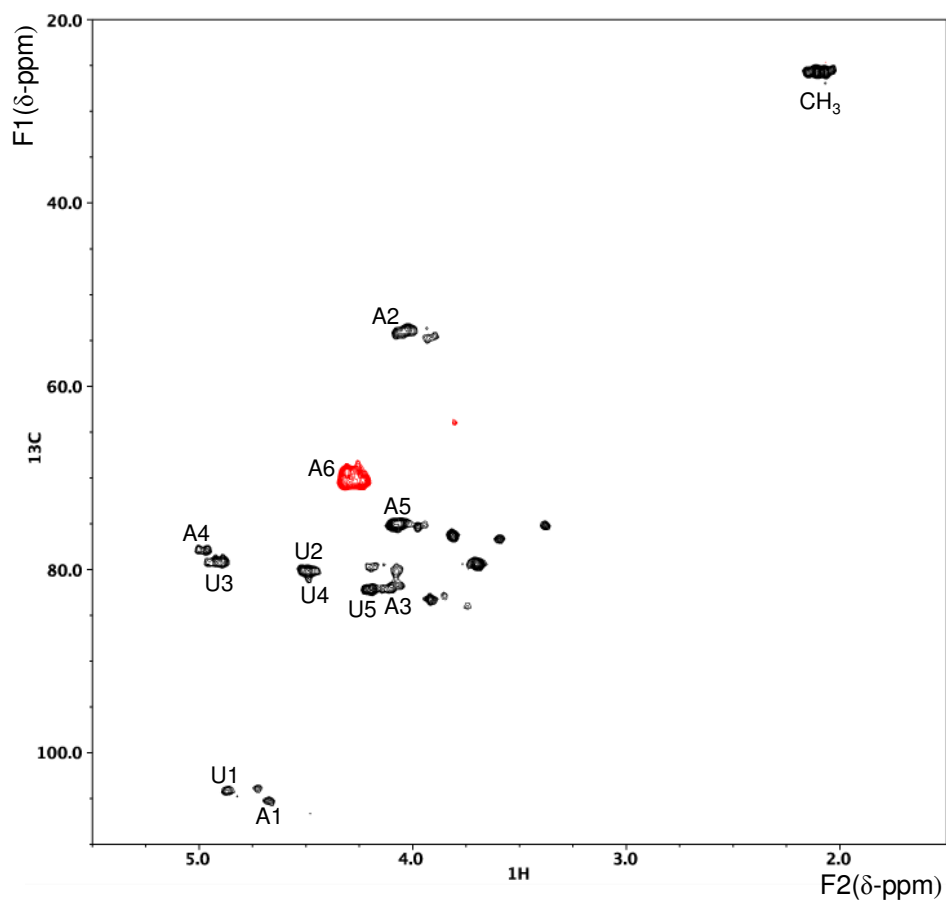


## Supplementary Material to Fonseca et al. “Effects of oversulfated and fucosylated chondroitin sulfates on coagulation: challenges for the study of anticoagulant polysaccharides” (Thromb Haemost 2010; 103.5)

### Structural analyses of oversulfated chondroitin sulfate by heteronuclear 2D NMR

**Method:** The two-dimensional  $^1\text{H}$ - $^{13}\text{C}$  Multiplicity-Edited Heteronuclear Single Quantum Coherence (HSQC) spectrum of the oversulfated chondroitin sulfate was recorded using a Bruker DRX 800 MHz spectrometer operating with a triple resonance probe. About 5 mg of the sample was dissolved in 0.5 mL 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA). The  $^1\text{H}/^{13}\text{C}$  Edited HSQC spectrum was recorded at 35°C with HOD suppression by presaturation, with 256 scans and increment number setup as 64, using states-time proportion phase incrementation (states-TPPI) for quadrature detection in the indirect dimension and run with 1024 x 256 points with globally optimized alternating phase rectangular pulses (GARP) for decoupling. Chemical shifts are displayed relative to external trimethylsilylpropionic acid at 0 ppm for  $^1\text{H}$  and relative to methanol for  $^{13}\text{C}$ .

**Results:** The 2D  $^1\text{H}$ - $^{13}\text{C}$  Edited HSQC (Fig. S1) revealed useful structural information about the oversulfated chondroitin sulfate sample used in this study. This chondroitin sulfate is highly sulfated, reaching the level of up to 72.1% of the total sites available for sulfation on the entire chondroitin backbone. The sulfate groups are exclusively positioned at carbons-2 and -3 of the glucuronic acids, and carbons-4 and -6 of the galactosamines (Figure S2). The integral of the peak assigned as carbon 6 of the galactosamine units (in red) accounts for 96.4% of the total 6-sulfation in the current oversulfated chondroitin sulfate. The majority of unlabeled peaks come from incomplete sulfation at glucuronic acid units, which are responsible for almost 22% out of 27.9 % of the non-sulfated sites. This low heterogeneity arises both from single or double absence of sulfation in glucuronic acid residues. Finally, both proton and carbon chemical shifts of the entirely sulfated disaccharide units of the oversulfated chondroitin sulfate (Fig. 1A) showed perfect agreement with the chemical shift values of this glycosaminoglycan reported in the literature (Table S1), indicating a quite homogeneous compound with no drastic chemical shift changes. Overall, this oversulfated chondroitin sulfate is quite similar to those described in the literature (Guerrini et al., 2008; Maruyama et al., 1998), even though non-sulfated positions still remain.



**Figure S1.** Structural NMR assignment of the major components of oversulfated chondroitin sulfate by  $^1\text{H}$ - $^{13}\text{C}$ -edited HSQC at 800 MHz for  $^1\text{H}$ -resonance. The positive cross-peaks in black are related to CH and CH<sub>3</sub>, while the negative cross-peaks in red are related to CH<sub>2</sub>. The signals of 4,6-di-*O*-sulfo-*N*-acetylgalactosamine and of 2,3-di-*O*-sulfo-glucuronic acid are respectively labeled as A and U, and with numbers that correspond to their respective positions of  $^1\text{H}$ - $^{13}\text{C}$  on the sugar ring. The signal labeled CH<sub>3</sub> belongs to the acetyl group. The unlabeled peaks, mostly from glucuronic acids, represent minor components of oversulfated chondroitin sulfate (27.9 % of total) that were partially sulfated.

**Table S1.** Proton and carbon chemical shifts ( $\delta$ -ppm)<sup>a</sup> of the major components of oversulfated chondroitin sulfate from this work and from literature references.

Monosaccharide	Current work		Guerrini et al. 2008 <sup>b</sup>		Maruyama et al. 1998 <sup>c</sup>
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H
4,6-di- <i>O</i> -Sulfo- <i>N</i> -acetyl-galactosamine (A)					
H1/C1	4.67	105.37	4.77	105.0	4.86
H2/C2	4.02	53.94	4.06	54.0	4.10
H3/C3	4.09	82.06	4.05	80.7	4.10
H4/C4	4.95	78.03	4.98	78.0	5.02
H5/C5	4.06	75.24	4.05	74.8	4.06
H6/C6	4.34-4.19	71.60-68.9	4.28	69.3	4.29
<i>N</i> -acetyl CH <sub>3</sub>	2.16-2.01	26.68-25.04	2.12	25.6	2.16
2,3-di- <i>O</i> -Sulfo-glucuronic acid (U)					
H1/C1	4.86	104.6	4.87	104.5	4.97
H2/C2	4.48	80.33	4.47	80.0	4.53
H3/C3	4.89	79.37	4.95	79.3	4.94
H4/C4	4.48	80.42	4.46	80.9	4.55
H5/C5	4.19	82.44	4.12	82.0	4.20

<sup>a</sup>The chemical shifts were measured at 35°C and referenced relative to external trimethylsilylpropionic acid at 0 ppm for <sup>1</sup>H, and relative to methanol for <sup>13</sup>C.

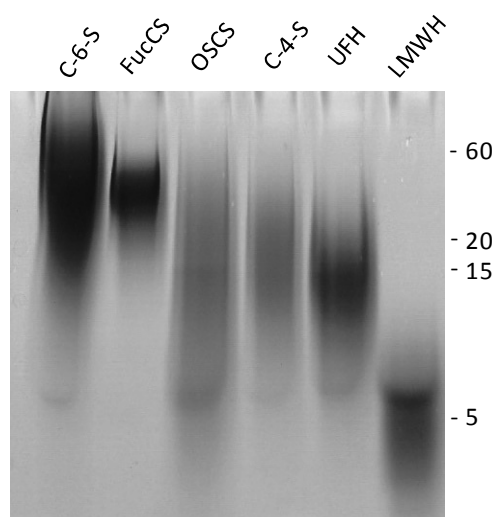
<sup>b</sup>Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A, Capila I, Lansing JC, Guglieri S, Fraser B, Al-Hakim A, Gunay NS, Zhang Z, Robinson L, Buhse L, Nasr M, Woodcock J, Langer R, Venkataraman G, Linhardt RJ, Casu B, Torri G, Sasisekharan R. *Nat Biotechnol.* 2008; 26: 669-675.

<sup>c</sup>Maruyama T, Toida T, Imanari T, Yu G, Linhardt RJ. *Carbohydr Res.* 1998; 306: 35-43.

## Molecular masses of oversulfated and fucosylated chondroitin sulfates

**Method:** The molecular masses of the highly sulfated chondroitin sulfates were estimated by polyacrylamide gel electrophoresis (31). In this experiment, glycosaminoglycans (~10  $\mu\text{g}$  of each) were applied to a 6% 1-mm-thick polyacrylamide slab gel in 0.02 M sodium barbital, pH 8.6, and run for 30 min at 100 V. The gels were stained with 0.1% toluidine blue in 1% acetic acid and then washed for 4 h in 1% acetic acid. The molecular masses of highly sulfated chondroitin sulfates were determined by comparison with the electrophoretic mobility of standard compounds.

**Results:** Fucosylated chondroitin sulfate has a molecular mass of ~40 kDa and a smaller dispersion in size compared to other glycosaminoglycans (Fig. S2). Oversulfated chondroitin sulfate shows a reduced mass and a wide dispersion when compared to native chondroitin 6-sulfate, possibly as a consequence of the chemical sulfonation reaction. Both highly sulfated chondroitin sulfates have a significantly larger size than unfractionated heparin (~15 kDa).



**Figure S2:** Sizes of the highly sulfated chondroitin sulfates used in this study. Chondroitin 6-sulfate from shark cartilage (C-6-S, average size 60 kDa), fucosylated chondroitin sulfate (FucCS), oversulfated chondroitin sulfate (OSCS), chondroitin 4-sulfate from whale cartilage (C-4-S, average size 20 kDa), unfractionated heparin from porcine intestine (UFH, average size 15 kDa) and low-molecular-weight heparin (LMWH, average size 15 kDa) (10  $\mu\text{g}$  of each) were separated by polyacrylamide gel electrophoresis. The molecular masses of standard compounds are indicated to the right of the panel.