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

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Abstract

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

Keywords: chitosan; sulfonated chitosan; reductive amination; polyampholyte; anticoagulant

1. INTRODUCTION

Chitosan (CHT) is a natural biopolymer known for its excellent biocompatibility, biodegradability, hemocompatibility, wound healing and antibacterial properties. Therefore, chitosan is a material of choice in the design of a wide range of applications in the biomaterials field, such as wound dressings (Mohandas, Deepthi, Biswas & Jayakumar, 2017), injectable hydrogels (Liu, Gao, Lu & Zhou, 2016), and scaffolds for tissue engineering (Oryan & Sahvieh, 2017). In order to further enhance the biological properties and enlarge the range of potential uses of CHT in the biomedical field, many chemical modifications have been attempted, such as alkylation (Desbrieres, Martinez & Rinaudo, 1996), carboxymethylation (Kong, Kim, Ahn, Byun & Kim, 2010) or quaternisation (Sajomsang, Gonil, Saesoo & Ovatlarnporn, 2012), all aiming improving or even endow further functionalities to chitosan such as mucoadhesivity, antibacterial properties and hemocompatibility (Balan & Verestiuc, 2014). In particular, in addition to the numerous possible chitosan modification routes mentioned above, sulfonation or sulphation of chitosan are also widely reported in the literature. The consequences of such chemical modifications is that these reactions give to chitosan chemical compositions comparable to that of the class of sulphated glycosaminoglycans (GAGs), components of the extracellular matrix (ECM) in the animal tissues. Thererefore, sulfated chitin or chitosan studied in literature were reported to advantageously serve as ECM analogs with advanced properties such as antibacterial (Jung, Kim, Choi, Lee & Kim, 1999) anti-HIV-1 properties (Artan, Karadeniz, Karagozlu, Kim & Kim, 2010), reduction of blood protein absorption (Lima et al., 2013), anticoagulant (Campelo, Lima, Rebêlo, Mantovani, Beppu & Vieira, 2016) and anti-thrombogenic properties which were claimed in some cases to be at least as performing as heparin (Ma, Huang, Kang & Yan, 2007). Chitosan sulphation can be carried out by direct modification of the chitosan repeat units in their 3-O and/or 6-O positions, and/or to modify the N position, by using sulphuric anhydride SO_3 / pyridine mixture (Hirano, Tanaka, Hasegawa, Tobetto & Nishioka, 1985; Jung, Na & Kim, 2007; Nishimura et al., 1998; Yeh & Lin, 2008) or chlorosulfonic acid/formamide (Gamzazade, Sklyar, Nasibov, Sushkov, Shashkov & Knirel, 1997; Hirano, Tanaka, Hasegawa, Tobetto & Nishioka, 1985; Ma, Huang, Kang & Yan, 2007). In order to target O positions, the regioselectivity could be controlled by using protection-deprotection of the primary amino groups strategies (Nishimura et al., 1998; Yeh & Lin, 2008). Besides, chitosans carrying sulfonate groups on N position could be obtained by using reactants such as vinylsulfonate (Jung, Kim, Choi, Lee & Kim, 1999), propane sultone (Jung, Na & Kim, 2007) or 3-chloro-2-hydroxy propanesulfate (Jayakumar, Nwe, Tokura & Tamura, 2007; Yin, Li, Yin, Miao & Jiang, 2009). In particular, chitosan modification can be advantageously achieved by using the reductive amination reaction path proposed by Hall and Yalpani (Hall & Yalpani, 1980; Roberts, 1992), which consists in reacting alkyl or aryl aldehydic compounds on chitosan substrates yielding Schiff's bases intermediates directly reduced into secondary amines in the presence of sodium cyanoborohydride. In 1992, Muzzarelli applied this reaction using 5-formyl-2-furansulfonic acid sodium salt (Muzzarelli, 1992) and the obtained N-sulfofurfuryl chitosan derivative was recently exploited in the biomaterials field for the elaboration of films with blood anti-coagulant properties (Amiji, 1998; Campelo, Chevallier, Vaz, Vieira & Mantovani, 2017; Campelo, Lima, Rebêlo, Mantovani, Beppu & Vieira, 2016; Huang, Du, Yang & Fan, 2003; Lima et al., 2013).

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

2. EXPERIMENTALS

2.1. Materials

Chitosan (CHT), *low molecular weight* grade batch SLBG1673V, 80.3% degree of deacetylation (supplier value), was supplied by Sigma-Aldrich with an intrinsic viscosity measured by capillary viscosimetry of [η]= 896 dL/g and molecular weight of Mv= 130 000 g.mol^{-1} determined according to Mark-Houwink equation [η]= K . M_v^{α} with K = 74 10^{-5} dL/g, α= 0.76 and 0.3M HAc/0.2M NaAc as solvent (Rinaudo, Milas & Dung, 1993). 2-formylbenzenesulfonic acid sodium salt (mono sulfonic, BZ1S), 2,4 formylbenzenesulfonic acid sodium salt dihydrate (di sulfonic, BZ2S), glacial acetic acid, sodium cyanoborohydride ($NaBH_3CN$) were purchased from Aldrich chemicals and used without further purification and methanol with HPLC gradient was purchased from Carlo Erba reagent S.A.S.

2.2. Methods

2.2.1. Synthesis of CHT2S and CHT1S series

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

2.2.2. Characterization by elemental analysis

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

Based on the above mentioned parameters, two theoretical relations between DS and %S were firstly established and plotted in figure S1 (supplementary data); the obtained second degree equations were $DS = 5.4.10^{-3}.(\%S)^2 + 4.59.10^{-2}.(\%S)$ (eq(1)) for CHT1S, and $DS = 3.10^{-3}.(\%S)^2 + 1.77.10^{-2}.(\%S)$ (eq(2)) for CHTS2, where %S is the theoretical sulphur weight percentage. The repeat unit's molecular weights of acylated glucosamine, glucosamine, and glucosamine reacted with BZ1S and BZ2S were 203 g/mol, 161 g/mol, 339 g/mol and 455 g/mol respectively. Thanks to both theoretical equations mentioned above, it was possible to calculate experimental DS values as well as the sulfonate groups (in mmol/g) of all synthesized chitosan derivatives from their sulphur contents measured by elemental analysis.

2.2.3. NMR Analyses

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

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

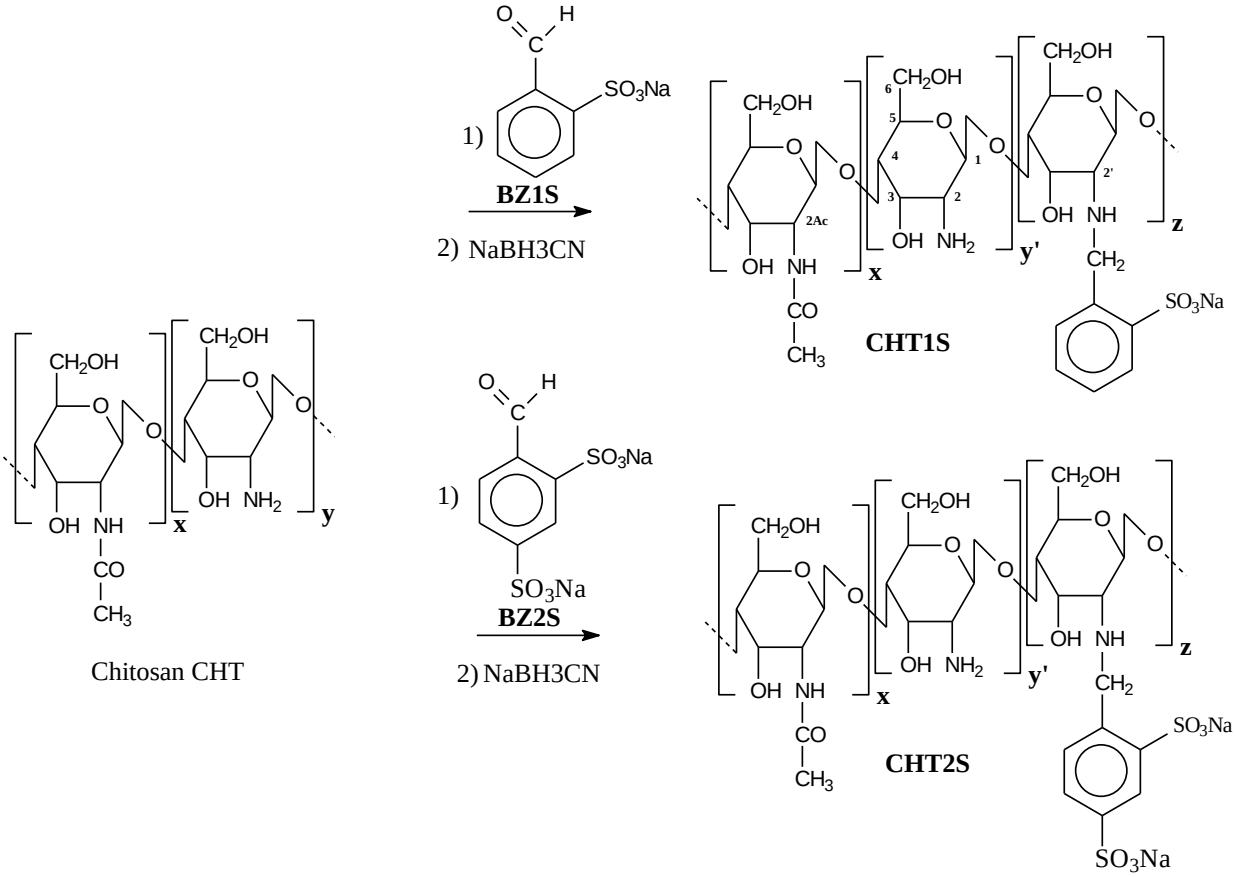

Figure 1. Syntheses routes of benzyl mono and di-sulfonate derivatives CHT1S and CHT2S where x is the relative molar ratio in acylated repeat units (considered constant at 0.197), y' the relative molar ratio of residual glucosamine units after reaction, and z is the relative molar ratio of sulfonated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

2.2.4. Characterization by coupled potentiometry and conductometry

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

2.2.5. Fourier transform infrared spectroscopy (FT-IR)

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

2.2.6. UV- visible spectrometry

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

2.2.7. Zeta potential

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

2.2.8. pH solubility domain

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

2.2.9. SEC-MALLS

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

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

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

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

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

2.2.9.3. Taylor dispersion analysis (TDA)

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

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

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

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

where k_B is the Boltzmann constant, T is the temperature (in Kelvin), R_c is the capillary diameter, η is the viscosity of the eluent, t_0 is the average elution time and σ^2 is the temporal variance of the elution profile.

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

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

2.2.10. Coagulation assays

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

For aPTT assay, 50 µL of each citrated normal human plasma were incubated at 37°C for 1 min and 50 µL of aPTT reagent (TriniCLOT^{tm} aPTT HS, Tcoag®**)** was added to the mixture and incubated at 37°C for 5 min. Thereafter, 100 µL of $CaCl_2$ (0.025 mol. mL^{-1}) were added and the clotting time was measured.

For PT assay, 100 µL of plasma samples were incubated at 37°C for 2 min. 200 µL of tissue thromboplastin reagent (NEOPLATINE® R, Diagnostica Stago, Inc., France), pre-incubated at 37°C for 10 min, were introduced and clotting time was recorded.

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

3. RESULTS AND DISCUSSION

3.1. Characterization and influence of R on DS by ^{1}H NMR

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

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

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

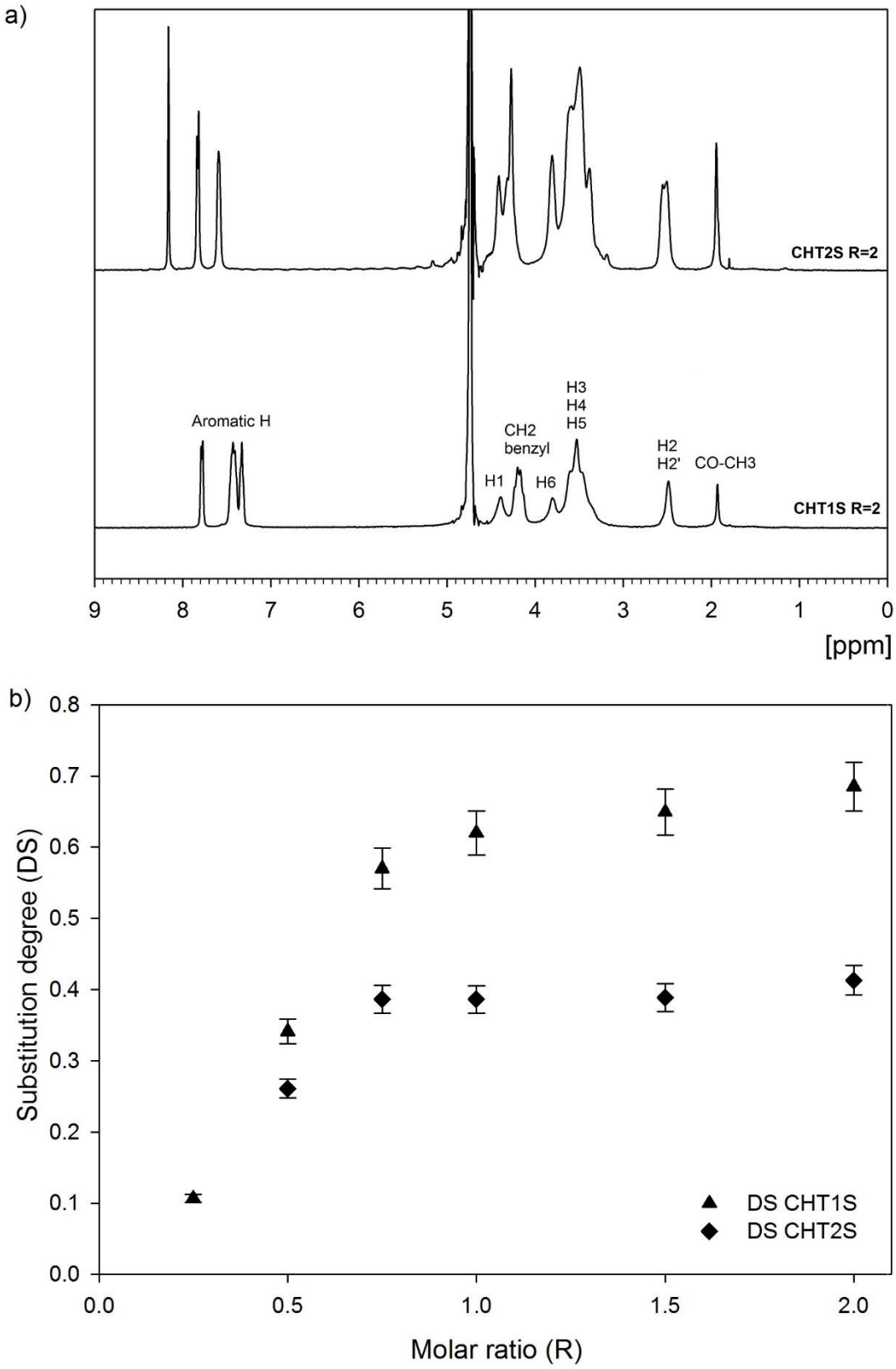

Figure 2. a) ^{1}H NMR spectra measured in 0.01M NaOD at 293 K; integrals values of signals of aromatic protons (visible in table S1) and of H2 and H'2 (2.5 ppm) signals were used for the calculations of DS reported in figure S1; b) DS of CHT1S and CHT2S versus R calculated from integrals of signals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

3.2. Elemental analysis

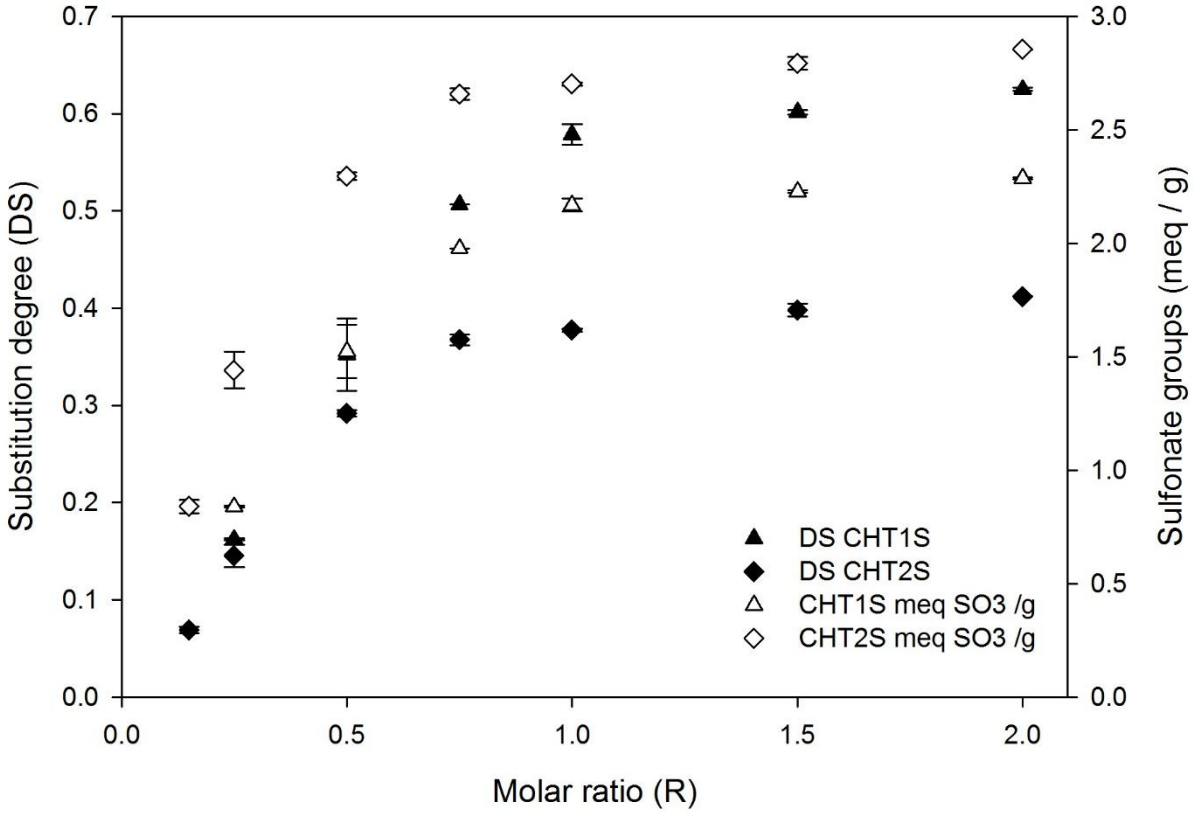

Figure 3. Degree of substitution DS (in molar ratio) and sodium sulfonate groups content (in mmol/g) calculated from elemental analysis in CHT1S and CHT2S series versus R = molar ratio of BZ1S and BZ2S versus amino groups of CHT in the reaction mixture. DS was determined from eq (1) and eq (2).

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

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

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

3.3. Characterization by FTIR

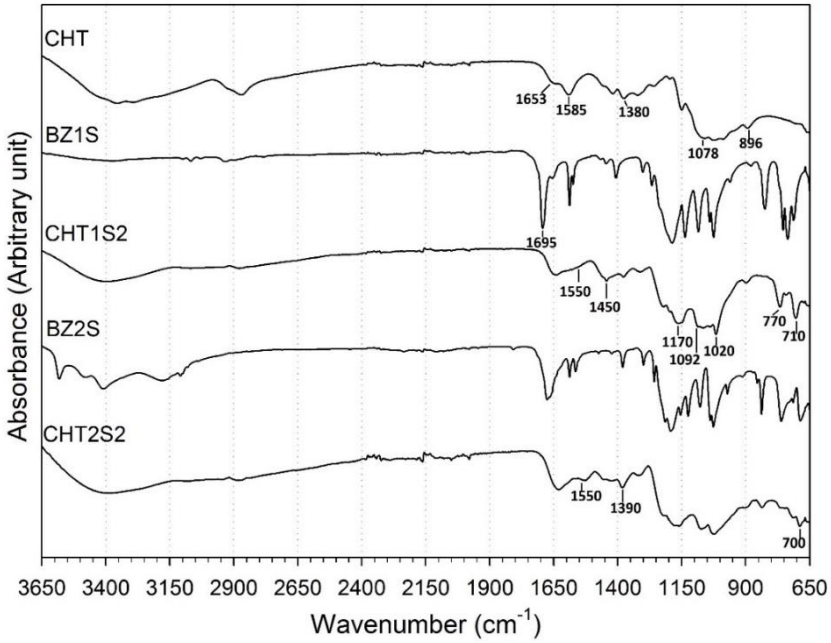

Figure 4. FTIR-ATR spectra of a) raw chitosan, aryl mono and di sulfonic reactants BZ1S, BZ2S, and mono and di arylsulfonic chitosans CHT1S2, CHT2S2

FTIR spectrum of chitosan in figure 4 displays a wide absorption band between 3000 and 3600 cm^{-1} related to O-H stretching from the hydroxyl and between 2800 and 3000 cm^{-1} related to the C-H asymmetric stretching. CHT presented bands at 1653 cm^{-1} related to the axial C=O stretching (amide group), at 1585 cm^{-1} related to the bending vibration of the N-H bonds of primary amines, at 1380 cm^{-1} linked to the CH_3 symmetrical deformation mode and at 1078 and 896 cm^{-1} related to the C–O and C–O–C stretching and the vibration of the glycosidic bonds (Corazzari et al., 2015).

Compared to chitosan, both sulfonated chitosan spectra display a decrease of the relative intensity of the N-H band at 1585 cm^{-1} and a new band appears at 1550 cm^{-1}. This reveals the conversion of the primary amino groups into secondary amino groups.

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

3.4. Coupled potentiometry and conductometry

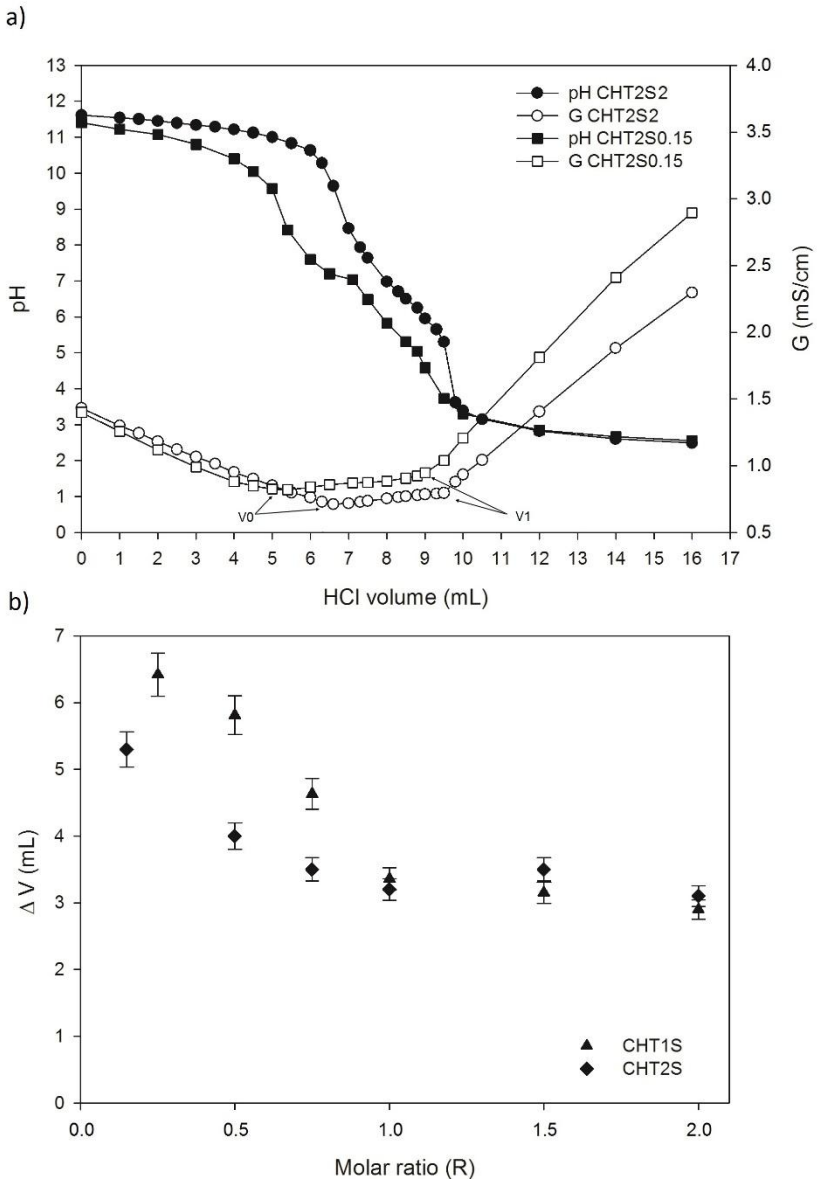

Figure 5. a) plots of simultaneous titration of CHT2S samples (R=0.15 and R=2) dissolved in NaOH 0.01M followed by pH-metry and conductometry; b) Evolution of the difference between the equivalent volumes ($\Delta V = V_1 - V_0$ in mL) measured from conductometric titrations of CHT1S and CHT2S series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

Solutions of products of CHT1S and CHT2S series dissolved in 0.01M NaOH were titrated by 0.1M HCl followed simultaneously by pHmetry and conductometry techniques. The potentiometric curves present two successive pH jumps whose first one is attributed to the neutralization of the excess of NaOH, and the second one to the protonation of free amino groups. Due to the strong acid character of sulfonic acids, their sodium salts form could not be converted into their sulfonic acid form in titration experimental conditions. In parallel, the conductometric plots display three sections with well-marked slope changes occurring simultaneously with the pH jumps observed by potentiometry and corresponding to added volumes of the titrant V0 and V1. For the good legibility of figure 5a, only the titration plots of CHT2S obtained from R values equal to 0.15 and 2 are displayed. For the same reason, the CHT1S series titration plots are not displayed as they present the same pattern as polymers of the CHT2S series. Figure 5b displays the difference of the equivalent volumes ($\Delta V = V1 - V0$) measured for all CHT2S and CHT1S compounds versus R. Interestingly, ΔV decreased down as R increased up to 1, and then stabilized for $R > 1$. Such an evolution of ΔV confirms the partial substitution of primary amino groups into secondary amino groups. No pH jump relative to neutralization of these secondary amino groups appeared on the conductimetry neither on pH titration plots because of the strong attractive inductive effect of the aryl sulfonate group which strongly decreased their basicity, preventing their protonation. However, ΔV values reached identical level for both series prepared in conditions where $R > 1$. For this reason, this titration method was not considered as quantitative contrary to NMR and elemental analyses afore mentioned.

3.5. UV spectrometry

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

3.6. Zeta potential measurements

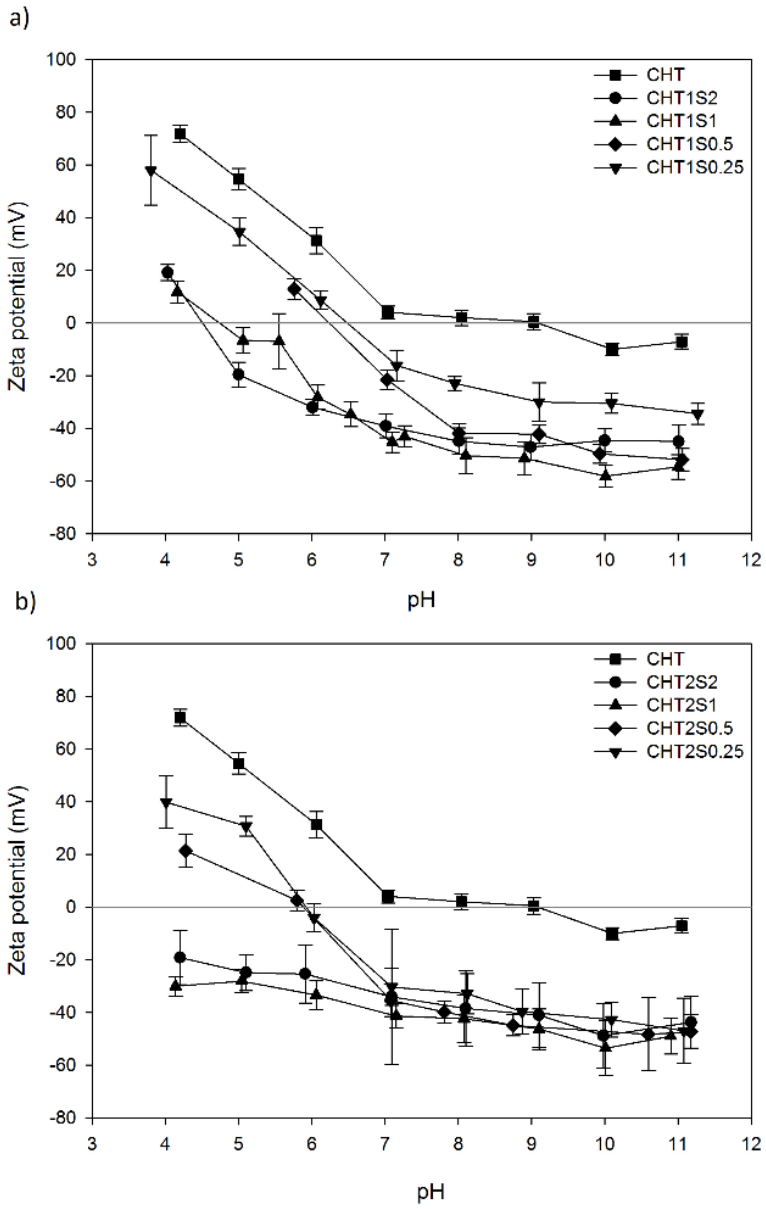

Figure 6. Evolution of zeta potential (ZP) for raw CHT and CHT1S and CHT2S series against pH. CHT1S and CHT2S were initially dissolved in 0.01M NaOH and raw CHT in 1%v/v CH_3COOH. Solutions pH were gradually varied with 0.05M NaOH and 0.05M HCl.

The zeta potential (ZP) of raw and sulfonated chitosans were measured in function of pH adjusted by addition of NaOH, or HCl, respectively. The ZP of raw chitosan initially solubilized in diluted 1%v/v CH_3COOH was stabilized at +70 mV at pH 4, and decreased down to -10mV as pH increased up to 11, due to gradual conversion of ammonium groups into amino groups (Yeh & Lin, 2008). In contrast, the ZP of CHT1S and CHT2S series initially solubilized in 0.01M NaOH presented strongly negative values of -60 mV and -50 mV for CHT1S and CHT2S respectively. Figure 6 displays that ZP

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

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

3.7. CHT1S and CHT2S solubility in water versus pH

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

present on polymer chains. While even lowest sulfonate contents ensured solubility in basic pH, the presence of residual ammonium groups in a large extent also ensured solubility at acidic pH. It is worth mentioning that the CHT1S-0.25, CHT1S-0.5 and CHT2S-0.25 precipitation zone was observed in the range of pH 5-7 and pH 3.5-7 respectively, where ZP values were around zero as observed in figure 6. As samples DS increased, residual ammonium groups density decreased inducing insolubility in acidic conditions (CHT1S-1 and CHT1S-2, CHT2S-0.5 and CHT2S-1). In this case the π-π stacking of aryl sulfonate groups promoted the chains coils collapsing. Finallly, due to the highest DS and subsequently highest sulfonate groups content (> 2.7 mmol of $-SO_3Na$ per gram), CHT2S-2 sample remained water soluble within the whole pH range, in particular in acidic conditions thanks to the repulsive effect between di sulfonated aryl substituents.

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

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

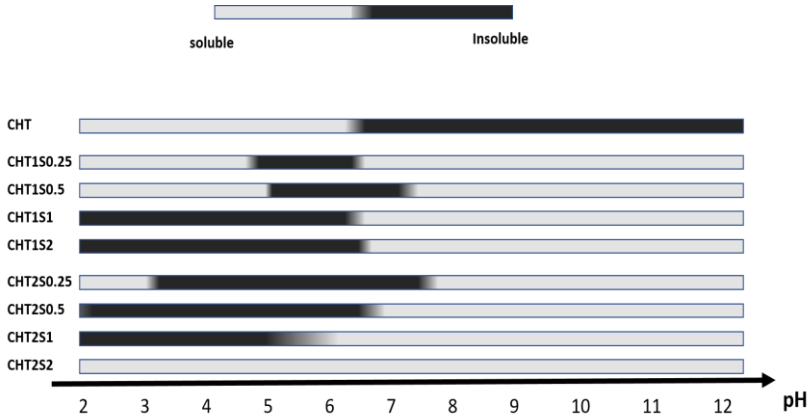

Figure 7. pH solubility domains of CHT1S and CHT2S series. Solubility was determined both by eye observation and by transmittance of the solution at 300 nm (see plots Transmittance% = f(pH) for CHT1S and CHT2S series in supplementary informations, figures S3A and S3B, respectively.

3.8. Molar mass determination by SEC-MALLS

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

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

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

Polymer	Molar ratio (R)	$(\delta n/\delta c)_{\mu}$	M_w (g.mol^{-1})	M_n (g.mol^{-1})	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

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

3.9. Taylor dispersion analysis experiments (TDA)

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

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

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

3.10. Anticoagulation activity

Table 2. Activated Partial thromboplastin time (aPTT), Prothrombin Time (PT) and anti-Xa factors of normal human platelet-poor plasma containing CHT and CHT1S and CHT2S with variable R values concentrated at 1 mg. mL^{-1}.

	Molar ratio R	**APTT (sec)**	**PT (sec)**	**Anti-Xa (UI/mL)**
CHT		29	12.7	0.09
CHT2S	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
CHT1S	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
Heparin (10UI/mL = 0.1mg/mL)		>250	120.8	> 2
Blood		29	12.8	0.09

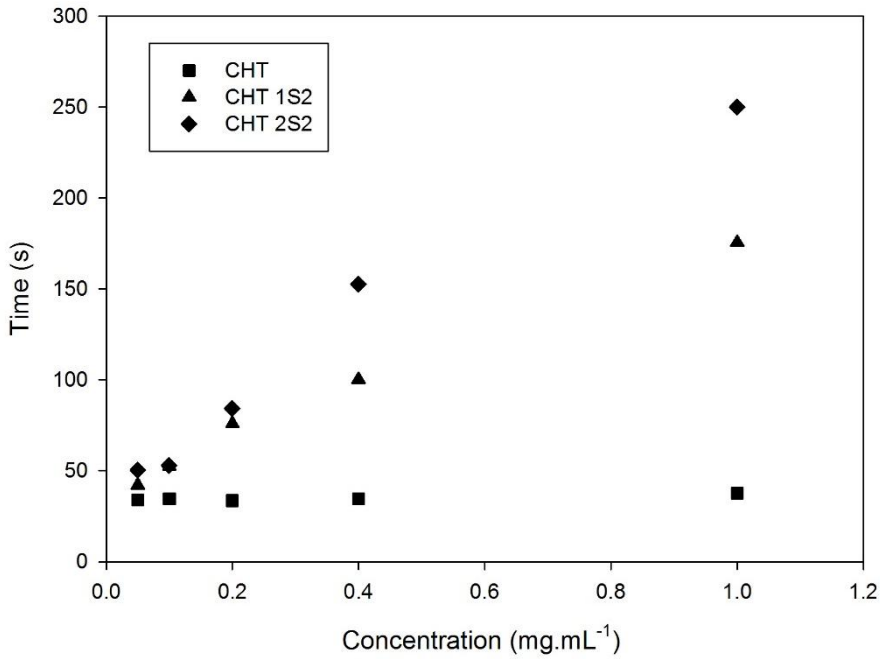

Figure 8. Activated Partial thromboplastin time (aPTT) of CHT, CHT1S and CHT2S at different concentrations. aPTT values for normal blood = 33 s, and 10U/mL heparin > 250 s.

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

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

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

As a matter of fact, on the one hand it is well known that the chitosan structure rich in amine residues can interfere with negatively charged plasma proteins, contributing to thrombus development, especially in acidic conditions (Amiji, 1998), on the other hand sulfonated chitosans based materials usually demonstrate anticoagulant properties explained by the presence of anionic SO_3^- groups capable of preventing protein absorption involved in the clotting cascade initiation (Amiji, 1998; Bauer, Schmuki, von der Mark & Park, 2013; Lima et al., 2013). The results from PT and anti-Xa assays showed that the activity of sulfonated chitosans on the extrinsic pathway was very low and the factor Xa was completely inhibited. These results suggested that the highly sulfated chitosan mainly interfered with the intrinsic pathway.

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

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

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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