti-iXase. Direct binding assay by BLI technology showed that FIXa could bind to immobilized nHG with high affinity (►**Supplementary Fig. S5**, ►**Supplementary Table S1**, available in the online version). To assess the apparent K_D of soluble nHG, dHG-5, oHG-8, and LMWH to compete with the immobilized nHG for binding to FIXa, the competition binding assays were

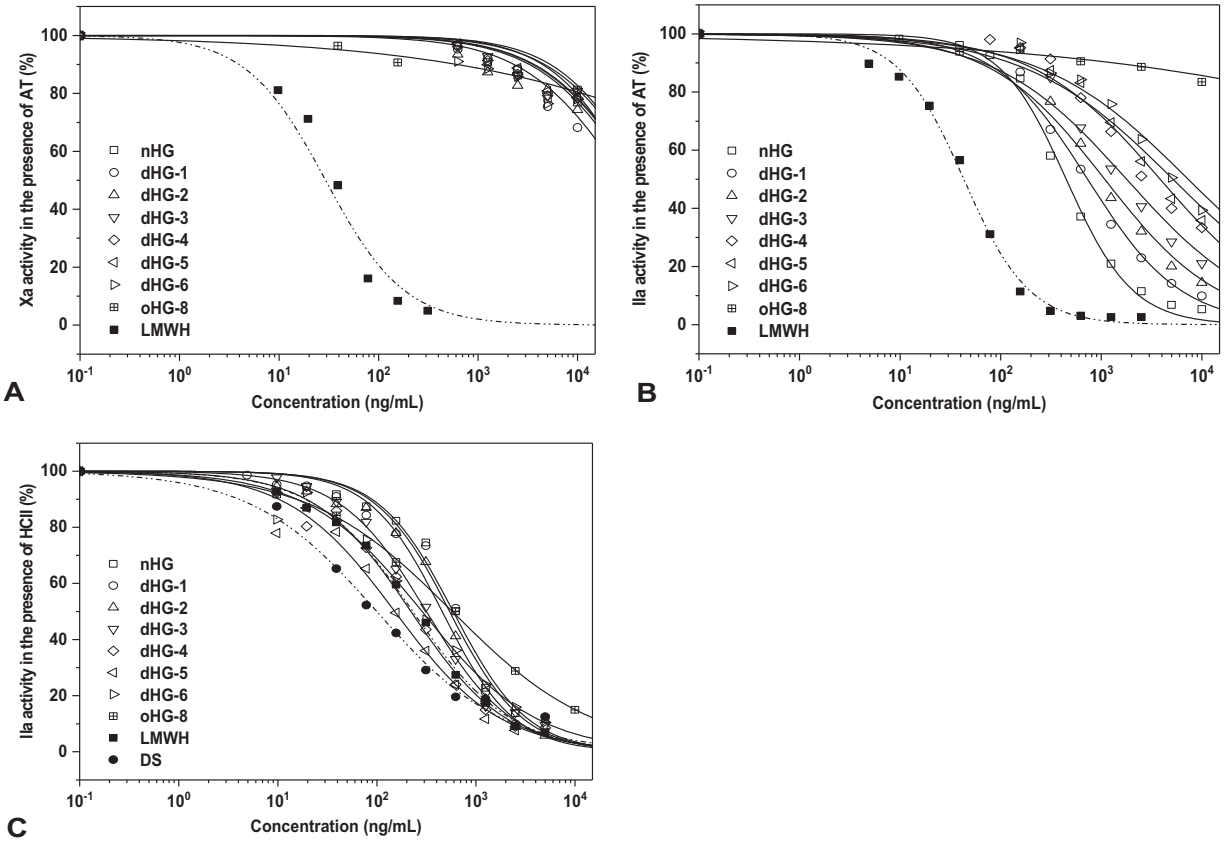

Fig. 3 Effects of native FG (nHG), depolymerized nHGs (dHGs), and octasaccharide (oHG-8) on factor Xa (FXa) activities in the presence of antithrombin (AT) (**A**), FIIa activities in the presence of AT (**B**), or heparin cofactor II (HCII) (**C**). AT (**A**, **B**) or HCII (**C**) were incubated with FXa or FIIa (**B**) in the presence of increasing concentrations of each compound. After incubation for 1 minute, the remaining FXa or FIIa activity was determined using a chromogenic substrate. Substrate hydrolysis was detected using a BioTek Microplate Reader. The data were nonlinear fitted by the Origin 2017 software and the IC_{50} values (mean ± standard error [SE]) are showed in ►**Table 1**.

performed to determine their respective EC_{50} values (►**Fig. 4**). The EC_{50} values for nHG, dHG-5, oHG-8, and LMWH were approximately 21.0, 482, 1,810, and 2,460 nM, respectively (►**Fig. 5**, ►**Supplementary Table S2**, available in the online version). The results showed that nHG, dHG-5, oHG-8, or LMWH could dose-dependently inhibit the binding of FIXa to the immobilized nHG, suggesting that nHG, dHG-5, and oHG-8 may have the same FIXa binding site with LMWH. The relative FIXa binding affinity of dHG-5 was approximately three- and fourfold higher than that of oHG-8 and LMWH, respectively, based on their EC_{50} values, consistent with the potent tendency of their anti-iXase activities. Although dHG-5 had similar anti-iXase activity with nHG (based on IC_{50} values), its relative FIXa binding affinity was weaker. Since the apparent affinity of protease binding to UFH will increase with chain length, we suspect that the stronger FIXa binding affinity of nHG might be attributed to its longer chain length.[45,46]

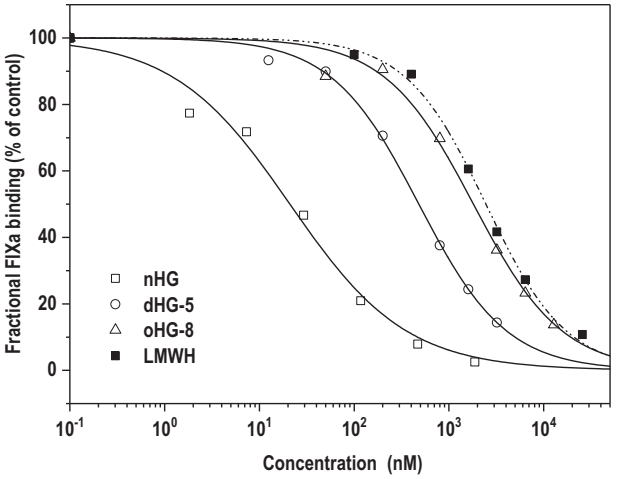

Fig. 5 Competitive bindings of soluble native FG (nHG), depolymerized nHG (dHG)-5, and octasaccharide (oHG-8) to factor IXa (FIXa) with Immobilized nHG. The data were nonlinearly fitted by the Origin 2017 software and the results were expressed as mean ± standard error (SE). The EC_{50} values of nHG, dHG-5, oHG-8, and low molecular weight heparin (LMWH) were 21.0 ± 3.20, 482 ± 22.2, 1,810 ± 190, and 2,460 ± 106 nM, respectively.

FXII Activation and Platelet Aggregation

It has been reported that FGs have the undesired effects of inducing FXII activation and platelet aggregation, which were reduced with the decrease of their molecular weight.[26,34] The nHG with the high molecular weight of 42.6 kDa showed potent FXII activation activity at the concentration range from 0.38 to 192 µg/mL, comparable to the positive control OSCS (►**Fig. 6**). While the depolymerized products dHG-5 (Mw ~5.30 kDa) and oHG-8 (Mw ~2.46 kDa) showed no obvious effect on FXII activation at the concentration up to 192 µg/mL.

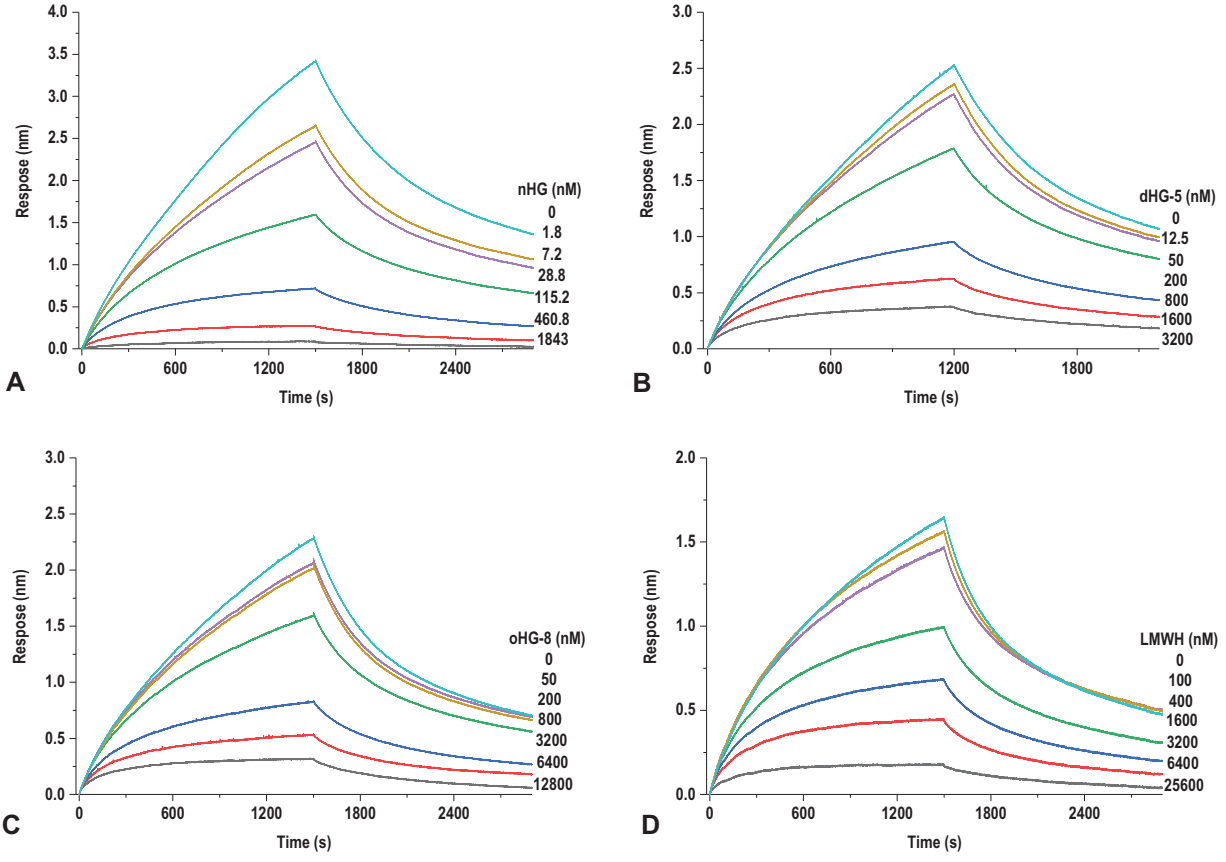

Fig. 4 Biolayer interferometry (BLI) response diagrams of factor IXa (FIXa) binding to immobilized native FG (nHG) competed by soluble nHG (**A**), depolymerized nHG (dHG)-5 (**B**), octasaccharide (oHG-8) (**C**), and low molecular weight heparin (LMWH) (**D**). Increasing concentrations of nHG, dHG-5, oHG-8, or LMWH were preincubated with 25 nM FIXa prior to interaction with immobilized nHG. The experiments were conducted at 30°C in HEPES buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, and 0.1% bovine serum albumin [BSA]) by BLI technology using an Octet Red 96 instrument.

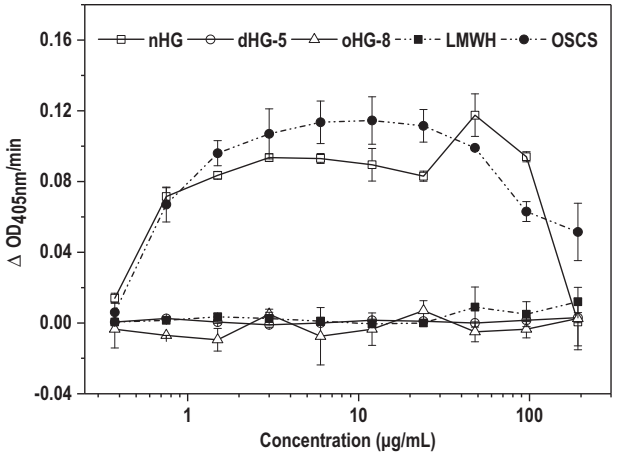

Fig. 6 Effects of native FG (nHG), depolymerized nHG (dHG)-5, and octasaccharide (oHG-8) on factor XII (FXII) activation. 40 μL diluted plasma and 30 μL different concentrations of each compound were mixed and incubated at 37°C for 1 minute. Then, 30 μL of 0.3 mM kallikrein chromogenic substrate CS-31(02) was added and the absorbance at 405 nm was recorded for 5 minutes. The method to determine the activity is based on the difference in absorbance between the p-nitroanilide formed and the original substrate. The concentration range of each compound was from 0.38 to 192 μg/mL and the data were expressed as mean ± standard deviation (SD).

Additionally, nHG could induce the aggregation of human platelets and rat-washed platelets at the concentration of 30 μg/mL, while dHG-5 and oHG-8 had no observable effects on platelet aggregation at the same concentration (►**Fig. 7**). The maximum aggregation rates of human platelets induced by nHG, dHG-5, oHG-8, and ADP were 43.5 ± 3.5%, 2.5 ± 0.7%, 1.5 ± 0.7%, and 72.0 ± 5.7%, respectively. The maximum aggregation rates of rat-washed platelets induced by nHG, dHG-5, oHG-8, and ADP were 82.5 ± 2.1%, 1.5 ± 0.7%, 1.5 ± 0.7%, and 46.5 ± 4.9%, respectively. These results suggested that dHG-5 and oHG-8, without the undesired effects of FXII activation and platelet aggregation, may be the promising candidates for anticoagulant development.

The Effects of nHG, dHG-5, and oHG-8 on the Coagulation of Human, Rat, and Rabbit Plasma

To assess the anticoagulant activity and the correlation with anti-iXase activities, the effects of nHG, dHG-5, and oHG-8 on the APTT, PT, and TT of human, rat, and rabbit plasma were detected, and compared with LMWH (►**Fig. 8**, ►**Table 2**).

nHG, dHG-5, and oHG-8 could significantly prolong the APTT of human, rat, and rabbit plasma. In human plasma, the concentration required to double APTT for dHG-5 was 7.67 μg/mL. Its activity was approximately twofold stronger than oHG-8 (16.9 μg/mL), and slightly stronger than LMWH (8.11 μg/mL). In rat plasma, the APTT prolonging effects of nHG, dHG-5, oHG-8, and LMWH had much less discrepancy, and the concentrations of them required for double APTT were 12.4, 11.7, 17.6, and 12.3 μg/mL, respectively. Moreover, the APTT prolonging activities of nHG, dHG-5, and oHG-8 in rabbit plasma had the same activity trend with that in human plasma, while the activity of dHG-5 (67.6 μg/mL) was slightly weaker than LMWH (50.6 μg/mL).

nHG, dHG-5, and oHG-8 showed no significant effects on PT of human, rat, and rabbit plasma at the concentration of up to 128 μg/mL (►**Supplementary Fig. S6**, available in the online version). And dHG-5 and oHG-8 also had no significant effect on TT of those plasmas. However, nHG showed significant TT prolonging activities in human, rat, and rabbit plasmas (the concentrations required for double TT were 6.46, 48.0, and 20.6 μg/mL, respectively), although much weaker than LMWH (the concentrations required for double TT were 1.50, 6.00, and 6.00 μg/mL, respectively) (►**Supplementary Fig. S7**, available in the online version).

Overall, it indicated that the anticoagulation effects of dHG-5 and oHG-8 are mainly on the intrinsic coagulation pathway and the APTT prolonging activities could be mainly

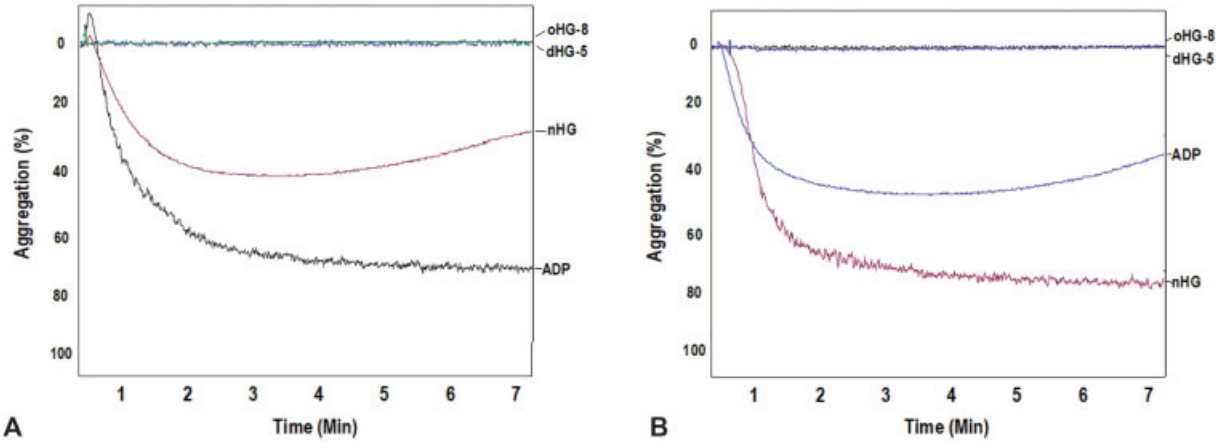

Fig. 7 The effects of native FG (nHG), depolymerized nHG (dHG)-5, octasaccharide (oHG-8), and adenosine diphosphate (ADP) on human platelets (platelet-rich plasma [PRP], **A**) and rat washed platelets (**B**). Human blood was drawn from cubital vein of the volunteer and PRP was collected after centrifugation (180 × *g*, 10 minutes, 15°C). Rat platelets were washed by calcium-free Tyrode's buffer and suspended in Tyrode's buffer with 0.5 mM Ca^{2+}. The aggregation curve of each compound (30 μg/mL) was detected using Chrono-log 700 aggregometer, and ADP (10 μM) was used as control. The maximum aggregation rates of human PRP induced by nHG, dHG-5, oHG-8, and ADP were 43.5 ± 3.5%, 2.5 ± 0.7%, 1.5 ± 0.7%, and 72.0 ± 5.7%, respectively. The maximum aggregation rates of washed platelet from rats induced by nHG, dHG-5, oHG-8, and ADP were 82.5 ± 2.1%, 1.5 ± 0.7%, 1.5 ± 0.7%, and 46.5 ± 4.9%, respectively.

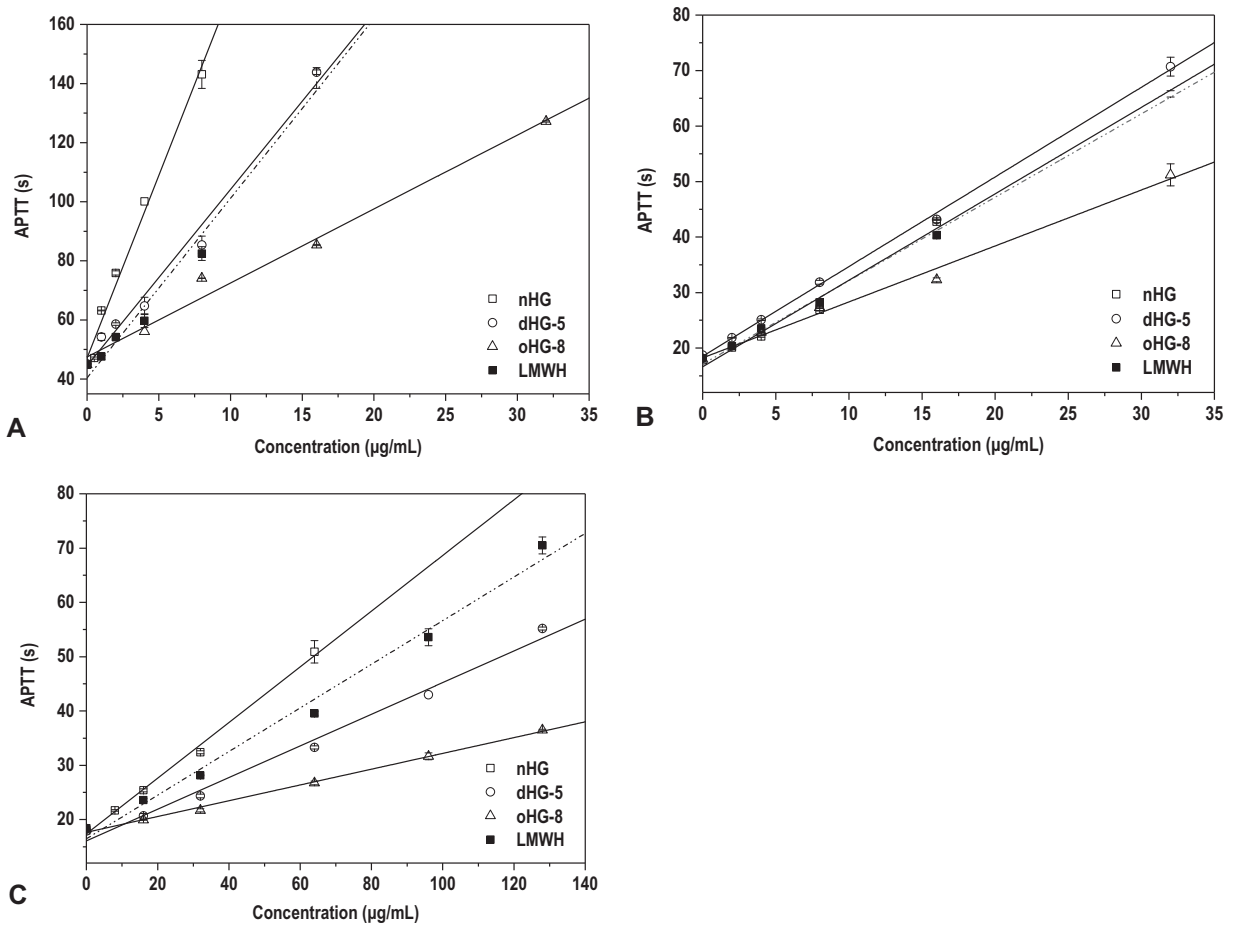

Fig. 8 Effects of native FG (nHG), depolymerized nHG (dHG)-5, and octasaccharide (oHG-8) on the activated partial thromboplastin time (APTT) of human coagulation control plasma (**A**), rat plasma (**B**), and rabbit plasma (**C**). 5 μL samples solution or Tris-HCl buffer (as control) and 45 μL plasma was mixed and incubated at 37°C for 2 minutes, then 50 μL APTT solution was added, after incubation for 3 minutes, timing was started after the addition of 50 μL $CaCl_2$ (0.02 M). Mean values for APTT were plotted versus concentration with error bars representing ± standard deviation (SD) ($n = 3$). The data were linearly fitted by the Origin 2017 software.

ascribed to their anti-iXase effects. nHG and LMWH also had TT prolonging activity, which might be due to its inhibition activity of FXa or FIIa in the presence of AT.

The Antithrombotic Effects of nHG, dHG-5, and oHG-8

Generally, arterial thrombotic disease is treated with antiplatelet drugs, while venous thrombosis is treated with anticoagulants that target proteins of the coagulation cascade.[2] Considering the potent anticoagulation effects of nHG, dHG-5, and oHG-8, a deep venous thrombosis model was used to evaluate their antithrombotic activities. The thrombus formation was initiated by tissue thromboplastin (intravenously) after subcutaneous injection of the tested compounds for 1 hour. The results showed that dHG-5 (4.0 ~ 9.0 mg/kg) and oHG-8 (3.3 ~ 7.5 mg/kg) can significantly inhibit thrombus formation in a dose-dependent manner

Table 2 The APTT, PT, and TT prolonging effects of nHG, dHG-5, and oHG-8 ($n = 3$)

Compounds	Mw (kDa)	APTT (μg/mL)[a]			PT (μg/mL)[b]			TT (μg/mL)[c]		
		Human	Rat	Rabbit	Human	Rat	Rabbit	Human	Rat	Rabbit
nHG	42.6	3.45	12.4	36.3	> 128	> 128	> 128	6.46	48.0	20.6
dHG-5	5.30	7.67	11.7	67.6	> 128	> 128	> 128	> 128	> 128	> 128
oHG-8	2.46	16.9	17.6	> 128	> 128	> 128	> 128	> 128	> 128	> 128
LMWH	4.50	8.11	12.3	50.6	> 128	> 128	> 128	6.00	1.50	6.00

Abbreviations: APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; LMWH, low molecular weight heparin; Mw, molecular weight.
[a]The concentrations required to double APTT.
[b]The concentrations required to double PT.
[c]The concentrations required to double TT.

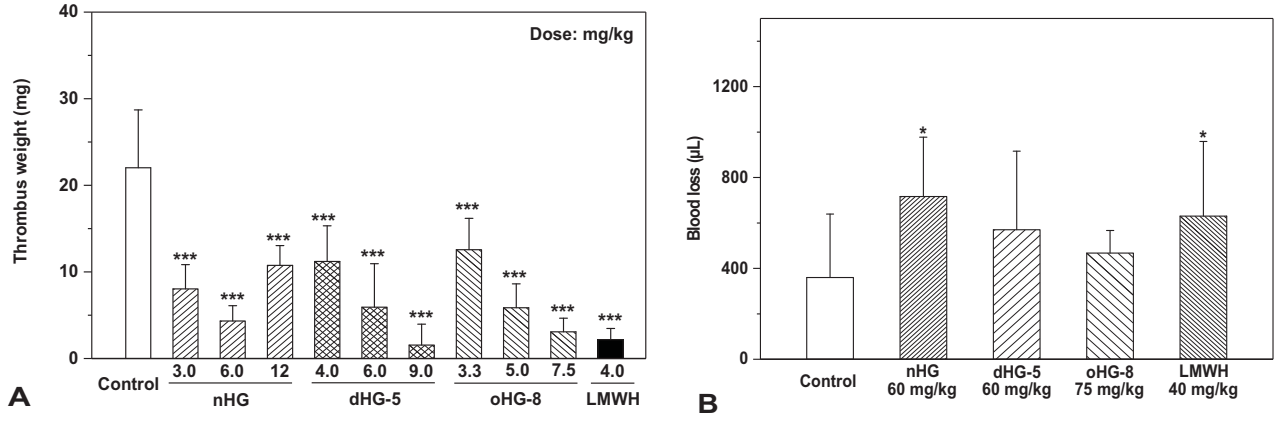

Fig. 9 (A) The effects of native FG (nHG), depolymerized nHG (dHG)-5 and octasaccharide (oHG-8) in rat venous thrombus formation. Antithrombotic activity was investigated in male Sprague-Dawley rats with the tissue thromboplastin-induced venous thrombosis model. The inhibition of thrombus formation in the presence of each compound was described in detail in "Materials and Methods." The results were expressed as thrombus weight (mean ± standard deviation [SD], $n = 8$, $^{***}p < 0.001$ and $^{**}p < 0.01$ vs. control). (**B**) Blood loss of mice treated with nHG, dHG-5, and oHG-8. Samples were injected subcutaneously into Kunming mice. After 60 minutes, the tails of the mice were cut 5 mm from the tip and immersed in 40 mL of distilled water for 90 minutes at 37°C with stirring. Blood loss was determined by measuring the hemoglobin present in the water. The results were expressed as blood loss (mean ± SD, $n = 6$, $^{*}p < 0.05$ vs. control).

(►**Fig. 9A**). Based on the dry weight of the thrombus, and compared with the control group, the inhibitory rates of dHG-5 at 6.0 and 9.0 mg/kg were approximately 77.2 and 92.9%, respectively ($p < 0.001$). The inhibitory rates of oHG-8 at 5.0 and 7.5 mg/kg were approximately 73.4 and 86.0%, respectively ($p < 0.001$). The inhibitory rates of nHG at 3.0 and 6.0 mg/kg were approximately 63.5 and 80.3%, respectively ($p < 0.001$). However, the inhibitory rate of nHG at 12 mg/kg (~ 51.2%) was markedly lower than that at 6.0 mg/kg, which might be due to the partial offset by its procoagulant effects produced by FXII activation and platelet aggregation.

The Hemorrhagic Tendency of nHG, dHG-5, and oHG-8

The risk of hemorrhagic complications is still a major concern with anticoagulants that are used for antithrombosis. Herein, 10-fold effective doses of nHG, dHG-5, and oHG-8 for thrombus inhibition (~80% inhibition rate) were used in the bleeding assay. The bleeding volumes of mice treated with nHG (60 mg/kg), dHG-5 (60 mg/kg), oHG-8 (75 mg/kg), and LMWH (40 mg/kg) were analyzed and compared (►**Fig. 9B**). The bleeding volumes of mice treated with dHG-5 at 60 mg/kg or oHG-8 at 75 mg/kg did not increase significantly compared with the control group ($p > 0.05$). Whereas the bleeding volumes of mice treated with nHG at 60 mg/kg or LMWH at 40 mg/kg were significantly increased compared with the control group ($p < 0.05$). It suggested that dHG-5 and oHG-8 have lower hemorrhagic risk than nHG and LMWH.

Discussion and Conclusion

The target of anticoagulants is crucial for their characteristics of anticoagulant activity and effects on hemostatic function. Current clinical anticoagulant drugs, such as heparins, direct FXa inhibitors, and direct thrombin inhibitors, target the coagulation factors in the common pathway which are also essential for hemostasis. Therefore, therapeutic and prophylactic effects of these anticoagulants may be associated with potentially severe and fatal bleeding complications.[4,7] In the past decade, increasing studies have demonstrated that selectively targeting intrinsic coagulation pathway may represent a promising safer anticoagulant strategy. And as the final and rate-limiting enzyme of the intrinsic coagulation pathway, iXase becomes one of such anticoagulant targets for prevention of thrombosis with low hemorrhagic risk.[14,24]

FG is a structurally unique glycosaminoglycan derivative with fucose side chains extracted from the body wall of sea cucumbers. FG has been studied in several aspects, including the discovery of various structures from different sea cucumber species, the preparation of oligosaccharides via selective depolymerization for further structure illumination, and the studies of structure–anticoagulant activity relationship. It was found that native FGs have potent iXase inhibitory activity, but with low selectivity.[25,28,31] Native FG could target the coagulation factors in both intrinsic and common coagulation pathway, and it also had the undesired effects of inducing platelet aggregation and FXII activation.[26–28] Compared with native FG, its depolymerized products have higher selectivity of iXase inhibition, while maintaining the anticoagulant activity. In the 1990s, DHG, an oxidative depolymerized product of FG from *Stichopus japonicus*, was systematically studied on chemical structure, anticoagulant activity, and anticoagulant mechanism. The antithrombotic mechanism of DHG was demonstrated to be serpin-independent, which was largely accounted to the inhibition of the iXase complex formation. And DHG inhibit the activity of iXase through binding to the FIXa exosite directly.[24,29,30] However, the molecular weight and structural features of FG could affect the anticoagulant potency and target selectivity.[34] DHG might have platelet and FXII activation activities due to its relatively high molecular weight of approximately 12,500 Da. Additionally, the limited selectivity of free radical depolymerization leads to the complexity of the structural characteristics.[47] Besides, Nitrophorin

2, as a 20-kDa lipoprotein identified from the salivary glands of the blood sucking insect, has been characterized as an inhibitor of the intrinsic pathway of coagulation upon binding to FIXa. And it has been demonstrated as a potent antithrombotic agent in rat models of thrombosis which do not cause excessive bleeding in the rat tail transection model.[48]

Herein, the native fucosylated glycosaminoglycan (nHG), which was mainly branched with Fuc_{3S4S} residues, was extracted from the sea cucumber *H. fuscopunctata*. Compared with other FGs bearing various types of Fuc branches,[49,50] the highly regular structure of nHG was conducive to its quality control. Moreover, its low molecular weight products were composed of well-defined oligosaccharides with regular and clear nonreducing and reducing terminals prepared by β-eliminative depolymerization method. This selective cleavage method also facilitated to remove the non-FG polymers simultaneously. The pharmacological and pharmacodynamic characteristics of nHG and its depolymerized products were studied and compared in vitro and in vivo, to find a safe and effective anticoagulant candidate with negligible hemorrhagic risk.

nHG had potent anticoagulant activity associated with multiple targets and could induce platelet aggregation and FXII activation, which is consistent with the activities of native FG from other sea cucumber species.[26–28] Its depolymerized products showed potent iXase inhibitory activities compared with LMWH. The iXase inhibitory activities of dHG-6 and oHG-8 were similar to that of LMWH, while anticoagulant and antithrombotic activity of LMWH was considered to be dependent on its effects on FIIa and FXa due to its far stronger AT-dependent FIIa and FXa inhibitory activities. Compared with LMWH, the oligosaccharides from FG were selective iXase inhibitors. Because FIIa and FXa are necessary for physiological hemostasis, LMWH could impair hemostatic function when it is used to inhibit thrombosis. Currently, it was found that intrinsic coagulation pathway is related to pathological thrombosis but at least partly dispensable for normal hemostasis, thus the intrinsic coagulation pathway inhibitors could inhibit pathological thrombosis without or slightly affecting hemostatic function.

Then, dHG-5 and oHG-8 were selected to compare the effects of anticoagulation and antithrombosis with nHG and LMWH. They showed potent iXase inhibitory activities that are higher than LMWH but slightly lower than nHG, and the activity of dHG-5 was approximately threefold of oHG-8. This activity might be attributed to the binding of dHG-5 and oHG-8 to FIXa, which disrupts the interaction between FIXa and FVIIIa A2 domain. The dHG-5 and oHG-8 lack FXII activation and platelet aggregation activities, thus they may not cause allergic reaction and affect antithrombotic effects, respectively. Compared with nHG and LMWH, dHG-5 and oHG-8 had no obvious effect on TT but showed significant APTT prolonging activity. It indicated that dHG-5 and oHG-8 target the intrinsic coagulation pathway and have potent anticoagulation activity. The APTT prolonging activity of dHG-5 was about one time stronger than oHG-8 in three detected plasma. Combining the results of their anti-iXase activities, the effects of dHG-5 and oHG-8 on the intrinsic coagulation pathway could be mainly ascribed to their iXase inhibitory activities.

In vivo, dHG-5 (4.0 ~ 9.0 mg/kg) and oHG-8 (3.3 ~ 7.5 mg/kg) could dose-dependently inhibit the deep venous thrombosis induced by the tissue thromboplastin. However, compared with the inhibitory effects of nHG at 6 mg/kg, the inhibition rate of nHG at 12 mg/kg decreased significantly. The weaken effect of nHG at high doses on the inhibition of thrombus formation may be attributed to its FXII activation and platelet aggregation activities. Like other anticoagulants under study targeting intrinsic coagulation pathway factors such as FXIIa, FXIa, and FIXa, dHG-5 and oHG-8 did not result in increased bleeding risk, with lower bleeding tendency than LMWH.[12,15] Though the octasaccharide oHG-8 displayed potent anti-iXase activity, its preparation yield was low and the production cost was much higher than dHG-5.

In conclusion, dHG-5, a potent iXase inhibitor, may be a promising anticoagulant candidate for antithrombotic therapies without increasing bleeding complications. Although dHG-5 is a multicomponent agent, our group has established effective preparation technics and quality control methods, and these studies will be published elsewhere. And the pharmacokinetic studies of dHG-5 are undergoing.

What is known about this topic?

- The inhibitors of intrinsic coagulation pathway can prevent thrombosis with low bleeding risks, and intrinsic factor Xase complex (iXase) is the rate-limiting enzyme of the intrinsic coagulation pathway.
- Depolymerization is an effective method to reduce the side effects of FG, and depolymerized FGs show higher selectivity in inhibiting the intrinsic coagulation pathway than native FGs.

What does this paper add?

- dHG-5 and oHG-8, depolymerized products of nHG, show potent anti-iXase activity with no AT-dependent FXa or FIIa inhibition, FXII activation, and platelet aggregation activities.
- dHG-5 and oHG-8 inhibit deep venous thrombosis in a dose-dependent manner, and they show negligible hemorrhagic risk compared with nHG and LMWH.
- dHG-5 may be a promising anticoagulant candidate for venous thrombosis treatment.

Authors' Contributions

L.Z. and N.G. performed research, analyzed data, and wrote the manuscript. H.S., C.X., L.Y., and Z.L. performed research. L.L. and R.Y. revised the manuscript. H.Z., X.J., and J.Z. designed the research, analyzed data, and revised the manuscript.

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Conflict of Interest
None declared.

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