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## **Synthesis and characterization of polyampholytic aryl-sulfonated chitosans and their in vitro anticoagulant activity**

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1 SYNTHESIS AND CHARACTERIZATION OF POLYAMPHOLYTIC ARYL-  
2 SULFONATED CHITOSANS AND THEIR IN VITRO ANTICOAGULANT ACTIVITY

3  
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16  
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18  
19 **Abstract**

20  
21 This work firstly aimed to synthesize mono- and di- sulfonic derivatives of chitosan by reductive amination  
22 reaction using respectively 2-formyl benzene sulfonic acid and 2,4 formyl benzene sulfonic acid sodium salts.  
23 The influence of the reactants molar ratio (R), aryl - substituted amino groups versus chitosan free amino groups,  
24 on the degree of substitution (DS) of both sulfonated chitosans was assessed by <sup>1</sup>H NMR, elemental analysis,  
25 coupled conductometry-potentiometry analysis and UV spectrometry and FTIR. The influence of pH on  
26 sulfonated chitosans' properties in solution were investigated by solubility and zeta potential (ZP) studies, size  
27 exclusion chromatography equipped with MALLS detection (SEC-MALLS) and Taylor dispersion analysis  
28 (TDA). The polyampholytic character of both series was evidenced and strongly modified the solutions  
29 properties compared to chitosan. Then, the anticoagulant properties of mono- and di- sulfonic polymers were  
30 investigated by the measurement of the activated partial thromboplastin time (aPTT), Prothrombin-time (PT) and  
31 anti-(factor Xa).

32  
33 *Keywords: chitosan; sulfonated chitosan; reductive amination; polyampholyte; anticoagulant*

44

## 45 1. INTRODUCTION

46 Chitosan (CHT) is a natural biopolymer known for its excellent biocompatibility, biodegradability,  
47 hemocompatibility, wound healing and antibacterial properties. Therefore, chitosan is a material of choice in the  
48 design of a wide range of applications in the biomaterials field, such as wound dressings (Mohandas, Deepthi,  
49 Biswas & Jayakumar, 2017), injectable hydrogels (Liu, Gao, Lu & Zhou, 2016), and scaffolds for tissue  
50 engineering (Oryan & Sahvieh, 2017). In order to further enhance the biological properties and enlarge the range  
51 of potential uses of CHT in the biomedical field, many chemical modifications have been attempted, such as  
52 alkylation (Desbrieres, Martinez & Rinaudo, 1996), carboxymethylation (Kong, Kim, Ahn, Byun & Kim, 2010)  
53 or quaternisation (Sajomsang, Gonil, Saesoo & Ovatlarnporn, 2012), all aiming improving or even endow further  
54 functionalities to chitosan such as mucoadhesivity, antibacterial properties and hemocompatibility (Balan &  
55 Verestiuc, 2014). In particular, in addition to the numerous possible chitosan modification routes mentioned  
56 above, sulfonation or sulphation of chitosan are also widely reported in the literature. The consequences of such  
57 chemical modifications is that these reactions give to chitosan chemical compositions comparable to that of the  
58 class of sulphated glycosaminoglycans (GAGs), components of the extracellular matrix (ECM) in the animal  
59 tissues. Therefore, sulfated chitin or chitosan studied in literature were reported to advantageously serve as  
60 ECM analogs with advanced properties such as antibacterial (Jung, Kim, Choi, Lee & Kim, 1999) anti-HIV-1  
61 properties (Artan, Karadeniz, Karagozlu, Kim & Kim, 2010), reduction of blood protein absorption (Lima et al.,  
62 2013), anticoagulant (Campelo, Lima, Rebêlo, Mantovani, Beppu & Vieira, 2016) and anti-thrombogenic  
63 properties which were claimed in some cases to be at least as performing as heparin (Ma, Huang, Kang & Yan,  
64 2007). Chitosan sulphation can be carried out by direct modification of the chitosan repeat units in their 3-O  
65 and/or 6-O positions, and/or to modify the N position, by using sulphuric anhydride SO<sub>3</sub> / pyridine mixture  
66 (Hirano, Tanaka, Hasegawa, Tobetto & Nishioka, 1985; Jung, Na & Kim, 2007; Nishimura et al., 1998; Yeh &  
67 Lin, 2008) or chlorosulfonic acid/formamide (Gamzazade, Sklyar, Nasibov, Sushkov, Shashkov & Knirel, 1997;  
68 Hirano, Tanaka, Hasegawa, Tobetto & Nishioka, 1985; Ma, Huang, Kang & Yan, 2007). In order to target O  
69 positions, the regioselectivity could be controlled by using protection-deprotection of the primary amino groups  
70 strategies (Nishimura et al., 1998; Yeh & Lin, 2008). Besides, chitosans carrying sulfonate groups on N position  
71 could be obtained by using reactants such as vinylsulfonate (Jung, Kim, Choi, Lee & Kim, 1999), propane  
72 sultone (Jung, Na & Kim, 2007) or 3-chloro-2-hydroxy propanesulfate (Jayakumar, Nwe, Tokura & Tamura,  
73 2007; Yin, Li, Yin, Miao & Jiang, 2009). In particular, chitosan modification can be advantageously achieved by  
74 using the reductive amination reaction path proposed by Hall and Yalpani (Hall & Yalpani, 1980; Roberts,  
75 1992), which consists in reacting alkyl or aryl aldehydic compounds on chitosan substrates yielding Schiff's  
76 bases intermediates directly reduced into secondary amines in the presence of sodium cyanoborohydride. In  
77 1992, Muzzarelli applied this reaction using 5-formyl-2-furansulfonic acid sodium salt (Muzzarelli, 1992) and  
78 the obtained N-sulfofurfuryl chitosan derivative was recently exploited in the biomaterials field for the  
79 elaboration of films with blood anti-coagulant properties (Amiji, 1998; Campelo, Chevallier, Vaz, Vieira &  
80 Mantovani, 2017; Campelo, Lima, Rebêlo, Mantovani, Beppu & Vieira, 2016; Huang, Du, Yang & Fan, 2003;  
81 Lima et al., 2013).

82 In 1995, our group used the aforementioned reductive amination pathway for the modification of a textile  
83 substrate coated with chitosan by using sodium salts of 2,4-formyl benzene sulfonic acid and 2-formyl benzene  
84 sulfonic acid. A strong cation exchange filter efficient in the removal of heavy metals from acidic media was  
85 obtained (Martel, Weltrowski, Morcellet & Scheubel, 1995). In parallel, this pathway was also applied to  
86 chitosan in solution and the resulting mono- and di- sulfonic chitosans (called here CHT1S and CHT2S  
87 respectively) derivatives were characterized by advanced NMR techniques and used as powdery sorbents for  
88 trapping heavy metals and textile dyestuffs in aqueous media (Crini et al., 2008; Crini, Martel & Torri, 2008;  
89 Crini, Torri, Guerrini, Morcellet, Weltrowski & Martel, 1997; Crini, Torri, Martel, Weltrowski, Morcellet &  
90 Cosentino, 1997; Weltrowski, Martel & Morcellet, 1996). In the frame of functional materials for environmental  
91 applications, large molar excesses of sulfonic aldehydes versus chitosan amino groups were used in order to graft  
92 the maximal amount of sulfonate groups on chitosan and to reach the maximal cation exchange capacity of the  
93 sorbent. However, for biomaterials applications of chitosan and chitosan derivatives, free primary amino groups  
94 present on glucosamine repeat units are thought as the major character resulting in special biological properties  
95 associated with chitosan (Yeh & Lin, 2008). Consequently, chitosan modification rate on N position should be  
96 controlled in order to maintain sufficient residual free amino groups. Therefore, the goal of the present study was  
97 to define the experimental conditions for the reductive amination reaction conditions in order to control the  
98 chitosan chains substitution degree and subsequently their residual glucosamine repeat units content. To reach  
99 this purpose, variable molar ratios of both sulfonic aldehydes BZ1S and BZ2S versus chitosan free amino groups  
100 in the reaction vessel (designed by R ratio) were applied in order to control the degree of substitution (DS) of the  
101 obtained chitosan sulfonic derivatives. Sulfonated products were characterized by elemental analysis, <sup>1</sup>H NMR,  
102 coupled potentiometry and conductometry, size exclusion chromatography, Taylor dispersion analysis, FTIR,  
103 UV-visible spectrometry and zeta potential. Based on literature cited above, these sulfonated chitosans due to  
104 their ampholytic characteristics mimicking heparin structure were expected to display anticoagulant properties.  
105 Therefore, the subsequent goal of the study aimed to investigate the anticoagulant properties of these sulfonic  
106 chitosans powders dispersed in total blood, in function of their DS, of the nature of their substituent (mono- or  
107 disulfonic aryl groups) and of their concentration.

108

## 109 **2. EXPERIMENTALS**

110

### 111 **2.1. Materials**

112 Chitosan (CHT), *low molecular weight* grade batch SLBG1673V, 80.3% degree of deacetylation (supplier  
113 value), was supplied by Sigma-Aldrich with an intrinsic viscosity measured by capillary viscosimetry of  $[\eta]=$   
114 896 dL/g and molecular weight of  $M_v= 130\ 000\ \text{g}\cdot\text{mol}^{-1}$  determined according to Mark-Houwink equation  $[\eta]=$   
115  $K \cdot M_v^\alpha$  with  $K = 74 \cdot 10^{-5}\ \text{dL/g}$ ,  $\alpha= 0.76$  and 0.3M HAc/0.2M NaAc as solvent (Rinaudo, Milas & Dung, 1993).

116 2-formylbenzenesulfonic acid sodium salt (mono sulfonic, BZ1S), 2,4 formylbenzenesulfonic acid sodium salt  
117 dihydrate (di sulfonic, BZ2S), glacial acetic acid, sodium cyanoborohydride (NaBH<sub>3</sub>CN) were purchased from  
118 Aldrich chemicals and used without further purification and methanol with HPLC gradient was purchased from  
119 Carlo Erba reagent S.A.S.

120

### 121 **2.2. Methods**

### 122 **2.2.1. Synthesis of CHT2S and CHT1S series**

123 The method for the preparation of N-arylsulfonate derivatives of chitosan by reductive amination was described  
124 previously (Weltrowski, Martel & Morcellet, 1996). 5 g of chitosan were dissolved in 500 mL of 1% (v/v) of  
125 aqueous acetic acid. This solution was then diluted by addition of 450 mL of methanol. The appropriate amount  
126 of sodium salt of mono sulfonic or disulfonic aldehydic compound, respectively 2-formyl benzene sulfonic acid  
127 (BZ1S) or 2,4-formylbenzenesulfonic acid (BZ2S) dissolved in 50 mL of water was added to the chitosan hydro-  
128 methanolic solutions. The amounts of BZ1S and BZ2S introduced in the reaction vessel were predetermined on  
129 the basis of the molar ratio (R) of BZ1S or BZ2S versus the free amino groups in chitosan (4.8 mmol of amino  
130 groups per gram of chitosan) dissolved in the reaction medium. Two series of chitosan derivatives, were  
131 obtained, called CHT1S and CHT2S depending on the use of BZ1S or BZ2S, respectively. Molar ratios  $R =$   
132  $[BZ1S] \text{ or } [BZ2S]/[CHT \text{ amino groups}]$  in the reaction mixtures were adjusted to 0.25, 0.5, 0.75, 1, 1.5 and 2 in  
133 each series. Within 3 minutes after BZ1S or BZ2S addition, the viscosity of the chitosan solution sharply  
134 decreased and a white precipitate appeared. Then 3g of sodium cyanoborohydride were added under vigorous  
135 stirring that was maintained during one hour at ambient temperature. The suspension was then directly poured in  
136 dialysis cellulosic membranes (SpectraPor 12-14 kDa, diameter 50mm) and dialyzed against distilled water  
137 (renewed twice a day) during 5 days. Finally, the powdered purified CHT1S and CHT2S products were  
138 recovered after freeze drying and grinding with a mortar. For clarity, the nomenclature CHT1S-1 or CHT2S-1  
139 was used for batches issued from molar ratio  $R= 1$ , and this was extended to all R values.

140

### 141 **2.2.2. Characterization by elemental analysis**

142 Elemental analysis of carbon, hydrogen, nitrogen and sulphur in mono and disulfonic chitosan derivatives was  
143 determined at **Service central d'analyse (SCA) USR59**, CNRS, Vernaison France. The experimental weight  
144 sulphur contents (%S) were converted into degree of substitution values ( $0 < DS < 0.803$ ) which is defined as the  
145 molar ratio of repeat units of chitosan modified by the aryl sulfonic groups reported as index z in Scheme 1,  
146 while index x is the degree of acylation of CHT considered as constant ( $x = 0.197$ ), and indexes y and y' are the  
147 free glucosamine repeat unit's ratio ( $y = 0.803$ ), before and after reaction.

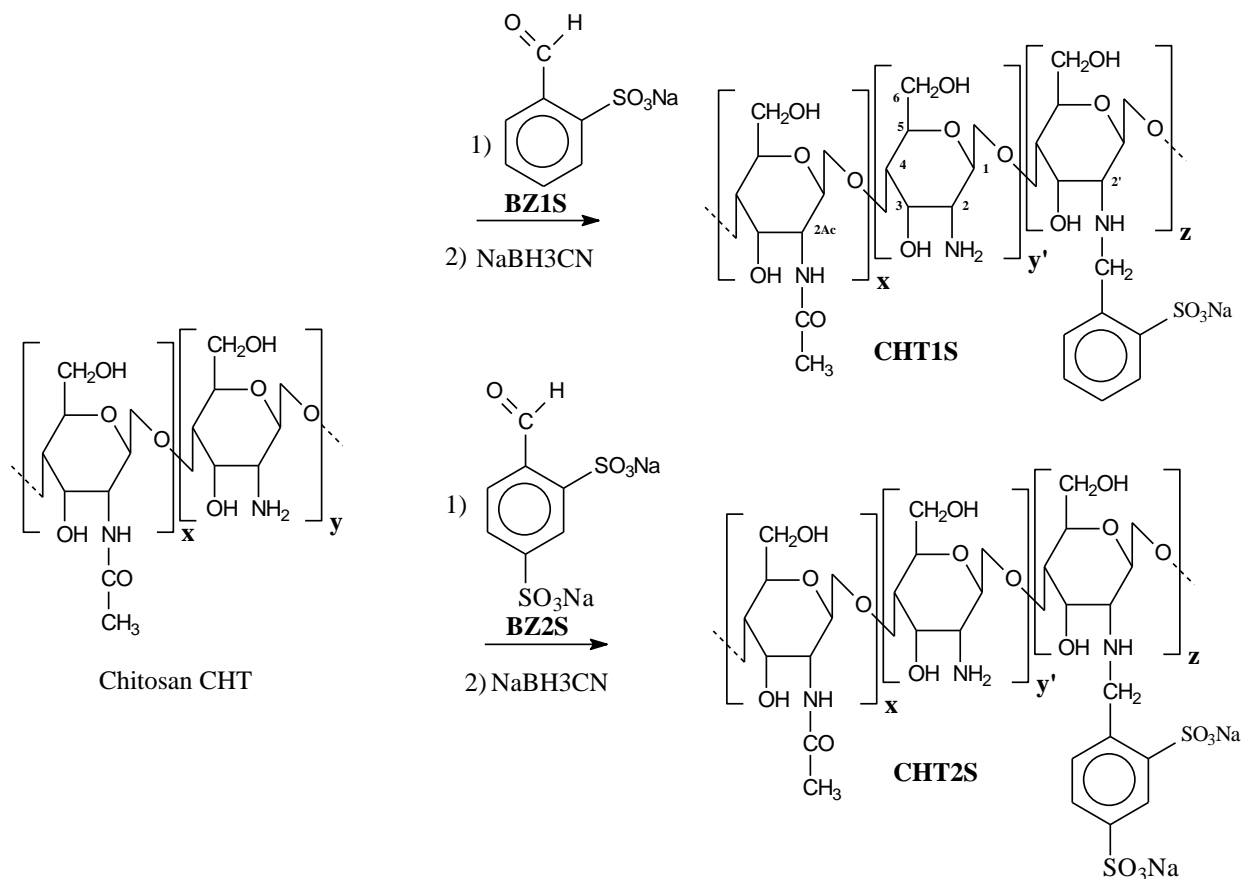
148 Based on the above mentioned parameters, two theoretical relations between DS and %S were firstly established  
149 and plotted in figure S1 (supplementary data); the obtained second degree equations were  $DS = 5.4 \cdot 10^{-3} \cdot (\%S)^2 +$   
150  $4.59 \cdot 10^{-2} \cdot (\%S)$  (eq(1)) for CHT1S, and  $DS = 3 \cdot 10^{-3} \cdot (\%S)^2 + 1.77 \cdot 10^{-2} \cdot (\%S)$  (eq(2)) for CHTS2, where %S is the  
151 theoretical sulphur weight percentage. The repeat unit's molecular weights of acylated glucosamine,  
152 glucosamine, and glucosamine reacted with BZ1S and BZ2S were 203 g/mol, 161 g/mol, 339 g/mol and 455  
153 g/mol respectively. Thanks to both theoretical equations mentioned above, it was possible to calculate  
154 experimental DS values as well as the sulfonate groups (in mmol/g) of all synthesized chitosan derivatives from  
155 their sulphur contents measured by elemental analysis.

156

### 157 **2.2.3. NMR Analyses**

158  $^1H$  NMR spectra were obtained at 300 MHz on a Bruker AC300 spectrometer (Bruker, Karlsruhe, Germany) or  
159 at 400 MHz (9.4T) on a Bruker Av II 400 spectrometer (Bruker, Karlsruhe, Germany). Chitosan sulfonate  
160 derivatives were dissolved in 0.01M NaOD solutions. All measurements were performed at 293 K. An advanced  
161 study previously reported the complete analysis of CHT1S and CHT2S derivatives  $^{13}C$  and  $^1H$  NMR spectra, and

162 presented the detailed attribution of NMR signals of all carbons and protons groups observed in the chemical  
 163 structures displayed in scheme 1 (Crini, Torri, Martel, Weltrowski, Morcellet & Cosentino, 1997). The degrees  
 164 of substitution of CHT1S and CHT2S series were calculated from the area of the proton signals at 2.65 ppm  
 165 corresponding to H2 and H2' (see labeling in scheme 1) present on unsubstituted and substituted glucosamine  
 166 repeat units on the one hand, and from the area of the aromatic protons of benzyl monosulfonate and disulfonate  
 167 observed in the 7-8 ppm range, on the other hand. As for elemental analysis method, DS values were relative to  
 168 the ratio of the aryl sulfonated repeat units represented by indexes z in Figure 1.



169  
 170 **Figure 1.** Syntheses routes of benzyl mono and di-sulfonate derivatives CHT1S and CHT2S where  $x$  is the relative molar ratio in acylated  
 171 repeat units (considered constant at 0.197),  $y'$  the relative molar ratio of residual glucosamine units after reaction, and  $z$  is the relative molar  
 172 ratio of sulfonated repeat units, also reported as DS, substitution degree. Initial molar ratio ( $R$ ) between sulfonic aldehyde compounds and  
 173 free amino group of CHT was the variable parameter ( $0.15 \leq R \leq 2$ )

174

#### 175 2.2.4. Characterization by coupled potentiometry and conductometry

176 100 mg of sulfonated chitosan derivatives were solubilized in 100 mL of 0.01 M NaOH solution, and 0.2 M KCl  
 177 and titrated with standardized 0.1 N HCl simultaneously by pHmetry (inolab (WTW)) and conductometry  
 178 (MeterLab®, CDM210) at ambient temperature. A dilution factor was applied on data before plotting the  
 179 conductance in mS versus added volume of the titrant HCl solution.

180

#### 181 2.2.5. Fourier transform infrared spectroscopy (FT-IR)

182 A PerkinElmer spectrometer (Spectrum One) equipped with Spectrum software was used to perform the FTIR  
183 analyses. The attenuated total reflectance (ATR) FTIR spectra were collected from 16 scans in the 400-4000  $\text{cm}^{-1}$   
184 range with a resolution of 4  $\text{cm}^{-1}$ .

185  
186

#### 187 **2.2.6. UV- visible spectrometry**

188 100 mg of CHT1S and CHT2S samples were solubilised in 50 mL 0.01M NaOH, an aliquot was transferred into  
189 a 10 mm path quartz cell and analyzed at ambient temperature between 250 and 300 nm using UV-visible  
190 spectrophotometer (Shimadzu UV-1800).

191

#### 192 **2.2.7. Zeta potential**

193 The zeta potential was evaluated using a Zetameter (Zetasizer nano Zs model, Malvern). A wide range of pH  
194 between 4 and 11 was studied. CHT1S and CHT2S samples were solubilized in NaOH (0.01M) at a  
195 concentration of 2  $\text{mg. mL}^{-1}$  and then, HCl (0.05M) was added in order to adjust the desired pH. In the  
196 meantime, a CHT solution was prepared by dissolving raw CHT in acetic acid solution 0.1% (v/v) at the same  
197 concentration as previous, 2  $\text{mg. mL}^{-1}$ , whose pH was adjusted with NaOH 0.05M increments.

198

#### 199 **2.2.8. pH solubility domain**

200 The samples were prepared according to the protocol followed for zeta potential measurement. UV-vis  
201 spectrophotometer (UV-1800, Shimadzu France) was used to measure the transmittance at each pH point.

202

#### 203 **2.2.9. SEC-MALLS**

##### 204 **2.2.9.1 Size Exclusion Chromatography (SEC) coupled with multi-angle laser light scattering (MALS)** 205 **detection**

206 The SEC-MALLS experiments were performed on a THERMO SCIENTIFIC ULTIMATE 3000 module  
207 equipped with a OHpak SBG SHODEX column guard (50 × 6 mm) and two SB-806M-HQ SHODEX columns  
208 (300 × 8 mm) connected in series in association with a miniDAWN-TREOS three-angle laser light scattering  
209 detector (41.5°, 90° and 138.5°) having a 658 nm laser (from WYATT, Santa Barbara, CA, USA) and with a  
210 RID-6A refractive index monitor (from SHIMADZU, Kyoto, Japan) at a thermostated temperature of 35°C. The  
211 eluent used was composed of 30 mM borate buffer at pH = 8.5. The eluent was filtered using DURAPORE®  
212 membrane filters of 0.1  $\mu\text{m}$  cut-off. The polymer samples (100  $\mu\text{L}$  injection volume at a concentration of 2  
213  $\text{g.L}^{-1}$ ) were eluted at a 0.8  $\text{mL.min}^{-1}$  flowrate. The data were analyzed using the Astra software (v6.1.1.17, from  
214 Wyatt Technology Corp.) considering an elution volume range comprised between 15 and 23 mL for all  
215 chromatograms.

##### 216 **2.2.9.2. Incremental refractive index $(\delta n/\delta c)_\mu$ determination**

217 The incremental refractive index  $(\text{dn}/\text{dc})_\mu$  of each polymer was determined experimentally using the  
218 refractometer used for the SEC-MALLS analysis. Mother polymer solutions were prepared at 2.0  $\text{g.L}^{-1}$  in the  
219 same eluent by weighting the amounts of dry polymer (including the counter-ions) and eluent. The amount of  
220 water weighted with the polymer was considered as negligible. Mother polymer solutions were dialyzed

221 overnight against the eluent before dilution for final polymer concentrations of 0.15, 0.25, 0.5, 0.75, 1.0, 1.5 and  
222 2.0 g.L<sup>-1</sup> (MILLIPORE membrane, ref. 131414, cutoff 100 Da). Note that the term *c* in the refractive index  
223 increment represents the polyelectrolyte mass concentration including the counter-ions. All polymers were under  
224 their sodium salt form.

### 225 2.2.9.3. Taylor dispersion analysis (TDA)

226 Taylor dispersion analysis (TDA) is an absolute method for the determination of diffusion coefficient  
227 (and thus hydrodynamic radius,  $R_h$ ) based on the band broadening of short initial solute plug under a  
228 laminar Poiseuille flow in an open tube. Due to the parabolic velocity profile, the analyte initial band  
229 is dispersed according to the combination of a convection/diffusion process, also named Taylor-Aris  
230 dispersion (Taylor, 1953). The analytes are redistributed along the tube cross-section owing to the  
231 molecular diffusion. When the characteristic diffusion time is lower than the average detection time,  
232 the Taylor-Aris dispersion leads to a Gaussian peak for a monodisperse sample. The elution profile is  
233 obtained by online UV detection through the capillary tube at a given distance from the injection end  
234 of the capillary

235 The diffusion coefficient  $D$  of the solute and the corresponding hydrodynamic radius  $R_h$  are given by  
236 equations (1) and (2):

$$237 \quad D = \frac{R_c^2 t_0}{24\sigma^2} \quad (1)$$

$$238 \quad R_h = \frac{k_B T}{6\pi\eta D} = \frac{4\sigma^2 k_B T}{\pi\eta R_c^2 t_0} \quad (2)$$

239 where  $k_B$  is the Boltzmann constant,  $T$  is the temperature (in Kelvin),  $R_c$  is the capillary diameter,  $\eta$  is  
240 the viscosity of the eluent,  $t_0$  is the average elution time and  $\sigma^2$  is the temporal variance of the elution  
241 profile.

242 The broader the peak, the higher the  $\sigma^2$  value and the higher the  $R_h$  value. Equations (1) and (2) are  
243 valid provided that  $t_0 \geq 1.25R_c^2/D$  and  $R_c u / D > 40$  (with  $u$  being the average linear mobile phase  
244 velocity), as mentioned in the literature (Cottet, Biron & Martin, 2014). It is worth noting that TDA  
245 leads to the weight-average  $R_h$  which is not biased toward the larger aggregates or nanoparticles  
246 contained in the sample as observed for the intensity-average  $R_h$  measured by DLS (Cottet, Biron &  
247 Martin, 2007; Hawe, Hulse, Jiskoot & Forbes, 2011). TDA experiments were performed on a capillary  
248 electrophoresis apparatus from AGILENT (Waldbronn, Germany) using 50  $\mu\text{m}$  i.d.  $\times$  38 cm ( $\times$  29.5  
249 cm to the detector window) capillaries. Solutes were monitored by UV absorbance at 214 nm. TDA  
250 experiments were carried out using 20 mbar mobilization pressure. Capillaries were prepared from  
251 bare silica tubing purchased from Composite Metal Services (Worcester, United Kingdom). All TDA  
252 experiments were carried out at 25°C. Each polymer sample is prepared at 2 g.L<sup>-1</sup> in the background



253 electrolyte (0.01 M NaOH). Before sample analysis, the capillary was previously filled with the same  
254 electrolyte. All TDA experiments were realized in duplicates.

255  
256  
257

### 258 **2.2.10. Coagulation assays**

259 The anticoagulation activity of mono- and di-sulfonated chitosans was evaluated by classic  
260 coagulation assays: activated partial thromboplastin time (aPTT), prothrombin-time (PT) and anti-  
261 factor Xa (anti-FXa), which correspond respectively to the intrinsic pathway, the extrinsic pathway  
262 and the common pathway of the coagulation cascade of the blood. Human blood was collected from  
263 healthy adult volunteer into citrate blood tubes. Two series of studies were conducted: the first one  
264 was to compare the anticoagulant activity between the different ratios of CHT1S and CHT2S at a  
265 constant concentration ( $1 \text{ mg}\cdot\text{mL}^{-1}$ ); the second one was to evaluate the concentration-dependent  
266 anticoagulant activity of CHT1S and CHT2S at different concentrations (0.05, 0.1, 0.2, 0.4 and 1  
267  $\text{mg}\cdot\text{mL}^{-1}$ ). For both studies, the anticoagulant activity of test samples was compared to that of the  
268 standard therapeutic heparin (Heparin sodium (5000 IU/mL), SANOFI, Paris, France) and non-  
269 supplemented complete citrated blood. All blood suspensions were incubated at  $37^\circ\text{C}$  for 30 minutes  
270 at 80 rpm. Then the platelet-poor plasma was obtained, according to the guidelines for preparing  
271 citrated plasma for hemostaseological analysis (centrifuged at 2500 g for 15 minutes at  $14^\circ\text{C}$ ), for  
272 following coagulation tests.

273 For aPTT assay, 50  $\mu\text{L}$  of each citrated normal human plasma were incubated at  $37^\circ\text{C}$  for 1 min and  
274 50  $\mu\text{L}$  of aPTT reagent (TriniCLOT<sup>tm</sup> aPTT HS, Tcoag<sup>®</sup>) was added to the mixture and incubated at  
275  $37^\circ\text{C}$  for 5 min. Thereafter, 100  $\mu\text{L}$  of  $\text{CaCl}_2$  ( $0.025 \text{ mol}\cdot\text{mL}^{-1}$ ) were added and the clotting time was  
276 measured.

277 For PT assay, 100  $\mu\text{L}$  of plasma samples were incubated at  $37^\circ\text{C}$  for 2 min. 200  $\mu\text{L}$  of tissue  
278 thromboplastin reagent (NEOPLATINE<sup>®</sup> R, Diagnostica Stago, Inc., France), pre-incubated at  $37^\circ\text{C}$   
279 for 10 min, were introduced and clotting time was recorded.

280 For anti-Xa assay, the assay was applied as indicated by the manufacturer (HYPHEN BioMed,  
281 France). Briefly, Human factor Xa ( $2.4 \text{ nkat/mL}$ ) and human antithrombin III ( $0.17 \text{ U/mL}$ ) was added  
282 to 100  $\mu\text{L}$  of prewarmed human plasma containing heparin standard or chitosan or sulfonated chitosan.  
283 The mixture was incubated for exactly 2 min at  $37^\circ\text{C}$ . To measure the residual factor Xa activity, the  
284 chromogenic substrate (SXa-11) was added. The increase of absorbance at 405 nm per minute was  
285 recorded. The anticoagulant activity was reported in equivalents to therapeutic heparin by converting  
286 clotting time to heparin IU/ml plasma using a standard heparin calibration curve.

287

## 288 **3. RESULTS AND DISCUSSION**

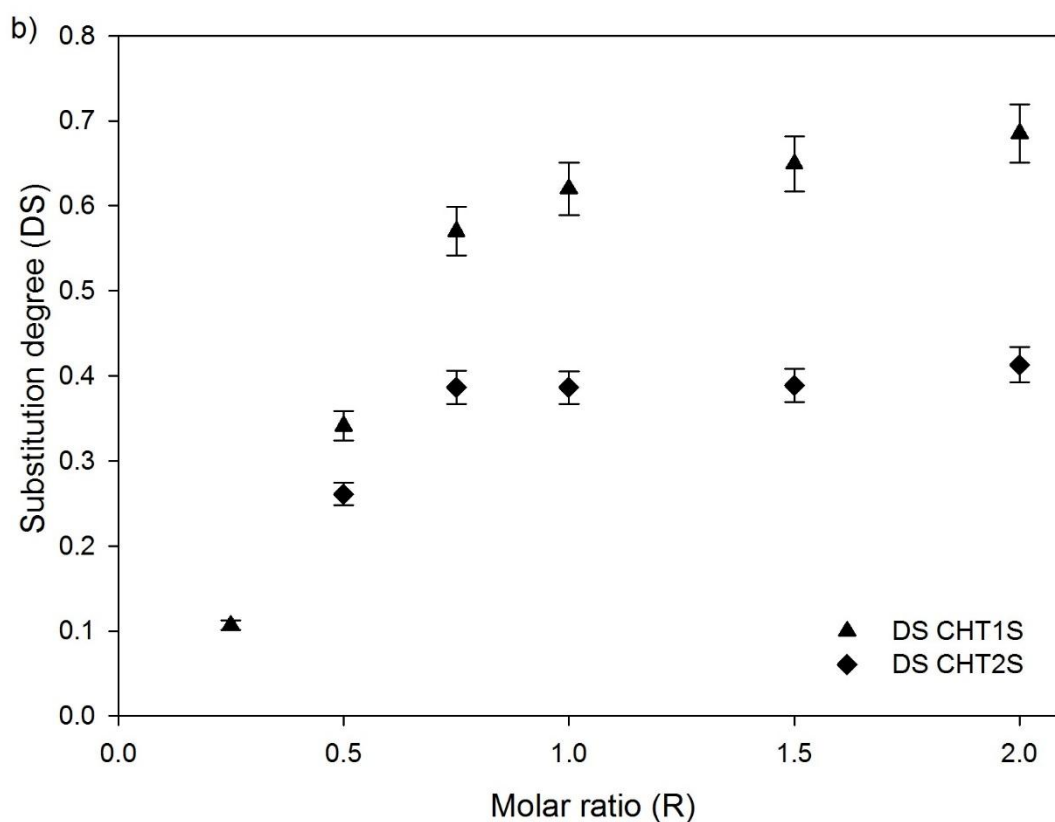
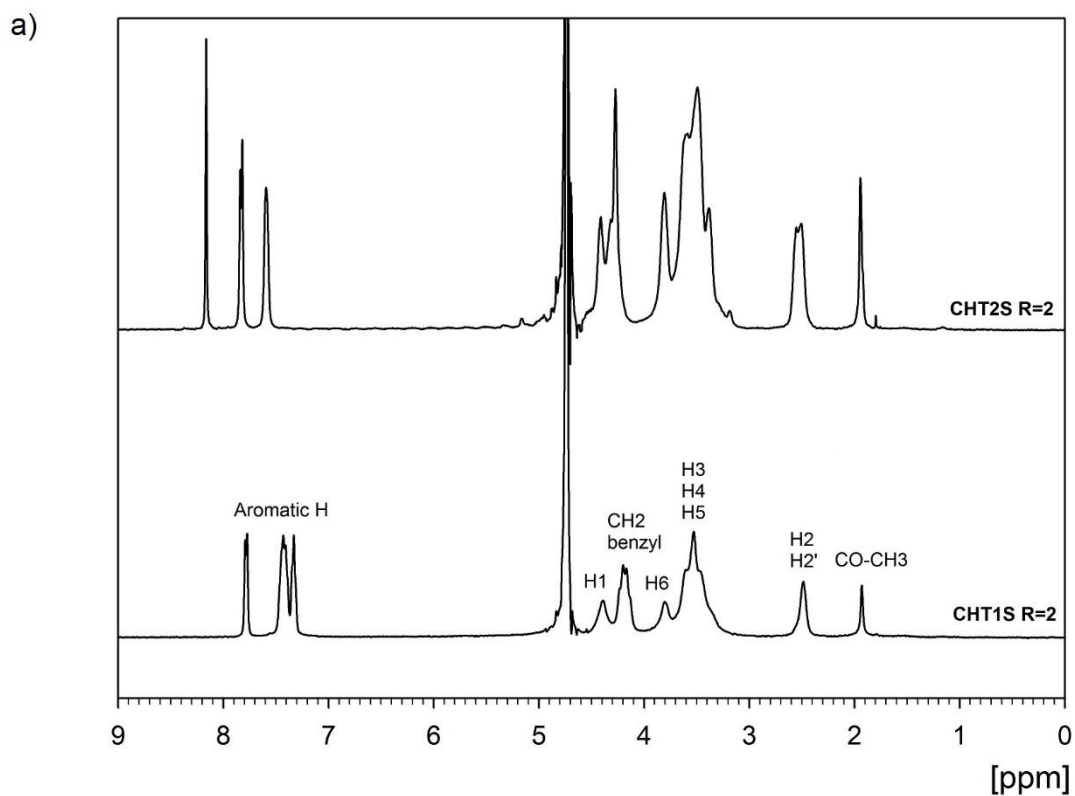
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### 290 **3.1. Characterization and influence of R on DS by <sup>1</sup>H NMR**

291 In prepared batches, increasing amounts of BZ1S and BZ2S were added to 5g of CHT, from R = 0.15  
292 (aldehyde in default) up to R = 2 (aldehyde in excess). *Schiff* reaction readily occurred between both  
293 aldehydic compounds and amino groups of chitosan which were converted to N-benzylsulfonate  
294 derivatives after reduction with sodium cyanoborohydride. The white precipitate appearing within 5  
295 minutes after BZ1S and BZ2S addition was accompanied by a sharp decrease of the solution viscosity  
296 that revealed a change of the physico-chemical characteristics of the reaction medium provoked by a  
297 fast reaction. After dialysis and freeze drying, the reaction products were collected and the influence of  
298 R parameter on the CHT1S and CHT2S composition were firstly studied by <sup>1</sup>H NMR spectroscopy. It  
299 is worth mentioning that on the contrary of native chitosan, all modified samples were soluble in basic  
300 medium. Therefore 0.01M NaOD was used as solvent in the NMR investigation.

301 An extensive NMR study of similar CHT1S and CHT2S compounds obtained only from R = 4  
302 conditions has been extensively reported in a former publication (Crini, Torri, Guerrini, Morcellet,  
303 Weltrowski & Martel, 1997; Crini, Torri, Martel, Weltrowski, Morcellet & Cosentino, 1997). The  
304 main feature of this former study was i) that chitosan substitution could be observed through the  
305 appearance of the aromatic groups signals and ii) the degrees of substitution (DS) of the derivatives  
306 could be calculated from the ratio of the integration of the latter signal versus that of the well resolved  
307 peak relative to the 2 position on the free and substituted glucosamine repeat units on the polymer  
308 backbone.

309 In the present paper, the DS of CHT1S and CHT2S series obtained from variable reactants molar  
310 ratios ( $0.15 < R < 2$ ) were calculated from the areas of signals mentioned above that appear on spectra  
311 displayed in figure 2a and situated between 7.1 ppm for CHT1S and 7.55 ppm for CHT2S (protons of  
312 aryl groups), and at 2.5 ppm (H2 and H2' signals). The protons signals integrations and resulting DS  
313 are reported in table S1. Figure 2b displays the DS increase as R was increased from 0.25 up to 1, and  
314 then a levelling-off at DS = 0.69 and 0.41 in CHT1S and CHT2S series respectively, when sulfonic  
315 aldehydes were put in excess compared to free glucosamine repeat units in the reaction medium.

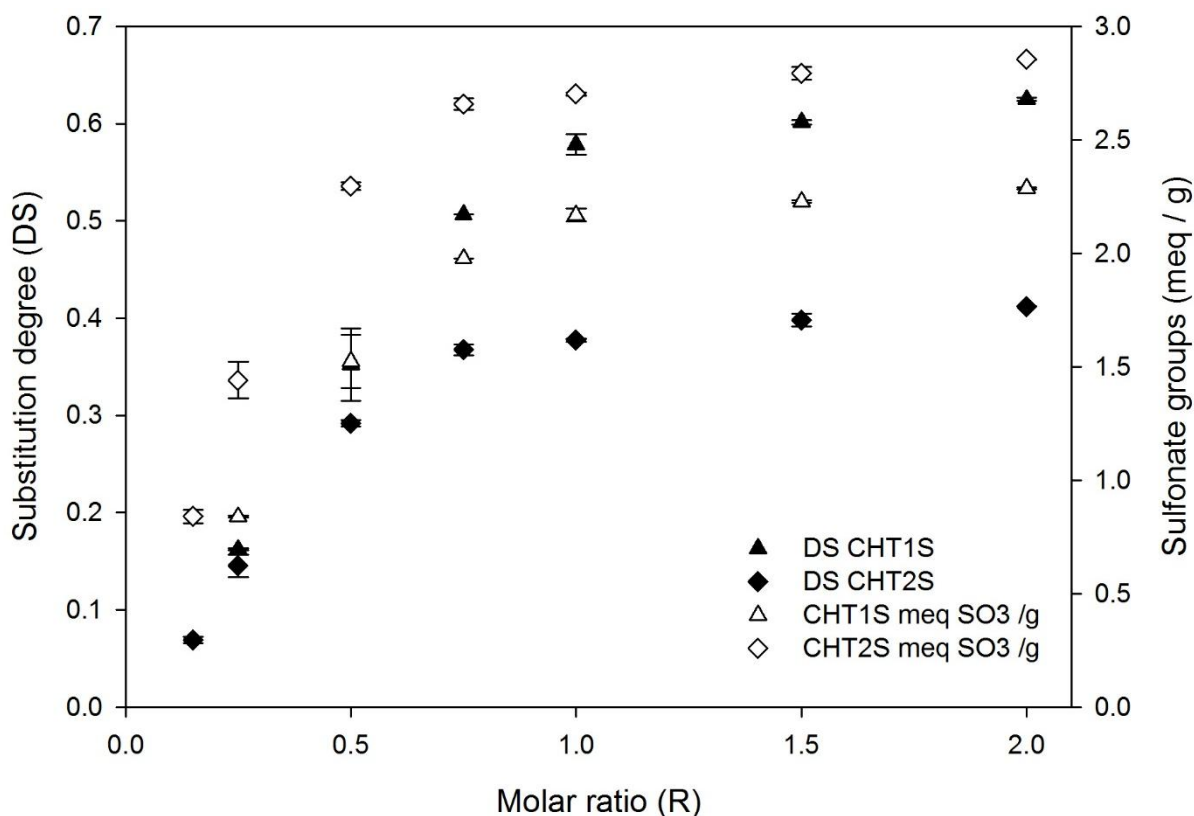


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**Figure 2.** a)  $^1\text{H}$  NMR spectra measured in 0.01M NaOD at 293 K; integrals values of signals of aromatic protons (visible in table S1) and of H2 and H'2 (2.5 ppm) signals were used for the calculations of DS reported in figure S1; b) DS of CHT1S and CHT2S versus R calculated from integrals of signals of aromatic protons (7-8 ppm) and H2 and H'2 (2.5 ppm) reported in table S1

322 **3.2. Elemental analysis**

323



324

325 **Figure 3.** Degree of substitution DS (in molar ratio) and sodium sulfonate groups content (in mmol/g) calculated from elemental analysis in  
 326 CHT1S and CHT2S series versus R = molar ratio of BZ1S and BZ2S versus amino groups of CHT in the reaction mixture. DS was  
 327 determined from eq (1) and eq (2).

328

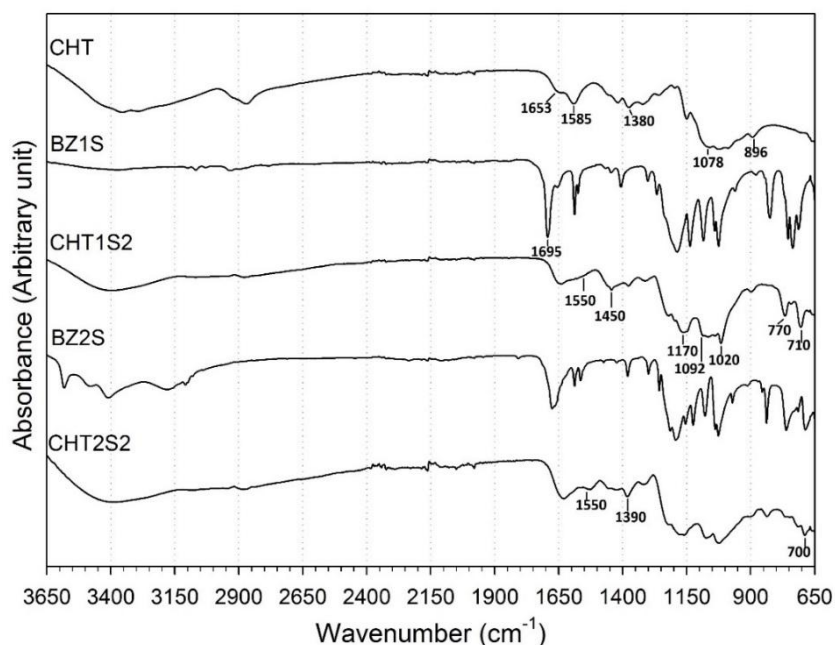
329 Sulphur weight content of CHT1S and CHT2S series, on the contrary of carbon, hydrogen and  
 330 nitrogen, increased with increasing R values up to R = 1 and then levelled off in presence of excess of  
 331 both aldehyde reactants (see C, H, N, S weight composition of chitosan and CHT1S and CHT2S in  
 332 supplementary information, table S2). Figure 3 displays that S% converted in DS from equations (1)  
 333 and (2) (see experimental part) gradually increased up to R = 1, and then levelled off to reach 0.6 and  
 334 0.4 for CHT1S-2 and CHT2S-2 respectively. It is worth mentioning that these results are in agreement  
 335 with values calculated by NMR (DS = 0.69 and 0.41 respectively). As observed in figure 3, amount of  
 336 sulfonate groups followed the same pattern, i.e. increased up to R = 1 and levelled off and raised  
 337 maximal values of 2.3 and 2.85 mmol per gram in CHT1S-2 and CHT2S-2 respectively. NMR and  
 338 elemental analysis can be considered as reliable quantitative methods that reveal that even though  
 339 displaying inferior maximal DS value, the CHT2S copolymers contained more sulfonate groups than  
 340 CHT1S, due to the presence of two sulfonate groups per benzyl group.

341 Thus NMR and elemental techniques could provide a quantitative analysis of the sulfonated products  
 342 and highlighted that: i) the CHT1S and CHT2S compositions were controlled by the molar ratio R,  
 343 especially for  $0 < R < 1$ ; ii) the twofold excess ( $R = 2$ ) of aryl sulfonic aldehydes yielded products  
 344 with maximal weight sulphur contents raising 7.32% and 9.14% and DS values up to 0.6 and 0.4 for

345 CHT1S and CHT2S respectively. For comparison, Amiji et al. - who took inspiration from Muzzarelli  
346 to modify chitosan with 5-Formyl-2-furansulfonic acid sodium salt by the same reductive amination  
347 pathway - found 5.2% sulfur content and a DS of 23.4% by using a molar ratio  $R = 0.63$  (Amiji, 1998;  
348 Muzzarelli, 1992). From figure 3, it is possible to observe that the reaction yield obtained by Amiji et  
349 al. was slightly inferior to those we obtained as those theoretically obtained in the same conditions for  
350 CHT1S and CHT2S would be approximately 0.4 and 0.3, respectively. Besides Yin et al., according to  
351 a different reaction path, used variable ratios of 3-chloro-2-hydroxy propanesulfate in the range 1-5  
352 compared to chitosan amino groups, measured DS in the range 0.17- 0.89 (Yin, Li, Yin, Miao & Jiang,  
353 2009).

354

### 355 3.3. Characterization by FTIR



356

357 **Figure 4.** FTIR-ATR spectra of a) raw chitosan, aryl mono and di sulfonic reactants BZ1S, BZ2S, and mono and di arylsulfonic chitosans  
358 CHT1S2, CHT2S2

359

360 FTIR spectrum of chitosan in figure 4 displays a wide absorption band between 3000 and 3600 cm<sup>-1</sup>  
361 related to O-H stretching from the hydroxyl and between 2800 and 3000 cm<sup>-1</sup> related to the C-H  
362 asymmetric stretching. CHT presented bands at 1653 cm<sup>-1</sup> related to the axial C=O stretching (amide  
363 group), at 1585 cm<sup>-1</sup> related to the bending vibration of the N-H bonds of primary amines, at 1380 cm<sup>-1</sup>  
364 linked to the CH<sub>3</sub> symmetrical deformation mode and at 1078 and 896 cm<sup>-1</sup> related to the C-O and C-  
365 O-C stretching and the vibration of the glycosidic bonds (Corazzari et al., 2015).

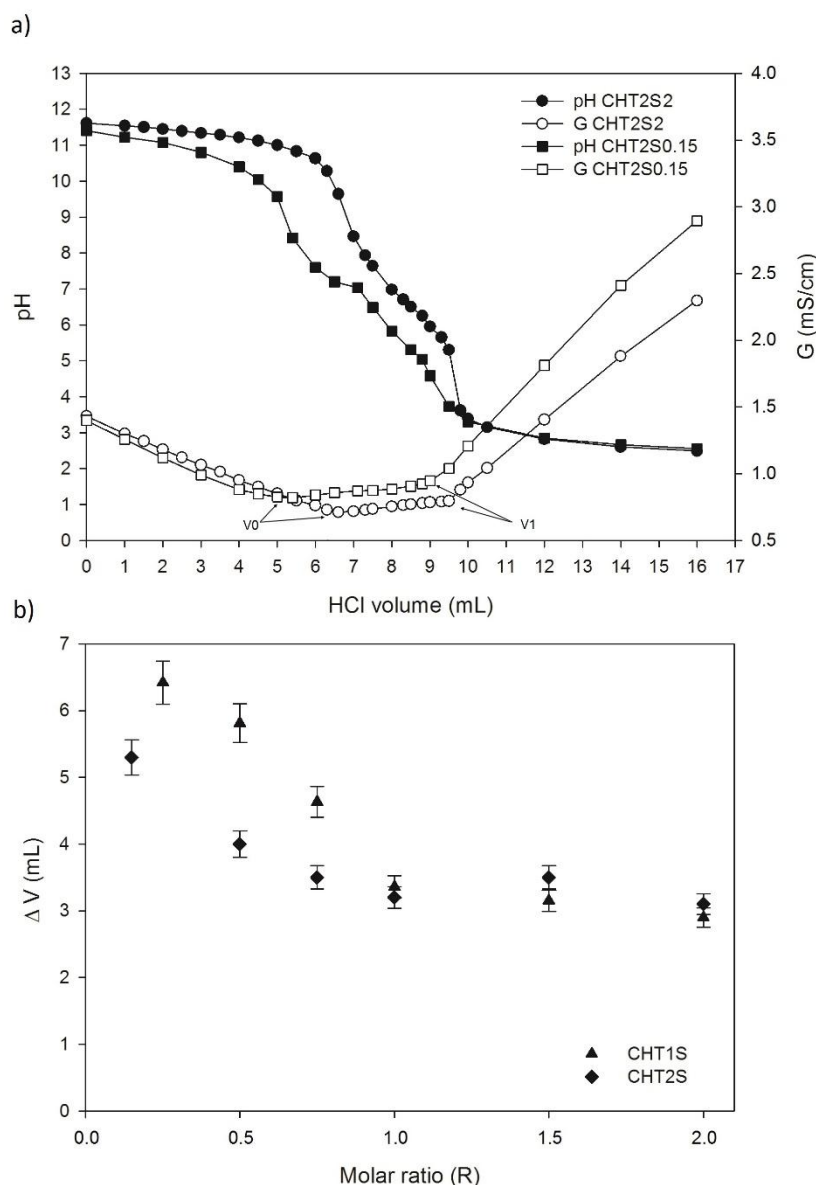
366 Compared to chitosan, both sulfonated chitosan spectra display a decrease of the relative intensity of  
367 the N-H band at 1585 cm<sup>-1</sup> and a new band appears at 1550 cm<sup>-1</sup>. This reveals the conversion of the  
368 primary amino groups into secondary amino groups.

369 Comparing the spectra of CHT1S and CHT2S with those of their precursor reactants BZ1S and BZ2S  
 370 confirms the substitution reaction by the appearance of additional peaks in the polymers relative to  
 371 stretching of sulfonate groups ( $1170\text{ cm}^{-1}$ ), symmetric O=S=O stretching ( $1020\text{ cm}^{-1}$ ) while the  
 372 aromatic groups are evidenced by ring vibration ( $1107\text{-}1092\text{ cm}^{-1}$ ) and C-H bending ( $770\text{ cm}^{-1}$ ,  $710$   
 373  $\text{cm}^{-1}$  in CHT1S, and  $700\text{ cm}^{-1}$  in CHT2S).

374

### 375 3.4. Coupled potentiometry and conductometry

376



377  
 378 **Figure 5.** a) plots of simultaneous titration of CHT2S samples ( $R=0.15$  and  $R=2$ ) dissolved in NaOH 0.01M followed by pH-metry and  
 379 conductometry; b) Evolution of the difference between the equivalent volumes ( $\Delta V = V_1 - V_0$  in mL) measured from conductometric titrations  
 380 of CHT1S and CHT2S series prepared from  $0.15 < R < 2$ . Decreasing  $\Delta V$  values reveals a decrease of free glucosamine groups and an  
 381 increase of DS.

382

383 Solutions of products of CHT1S and CHT2S series dissolved in 0.01M NaOH were titrated by 0.1M  
384 HCl followed simultaneously by pHmetry and conductometry techniques. The potentiometric curves  
385 present two successive pH jumps whose first one is attributed to the neutralization of the excess of  
386 NaOH, and the second one to the protonation of free amino groups. Due to the strong acid character of  
387 sulfonic acids, their sodium salts form could not be converted into their sulfonic acid form in titration  
388 experimental conditions. In parallel, the conductometric plots display three sections with well-marked  
389 slope changes occurring simultaneously with the pH jumps observed by potentiometry and  
390 corresponding to added volumes of the titrant  $V_0$  and  $V_1$ . For the good legibility of figure 5a, only the  
391 titration plots of CHT2S obtained from R values equal to 0.15 and 2 are displayed. For the same  
392 reason, the CHT1S series titration plots are not displayed as they present the same pattern as polymers  
393 of the CHT2S series. Figure 5b displays the difference of the equivalent volumes ( $\Delta V = V_1 - V_0$ )  
394 measured for all CHT2S and CHT1S compounds versus R. Interestingly,  $\Delta V$  decreased down as R  
395 increased up to 1, and then stabilized for  $R > 1$ . Such an evolution of  $\Delta V$  confirms the partial  
396 substitution of primary amino groups into secondary amino groups. No pH jump relative to  
397 neutralization of these secondary amino groups appeared on the conductimetry neither on pH titration  
398 plots because of the strong attractive inductive effect of the aryl sulfonate group which strongly  
399 decreased their basicity, preventing their protonation. However,  $\Delta V$  values reached identical level for  
400 both series prepared in conditions where  $R > 1$ . For this reason, this titration method was not  
401 considered as quantitative contrary to NMR and elemental analyses afore mentioned.

402

### 403 **3.5. UV spectrometry**

404 In the UV domain, compared to chitosan, sulfonated chitosan solutions displayed additional absorption  
405 bands that confirmed the presence of the aryl sulfonate chromophore groups at 260, 266 and 273 nm  
406 for CHT1S series, and 263, 269 and 277 nm for CHT2S series (see UV spectra in supplementary data  
407 figure S2A). Plots of the maximum absorbances of bands centered at 266 nm and at 269 nm  
408 (concentrations fixed at  $C=1\text{g/L}$ ) confirmed the increase of DS when R was increased up to 1, and then  
409 a leveling off was observed for  $R > 1$  (see figure S2B in supplementary data), in accordance with  
410 elemental analysis, NMR and titrations techniques afore mentioned.

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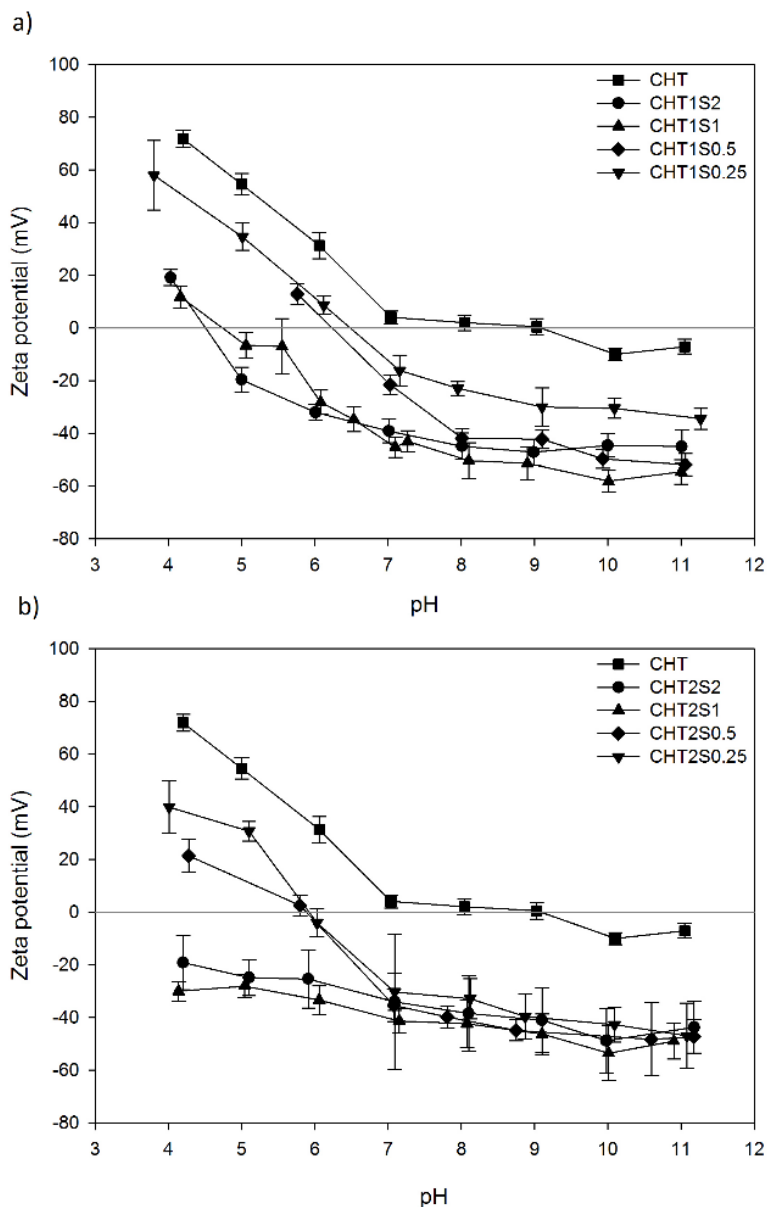
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### 3.6. Zeta potential measurements



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**Figure 6.** Evolution of zeta potential (ZP) for raw CHT and CHT1S and CHT2S series against pH. CHT1S and CHT2S were initially dissolved in 0.01M NaOH and raw CHT in 1%v/v CH<sub>3</sub>COOH. Solutions pH were gradually varied with 0.05M NaOH and 0.05M HCl.

428 The zeta potential (ZP) of raw and sulfonated chitosans were measured in function of pH adjusted by  
429 addition of NaOH, or HCl, respectively. The ZP of raw chitosan initially solubilized in diluted 1%v/v  
430 CH<sub>3</sub>COOH was stabilized at +70 mV at pH 4, and decreased down to -10mV as pH increased up to 11,  
431 due to gradual conversion of ammonium groups into amino groups (Yeh & Lin, 2008). In contrast, the  
432 ZP of CHT1S and CHT2S series initially solubilized in 0.01M NaOH presented strongly negative  
433 values of -60 mV and -50 mV for CHT1S and CHT2S respectively. Figure 6 displays that ZP



434 progressively increased as pH decreased from 11 down to 4 for all samples. This tendency can be  
435 related to the gradual protonation of the residual glucosamine residues in polymers upon HCl addition  
436 in the medium. As reported in table S3, samples presented an isoelectric point ( $ZP = 0$ ) except for  
437 CHT2S-1 and CHT2S-2 which revealed that equality between positive amino groups and negative  
438 sulfonate groups could be reached at these specific pH values. On the contrary, ZP values of CHT2S1  
439 and CHT2S2 samples remained negative (in the range of - 20 to - 30 mV) in the pH range from 11  
440 down to 4. This is due to their highest sulfonate groups contents (2.85 mmol/g for CHT2S-2 compared  
441 against 2.29 mmol/g for CHT1S-2 as reported in table S2) that are not counterbalanced by ammonium  
442 groups present in these polymers even at lowest pH values.

443 As these polymers are designed for biomaterials application, their ZP values at pH 7.4 are reported in  
444 table S3. All sulfonated derivatives presented strongly negative ZP at physiological pH (comprised  
445 between -19 and -47 mV), in contrast with raw chitosan which has neutral electric charge around pH =  
446 8.0 (which is close the physiological pH value). This feature should be of importance with regard to  
447 the interactions of these polymers with components of physiologic media such as blood plasma  
448 proteins, by potentially stimulating an anticoagulant activity mimicking that of heparin (Yeh & Lin,  
449 2008).

450

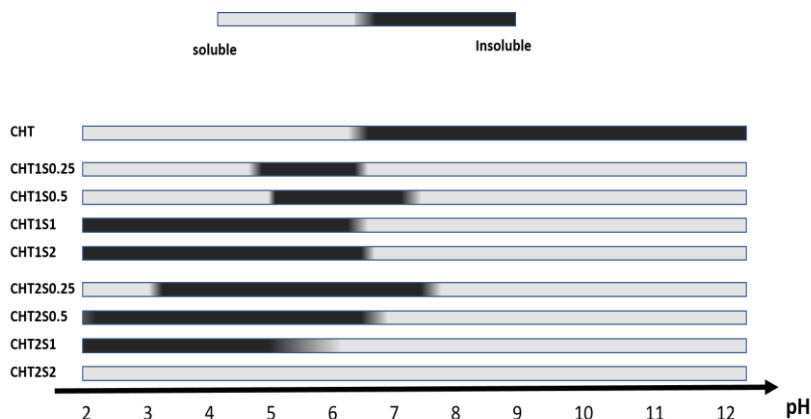
### 451 **3.7. CHT1S and CHT2S solubility in water versus pH**

452 Samples solubility versus pH was determined in parallel by eye observation and by transmittance  
453 measurement of the solution at 300 nm (see plots Transmittance % = f(pH) for CHT1S series in  
454 supplementary information, figure S3A and S3B). As observed in figure 7, unlike raw chitosan which  
455 is water soluble below pH = 5, all mono and di-sulfonated sulfonated CHT samples were soluble in  
456 basic to neutral medium (0.01M NaOH). A fast solubilization of CHT1S samples obtained for R =  
457 0.25, 0.5 and 0.75 was observed in NaOH 0.01M while a longer time (24h) was necessary to solubilize  
458 all other samples. Upon acidification, the initially clear alkaline solutions became trouble appearing as  
459 colloidal suspensions, and then flocculation occurred upon further decreasing pH below pH 8-6.  
460 Interestingly, flakes of CHT1S-0.25 CHT1S-0.5 and CHT2S-0.25 formed around neutral pH could be  
461 re-solubilized when pH was further decreased. So these three compounds displayed a dual domain of  
462 solubility at extreme pHs domains. Differently, samples CHT1S with R = 1 and 2 and CHT2S with R  
463 = 0.5 and 1 presented solubility at basic pH and precipitated below pH 7. Finally, the CHT2S-2 sample  
464 was soluble in the whole investigated pH domain. Such particular results can be related to the  
465 simultaneous presence of amino and aryl sulfonate groups on the polymers that endowed them a more  
466 or less marked polyampholytic character, depending on samples DS and on the presence of one or two  
467 sulfonate groups per aryl substituent. Indeed, while unsubstituted glucosamine (weak base) repeat  
468 units are largely protonated below pH 5 (pKa of chitosan = 6.5), aryl sulfonate substituents (pKa =  
469 2.5) remained ionized in the whole pH domain. Our results clearly show that samples solubility  
470 domains were mainly dependent from the balance between both ammonium and sulfonate groups

471 present on polymer chains. While even lowest sulfonate contents ensured solubility in basic pH, the  
 472 presence of residual ammonium groups in a large extent also ensured solubility at acidic pH. It is  
 473 worth mentioning that the CHT1S-0.25, CHT1S-0.5 and CHT2S-0.25 precipitation zone was observed  
 474 in the range of pH 5-7 and pH 3.5-7 respectively, where ZP values were around zero as observed in  
 475 figure 6. As samples DS increased, residual ammonium groups density decreased inducing insolubility  
 476 in acidic conditions (CHT1S-1 and CHT1S-2, CHT2S-0.5 and CHT2S-1). In this case the  $\pi$ - $\pi$  stacking  
 477 of aryl sulfonate groups promoted the chains coils collapsing. Finally, due to the highest DS and  
 478 subsequently highest sulfonate groups content ( $> 2.7$  mmol of  $-\text{SO}_3\text{Na}$  per gram), CHT2S-2 sample  
 479 remained water soluble within the whole pH range, in particular in acidic conditions thanks to the  
 480 repulsive effect between di sulfonated aryl substituents.

481 To summarize, the solubility study displayed a noticeable change of the solubility domains of both  
 482 sulfonated CHT series compared to native CHT. This is due to the partial substitution of the amino  
 483 groups by aryl sulfonic groups, endowing the polyampholytic character to sulfonated chitosans where  
 484 cationic glucosamine repeat units are counterbalanced by anionic repeat units substituted by  
 485 benzylsulfonate groups.

486 Such changes of pH solubility domains of their chitosan sulfonated derivatives was also abundantly  
 487 noticed in literature (Amiji, 1998; Muzzarelli, 1992), in particular Yin et al, who observed by light  
 488 transmittance a precipitation around the isoelectric point of their polymers situated around  $\text{pH} = 6$   
 489 (Yin, Li, Yin, Miao & Jiang, 2009).



490  
 491 **Figure 7.** pH solubility domains of CHT1S and CHT2S series. Solubility was determined both by eye observation and by transmittance of  
 492 the solution at 300 nm (see plots  $\text{Transmittance}\% = f(\text{pH})$  for CHT1S and CHT2S series in supplementary informations, figures S3A and  
 493 S3B, respectively.

### 494 3.8. Molar mass determination by SEC-MALLS

495 Preliminarily to SEC experiments, the measured  $(\delta n/\delta c)_\mu$  values were independent of the R value (thus  
 496 of the degree of sulfonation and the polymer molar mass) and depended only on the nature of the  
 497 polymer (CHT1S or CHT2S). Numerical values are given in Table 1. An average  $(\delta n/\delta c)_\mu$  value of  
 498 0.1507 for CHT1S and 0.1356 for CHT2S series were found, which is slightly less than the 0.165  
 499 value generally obtained in the literature for unmodified chitosan samples (Morris, Castile, Smith,

500 Adams & Harding, 2009; Schatz, Viton, Delair, Pichot & Domard, 2003; Theisen, Johann, Deacon &  
 501 Harding, 2000).

502

503 **Table 1.** Physicochemical characteristics of CHT1S and CHT2S polymers. All  $(dn/dc)_\mu$  values are given with a standard deviation of 0.002.  
 504 All  $R_h$  values obtained by Taylor dispersion analysis (TDA) are given with a standard deviation of 3 %.

505

Polymer	Molar ratio (R)	$(\delta n/\delta c)_\mu$	$M_w$ (g.mol <sup>-1</sup> )	$M_n$ (g.mol <sup>-1</sup> )	PDI	$R_h$ (nm)
CHT1S	0.5	0.136	282 000	164 000	1.72	54
	0.75	0.135	299 000	170 000	1.76	53
	1	0.134	297 000	149 000	1.99	54
	1.5	0.134	285 000	151 000	1.89	54
	2	0.137	235 000	121 000	1.94	52
CHT2S	0.5	0.149	89 000	66 000	1.35	62
	0.75	0.154	86 000	65 000	1.32	65
	1	0.150	78 000	62 000	1.26	63
	1.5	0.151	80 000	61 000	1.31	64
	2	0.150	56 000	46 000	1.22	62

506

507 Figures S4A and S4B show the SEC-MALLS chromatograms of all samples where RI and LS (90°)  
 508 traces are displayed. All the SEC-MALLS chromatograms of CHT1S and CHT2S series showed  
 509 similar behavior. The LS trace (in black) shows the presence of large aggregates or particles at lower  
 510 elution volume (i.e., at low retention time), making it quite difficult to accurately determine the molar  
 511 masses of the polymers as LS detection is very sensitive to aggregates that diffuse light even at low  
 512 concentration, therefore a double distribution can be observed especially for  $R \leq 1$  in CHT1S series,  
 513 and  $R \leq 0.5$  in CHT2S series. On contrary, the RI trace (in grey) is related to the concentration of the  
 514 species and displayed a single distribution. All the corresponding chromatograms are displayed in  
 515 Supplementary Information (Figures S4A and S4B).

516

### 517 3.9. Taylor dispersion analysis experiments (TDA)

518 Taylorgrams of all samples of the CHT1S and CHT2S series showed similar profiles (Figure S5) and  
 519 resulting numerical values of hydrodynamic radius ( $R_h$ ) are reported in Table 1. Contrary to the  
 520 Diffusion Light Scattering (DLS) method that provides intensity-average  $R_h$  values (harmonic z-  
 521 average values), weight-average  $R_h$  values are determined by TDA using a mass-concentration  
 522 sensitive UV detector. In the case of polydisperse samples, TDA leads to the weight-average  $R_h$  which  
 523 is not biased toward the larger aggregates or nanoparticles contained in the samples as observed for the  
 524 intensity-average  $R_h$  measured by DLS. Taylorgrams presented in Figure S5 show a single Gaussian  
 525 profile, confirming the low proportion of aggregates detected by SEC-MALLS especially for samples  
 526 with lowest R values. For a given R value, the UV absorbance was higher in the case of CHT1S

527 polymers than in the case of CHT2S polymers, which is in agreement with the higher content of  
 528 aromatic groups found in CHT1S polymers.

529 From Table 1, it can be concluded that CHT1S samples have similar hydrodynamic radii (53 nm) and  
 530 CHT2S samples also have similar hydrodynamic radii (63 nm). The higher hydrodynamic size of  
 531 CHT2S samples is probably due to the presence of two sulfonate groups instead of only one in CHT1S  
 532 samples, extending the coil by intramolecular repulsive forces. The hydrodynamic size values of both  
 533 CHT1S and CHT2S samples are in agreement with the values found for other hydrophobic derivatives  
 534 of chitosan (Philippova & Korchagina, 2012).

### 535 3.10. Anticoagulation activity

536

537 **Table 2.** Activated Partial thromboplastin time (aPTT), Prothrombin Time (PT) and anti-Xa factors of normal human platelet-poor plasma  
 538 containing CHT and CHT1S and CHT2S with variable R values concentrated at 1 mg. mL<sup>-1</sup>.

	<b>Molar ratio R</b>	<b>APTT (sec)</b>	<b>PT (sec)</b>	<b>Anti-Xa (UI/mL)</b>
<b>CHT</b>		29	12.7	0.09
<b>CHT2S</b>	0.25	31	11.2	0.09
	0.5	53	12.7	0.09
	0.75	142	13.9	0.09
	1	190	15.5	0.09
	1.5	225	21.8	0.09
	2	>250	26.6	0.09
<b>CHT1S</b>	0.25	28	12.5	0.09
	0.5	39	12.5	0.09
	0.75	77	11.7	0.09
	1	135	16	0.09
	1.5	202	18.2	0.09
	2	219	17.8	0.09
<b>Heparin (10UI/mL = 0.1mg/mL)</b>		>250	120.8	> 2
<b>Blood</b>		29	12.8	0.09

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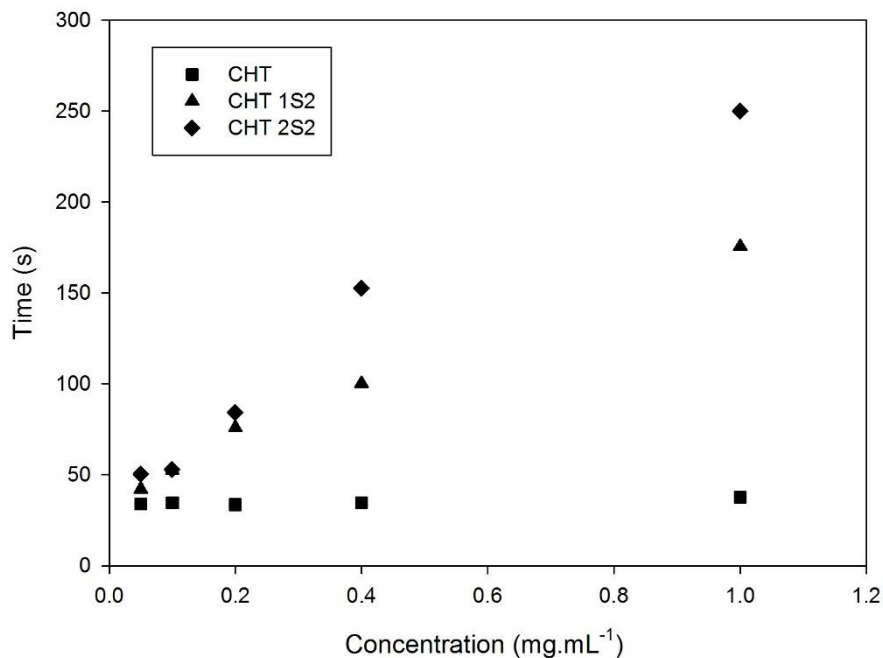
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573 **Figure 8.** Activated Partial thromboplastin time (aPTT) of CHT, CHT1S and CHT2S at different concentrations. aPTT values for normal  
574 blood = 33 s, and 10U/mL heparin > 250 s.  
575

576 The anticoagulant activity of CHT and sulfonated CHT were evaluated by *in vitro* coagulation assays  
577 of activated partial thromboplastin time (aPTT), prothrombin time (PT) and anti-factor Xa (Anti-FXa)  
578 to elucidate possible cause of anticoagulant property. As observed in Table 2, raw CHT did not exhibit  
579 any anticoagulant activity as its aPTT, PT, and anti-FXa were identical to those of the negative  
580 controls, i.e 30 seconds, 12.8 seconds and 0.09 UI/mL. By contrast, APTT and PT of both heparin and  
581 some sulfonated chitosan were effectively prolonged compared to negative control. While for both  
582 series of sulfonated chitosan, at constant concentration (1 mg/mL), the extent of prolongation of aPTT  
583 and PT depended on the R values. In fact, with gradually increasing R (from 0.25 up to 2), an increase  
584 of aPTT from 28 up to 219 seconds for the CHT1S series, and from 31 up to 250 seconds for the  
585 CHT2S series, were observed. Similarly, an increase of PT from 12.5 up to 18 seconds for the CHT1S  
586 series, and from 11 up to 26.6 seconds for the CHT2S series, were noticed. It evidenced the influence  
587 of the introduction of the aryl sulfonate groups to chitosan on its anticoagulant property. According to  
588 the literature (Huynh, Chaubet & Jozefonvicz, 2001; Yang et al., 2013), in contrast with raw CHT that  
589 possess hemostatic property due to the interaction between the positively charged amine residues and  
590 negatively charged plasma proteins, sulfated polysaccharides benefit from anticoagulant activity  
591 thanks to the strong interaction between the negatively charged sulfate groups and some positively

592 charged peptidic sequences of proteins implied in the coagulation system. The chemical modification  
593 of CHT induced a decrease of its positive charge density by the glucosamine group substitution, and  
594 an increased level of negative charge density produced by the sulfonate groups. The higher  
595 anticoagulant activity observed for samples with higher R value is probably related to their increased  
596 negative charge density, which enabled them stronger capacity to neutralize the positively charged  
597 amino acid residues from thrombin hindering the fibrinogen transformation and improving the  
598 anticoagulant activity (Wang, Zhou, Xie, Chen, Zheng & Fan, 2012). It is worth noting that, for the  
599 same R value, the anticoagulant activity of CHT2S was stronger than that of CHT1S.

600 This feature was elucidated by ZP measurements, which clearly showed that i) the polyampholyte  
601 character of the derivatives where protonated amino groups coexisted with anionic sulfonate groups as  
602 pH was varied; ii) derivatives with lowest DS displayed an isoelectric point on the contrary of samples  
603 with higher DS, iii) at the physiological pH = 7.4 all modified samples presented negative ZP  
604 whatever their DS.

605 As a matter of fact, on the one hand it is well known that the chitosan structure rich in amine residues  
606 can interfere with negatively charged plasma proteins, contributing to thrombus development,  
607 especially in acidic conditions (Amiji, 1998), on the other hand sulfonated chitosans based materials  
608 usually demonstrate anticoagulant properties explained by the presence of anionic  $\text{SO}_3^-$  groups capable  
609 of preventing protein absorption involved in the clotting cascade initiation (Amiji, 1998; Bauer,  
610 Schmuki, von der Mark & Park, 2013; Lima et al., 2013). The results from PT and anti-Xa assays  
611 showed that the activity of sulfonated chitosans on the extrinsic pathway was very low and the factor  
612 Xa was completely inhibited. These results suggested that the highly sulfated chitosan mainly  
613 interfered with the intrinsic pathway.

614 The concentration-dependent anticoagulant activity of sulfonated chitosans is demonstrated in Figure  
615 8. aPTT prolonged from 51 to beyond 250 seconds for CHT2S-2 and from 42 to 176 seconds for  
616 CHT1S-2 with increasing concentrations from 0.05 to 1 mg/mL, while the clotting time of raw CHT  
617 remained unchanged with varying concentrations. It again displayed that the anticoagulant activity of  
618 CHT1S-2 was weaker than that of CHT2S-2. Based on the above results, it can be concluded, in  
619 agreement with previous results obtained by Wang et al. (Wang, Zhou, Xie, Chen, Zheng & Fan,  
620 2012), that the anticoagulant activity increased with the polymers concentrations and with their degree  
621 of substitution, and was superior for disulfonated derivatives compared with monosulfonated ones.

622 Interestingly Yeh et al. (Yeh & Lin, 2008) reported that surface sulfonated chitosan membrane with  
623 amino group protection-deprotection strategy displayed anticoagulant properties in which platelet  
624 adhesion was promoted, but the adhered platelets were not activated after the chemical modification.  
625 Based on this background, the sulfonated chitosans described here are intended for the processing of  
626 electrospun biomaterials and considering the present results, such nanofibrous mats would be very  
627 promising candidates for blood contact medical devices with antithrombogenic properties.

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## **Conclusion**

This work described the chemical modification of chitosan through reductive amination reaction by using mono- and di-sulfonated formylbenzene reactants. The influence of the reactants ratio was systematically investigated by different techniques that all displayed the increase of the CHT chain substitution up to equimolarity between the sulfonic aldehydes and CHT amino groups in the reaction medium. As substitution was uncomplete (even in presence of two-fold excess of the sulfonic aldehydes) the resulting CHT1S and CHT2S contained residual free amino groups that provided a polyampholytic character evidenced by zeta potential measurements. In particular, on contrary to parent CHT, the sulfonated derivatives presented a negative zeta potential at physiologic pH. CHT1S and CHT2S were soluble in basic medium on contrary of parent CHT, and interestingly, samples with low substitution degrees presented also solubility at low pH while they were insoluble at intermediate pH. The existence of polymer aggregates formed in solution was detected by size exclusion chromatography preventing the absolute molecular masses measurements; Taylor dispersion analysis allowed to observe hydrodynamic radii of 53 and 63 nm in average for CHT1S and CHT2S samples, respectively. Finally, through aPTT, and PT measurements, it was observed that both sulfonated series displayed anticoagulant activities increasing with the polymers concentrations and with their degrees of substitution. It was also observed that clotting times were superior for disulfonated derivatives compared with monosulfonated ones. The next step will be to convert the present sulfonated chitosans into nanofibers by electrospinning. The investigation will then be oriented toward the study of the anticoagulant properties of these materials through a different and complementary approach that will consist of determining the platelet adhesion and activation after being put in contact with blood.

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