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Synthesis and properties of functional glycomimetics through click grafting of fucose onto chondroitin sulfates

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ABSTRACT:

Fucosylated chondroitin sulfate (fCS), a representative marine polysaccharide isolated from sea cucumber, possesses diverse biological functions especially as a promising anticoagulant. However, its supply suffers from the challenges of high-cost materials, different species and batch-to-batch variability. In the present study, we designed a concise route for the synthesis of functional glycomimetics by using natural fCS as

template. DMTMM-mediated amidation was applied on chondroitin sulfates for site-selective alkynylation with controllable ratios between 0.15~0.78. A small library of 12 fCS glycomimetics with specific sulfation patterns and fucose branches was prepared through copper-catalyzed azide–alkyne cycloaddition (CuAAC), which was fully characterized by NMR and SEC-MALLS-RI. Through screening of their biological activities, CSE-F1 and CSE-SF1 exhibited anticoagulant activities through intrinsic pathway and inhibition of FXa by ATIII. The concise approach developed herein supplies novel glycopolymers to mimic the distinct functions of natural polysaccharides and promote the development of marine carbohydrate-based drugs.

KEYWORDS: Fucosylated chondroitin sulfates (fCS), alkynylation, azide-alkyne click reaction, fucose, glycomimetics, anticoagulation.

INTRODUCTION

As a representative L-hexose, fucose is widely present in nature especially as a component of *N*-glycans[1-3] involved in signal transduction and cell adhesion, as well as being the fundamental building blocks of natural polysaccharides[4-6] that exhibit antivirus, anticoagulant and antitumor activities. Fucosylated chondroitin sulfate (fCS) is exclusively isolated from sea cucumber,[7] and the structural properties of fCS are interesting with fucosylation at the *O*-3 position of D-glucuronic acid (GlcA)[8, 9] or *O*-6 position of *N*-acetyl-D-galactosamine (GalNAc)[10-12] on the backbone of chondroitin sulfate (CS) (Figure 1A). fCS has anticoagulant and antithrombotic

activity through inhibition of thrombin (FIIa) and factor Xa (FXa) mediated by antithrombin (AT) and heparin cofactor II (HC-II),[13-15] whereas the counterpart CS exhibits no parallel activity.[16] It is noteworthy that fCS exhibits anticoagulant activity even when it is administered orally, with low bleeding risk,[17] promoting fCS development as a potential drug candidate. The oligosaccharides depolymerized from natural fCS exhibit selective inhibition of intrinsic tenase,[18, 19] and a nonasaccharide bearing a 2,4-di-*O*-sulfated fucosyl residue is the minimum fragment for intrinsic tenase inhibition.[20] The anticoagulant activity of natural fCS is strongly related to its molecular weight, fucose branches, and sulfation patterns.[16] Furthermore, fCS isolated from sea cucumber *Holothuria Mexicana* exhibited high affinity to fibroblast growth factor (FGF) 1 and 2 influenced by the specific linkage types.[10] Chondroitin sulfates are easily obtained with high purity by extraction[21-23] and fermentation,[24, 25] allowing them to be commercially available, while natural fCS is still rare and relatively expensive with complex and diverse structures. Clinical studies of fCS polysaccharides have been thoroughly performed in various countries,[5, 16] but the structure-activity relationships (SARs) are still unclear and must be clarified. Furthermore, potential contaminations including other types of GAGs and proteins will also affect the purity and SAR studies of natural polysaccharides.

To decipher the structure-activity relationship (SAR) of fCS, chemical synthesis is an alternative way to obtain structurally well-defined fCS oligosaccharides for anticoagulant activity studies. To the best of our knowledge, although synthesis of the

chondroitin sulfate backbone is increasingly reported,[26] the chemical synthesis of fCS remains a significant challenge. Tamura et al.[27] reported a total synthesis of the fCS repeating trisaccharide via a stepwise coupling strategy that resulted in a monosulfated trisaccharide. To study the effects of the sulfation patterns on the bioactivity, Nifantiev et al. synthesized regioselectively sulfated fCS oligosaccharides[28] and analyzed their conformations for SAR.[29] However, bioactivities of the total synthesized fCS fragments were not evaluated. To circumvent the tremendous effort and time consuming process of total synthesis, semi-synthetic approaches have attracted attention to reduce glycosylation coupling steps and manipulations of protecting groups. Recently, Li et al.[30] reported the synthesis of fCS fragments by combining enzymatic degradation of chondroitin and chemical fucosylation over 12 linear steps. The synthetic nonasaccharide exhibited selective intrinsic tenase inhibition, as previously reported.[20] This approach proved that semi-synthesis could serve as an efficient tool for mimicking natural fCS polysaccharides.

The synthesis of glycomimetics, which preserve the structural and biological features of natural polysaccharides, has received considerable attention in recent years for assisting the SAR study of complex polysaccharides as well as for the development of carbohydrate-derived drugs.[31-33] As for fCS, Li et al.[34] also reported the synthesis of glycoclusters as fCS mimetics through a click reaction by forming multivalent scaffolds from the trisaccharide motifs. The anticoagulant activities of the

synthetic glycoclusters were relatively consistent with natural polysaccharides. These synthetic glycoclusters with non-saccharide backbone had distinct structure from natural fCS but exhibited a similar level of biological effects. In addition, the semi-synthesis of fCS polysaccharides was reported by Bedini et al.[35, 36] with direct chemical fucosylation on microbial-sourced chondroitin backbones. Through orthogonal manipulation of protecting groups, semi-synthetic fCSs were achieved with relatively defined structures that exhibited moderate bioactivities compared to natural fCS. However, the structures of semi-synthetic fCS in their study were still heterogeneous with linkage sites and sulfation patterns that differ from natural fCS.

Structurally, fCS is composed of a CS backbone with sulfated or nonsulfated α-fucose branches linked to the *O*-3 position of D-glucuronic acid (GlcA) or *O*-6 position of *N*-acetyl-D-galactosamine (GalNAc). The branches are necessary for anticoagulant activity (Figure 1A). We hypothesize that preservation of the CS backbone and fucose branches on glycomimetics could achieve the anticoagulant activity of natural fCS. Therefore, novel fCS mimetics (Figure 1B) were designed and synthesized through chemical grafting of fucose branches onto CS in this study.

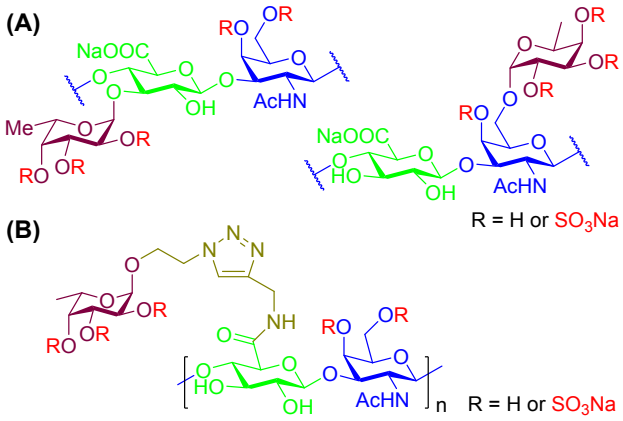


Figure 1. Chemical structures of (A) natural fucosylated chondroitin sulfates (fCS) and (B) designed fCS mimetics.

Chondroitin sulfate (CS) contains hydroxyl, amine and carboxyl groups that could serve as anchors for covalent modification. We chose the carboxyl groups for coupling with the fucose branches to access the fCS mimetics, and their anticoagulant activities were evaluated including APTT, TT, PT, and FXa assays.

Experimental section

Materials. Chondroitin sulfate A (CSA, M_w: 18.9 kDa, sulfate content 16.5%) was purchased from BeiErTe Biotechnology Co., Ltd (Qingdao, China). Chondroitin sulfate E (CSE, M_w: 9.1 kDa, sulfate content 20.3%) was obtained as previously reported.[37] Natural fucosylated chondroitin sulfate (fCS, M_w: 41.9 kDa, sulfate content 32.3%) was extracted from *Holothuria Polli* in our laboratory. Heparin (HP, M_w: 12 kDa, sulfate content 29.8%) and low-molecular-weight heparin (LMWH) from porcine intestinal mucosa and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 95%) were purchased from Sigma (St. Louis, MO).

N-(3-(dimethylamino)propyl)-*N*'-ethylcarbodiimide (EDC, 98.0%), *N*-hydroxysuccinimide (NHS, 98%), 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 97%), *N*-methylmorpholine (NMM, 99.5%), propargylamine (97%), 2-(*N*-morpholino)ethanesulfonic acid (MES, 99%), sulfur trioxide pyridine complex (SO_3·Py, 97%) and Amberlite® IR-120 cation exchange resin (Na^+ form) were purchased from Aladdin and used without further purification. Other chemical reagents were purchased from Energy Chemical and used directly. Deionized water with a resistivity of 18 MΩ·cm^{-1} was used as solvent. Dialysis was performed using cellulose membranes with a molecular weight cutoff of 3.5 kDa with water as solvent. The activated partial thromboplastin time (APTT, F008-1) kit, prothrombin time (PT, F007) kit and thrombin time (TT, F009) kit reagents were purchased from NanJing JianCheng Bioengineering Institute, NanJing, China. Sheep plasma was purchased from Jiulong Biological Co., Ltd, Shanghai, China. Antithrombin III (ATIII), bovine coagulation factor Xa (FXa) FXa, human coagulation factor IIa (FIIa), chromogenic substrate S-2765 and S-2238 were purchased from Adhoc International Technologies Co., Ltd (Beijing, China).

Characterization. Nuclear magnetic resonance (NMR) spectra were recorded on an Agilent DD2 spectrometer (500 MHz). The chemical shifts of all the NMR spectra were reported in delta (δ) units and expressed as parts per million (ppm). The NMR spectra were referenced using CD_3OD (^{1}H NMR δ = 3.31 ppm, ^{13}C NMR δ = 49.00 ppm), and D_2O (^{1}H NMR δ = 4.79 ppm). The peak and coupling constant assignments

are based on ^{1}H NMR, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC experiments. Multiplicities of the ^{1}H NMR data are denoted as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet).

Molecular weights (M_w) were characterized by size exclusion chromatography with multi-angle light scattering and refractive index (SEC-MALLS-RI) using a high performance liquid chromatography (HPLC, Agilent 1260) system equipped with two OHpak water columns (SB-804 HQ, SB-803 HQ, Shodex), a light scattering detector (miniDAWN, Wyatt Technology), and a refractive index detector (TREOS, Wyatt Technology). The column temperature was set at 35 °C, and 0.1 M Na_2SO_4 in H_2O was employed as an eluent at a flow rate of 0.6 mL·min^{-1}.

For Fourier transform infrared spectroscopy (FT-IR) spectra collection, the dried sample was mixed with dried KBr and pressed to make transparent film for FT-IR measurements using a Nicolet Nexus 470 instrument (Thermo Electron Corp., Madison, WI, USA) with a frequency resolution of 1 cm^{-1} and 64 scans between 4000 and 500 cm^{-1}.

Sulfur content was determined by ion chromatography.[38] Briefly, ~1.5 mg of sample was hydrolyzed in ampoule with 1 M HCl at 110 °C for 8 h. The hydrolysate was dried under vacuum before dissolved in deionized water (25 mL). Subsequently, sulfur quantification was performed by using ion chromatography (CIC-100, Qingdao ShengHan Chromatograph Technology Co., Ltd.) equipped with a ShengHan

SH-AC-3 column and a suppressed conductivity detector. The column temperature was set at 35 °C and 2 mM Na_2CO_3-8 mM $NaHCO_3$ aqueous solutions was employed as an eluent at a flow rate of 1.0 mL·min^{-1}.

Synthesis of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). The reported procedure was applied to synthesize DMTMM.[39] *N*-Methylmorpholine (NMM, 434.7 μL, 3.89 mmol) was added to a solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 751.4 mg, 4.28 mmol) in THF (15 mL) at room temperature. A white solid appeared within several minutes. After stirring for 30 min at room temperature, the solid was collected through filtration and washed with THF and dried to give DMTMM (1.03 g, 96%).

Synthesis of 1-azidoethyl-2,3,4-tri-*O*-sulfonato-α-L-fucopyranoside (F2). To a solution of 1-azidoethyl-α-L-fucopyranoside (**F1**, Scheme 1)[40] (40 mg, 0.1715 mmol) in 600 μL DMF, SO_3·Py (814.5 mg, 5.15 mmol) was added and stirred at 50 ºC for 8 h. Upon confirmation of complete conversion by TLC (CH_3Cl:MeOH:H_2O = 1:1:0.1, R_f = 0.4), the reaction mixture was cooled to room temperature and neutralized by adding saturated $NaHCO_3$ (aq.). The solvent was evaporated under reduced pressure, and the residue was redissolved in MeOH and filtrated. The filtration was concentrated under reduced pressure and the crude product was purified by Sephadex LH-20 gel filtration (MeOH). Subsequently, the crude product was passed through Amberlite® IR-120 cation exchange resin (Na^+ form) to give the desired product **F2**

as a white solid in Na^+ form (95.9 mg, 72%). ^1H NMR (500 MHz, CD_3OD): δ 5.21 (d, J = 3.6 Hz, 1H, H1), 5.00 (d, J = 2.8 Hz, 1H, H4), 4.74 (dd, J = 10.7, 3.1 Hz, 1H, H3), 4.62 (dd, J = 10.6, 3.6 Hz, 1H, H2), 4.19 (q, J = 6.5 Hz, 1H, H5), 3.86 (ddd, J = 11.2, 7.9, 3.6 Hz, 1H, O-C**H**$_2$-CH_2-N_3), 3.75 (ddd, J = 10.7, 5.2, 3.9 Hz, 1H, O-C**H**$_2$-CH_2-N_3), 3.57 (ddd, J = 11.6, 7.8, 3.7 Hz, 1H, O-CH_2-C**H**$_2$-N_3), 3.45 (ddd, J = 13.2, 5.1, 3.8 Hz, 1H, O-CH_2-C**H**$_2$-N_3), 1.32 (d, J = 6.5 Hz, 3H, H6). ^{13}C NMR (126 MHz, CD_3OD): δ 97.62 (C1), 78.35 (C4), 72.49 (C3), 71.88 (C2), 67.08 (O-**C**H_2-CH_2-N_3), 65.89 (C5), 50.40 (O-CH_2-**C**H_2-N_3), 15.65 (C6). HRMS: calcd for $[C_8H_{12}N_3NaO_{14}S_3]^{2-}$ 246.4695 ($[M+Na]^{2-}$), found 246.4691 ($[M+Na]^{2-}$) and 235.4782 ($[M+H]^{2-}$).

Alkynylation of CS. The CS was transformed into H^+ form before alkynylation. Amberlite® IR-120 cation exchange resin (Na^+ form) was transformed into H^+ form by eluted with 5% HCl and subsequent deionized water. CS (1.6 g) was dissolved in 40 mL deionized water and loaded on Amberlite® IR-120 cation exchange resin (H^+ form) column. Afterward, the column was eluted by 2000 mL deionized water. The eluent portions were collected and lyophilized to afford CS in H^+ form. CS (1 g) was dissolved in MES buffer (0.1 M, pH 5.0). The DMTMM (calculated based on the disaccharide repeating units) was added and stirred at room temperature for 30 min. The desired amount of propargylamine was mixed with MES buffer (pH 5.0) (v/v, 1/1) and then added to the solution (based on disaccharide repeating units). MES buffer (pH 5.0) was added to enable the desired final concentration of CS. The mixture was

stirred at set temperature for 24 h and then cooled to room temperature. Subsequently, NaCl was added to raise [NaCl] to 16% and stirred at room temperature for 30 min. The mixture was precipitated by the addition of ethanol and the precipitate was centrifuged at 4000 rpm. The residue was dialyzed against deionized water and lyophilized to afford the alkynylated CS as a white powder. The products were characterized by ^{1}H NMR and FT-IR. The degree of substitution of alkyne (DS_y) was calculated based on the disaccharide repeating units by ^{1}H NMR integration between the alkyne signals at 2.69 ppm[41] and the CH_3 signal of GalNAc at 2.05 ppm: $DS_y = [3\times A_{(alkyne)}]/A_{[CH3(G)]}$.

Grafting fucose onto alkynylated CS by copper-catalysed azide–alkyne cycloadditions (CuAAC).

*Alkynylated CS coupling with nonsulfated fucose **F1***. In a 10 mL round bottomed vessel, alkynylated CS (6 mg) and nonsulfated fucose **F1** (3.0 eq., based on alkyne) were dissolved in deionized water. Freshly prepared stock $CuSO_4\cdot 5H_2O$ solution (0.4 eq., 25 mg·mL^{-1}, based on alkyne) and stock Na-ascorbate solution (1.2 eq., 100 mg·mL^{-1}, based on alkyne) were added. Deionized water was added to adjust the final concentration of alkynylated CS to 10 mg·mL^{-1}. The mixture was stirred at 60 ºC for 24 h and then cooled to room temperature. Subsequently, NaCl was added to raise [NaCl] to 16% and stirred at room temperature for 30 min. The mixture was precipitated by the addition of ethanol and the precipitate was centrifuged at 4000 rpm.

The residue was dialyzed against deionized water and lyophilized to afford fucose grafted CS as white powder. The products were characterized by nuclear magnetic resonance (NMR), FT-IR and size exclusion chromatography with multi-angle light scattering and refractive index (SEC-MALLS-RI). The degree of substitution of fucose (DS_f) was calculated based on the disaccharide repeating units by ^1H NMR integration between the CH_3 signal of fucose at 1.19 ppm and the CH_3 signal of GalNAc at 2.05 ppm: $DS_f = A_{(F6)}/A_{[CH3(G)]}$.

Alkynylated CS coupling with trisulfated fucose ***F2***. In a 10 mL round bottomed vessel, alkynylated CS (6 mg) was dissolved in PBS buffer (pH 7.38). Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (4 eq., based on alkyne) was mixed with freshly prepared $CuSO_4 \cdot 5H_2O$ solution (0.4 eq., 25 $mg \cdot mL^{-1}$, based on alkyne) in PBS buffer (pH 7.38) and added to the reaction solution. The reaction mixture was placed under flow of nitrogen gas then to it was added freshly prepared Na-ascorbate solution (1.2 eq., 100 $mg \cdot mL^{-1}$, based on alkyne) in PBS buffer (pH 7.38). After stirring at room temperature for 30 min, trisulfated fucose **F2** (3.0 eq., based on alkyne) in PBS buffer (pH 7.38) was added and PBS buffer (pH 7.38) was added to adjust the final concentration of alkynylated CS to 10 $mg \cdot mL^{-1}$. The mixture was stirred at 60 ºC for 24 h and then cooled to room temperature. Subsequently, NaCl was added to raise [NaCl] to 16% and stirred at room temperature for 30 min. The mixture was precipitated by the addition of ethanol and the precipitate was centrifuged at 4000 rpm. The residue was dialyzed against deionized water and

lyophilized to afford fucose grafted CS as white powder. The products were characterized by nuclear magnetic resonance (NMR), FT-IR and size exclusion chromatography with multi-angle light scattering and refractive index (SEC-MALLS-RI). DS_f was calculated based on the disaccharide repeating units by ^1H NMR integration between the CH_3 signal of fucose at 1.19 ppm and the CH_3 signal of GalNAc at 2.05 ppm: $DS_f = A_{(F6)}/A_{[CH3(G)]}$.

Anticoagulant activity assay. For APTT assay, plasma (90 μL) was incubated at 37 °C with 10 μL of polysaccharide samples and APTT reagent (100 μL). After stirring 3 min, 2.5×10^{-2} mol L^{-1} $CaCl_2$ (100 μL) was added, and the clotting times were measured by an automated coagulometer. For PT assay, plasma (90 μL) was incubated at 37 °C with 10 μL of polysaccharide samples. After stirring 3 min, 200 μL of PT reagent was added and the clotting times were measured by an automated coagulometer. For TT assay, plasma (90 μL) was incubated at 37 °C with 10 μL of polysaccharide samples. After stirring 3 min, 100 μL of TT reagent was added, and the clotting times were measured by an automated coagulometer. Results were expressed for each group (n = 3) as mean APTT, PT or TT (s) ± standard deviation of the mean (SD).

Chromogenic Assays for the Measurement of Anti-FXa and anti-FIIa activity in the presence of ATIII. The anti-FXa and anti-FIIa activities of fCS mimetics in the presence of ATIII were estimated by following the previously published

methods.[42] Incubations were performed in 96-well plates and a mixture containing 20 μL sample and 20 μL of 0.5 IU/mL ATIII was incubated at 37 °C for 2 min. Then, 40 μL of 0.25 IU/mL FXa or 5 IU/mL FIIa was added. After incubation for 2 min, the residual FXa or FIIa activity was measured by the addition of 50 μL of 1 mM FXa chromogenic substrate S-2765 or FIIa chromogenic substrate S-2238. After incubation for 1 min, the reaction was terminated by adding 80 μL of 30% acetic acid and absorbance of the reaction mixture was recorded at 405 nm.

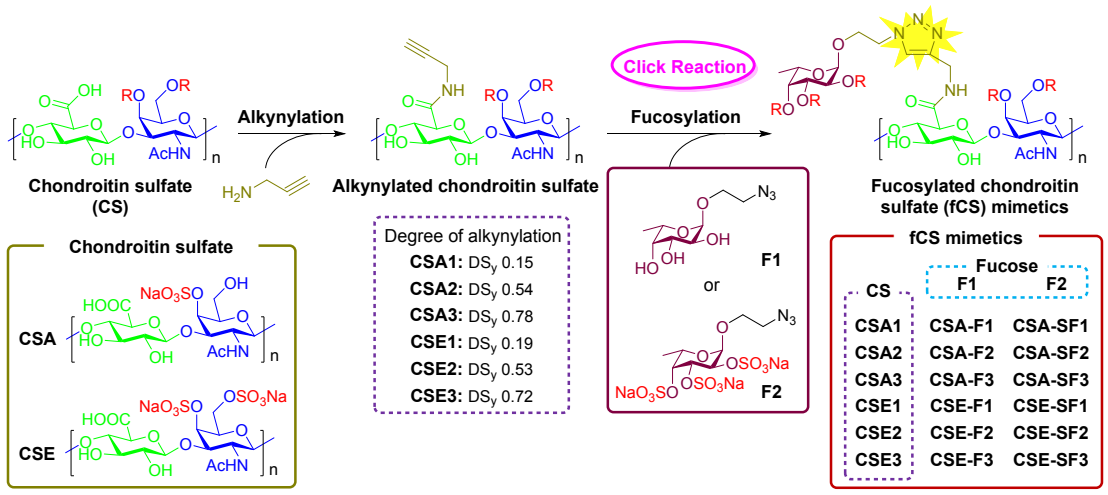
RESULTS AND DISCUSSION

Commercially available chondroitin sulfate A (CSA) was chosen as starting material for the preparation of fCS mimetics, and the carboxyl groups on CSA were employed as sites for regioselective amidation. The carboxyl groups on CSA were largely present in the Na^+ form, leading to low conversion of the amidation reaction.[43] Therefore, CSA was transformed into the proton (H^+) form by pretreatment with a cation exchange resin (H^+ form) prior to amidation. Nonsulfated fucose **F1**[40] with functionalized azide groups was utilized for grafting α-fucose onto CSA. After catalytic hydrogenation, we attempted direct amidation between the free amine on fucose and carboxyl groups on CSA with the conventional activators of *N*-(3-(dimethylamino)propyl)-*N*'-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS).[44] Unfortunately, fucose branches were not successfully grafted onto CSA as indicated by ^{1}H NMR. We speculated that steric

hindrance, especially for the carboxyl groups on the CSA, resulted in the failure of the coupling reaction.

To overcome the steric hindrance during the coupling reaction, a two-step strategy was adopted to graft fucose onto CSA through the copper-catalyzed azide-alkyne cycloaddition (CuAAC)[45] with lower sterically hindered molecule bearing alkyne groups (Scheme 1). To this end, propargylamine was used for alkyne functionalization of CSA, and the degree of substitution of the alkyne (DS_y) could be readily calculated based on the ^1H NMR integration between the alkyne signals at 2.69 ppm[41] and the CH_3 signal of GalNAc at 2.05 ppm (Figure 2).

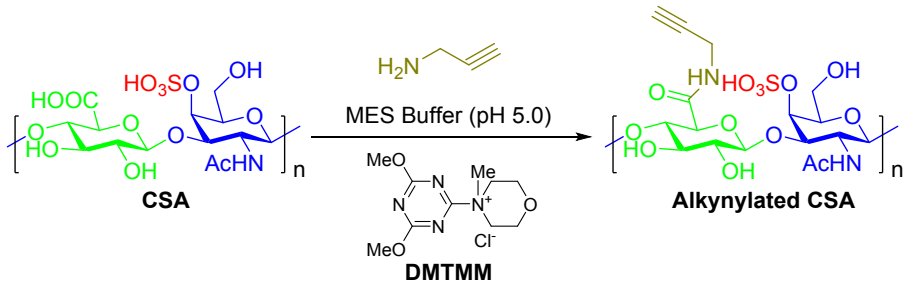
Scheme 1. Schematic Illustration of the Synthesis of fCS Mimetics with Various Sulfation and Fucose Densities Through the Click Grafting of Azide-Fucose onto Alkyne-Functionalized Chondroitin Sulfate



Alkynylation of chondroitin sulfate A and chondroitin sulfate E. Polysaccharides usually exhibit complex topological structures due to the strong

hydrogen bonds between hydroxyl, carboxyl and amine groups, which dramatically decrease the activation capability of the amidation reagents.[46] The amidation of polysaccharides with EDC/NHS for biomaterial application could result in a low efficiency, as previously reported.[47] Alternatively, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM),[48] an aqueous soluble activator, was shown to possess potent activation capabilities when initially used in peptide synthesis.[49] It had been applied for carboxyl polysaccharides modification with a well-controlled degree of substitution since 2007.[50-53] To the best of our knowledge, DMTMM mediated amidation of CS has not been reported yet. In our study, DMTMM was employed for the controllable alkynylation of CSA. To achieve a higher coupling efficiency, CSA was typically activated in the presence of DMTMM before the addition of propargylamine. To guarantee the H^+ form of the carboxyl groups during the reaction, the amidation was performed in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 5.0 with the premixing of propargylamine before addition. The alkynylation of CSA with propargylamine mediated by DMTMM is summarized in Table 1. When the reaction was conducted at room temperature at a carboxyl/DMTMM/propargylamine feed ratio of 1:2:0.5, the alkyne signals were not observed in the ^{1}H NMR spectrum (DS_y 0, Table 1, entry 1). Changing the carboxyl/DMTMM/propargylamine feed ratio to 1:2:1, the DS_y value was determined to be 0.15 (Table 1, entry 2).

Table 1. Alkynylation of Chondroitin Sulfate A (CSA) with Propargylamine by Using DMTMM as Condensation Reagent Under Various Conditions



entry	[carboxyl]/[DMTMM]/[amine][a]	T^b (ºC)	$C_{CSA}{}^c$ (mg/mL)	$DS_y{}^d$	product[e]
1	1:2:0.5	R. T.	20	0	-
2	1:2:1	R. T.	20	0.15	**CSA1**
3	1:4:1	R. T.	20	0.06	-
4	1:4:5	R. T.	20	0.54	**CSA2**
5	1:4:10	R. T.	20	0	-
6	1:4:15	R. T.	20	0	-
7	1:4:2	37	10	0.69	-
8	1:4:3	37	10	0.78	**CSA3**
9	1:4:4	37	10	0.75	-
10	1:4:5	37	10	0.69	-

[a]Molar ratio of [carboxyl group (carboxyl)]/[DMTMM]/[propargylamine (amine)]. [b]R. T. = room temperature. [c]The final concentration of CSA in MES buffer (pH 5.0). [d]Degree of substitution of alkyne, based on the carboxyl group of CSA, determined by ^{1}H NMR integration of the alkyne signal (2.69 ppm) to the GalNAc acetyl signal (2.05 ppm). [e]The alkynylated chondroitin sulfates with DS_y were determined to be 0.15 (**CSA1**), 0.54 (**CSA2**), and 0.78 (**CSA3**) for further fucosylation.

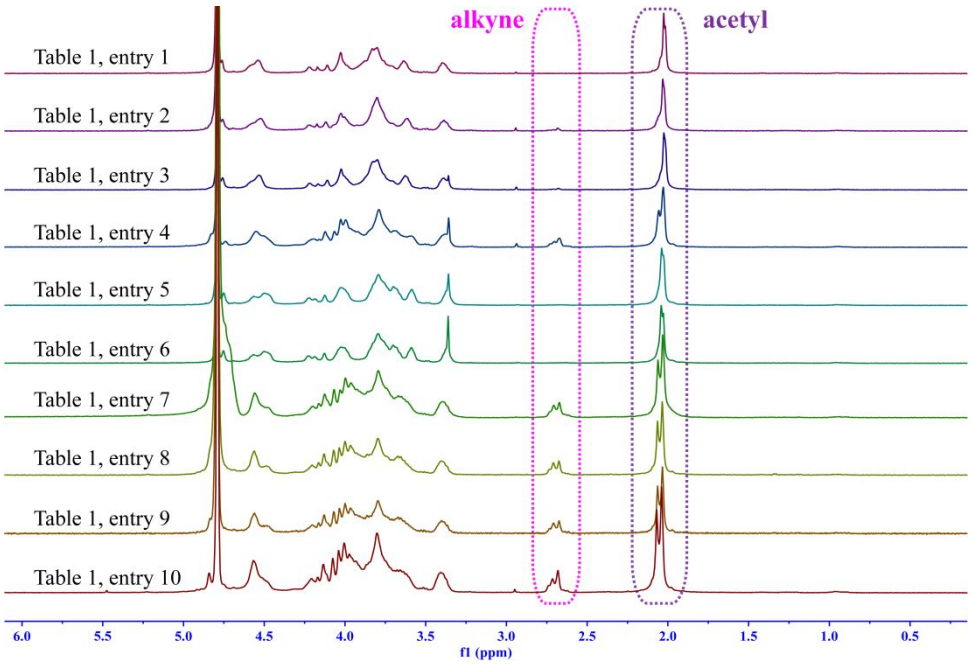


Figure 2. ^{1}H NMR spectra of the alkynylation of CSA (Table 1) in D_2O. The degree of substitution of the alkyne was determined by ^{1}H-NMR integration of the alkyne signal (2.69 ppm) to the GalNAc acetyl signal (2.05 ppm).

To enhance the DS_y value of the amidation on the CSA, the reaction conditions were further optimized including the carboxyl/DMTMM/propargylamine feed ratio, reaction temperature, and final concentration of CSA. When the feed ratio of carboxyl/DMTMM/propargylamine was set up to 1:4:5, the DS_y value clearly increased to 0.54, as shown by ^{1}H NMR (Table 1, entry 4). Thus, the enhancement of the loading of propargylamine and DMTMM dramatically improved DS_y. However, the further increase of the carboxyl/DMTMM/propargylamine feed ratio at 1:4:10 and 1:4:15 (Table 1, entries 5 and 6) resulted in the reaction mixture becoming turbid at 0 of DS_y. The high loading of propargylamine induced the precipitation of the starting

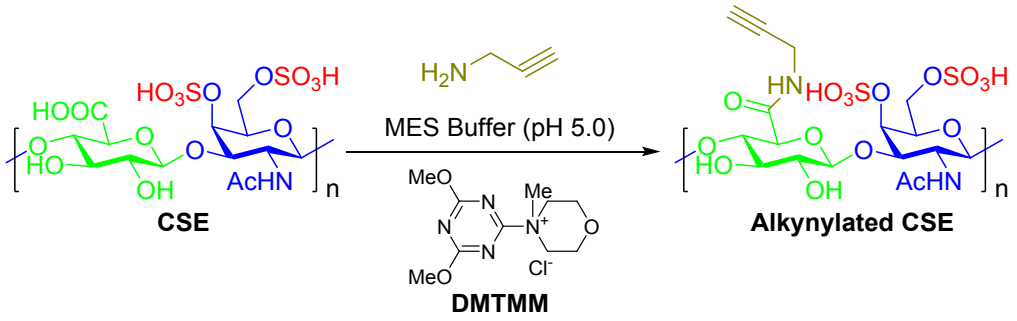
materials, retarding the amidation reaction. These results indicated that the carboxyl/DMTMM/propargylamine feed ratio should be below 1:4:5 to prevent this phenomenon.

As previously reported, coupling reaction mediated by DMTMM can result in the degree of HA substitution up to 0.76.[53] Meanwhile, decreasing the viscosity of the solution could also increase the derivatization efficiency of the polysaccharide. Thus, the final concentration of CSA was decreased to 10 mg/mL for amidation, and the reaction temperature was set at 37 ºC to avoid the decomposition of DMTMM (Table 1, entries 7-10). The DS_y values were determined to be 0.69, 0.78, 0.75, and 0.69 for the reactions at carboxyl/DMTMM/propargylamine feed ratios of 1:4:2, 1:4:3, 1:4:4 and 1:4:5, respectively. The DS_y values were not significantly increased as the loading of propargylamine increased. In particular, the carboxyl/DMTMM/propargylamine feed ratio of 1:4:3 yielded the product with highest DS_y of 0.78 (Table 1, entry 8). As shown in Table 1, alkynylated CSA with various DS_y values were obtained under different reaction conditions. To study the influence of the free carboxyl on the anticoagulant activity, we chose alkynylated CSA with DS_y values of 0.15 (**CSA1**), 0.54 (**CSA2**) and 0.78 (**CSA3**) for the next coupling with fucose.

Furthermore, to study the influence of different CS sulfation patterns on the anticoagulant activity, the CSE (chondroitin sulfate E) in the triethylammonium salt form obtained from our previous report[37] was applied for fucose grafting and

alkynylation through DMTMM mediated amidation with propargylamine. Alkynylation of the CSE yielded **CES1**, **CSE2** and **CSE3** according to the counterpart procedures for **CSA1**, **CSA2** and **CSA3** preparation (Table 2). The DS_y values of **CES1**, **CSE2** and **CSE3** were determined to be 0.19, 0.53, and 0.72, respectively, according to the ^1H NMR integration of the alkyne signal (2.69 ppm) to the CH_3 signal of GalNAc (2.05 ppm) (Figure S1).

Table 2. Synthesis of Alkynylated Chondroitin Sulfate E (CSE) with Propargyl Amine Using DMTMM as Condensation Reagent



entry	[carboxyl]/[DMTMM]/ [amine][a]	T^b (ºC)	$C_{CSE}{}^c$ (mg/mL)	$DS_y{}^d$	product
1	1:2:1	R. T.	20	0.19	**CSE1**
2	1:4:5	R. T.	20	0.53	**CSE2**
3	1:4:3	37	10	0.72	**CSE3**

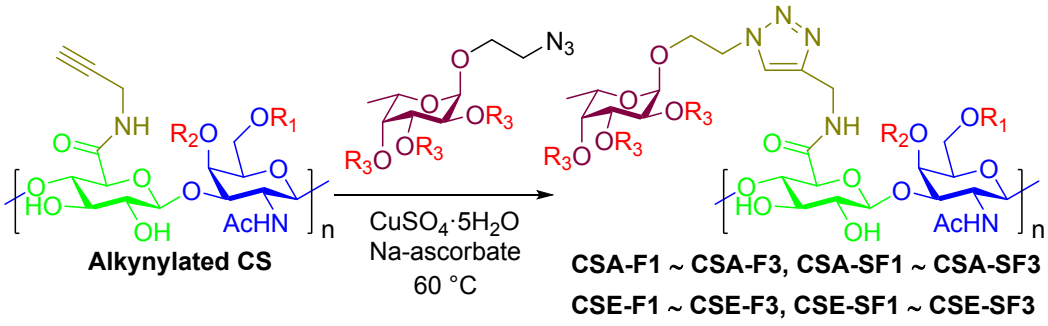
[a]Molar ratio of [carboxyl group (carboxyl)]/[DMTMM]/[propargylamine (amine)]. [b]R. T. = room temperature. [c]The final concentration of CSE in MES buffer (pH 5.0). [d]Degree of substitution of the alkyne, based on the carboxyl group of CSE, determined by ^1H NMR integration of the alkyne signal (2.69 ppm) to GalNAc acetyl signal (2.05 ppm).

Coupling of azide-fucose with alkynylated chondroitin sulfate through CuAAC. Upon obtaining alkynylated CSA and CSE, we next conducted the coupling reaction of alkynylated CS with azide functionalized fucose. CuAAC, an efficient click reaction to achieve molecular reagent, has been widely applied for the

modification of natural polysaccharides.[54] In our study, the CuAAC reaction in an aqueous solvent was employed for the coupling between alkynylated CS and azide-functionalized fucose.

The CuAAC reaction was conducted under conventional conditions[55] for condensation of alkynylated CSA (**CSA1**, **CSA2** and **CSA3**) and non-sulfated fucose **F1** (Table 3, entries 1-3). The alkynylated CSA were first dissolved in deionized water with the addition of non-sulfated fucose **F1**. The reaction was performed in the presence of $CuSO_4 \cdot 5H_2O$ and L-ascorbate at 60 ºC for 24 h. The formation of a triazole ring and grafting of fucose branches produced representative signals at around 8.33, 1.19, and 2.69 ppm in the ^{1}H NMR spectra with the disappearance of the alkyne peaks. The degree of substitution of fucose (DS_f) was calculated based on the carboxyl groups by ^{1}H NMR integration of the CH_3 signal of fucose (1.19 ppm) to the CH_3 signal of GalNAc (2.05 ppm). The DS_f values were determined to be 0.15, 0.50, and 0.71 for the products **CSA-F1**, **CSA-F2** and **CSA-F3** from **CSA1**, **CSA2**, and **CSA3**, respectively.

Table 3. Synthesis of fCS Mimetics by Copper-Catalyzed Cycloadditions of Azide-Fucose with Alkyne-Functionalized Chondroitin Sulfate



Entry	CS unit	solvent	R_1, R_2, R_3	alkyne conv.[a] (%)	M_w[b] (kDa)	M_n[b] (kDa)	PDI[b] (M_w/M_n)	DS_f[c]	product
1	**CSA1**	H_2O	H, SO_3^-, H	100	19.3	16.1	1.20	0.15	**CSA-F1**
2	**CSA2**	H_2O	H, SO_3^-, H	100	22.1	19.1	1.16	0.50	**CSA-F2**
3	**CSA3**	H_2O	H, SO_3^-, H	100	24.3	18.5	1.31	0.71	**CSA-F3**
4	**CSA1**	Buffer[d]	H, SO_3^-, SO_3^-	100	19.6	16.2	1.21	0.14	**CSA-SF1**
5	**CSA2**	Buffer	H, SO_3^-, SO_3^-	100	26.2	22.8	1.15	0.52	**CSA-SF2**
6	**CSA3**	Buffer	H, SO_3^-, SO_3^-	100	29.8	26.8	1.11	0.70	**CSA-SF3**
7	**CSE1**	H_2O	SO_3^-, SO_3^-, H	100	9.4	9.3	1.01	0.14	**CSE-F1**
8	**CSE2**	H_2O	SO_3^-, SO_3^-, H	100	10.2	8.4	1.21	0.51	**CSE-F2**
9	**CSE3**	H_2O	SO_3^-, SO_3^-, H	100	11.1	8.2	1.35	0.72	**CSE-F3**
10	**CSE1**	Buffer	SO_3^-, SO_3^-, SO_3^-	100	9.8	7.9	1.24	0.13	**CSE-SF1**
11	**CSE2**	Buffer	SO_3^-, SO_3^-, SO_3^-	100	11.6	11.0	1.05	0.49	**CSE-SF2**
12	**CSE3**	Buffer	SO_3^-, SO_3^-, SO_3^-	100	14.1	10.8	1.31	0.71	**CSE-SF3**

[a]Determined by disappearance of the alkyne signal (2.69 ppm) on ^{1}H NMR. [b]Number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI) were determined by SEC-MALLS-RI. [c]Degree of substitution of fucose unit, based on the carboxyl group of the CS. [d]Buffer = PBS buffer (pH 7.38).

The fucose branches in natural fCS are generally sulfated and play an important role in its anticoagulant activity. To mimic the sulfated branches in natural fCS, persulfated fucose **F2** with azide functionalization was synthesized from compound **F1** in the presence of SO_3·Py in DMF.[56, 57] Compound **F2** was finally obtained as the triethylammonium salt after neutralization, and the structure of compound **F2** was well characterized by ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC (Figure

S20). Trisulfated fucose **F2** was then applied to the CuAAC reaction under the same reaction condition with non-sulfated fucose **F1**. However, the ^{1}H NMR spectrum showed no signals for the triazole ring or CH_3 of fucose, which indicated that the grafting was unsuccessful (Table S1, entry 1; Figure S2). We speculated that the highly sulfated fucose with a negative charge could chelate the positive charged Cu(I) through static interaction, impeding the catalytic process of Cu(I). However, the reaction efficiency could be significantly accelerated in the presence of Cu(I)-stabilizing ligands.[58] The most commonly used Cu(I)-stabilizing ligands are hydrophobic tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)[59] and hydrophilic tris(3-hydroxypropyltriazolylmethyl)amine (THPTA).[60-62] Due to the aqueous solubility of the starting materials, hydrophilic THPTA was adopted as a Cu(I)-stabilizing ligand for the CuAAC reactions (Table S1, entry 2). The ^{1}H NMR spectrum still showed no signals for the triazole ring or CH_3 of fucose, although the signals of alkyne disappeared (Figure S2). During the process of the CuAAC reaction, the alkyne groups first react with Cu(I) to form intermediates, after which they react with the azide groups to form triazole rings (Scheme S1). This observation indicated that THPTA indeed hindered the chelation between the sulfate groups and Cu(I), but the intermediates did not react with trisulfated fucose **F2**. To this end, the trisulfated fucose **F2** was transformed into the Na^+ form after treatment with a cation exchange resin (Na^+ form) in a PBS buffer (pH 7.38). In addition, the reaction was performed under nitrogen gas to avoid oxidation of Cu(I) by O_2. A freshly prepared stock

solution of $CuSO_4 \cdot 5H_2O$ in a PBS buffer (pH 7.38) was premixed with THPTA and added to the mixed solution with L-ascorbate in a PBS buffer (pH 7.38). The mixture was stirred at room temperature for 30 min, and subsequently, trisulfated fucose **F2** in PBS buffer (pH 7.38) was added and stirred at 60 ºC for 24 h (Table 3, entry 4). The obtained product (**CSA-SF1**) showed representative signals for the triazole ring and CH_3 of fucose, and the DS_f was determined to be 0.14 (Figure S2) based on the ^1H NMR spectrum. According to the established protocol for the CuAAC reaction of **CSA1** with trisulfated fucose **F2**, the products **CSA-SF2** with a DS_f of 0.52 and **CSA-SF3** with a DS_f of 0.70 were obtained from **CSA2** and **CSA3,** respectively, with an alkyne conversion up to 100% (Table 3, entries 5 and 6).

The CuAAC reaction of alkynylated CSE was performed according to the above optimized procedure for the preparation of non- and tri-sulfated fucose grafted CSA. The grafting products of **CSE-F1**, **CSE-F2** and **CSE-F3** were obtained with DS_f values of 0.14, 0.51, and 0.72, respectively (Table 3, entries 7-9). Under optimized coupling reaction between alkynylated CSA and sulfated fucose **F2**, **CSE-SF1**, **CSE-SF2,** and **CSE-SF3** were obtained from **CSE1**, **CSE2,** and **CSE3** with DS_f values of 0.13, 0.49, and 0.71, respectively (Table 3, entries 10-12).

In contrast to Bedini's approach,[35, 36] we developed an alternative modular route to assemble fCS analogs without the need for protection/deprotection steps. 12 fCS mimetics were obtained with various sulfation patterns and fucose densities (DS_f

values in the 0.13~0.72 range) through the concise grafting protocols developed in this study (Table S4). The molecular weights of synthetic fCS mimetics were characterized by SEC-MALLS-RI. The fCS mimetics with higher DS_f values possessed higher molecular weights than those with lower DS_f values (Table 3). The sulfate contents of **CSA-F3**, **CSA-SF3**, **CSE-F3,** and **CSE-SF3** were consistent with grafting of fucose onto natural CS (Table S5) and FT-IR spectra confirmed complete reaction of alkyne with azide (Figure S21, S22). The integrated NMR analysis (^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC) for the fCS mimetics with the highest DS_f values of the fucose branches (**CSA-F3**, **CSA-SF3**, **CSE-F3,** and **CSE-SF3**) also confirmed the successful grafting of fucose onto alkynylated CS through observation of the triazole and CH_3 of the fucose signals (Figure 3, S47-S49; Table 4, S6-S8). Moreover, the anomeric proton signals of CS were around 4.5 ppm. For the fucose branches, chemical shifts of the anomeric protons for the trisulfated fucose were strongly shifted to 5.21 ppm compared with non-sulfated fucose at 4.83 ppm (**CSA-SF3** and **CSE-SF3** *vs* **CSA-F3** and **CSE-F3**). Taking **CSA-SF3** as an example (Figure 3, Table 4), the signals at 5.21/96.27, 4.62/104.29, and 4.55/100.39 were assigned to anomeric ^{1}H/^{13}C of fucose, GlcA, and GalNAc, respectively, according to the ^{1}H-^{13}C HSQC. The signals for the fucose sugar ring were deduced from the ^{1}H-^{1}H COSY at 4.52/4.61/4.87 ppm for H-2/H-3/H-4. The crosslinking signals of H-6/C-6 at 3.77/60.94 (GalNAc) were identified from the ^{1}H-^{13}C HSQC (blue circle) together with the signal of H-4/C-4 at 4.86/76.38 (GalNAc), which

confirmed that sulfation occurred at the C-4 position of the backbone. The H-2/H-3/H-4 signals at 3.40/3.67/3.86 ppm for GlcA were clearly observed from the ^{1}H-^{1}H COSY, which demonstrated the linkage of GalNAc at the C-4 position of GlcA. In addition, another three $^{1}H/^{13}C$ signals at 4.73/50.24, 4.15/66.25, and 4.01/66.06 (blue circles) were attributed to CH_2 residues of linkers based on the ^{1}H-^{13}C HSQC spectrum.

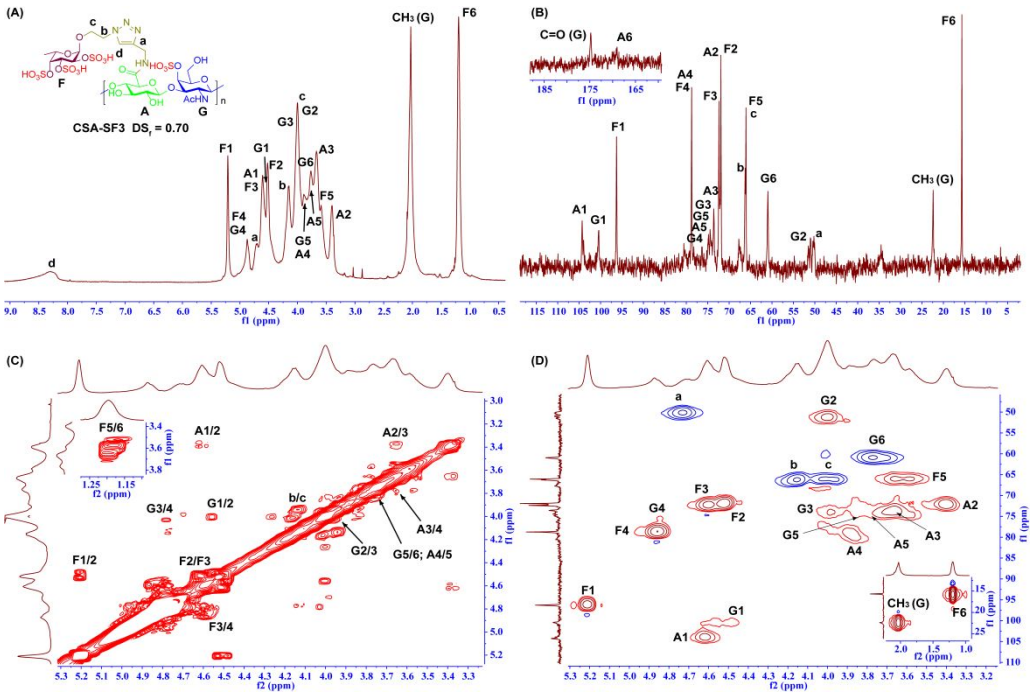


Figure 3. 1D and 2D-NMR spectra of **CSA-SF3**: (A) ^{1}H NMR, (B) ^{13}C NMR, (C) ^{1}H-^{1}H COSY, (D) ^{1}H-^{13}C HSQC. F, A, and G stand for fucose, GlcA and GalNAc, respectively.

Table 4. ^{1}H and ^{13}C NMR Chemical Shift Assignments of CSA-SF3 (500 MHz, 298 K, D_2O)[a]

position	GlcA	GalNAc	Fucose	Linker

	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	4.62	104.29	4.55	100.39	5.21	96.27	-	-
2	3.40	72.04	4.00	51.21	4.52	71.91	-	-
3	3.67	73.58	4.02	74.72	4.61	72.35	-	-
4	3.86	78.74	4.86	76.38	4.87	78.73	-	-
5	3.74	74.86	3.85	74.98	3.59	66.06	-	-
6	-	169.03	3.77	60.94	1.20	15.69	-	-
CH$_3$CO	-	-	2.03	22.38	-	-	-	-
CH$_3$**CO**	-	-	-	174.79	-	-	-	-
triazole	-	-	-	-	-	-	8.29	n.d.[b]
fucose-**CH$_2$**CH$_2$	-	-	-	-	-	-	4.01	66.06
fucose-CH$_2$**CH$_2$**	-	-	-	-	-	-	4.15	66.25
CS-CONH**CH$_2$**	-	-	-	-	-	-	4.73	50.24

[a]Chemical shifts expressed in δ. [b]Not determinable.

Anticoagulant activities of fCS mimetics. To evaluate the anticoagulant activities of synthetic fCS mimetics, preliminary activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) of plasma clotting assays, which are frequently applied to evaluate the intrinsic, extrinsic, and common pathways of the coagulation, were conducted as shown in Figure 4A-4C.[63] Deionized water was used as a negative control while heparin (HP) and low molecular weight heparin (LMWH) were used as positive controls. The fCS mimetics with CSA backbones exhibited no obvious anticoagulant activities, while **CSE-F1** and **CSE-SF1** with CSE backbones indicated significant effects on their anticoagulant activities. They exhibited significantly prolonged APTTs for more than 120 s, which were comparable to that of

natural fCS extracted from *Holothuria Polli*. Meanwhile, all fCS mimetics exhibited no significant capabilities for PT and TT prolongation. These preliminary results indicated that fCS mimetics exhibited intrinsic anticoagulant activities, which is clearly consistent with the natural fCS reported in the literature before.[8, 20, 34] The fCS mimetics developed in our study possess moderate anticoagulant activity, which is comparable to the substrates reported in Bedini's work.[35, 36] The degree of free carboxyl groups, which was calculated by 1-DS_f, decreased with the increment of DS_f values. Lower DS_f values (at ~0.20) for the fCS mimetics with CSE backbones exhibited longer APTTs, which suggested that fucose branches were required for anticoagulant activity, even at low DS_f values,[64] and free carboxyl groups were also important for the anticoagulant activity. However, higher DS_f values resulted in the elimination of their anticoagulation activities possibly due to the lower percentages of free carboxyl groups. Accordingly, **CSE-F1** and **CSE-SF1** exhibited anticoagulant activity due to highly reserved fCS structure properties including fucose branches, specific sulfation and carboxyl groups, which are inevitable for the biological activity of natural fCS. The fCS mimetic **CSE-SF1** with higher sulfated fucose branches exhibited slightly better anticoagulant activity compared to **CSE-F1** with non-sulfated fucose branches, which revealed that the increase of sulfation content on the fucose branches can improve the anticoagulant activities.

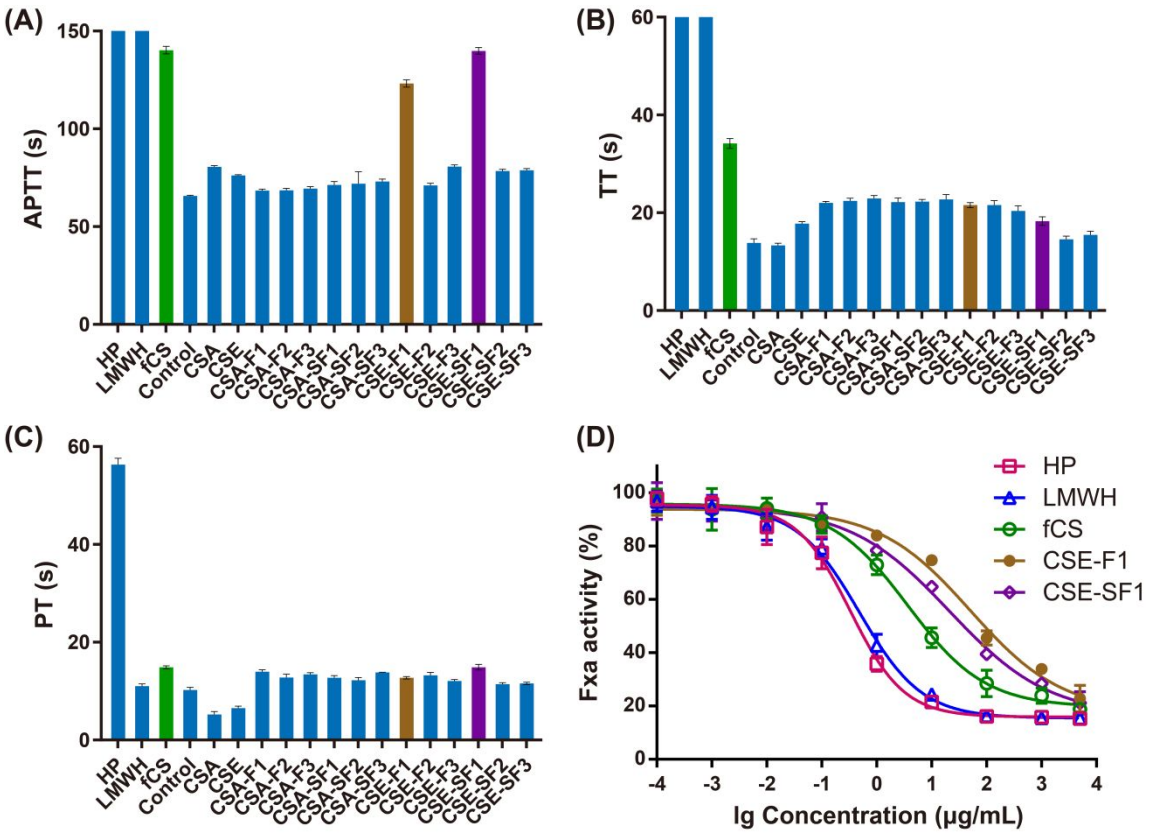


Figure 4. The anticoagulant activities of synthetic fCS mimetics. Effect of synthetic fCS mimetics on prolongation of (A) APTT, (B) TT, and (C) PT at a concentration of 500 μg/mL. APTT > 250s for HP and LMWH, and TT > 400s for HP and LMWH. (D) Inhibition of ATIII-mediated FXa activity by synthetic fCS mimetics.

To further understand the anticoagulant activity of **CSE-F1** and **CSE-SF1**, the AT III-dependent activity against factor Xa (Figure 4D) and IIa (Figure S4) were measured,[10] and the IC_{50} values are summarized in Table 5. The IC_{50} values of the anti-FXa activities for **CSE-F1** and **CSE-SF1** were determined to be 51.10 μg/mL and 21.26 μg/mL, respectively, while anti-FIIa activities for **CSE-F1** and **CSE-SF1** were not detected. Meanwhile, **CSE-SF1** also exhibited higher anti-Xa activities than

CSE-F1. These findings indicated that the fucose grafted CSE with low DS_f values could mimic the anticoagulant activity of natural fCS through intrinsic pathways and FXa inhibition.

Table 5. FXa and FIIa Inhibition Activity of Synthetic fCS Mimetics Mediated by ATIII

entry	compound	IC_{50} (μg/mL)[a]	
		anti-FXa	anti-FIIa
1	HP	0.332	0.104
2	LMWH	0.483	0.362
3	fCS	3.691	6.431
4	**CSE-F1**	51.10	n.d.[b]
5	**CSE-SF1**	21.26	n.d.

[a]IC_{50} value, concentration required to inhibit 50% activity of protease (FXa and FIIa) for synthetic fCS mimetics. [b]Not Detected.

CONCLUSIONS

Fucosylated chondroitin sulfate (fCS) is a remarkable fucose branched glycosaminoglycan (GAG) with a promising future for clinical use. In this study, a concise protocol was established for the design and synthesis of fCS mimetics through alkynylation of chondroitin sulfate and subsequent fucose grafting as branches without manipulation of protecting groups. DMTMM was determined to be a more efficient condensation reagent for amidation on CS with well-controlled degrees of

substitution between 0.15-0.78. The fucose branches were successfully grafted onto alkynylated CS though the CuAAC 'click' reaction, and the Cu(I)-stabilizing ligand THPTA was confirmed to be an efficient reagent for the CuAAC reaction of sulfated fucose. A small library of 12 fCS mimetics with various sulfation patterns and fucose branch densities was obtained and fully characterized by SEC-MALLS-RI and NMR spectra (Scheme S3). Screening of their biological activities showed that **CSE-F1** and **CSE-SF1** exhibited anticoagulant activities through intrinsic pathways and inhibition of FXa by ATIII. Thus we speculate that the CSE backbone and free carboxyl groups are crucial for the anticoagulant activity of these fCS glycomimetics. Moreover, the fucose branches were essential for anticoagulant activity even at low densities, while higher sulfation content was favorable for higher activity. The concise protocol developed in this study supplies a robust route for the fabrication of glycomimetics to achieve diverse functions of natural polysaccharides. Further SAR and biological studies should be done to explain anticoagulant mechanism of fCS mimetics as well as natural fCS polysaccharides, which potentially promote the development of novel marine carbohydrate-based drugs.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Additional structural characterization (^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC spectra, SEC curves, and FT-IR spectra) of grafting fCS mimetics and biological evaluation of natural polysaccharides.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Lin, C. W.; Tsai, M. H.; Li, S. T.; Tsai, T. I.; Chu, K. C.; Liu, Y. C.; Lai, M. Y.; Wu, C. Y.; Tseng, Y. C.; Shivatare, S. S.; Wang, C. H.; Chao, P.; Wang, S. Y.; Shih, H. W.; Zeng, Y. F.; You, T. H.; Liao, J. Y.; Tu, Y. C.; Lin, Y. S.; Chuang, H. Y.; Chen, C. L.; Tsai, C. S.; Huang, C. C.; Lin, N. H.; Ma, C.; Wu, C. Y.; Wong, C. H. A common glycan structure on immunoglobulin G for enhancement of effector functions. *P. Natl. Acad. Sci. U. S. A.* **2015,** *112*, 10611-10616.

(2) De Castro, C.; Speciale, I.; Duncan, G.; Dunigan, D. D.; Agarkova, I.; Lanzetta, R.; Sturiale, L.; Palmigiano, A.; Garozzo, D.; Molinaro, A.; Tonetti, M.; Van Etten, J. L. *N*-Linked glycans of chloroviruses sharing a core architecture without precedent. *Angew. Chem. Int. Ed.* **2016,** *55*, 654-658.

(3) Cheng, L.; Gao, S.; Song, X.; Dong, W.; Zhou, H.; Zhao, L.; Jia, L. Comprehensive *N*-glycan profiles of hepatocellular carcinoma reveal association of fucosylation with tumor progression and regulation of FUT8 by microRNAs. *Oncotarget* **2016,** *7*, 61199-61214.

(4) Fan, F.; Cai, C.; Wang, W.; Gao, L.; Li, J.; Li, J.; Gu, F.; Sun, T.; Li, J.; Li, C.; Yu, G. Synthesis of fucoidan-mimetic glycopolymers with well-defined sulfation patterns via emulsion ring-opening metathesis polymerization. *ACS Macro Lett.* **2018,** *7*, 330-335.

(5) Pomin, V. H. Holothurian fucosylated chondroitin sulfate. *Mar. Drugs* **2014,** *12*, 232-254.

(6) Liu, X.; Liu, Y.; Hao, J.; Zhao, X.; Lang, Y.; Fan, F.; Cai, C.; Li, G.; Zhang, L.; Yu, G. *In vivo* anti-cancer mechanism of low-molecular-weight fucosylated chondroitin sulfate (LFCS) from sea cucumber *Cucumaria frondosa*. *Molecules* **2016,** *21*, 625.

(7) Myron, P.; Siddiquee, S.; Al Azad, S. Fucosylated chondroitin sulfate diversity in sea cucumbers: a review. *Carbohydr. Polym.* **2014,** *112*, 173-178.

(8) Liu, X.; Hao, J.; Shan, X.; Zhang, X.; Zhao, X.; Li, Q.; Wang, X.; Cai, C.; Li, G.; Yu, G. Antithrombotic activities of fucosylated chondroitin sulfates and their depolymerized fragments from two sea cucumbers. *Carbohydr. Polym.* **2016,** *152*, 343-350.

(9) Chen, S.; Xue, C.; Yin, L. a.; Tang, Q.; Yu, G.; Chai, W. Comparison of structures and anticoagulant activities of fucosylated chondroitin sulfates from different sea cucumbers. *Carbohydr. Polym.* **2011,** *83*, 688-696.

(10) Li, Q.; Cai, C.; Chang, Y.; Zhang, F.; Linhardt, R. J.; Xue, C.; Li, G.; Yu, G. A novel structural fucosylated chondroitin sulfate from Holothuria Mexicana and its effects on growth factors binding and anticoagulation. *Carbohydr. Polym.* **2018,** *181*, 1160-1168.

(11) Ustyuzhanina, N. E.; Bilan, M. I.; Dmitrenok, A. S.; Tsvetkova, E. A.; Shashkov, A. S.; Stonik, V. A.; Nifantiev, N. E.; Usov, A. I. Structural characterization of fucosylated chondroitin sulfates from sea cucumbers *Apostichopus japonicus* and *Actinopyga mauritiana*. *Carbohydr. Polym.* **2016,** *153*, 399-405.

(12) Ustyuzhanina, N. E.; Bilan, M. I.; Dmitrenok, A. S.; Shashkov, A. S.; Nifantiev, N. E.; Usov, A. I. The structure of a fucosylated chondroitin sulfate from the sea cucumber *Cucumaria frondosa*. *Carbohydr. Polym.* **2017,** *165*, 7-12.

(13) Mourao, P.A.S.; Pereira, M.S.; Pavão, M.S.; Mulloy, B.; Tollefsen, D.M.; Mowinckel, M.C.; Abildgaard, U. Structure and anticoagulant activity of a fucosylated chondroitin sulphate from echinoderm. Sulphated fucose branches on the polysaccharide account for its high anticoagulant action. *J. Biol. Chem.* **1996**, *271*, 23973–23984.

(14) Nagase, H.; Enjyoji, K.; Minamiguchi, K.; Kitazato, K. T.; Kitazato, K.; Saito, H.; Kato, H. Depolymerized holothurian glycosaminoglycan with novel anticoagulant actions: antithrombin III- and heparin cofactor II-independent inhibition

of factor X activation by factor IXa-factor VIIIa complex and heparin cofactor II-dependent inhibition of thrombin. *Blood* **1995,** *85*, 1527.

(15) Glauser, B. F.; Pereira, M. S.; Monteiro, R. Q.; Mourão, P. A. S. Serpin-independent anticoagulant activity of a fucosylated chondroitin sulfate. *Thromb. Haemost.* **2008,** *100*, 420-428.

(16) Vitor, H. P. Medical gains of chondroitin sulfate upon fucosylation. *Curr. Med. Chem.* **2015,** *22*, 4166-4176.

(17) Fonseca, R. J. C.; Sucupira, I. D.; Oliveira, S. N. M. C. G.; Santos, G. R. C.; Mourão, P. A. S. Improved anticoagulant effect of fucosylated chondroitin sulfate orally administered as gastroresistant tablets. *Thromb. Haemost.* **2017,** *117*, 662-670.

(18) Wu, M.; Wen, D.; Gao, N.; Xiao, C.; Yang, L.; Xu, L.; Lian, W.; Peng, W.; Jiang, J.; Zhao, J. Anticoagulant and antithrombotic evaluation of native fucosylated chondroitin sulfates and their derivatives as selective inhibitors of intrinsic factor Xase. *Eur. J. Med. Chem.* **2015,** *92*, 257-269.

(19) Zhao, L.; Lai, S.; Huang, R.; Wu, M.; Gao, N.; Xu, L.; Qin, H.; Peng, W.; Zhao, J. Structure and anticoagulant activity of fucosylated glycosaminoglycan degraded by deaminative cleavage. *Carbohydr. Polym.* **2013,** *98*, 1514-1523.

(20) Zhao, L.; Wu, M.; Xiao, C.; Yang, L.; Zhou, L.; Gao, N.; Li, Z.; Chen, J.; Chen, J.; Liu, J.; Qin, H.; Zhao, J. Discovery of an intrinsic tenase complex inhibitor:

Pure nonasaccharide from fucosylated glycosaminoglycan. *P. Natl. Acad. Sci. U. S. A.* **2015,** *112*, 8284-8289.

(21) Murado, M. A.; Fraguas, J.; Montemayor, M. I.; Vázquez, J. A.; González, P. Preparation of highly purified chondroitin sulphate from skate (*Raja clavata*) cartilage by-products. Process optimization including a new procedure of alkaline hydroalcoholic hydrolysis. *Biochem. Eng. J.* **2010,** *49*, 126-132.

(22) Chascall, V.; Calabro, A.; Midura, R. J.; Yanagishita, M., [24] Isolation and characterization of proteoglycans. *Method. Enzymol.* **1994**, *230*, 390-417.

(23) Rodén, L.; Baker, J. R.; Anthony Cifonelli, J.; Mathews, M. B., [7] Isolation and characterization of connective tissue polysaccharides. *Method. Enzymol.* **1972**, *28*, 73-140.

(24) He, W.; Fu, L.; Li, G.; Andrew Jones, J.; Linhardt, R. J.; Koffas, M. Production of chondroitin in metabolically engineered *E. coli. Metab. Eng.* **2015,** *27*, 92-100.

(25) Bedini, E.; De Castro, C.; De Rosa, M.; Di Nola, A.; Iadonisi, A.; Restaino, O. F.; Schiraldi, C.; Parrilli, M. A microbiological-chemical strategy to produce chondroitin sulfate A,C. *Angew. Chem. Int. Ed. Engl.* **2011,** *50*, 6160-6163.

(26) Mende, M.; Bednarek, C.; Wawryszyn, M.; Sauter, P.; Biskup, M. B.; Schepers, U.; Brase, S. Chemical synthesis of glycosaminoglycans. *Chem. Rev.* **2016,** *116*, 8193-8255.

(27) Tamura, J.-I.; Tanaka, H.; Nakamura, A.; Takeda, N. Synthesis of β-D-GalNAc(4,6-diS)(1–4)[α-L-Fuc(2,4-diS)(1–3)]-β-D-GlcA, a novel trisaccharide unit of chondroitin sulfate with a fucose branch. *Tetrahedron Lett.* **2013,** *54*, 3940-3943.

(28) Ustyuzhanina, N. E.; Fomitskaya, P. A.; Gerbst, A. G.; Dmitrenok, A. S.; Nifantiev, N. E. Synthesis of the oligosaccharides related to branching sites of fucosylated chondroitin sulfates from sea cucumbers. *Mar. Drugs* **2015,** *13*, 770-787.

(29) Gerbst, A. G.; Dmitrenok, A. S.; Ustyuzhanina, N. E.; Nifantiev, N. E. Conformational analysis of the oligosaccharides related to side chains of holothurian fucosylated chondroitin sulfates. *Mar. Drugs* **2015,** *13*, 936-947.

(30) Zhang, X.; Liu, H.; Lin, L.; Yao, W.; Zhao, J.; Wu, M.; Li, Z. Synthesis of fucosylated chondroitin sulfate nonasaccharide as a novel anticoagulant targeting intrinsic factor Xase complex. *Angew. Chem. Int. Ed. Engl.* **2018,** *57*, 12880-12885.

(31) Zhang, G. L.; Ye, X. S. Synthetic glycans and glycomimetics: A promising alternative to natural polysaccharides. *Chem. Eur. J.* **2018,** *24*, 6696-6704.

(32) Zhang, G.-L.; Yang, L.; Zhu, J.; Wei, M.; Yan, W.; Xiong, D.-C.; Ye, X.-S. Synthesis and antigenic evaluation of oligosaccharide mimics of Vi Antigen from *Salmonella typhi*. *Chem. Eur. J.* **2017,** *23*, 10670-10677.

(33) Nagao, M.; Fujiwara, Y.; Matsubara, T.; Hoshino, Y.; Sato, T.; Miura, Y. Design of glycopolymers carrying sialyl oligosaccharides for controlling the interaction with the influenza virus. *Biomacromolecules* **2017,** *18*, 4385-4392.

(34) Zhang, X.; Yao, W.; Xu, X.; Sun, H.; Zhao, J.; Meng, X.; Wu, M.; Li, Z. Synthesis of fucosylated chondroitin sulfate glycoclusters: A robust route to new anticoagulant agents. *Chem. Eur. J.* **2017**, *24*, 1694-1700.

(35) Laezza, A.; Iadonisi, A.; Pirozzi, A. V.; Diana, P.; De Rosa, M.; Schiraldi, C.; Parrilli, M.; Bedini, E. A modular approach to a library of semi-synthetic fucosylated chondroitin sulfate polysaccharides with different sulfation and fucosylation patterns. *Chem. Eur. J.* **2016,** *22*, 18215-18226.

(36) Laezza, A.; Iadonisi, A.; Castro, C. D.; De Rosa, M.; Schiraldi, C.; Parrilli, M.; Bedini, E. Chemical fucosylation of a polysaccharide: a semisynthetic access to fucosylated chondroitin sulfate. *Biomacromolecules* **2015,** *16*, 2237-2245.

(37) Cai, C.; Solakyildirim, K.; Yang, B.; Beaudet, J. M.; Weyer, A.; Linhardt, R. J.; Zhang, F. Semi-synthesis of chondroitin sulfate-E from chondroitin sulfate-A. *Carbohydr. Polym.* **2012,** *87*, 822-829.

(38) Li J.; Li J.; Sun T.; Cai C.; Shao M.; Yu G. Concise chemoenzymatic synthesis of heparan sulfate analogues as potent BACE-1 inhibitors. *Carbohydr. polym.* **2019**, *217*, 232-239.

(39) Kunishima, M.; Kawachi, C.; Morita, J.; Terao, K.; Iwasaki, F.; Tani, S. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM): an efficient condensing agent leading to the formation of amides and esters. *Tetrahedron* **2000,** *55*, 13159-13170.

(40) Fan, F.; Cai, C.; Gao, L.; Li, J.; Zhang, P.; Li, G.; Li, C.; Yu, G. Microwave-assisted synthesis of glycopolymers by ring-opening metathesis polymerization (ROMP) in an emulsion system. *Polym. Chem.* **2017,** *8*, 6709-6719.

(41) Hassan, S.; Tschersich, R.; Müller, T. J. J. Three-component chemoenzymatic synthesis of amide ligated 1,2,3-triazoles. *Tetrahedron Lett.* **2013,** *54*, 4641-4644.

(42) Pomin, V. H. How to analyze the anticoagulant and antithrombotic mechanisms of action in fucanome and galactanome? *Glycoconjugate J.* **2014,** *31*, 89-99.

(43) Liu, L. L.; Zhang, Y. Q.; Han, X. W.; Shan, X. D.; Hou, Y. W.; Lang, Y. Z.; Zhu, H.; Yu, G. Modification of reduction method about uronic acids in acidic polysaccharides. *Chin. J. Mar. Drugs* **2014**, *33*, 1-7.

(44) Lee, J.-Y.; Chung, S.-J.; Cho, H.-J.; Kim, D.-D. Bile acid-conjugated chondroitin sulfate A-based nanoparticles for tumor-targeted anticancer drug delivery. *Eur. J. Pharm. Biopharm.* **2015,** *94*, 532-541.

(45) Huisgen R. Kinetics and mechanism of 1, 3-dipolar cycloadditions. *Angew. Chem. Int. Ed. Engl.* **1963**, *2*, 633-645.

(46) Duan, B.; Chang, C.; Ding, B.; Cai, J.; Xu, M.; Feng, S.; Ren, J.; Shi, X.; Du, Y.; Zhang, L. High strength films with gas-barrier fabricated from chitin solution dissolved at low temperature. *J. Mater. Chem. A* **2013,** *1*, 1867-1874.

(47) Strehin, I.; Nahas, Z.; Arora, K.; Nguyen, T.; Elisseeff, J. A versatile pH sensitive chondroitin sulfate-PEG tissue adhesive and hydrogel. *Biomaterials* **2010,** *31*, 2788-2797.

(48) Kunishima, M.; Kawachi, C.; Monta, J.; Terao, K.; Iwasaki, F.; Tani, S. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride: an efficient condensing agent leading to the formation of amides and esters. *Tetrahedron* **1999,** *55*, 13159-13170.

(49) Han, S.-Y.; Kim, Y.-A. Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron* **2004,** *60*, 2447-2467.

(50) Farkaš, P.; Bystrický, S. Efficient activation of carboxyl polysaccharides for the preparation of conjugates. *Carbohydr. Polym.* **2007,** *68*, 187-190.

(51) Nimmo, C. M.; Owen, S. C.; Shoichet, M. S. Diels-Alder Click cross-linked hyaluronic acid hydrogels for tissue engineering. *Biomacromolecules* **2011,** *12*, 824-830.

(52) Yu, F.; Cao, X.; Li, Y.; Zeng, L.; Yuan, B.; Chen, X. An injectable hyaluronic acid/PEG hydrogel for cartilage tissue engineering formed by integrating enzymatic crosslinking and Diels–Alder "click chemistry". *Polym. Chem.* **2014,** *5*, 1082-1090.

(53) Yu, F.; Cao, X.; Li, Y.; Zeng, L.; Zhu, J.; Wang, G.; Chen, X. Diels–Alder crosslinked HA/PEG hydrogels with high elasticity and fatigue resistance for cell encapsulation and articular cartilage tissue repair. *Polym. Chem.* **2014,** *5*, 5116-5123.

(54) Meng, X.; Edgar, K. J. "Click" reactions in polysaccharide modification. *Prog. Polym. Sci.* **2016,** *53*, 52-85.

(55) MacCormick, B.; Vuong, T. V.; Master, E. R. Chemo-enzymatic synthesis of clickable xylo-oligosaccharide monomers from hardwood 4-*O*-methylglucuronoxylan. *Biomacromolecules* **2018,** *19*, 521-530.

(56) Zong, C.; Huang, R.; Condac, E.; Chiu, Y.; Xiao, W.; Li, X.; Lu, W.; Ishihara, M.; Wang, S.; Ramiah, A.; Stickney, M.; Azadi, P.; Amster, I. J.; Moremen, K. W.; Wang, L.; Sharp, J. S.; Boons, G.-J. Integrated approach to identify heparan sulfate ligand requirements of Robo1. *J. Am. Chem. Soc.* **2016,** *138*, 13059-13067.

(57) Zong, C.; Venot, A.; Li, X.; Lu, W.; Xiao, W.; Wilkes, J.-S. L.; Salanga, C. L.; Handel, T. M.; Wang, L.; Wolfert, M. A.; Boons, G.-J. Heparan sulfate microarray reveals that heparan sulfate–protein binding exhibits different ligand requirements. *J. Am. Chem. Soc.* **2017,** *139*, 9534-9543.

(58) Soriano del Amo, D.; Wang, W.; Jiang, H.; Besanceney, C.; Yan, A. C.; Levy, M.; Liu, Y.; Marlow, F. L.; Wu, P. Biocompatible copper(I) catalysts for in vivo imaging of glycans. *J. Am. Chem. Soc.* **2010,** *132*, 16893-16899.

(59) Köhling, S.; Künze, G.; Lemmnitzer, K.; Bermudez, M.; Wolber, G.; Schiller, J.; Huster, D.; Rademann, J. Chemoenzymatic synthesis of nonasulfated tetrahyaluronan with a paramagnetic tag for studying its complex with interleukin-10. *Chem. Eur. J.* **2016,** *22*, 5563-5574.

(60) Finetti, C.; Sola, L.; Elliott, J.; Chiari, M. Synthesis of hydrogel via click chemistry for DNA electrophoresis. *J. Chromatogr. A* **2017,** *1513*, 226-234.

(61) Hong, V.; Presolski, S. I.; Ma, C.; Finn, M. G. Analysis and optimization of copper-catalyzed azide–alkyne cycloaddition for bioconjugation. *Angew. Chem. Int. Ed.* **2009,** *48*, 9879-9883.

(62) Liao, S.; Zhao, J.; Qin, Y.; Zhao, S. A novel fluorescence polarization assay for copper ions based on DNA-templated click chemistry and amplification of nanoparticles. *RSC Adv.* **2017,** *7*, 55668-55672.

(63) Martinichen-Herrero, J. C.; Carbonero, E. R.; Gorin, P. A. J.; Iacomini, M. Anticoagulant and antithrombotic activity of a sulfate obtained from a glucan component of the lichen Parmotrema mantiqueirense Hale. *Carbohydr. Polym.* **2005,** *60*, 7-13.

(64) Santos, G. R.; Glauser, B. F.; Parreiras, L. A.; Vilanova, E.; Mourao, P. A. Distinct structures of the alpha-fucose branches in fucosylated chondroitin sulfates do not affect their anticoagulant activity. *Glycobiology* **2015,** *25*, 1043-1052.

For Table of Contents Use Only

Synthesis and properties of functional glycomimetics through click grafting of fucose onto chondroitin sulfates

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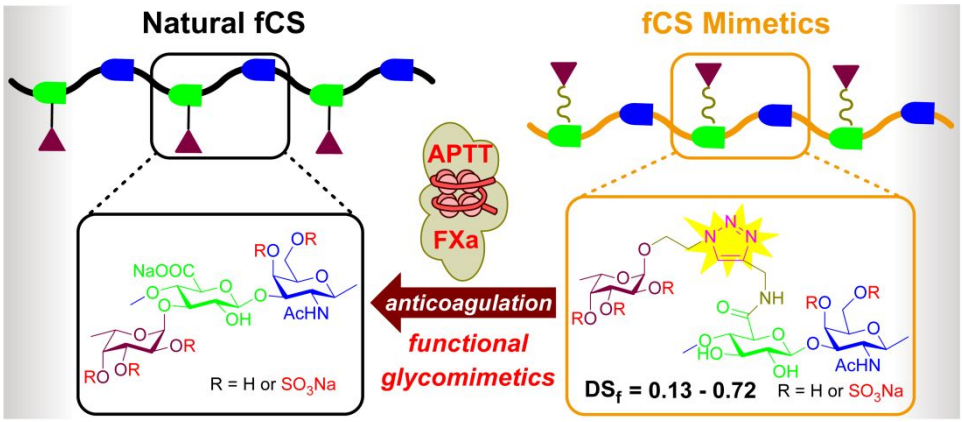

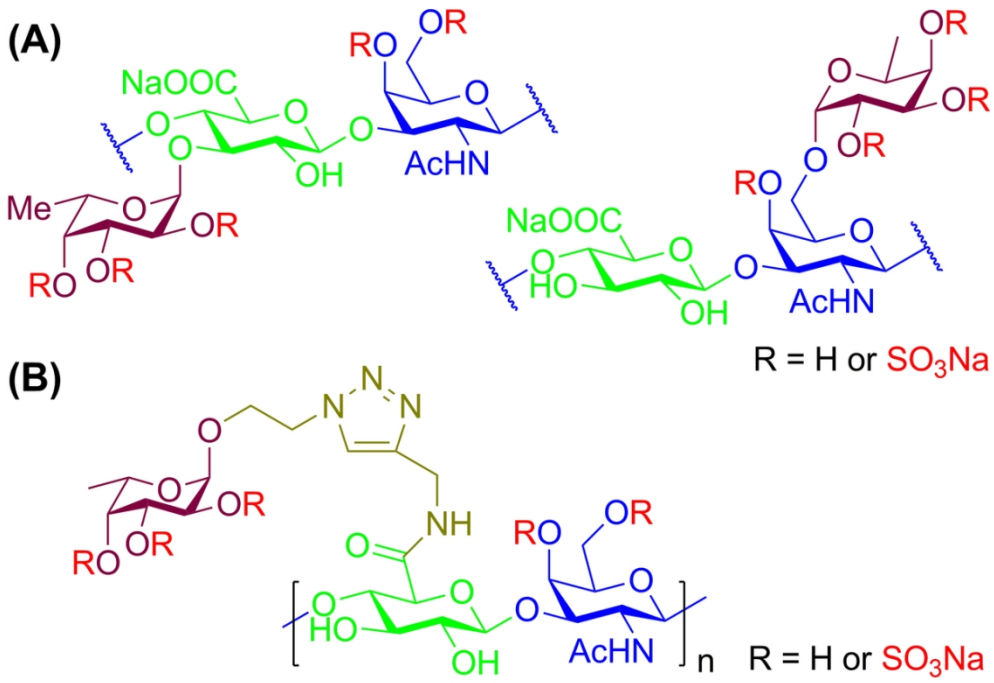

Figure 1. Chemical structures of (A) natural fucosylated chondroitin sulfates (fCS) and (B) designed fCS mimetics.

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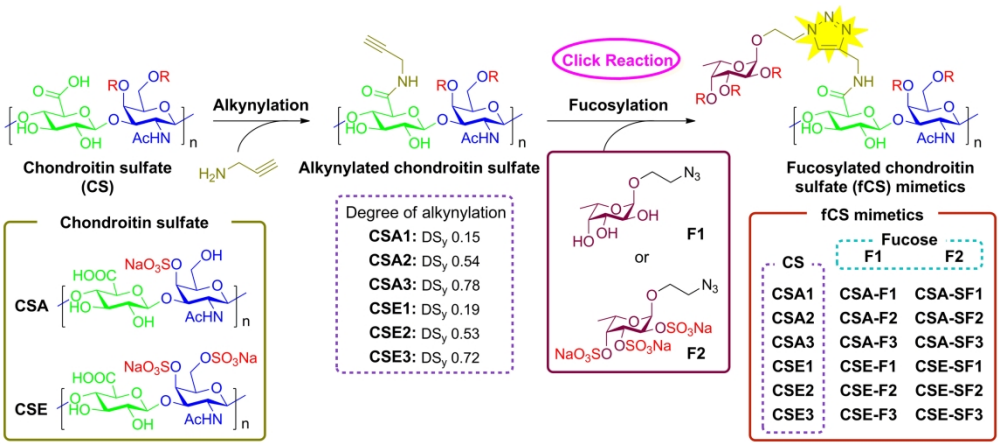

Scheme 1. Schematic Illustration of the Synthesis of fCS Mimetics with Various Sulfation and Fucose Densities Through the Click Grafting of Azide-Fucose onto Alkyne-Functionalized Chondroitin Sulfate

239x106mm (300 x 300 DPI)

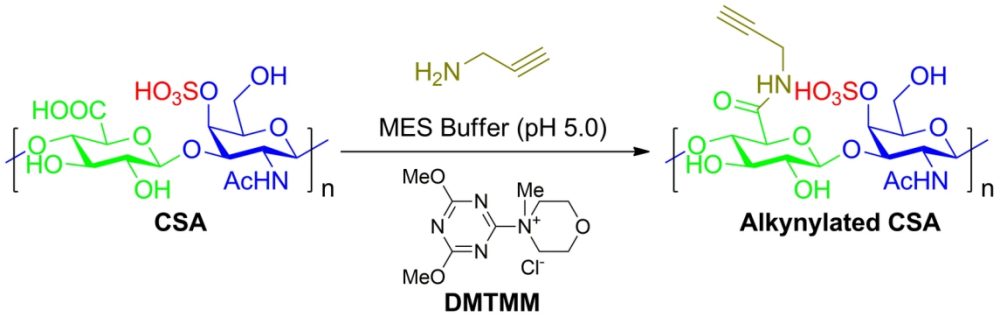

Figure in Table 1. Alkynylation of Chondroitin Sulfate A (CSA) with Propargylamine by Using DMTMM as Condensation Reagent Under Various Conditions

139x44mm (300 x 300 DPI)

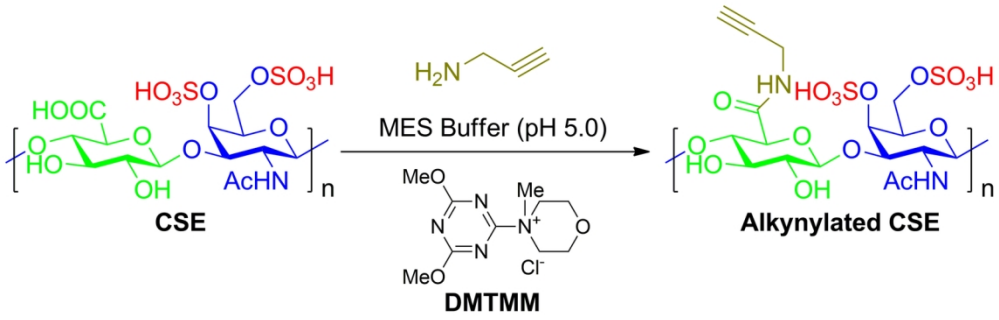

Figure in Table 2. Synthesis of Alkynylated Chondroitin Sulfate E (CSE) with Propargyl Amine Using DMTMM as Condensation Reagent

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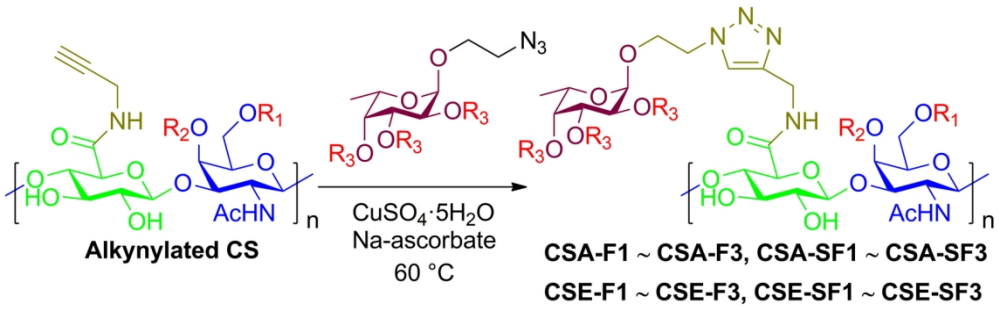
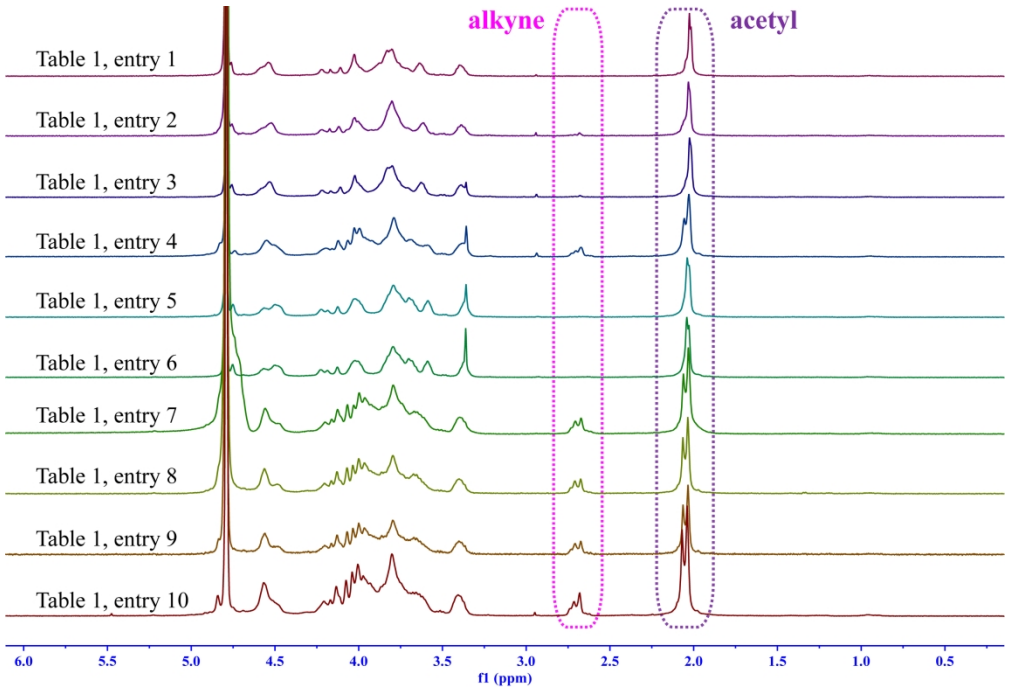


Figure in Table 3. Synthesis of fCS Mimetics by Copper-Catalyzed Cycloadditions of Azide-Fucose with Alkyne-Functionalized Chondroitin Sulfate

146x45mm (300 x 300 DPI)



^{1}H NMR spectra of the alkynylation of CSA (Table 1) in D_2O. The degree of substitution of the alkyne was determined by ^{1}H-NMR integration of the alkyne signal (2.69 ppm) to the GalNAc acetyl signal (2.05 ppm).

127x86mm (600 x 600 DPI)

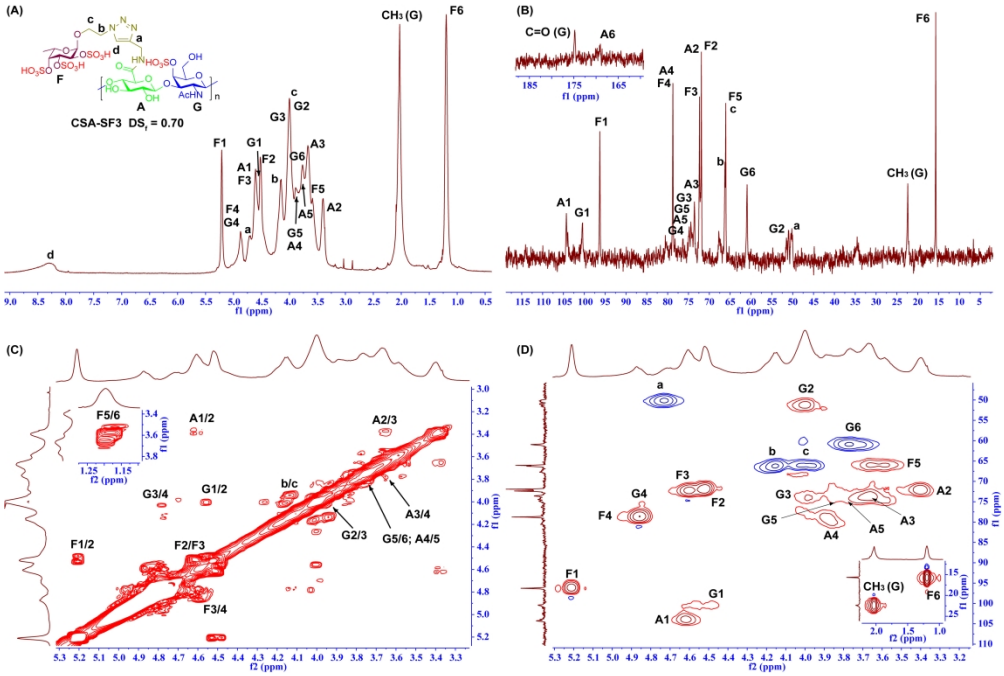


Figure 3. 1D and 2D-NMR spectra of CSA-SF3: (A) ^{1}H NMR, (B) ^{13}C NMR, (C) ^{1}H-^{1}H COSY, (D) ^{1}H-^{13}C HSQC. F, A, and G stand for fucose, GlcA and GalNAc, respectively.

177x118mm (600 x 600 DPI)

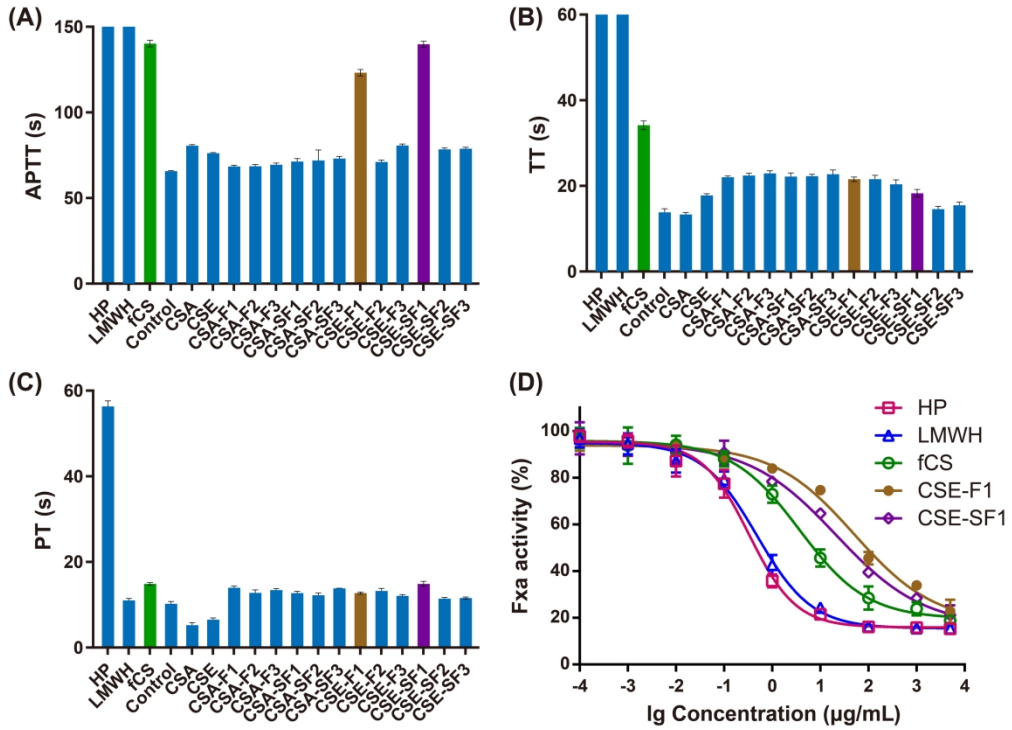


Figure 4. The anticoagulant activities of synthetic fCS mimetics. Effect of synthetic fCS mimetics on prolongation of (A) APTT, (B) TT, and (C) PT at a concentration of 500 μg/mL. APTT > 250s for HP and LMWH, and TT > 400s for HP and LMWH. (D) Inhibition of ATIII-mediated FXa activity by synthetic fCS mimetics.

150x109mm (600 x 600 DPI)