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NMR and FT Raman characterisation of regioselectively sulfated chitosan regarding the distribution of sulfate groups and the degree of substitution

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ABSTRACT

Chitosan sulfates (CHS) were prepared with chlorosulfonic acid homogeneously and non-homogeneously. The total degrees of substitution (DS) ascribed to sulfate groups (DS_S) were determined with elemental analysis and the partial DS at 6-*O*-position was estimated via ^{13}C NMR. CHS with diverse total DS_S and sulfation patterns were obtained according to the analysis. The effects of selected reaction parameters that can influence the distribution of sulfate groups were examined. The structure of CHS was then characterized with various NMR techniques, i.e. one- (1D-) and two-dimensional (2D-) NMR, and FT Raman spectroscopy. It was found that the primary hydroxyl groups were always predominantly sulfated for CHS prepared under homogeneous or non-homogeneous conditions and no sulfate groups at 2-*N*-position could be detected. Finally, the feasibility of using FT Raman spectroscopy as another alternative for determining the total DS_S of CHS was presented.

1. Introduction

Naturally occurring sulfated biopolymers, such as heparin and chondroitin sulfate, are widely spread in nature and demonstrate important functions in the regulation of cellular proliferation and differentiation [1,2]. Even polysaccharides without sulfate groups exhibit biological activities after sulfation, e.g. cellulose sulfate shows anticoagulant and antiviral functions [3–5]. Among others, the application of sulfated polysaccharides or polyanions to influence the biological activity of growth factors has attracted broad attention in tissue engineering. Besides being used as part of scaffolds or for the encapsulation of proteins, the sulfated polysaccharides were also directly applied to control the binding and activity of growth factors in cells [6–9]. It was evidenced that cellulose sulfate with high contents of sulfate groups could bind to fibroblast growth factor (FGF) and promote FGF2-induced proliferation intensively [10]. Attempts have been made with the aim to elucidate the role of sulfation using well-defined sulfated oligosaccharides depicting the structure of chondroitin sulfate [11]. These approaches allow a better understanding of the effects attributed to modified saccharides and can also be used to alter the activity of growth factors for bone repair or the treatment of bone related diseases [12–14].

Chitosan is a deacetylated derivative of chitin representing naturally occurring polysaccharides. Chitosan has some beneficial properties, such as antimicrobial activity, excellent biocompatibility and low toxicity that promote its applications in many fields including food industry and pharmaceutics [15–17]. Moreover, chitosan is readily soluble in some inorganic and organic acids, including hydrochloric acid and acetic acid, while cellulose is only very poorly soluble in such acids [17,18].

Chemical modification of chitosan and chitin has been frequently carried out, in order to prepare their derivatives with advantageous properties, such as carboxymethylated, oxidized and sulfated chitosan/chitin [15,19,20]. These derivatives have wide applications, e.g. as additives in food and cosmetic products, as drug and gene delivery system or as chiral stationary phases for HPLC [15,19–23]. Among many derivatives of chitosan, CHS have been proven to be anticoagulant, antiviral, antimicrobial, and antioxidant [24–27]. CHS are usually synthesized in aprotic organic solvents and most of its preparations were executed heterogeneously or quasi-homogeneously due to very poor solubility or non-solubility of chitosan in these solvents, provided that chitosan with a high degree of deacetylation was used for the sulfation [24,26,28,29]. In addition to that, CHS could also be obtained through homogeneous sulfation of chitosan. For this purpose, chitosan was firstly dissolved in dichloroacetic acid or formic acid, and

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then diluted with aprotic organic solvents such as *N*,*N*-dimethylformamide (DMF) before adding sulfating agents [28,30].

Polysaccharide derivatives can be characterized via various analysis methods including NMR and IR spectroscopy. Raman spectroscopy as a rapid and non-destructive analysis method has been successfully applied to characterize biological systems and cellulose derivatives because of its beneficial properties including ultra-sensitive characterisation and no requirement of sample preparation [31–33]. It displays not only a qualitative but also a quantitative method in analysing cellulose derivatives and determining the contents of substituents within these derivatives, such as carboxymethyl cellulose and cellulose sulfate [32,33].

However, little information about the effects of reaction conditions on the total DS_S and especially on the distribution of sulfate groups within repeating units was provided, and also little is known about the 2D-NMR characterisation of CHS with distinct distributions of sulfate groups. This report aims to give more insights into the synthesis and characterisation of CHS. Various NMR techniques and FT Raman spectroscopy of CHS were measured regarding above mentioned aspects. Moreover, FT Raman spectroscopy demonstrates another alternative for determining the total DS_S of CHS.

2. Experimental section

2.1. Materials

Chitosan with a degree of deacetylation of 95.7% and viscosity of 145 mPas was purchased from Heppe Medical Chitosan GmbH (Halle, Germany). Formic acid with a content of 98–100% was obtained from Riedel-de Haen AG (Seelze, Germany). *N*,*N*-Dimethylformamide (DMF) from Carl-Roth GmbH (Karlsruhe, Germany) was fresh distilled under vacuum and deionized water was applied in all experiments. Other chemicals are all of analytical grade and are used without further treatment. Dialysis membrane from Spectrum Laboratories Inc. (Rancho Dominquez, USA) has an approximate molecular weight cut off of up to 500 Da.

2.2. Preparation of chitosan formate

Chitosan (1 g) was dissolved in 30 ml formic acid under stirring at room temperature (RT). After 3, 5 and 24 h the chitosan formates were collected after precipitation in 150 ml diethyl ether. After 3 times washing with 50 ml diethyl ether or acetone, the products were air-dried for 1 h and then dried under vacuum for 1 week at RT.

2.3. Homogeneous sulfation of chitosan

During a typical homogeneous sulfation, chitosan (1 g) was dissolved in 20 ml formic acid within 3 h at RT. Then 156 ml DMF was added, followed by another 2 h of stirring. After that, chlorosulfonic acid in DMF was dropped slowly into the chitosan solution within 30 min and the mixture was kept at 50 °C for 3 h. After cooling down to RT, the yellow to brown solution was poured into 600 ml saturated alkaline ethanolic solution of anhydrous sodium acetate. The obtained precipitate was dissolved in water after washing with ethanol/water-mixture (8/2, v/v). The pH value of the solution was adjusted to 7.5. Subsequently, the solution was dialyzed against water and lyophilized to give CHS.

2.4. Non-homogeneous sulfation of chitosan

Chitosan had to be activated to conduct the non-homogeneous sulfation. Chitosan (1 g) was dissolved in 1% aqueous acetic acid and 100 ml of methanol was added afterwards. Subsequently, 100 ml of 4% sodium hydrogen carbonate in water was given to the solution followed by 2 h of stirring. After washing with methanol and DMF, the activated chitosan was dispersed in 50 ml DMF for subsequent sulfation. For a typical sulfation, the sulfating agent consisting of chlorosulfonic acid in DMF was added and the mixture was kept at 50 °C for 3 h. After reaction, CHS were obtained by precipitating in 250 ml saturated alkaline ethanolic solution of anhydrous sodium acetate, dissolving in water, adjusting pH value to 7.5, dialysing against water and lyophilizing.

2.5. Measurements

The determination of the contents of formyl groups in chitosan formates was carried out using a back-titration method after complete saponification. The DS_F could be calculated according to the amount of the formyl groups [34].

The sulphur content of the CHS was measured with Elemental Analyser Eltra CS 500 (Neuss, Germany). The contents of carbon, hydrogen and nitrogen were determined with Elemental Analyser vario El from Elementar (Hanau, Germany). The total DS_S was calculated according to: Total $DS_S = (S\%/32)/(N\%/14)$.

FT Raman spectra of the samples in small metallic discs were recorded on a Bruker MultiRam spectrometer (Bruker Optics, Etlingen, Germany) with a liquid-nitrogen cooled Ge diode as detector. A cw-Nd:YAG-laser with an exciting line of 1064 nm was applied as light source for the excitation of Raman scattering. The spectra were recorded over a range of 3500–100 cm^{-1} using an operating spectral resolution of 3 cm^{-1} and a laser power output of 100 mW. A double analysis per 400 scans was carried out for each sample. An average Raman spectrum was formed afterwards and the spectrum was vector-normalised using the operating spectroscopy software OPUS Ver. 6.5 (Bruker Optics).

The ^{13}C NMR spectra were recorded at RT using a Bruker DPX 400 spectrometer (Bruker Biospin) at a ^{13}C-frequency of 100.13 MHz and with 30° pulse width, 0.35 s acquisition time and a relaxation delay of 3 s. The samples were dissolved in D_2O and scans of up to 20,000 were accumulated. The ^1H NMR and 2D-NMR as COSY (^1H–^1H correlation spectroscopy), HSQC (^1H detected heteronuclear single quantum coherence) and ROESY (^1H–^1H Rotating-frame overhause effect spectroscopy) were conducted on a Bruker Avance III 600 spectrometer at RT with a frequency of 600 MHz and an acquisition time of 2.6 s. The samples were dissolved in D_2O and 16 to 32 scans were accumulated. The solid-state CP/MAS ^{15}N NMR was carried out using a Bruker Avance 400 WB spectrometer at RT with a contact time of 1 ms, an acquisition time of 20 ms and a relaxation delay of 1 s. 80,000 scans were accumulated and the chemical shifts were externally referred to ^{15}N-nitromethane.

3. Results and discussion

3.1. Preparation of CHS under homogeneous and non-homogeneous conditions

Chitosan can be sulfated under distinct reaction conditions, i.e. homogeneous or non-homogeneous as described [24,26,28–30]. These different conditions may affect the properties of CHS concerning their total DS_S and distributions of sulfate groups.

3.1.1. Preparation of chitosan formates

The dissolving of chitosan with formation of chitosan formate was the first step before the addition of sulfating agents during the homogeneous sulfation of chitosan and this intermediate was examined at first. Chitosan formates were prepared and their DS attributed to formyl groups (DS_F) were analysed (Table 1).

Table 1
Preparation of chitosan formates at RT.

Samples	Reaction duration (h)	Washing mediums	DS_F	Solubility	
				Water	DMSO
CHF1	3	Diethyl ether	1.48	+	−
CHF2	5	Diethyl ether	1.54	+	−
CHF3	24	Diethyl ether	1.64	+	−
CHF4	24	Acetone	1.21	+	−

Fig. 1 illustrates the Raman spectra of chitosan and obtained chitosan formates. Characteristic signal at 1720 cm^{-1} ascribed to stretching vibrations of C=O groups could be seen and its intensity rises with increasing DS_F. A new band at 1350 cm^{-1} emerges within the spectra of chitosan formates. As described previously, the signals attributed to deformation and stretching vibrations of CH_2, HCC, HCO and COH groups lie in the range of 1200 and 1415 cm^{-1} [32,35,36]. It can be assumed that the band at 1350 cm^{-1} is derived from the vibrations of HCO groups within formyl groups.

Other changes of Raman signals are visible between 1000 and 1200 cm^{-1}. The peaks at 1116 and 1094 cm^{-1} derived from the symmetric stretching vibrations of glycosidic bonds in chitosan backbones are dominant within the spectrum of chitosan, as known from other polysaccharides [35,36]. However, they demonstrate only shoulders of the new peak at 1123 cm^{-1} after the introduction of formyl groups. This new peak can be classified as the stretching vibrations of C=O groups [36]. Furthermore, the signal at 3311 cm^{-1} representing the vibrations of N–H groups disappeared within the spectra of chitosan formates, which indicates the substitution of the amino groups by formic acid. The band at 2885 cm^{-1} due to the stretching vibrations of C–H groups shifted to 2917 cm^{-1}. These notices suggest the successful introduction of formyl groups into GlcN units of chitosan.

It has been argued that chitosan could be converted into its salts and diverse polymorphs could be observed after being dissolved in acids. The salts of chitosan with carboxylic acids including formic acid were declared to be hydrated and were classified as Type II salt [37,38]. This Type II form should have a conformation that is different from chitosan due to the absence of intramolecular hydrogen bonds [38]. The hydrogen bond system within chitosan chains was possibly broken during the dissolving and hence, above discussed changes within the Raman spectrum due to introduction of formyl groups are notable.

According to Table 1, a DS_F of 1.48 was obtained after 3 h reaction with formic acid. A prolongation of the reaction duration to 24 h increased the DS_F only slightly. This fact implies that 3 h were already long enough for the esterification of most possible reaction

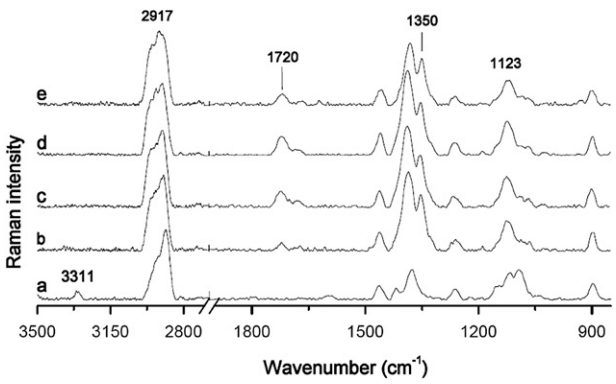

Fig. 1. FT Raman spectra (3500–850 cm^{-1}) of (a) chitosan, (b) CHF1, (c) CHF2, (d) CHF3 and (e) CHF4 at RT.

Table 2
Homogeneous sulfation of chitosan.

Samples	Amount of FA /dissolving duration	Molar ratio[a]	Reaction temperature /duration	DS_{S6}[b]	Total DS_S[b]	DS_{S3}[b]
CHS1	10 ml/0.5 h	6	RT/7.5 h	1	1.67	0.67
CHS2	10 ml/0.5 h	6	50°C/5 h	0.93	1.59	0.66
CHS3	10 ml/1 h	6	RT/5 h	1	1.58	0.58
CHS4	10 ml/1 h	6	50°C/5 h	0.87	1.36	0.49
CHS5	20 ml/1 h	6	RT/5 h	1	1.29	0.29
CHS6	20 ml/1 h	6	50°C/5 h	0.73	1.13	0.40
CHS7	20 ml/3 h	6	RT/3 h	0.65	1.23	0.58
CHS8	20 ml/3 h	6	50°C/3 h	0.79	1.29	0.50
CHS9	20 ml/3 h	10	50°C/3 h	0.91	1.33	0.42
CHS10	20 ml/3 h	13	50°C/3 h	0.87	1.35	0.48
CHS11	30 ml/3 h	6	50°C/3 h	0.80	1.25	0.45

[a] Molar ratio in mol chlorosulfonic acid per mol GlcN units.
[b] DS_{S6} was estimated by ^{13}C NMR; total DS_S was calculated with sulphur content determined by elemental analysis; DS_{S3}: DS_S at the 3-*O*-position, is equal the difference between the total DS_S and DS_{S6}.

positions. Because prepared chitosan formates all exhibit DS_F higher than 1, formic acid should have esterified not only total amino groups, but also parts of primary hydroxyl groups.

In addition, the utilized organic solvents as washing medium could affect the DS_F. As shown in Table 1, CHF3, which was washed with diethyl ether, exhibits a remarkably higher DS_F than CHF4 washed with acetone under other equal synthesis conditions.

3.1.2. Preparation of CHS under homogeneous conditions

CHS could be prepared under homogeneous conditions and the products were characterized. The total DS_S of CHS between 1.13 and 1.67 are obtained based on the sulphur contents determined by elemental analysis and the partial DS_S at 6-*O*-position (DS_{S6}) can be estimated via ^{13}C NMR (Table 2, Fig. 3).

Fig. 2 presents FT Raman spectra of chitosan and CHS with diverse DS_S and characteristic signals ascribed to sulfate groups are visible. New bands emerged at 1070, 1014, between 823 and 834 as well as between 580 and 610 cm^{-1}. The signals at 1070 and 1014 cm^{-1} are attributed to stretching vibrations of O=S=O groups, while the band between 580 and 610 cm^{-1} is ascribed to deformation vibrations of O=S=O groups. Another new band

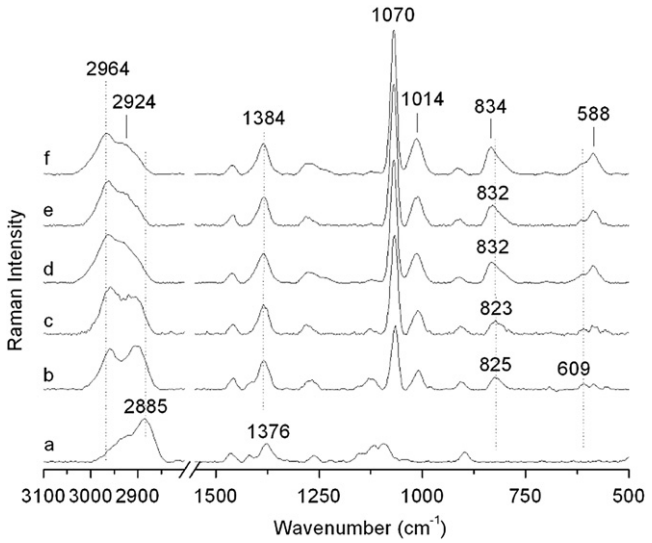

Fig. 2. FT Raman spectra (3100–500 cm^{-1}) of (a) chitosan (DS_S = 0), (b) CHS14 (DS_S = 0.86), (c) CHS6 (DS_S = 1.17), (d) CHS10 (DS_S = 1.35), (e) CHS3 (DS_S = 1.58) and (f) CHS20 (DS_S = 1.67) at RT.

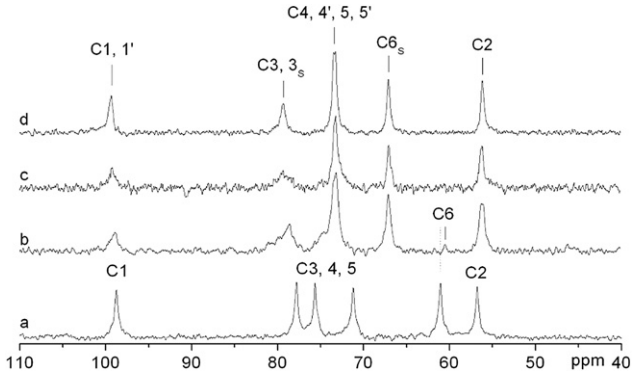

Fig. 3. ^{13}C NMR spectra (110–40 ppm) of (a) chitosan in 1% aqueous acetic acid at RT, (b) CHS2 ($DS_S = 1.59$), (c) CHS3 ($DS_S = 1.58$) and (d) CHS15 ($DS_S = 1.59$) in D_2O at RT.

between 823 and 834 cm^{-1} arises from stretching vibrations of C–O–S groups [29,33]. The presence of these new bands suggests a successful sulfation of chitosan.

In addition, the band at 2885 cm^{-1} within the spectrum of chitosan derived from stretching vibrations of CH groups was shifted to 2924 cm^{-1} after sulfation, while a new band at 2964 cm^{-1} exhibits a much higher intensity.

3.1.3. Preparation of CHS under non-homogeneous conditions

In addition to homogeneous sulfation, chitosan could also be non-homogeneously sulfated by chlorosulfonic acid after the activation (Figs. 2f and 3d). The total DS_S of prepared CHS were determined to be between 0.86 and 1.67 via elemental analysis and the DS_{S6} were estimated by ^{13}C NMR (Table 3).

Table 3
Non-homogeneous sulfation of chitosan.

Samples	Molar ratio[a]	Reaction temperature/duration	DS_{S6}[b]	Total DS_S[b]	DS_{S3}[b]
CHS12[c]	6	50°C/5 h	0	0	0
CHS13	6	40°C/5 h	1	1.33	0.33
CHS14	4	50°C/3 h	0.62	0.86	0.24
CHS15	6	50°C/3 h	1	1.59	0.59
CHS16	6	50°C/5 h	1	1.49	0.49
CHS17	6	70°C/3 h	1	1.21	0.21
CHS18	10	50°C/3 h	1	1.61	0.61
CHS19	10	50°C/5 h	1	1.48	0.48
CHS20	13	50°C/3 h	1	1.67	0.67

[a] Molar ratio in mol chlorosulfonic acid per mol GlcN units.
[b] DS_{S6} was estimated by ^{13}C NMR; total DS_S was calculated with sulphur content determined by elemental analysis; DS_{S3} is equal the difference between the total DS_S and DS_{S6}.
[c] CHS12 was prepared without previous activation..

3.2. 1D and 2D-NMR of CHS

In order to find out the relationship between structures and biological activities, it is important to clarify the exact distribution of the sulfate groups within the repeating units [11,29,39,40]. Therefore, an accurate understanding of the NMR signals is of importance. The 1D-NMR provides information about the structures of CHS, but some signals severely overlap and not all signals can be precisely assigned [24,26,28,30]. These limits can be overcome by 2D-NMR. Moreover, 2D-NMR offers the information about the correlation between the C–H and H–H inside or between repeating units. Thus, in addition to 1D-NMR as ^{13}C, ^{1}H and ^{15}N NMR, 2D-NMR including COSY, HSQC and ROESY was carried out to elucidate the structures of CHS.

Fig. 3 depicts ^{13}C NMR spectra of chitosan and CHS. The signal at 60 ppm attributed to C6 of the GlcN units was shifted to 67 ppm after sulfation [27,28]. Both signals are derived from C6 and the integrated peak areas under both signals are together 100%. Hence, DS_{S6} can be calculated according to the proportion of integrals at 67 ppm (Tables 2 and 3) [33,41]. Without the peak at 60 ppm, the DS_{S6} can be regarded as 1, e.g. CHS3 (Fig. 3c). The partial DS_S at another position, i.e. 3-*O*-position, can be calculated and is equal the difference between the total DS_S and DS_{S6}. The C2 showed a signal at 56 ppm. C3 and $C3_S$ displayed a combined peak at 78.3 ppm. However, the signals of C4 and C5 in the region of 70 and 75 ppm strongly overlap.

^{1}H NMR delivers accurate information about the protons at different positions and has been used to determine the degree of acetylation of chitin and chitosan [42–44].

Typical ^{1}H NMR spectra of CHS can be found in Fig. 4. The signals within ^{1}H NMR spectra of CHS were assigned based on 2D-NMR spectra of CHS in Figs. 5 and 6. As shown in Fig. 4, the protons on C1 (i.e. H1) show a detectable signal at 4.75 ppm that is close to the signal of HOD. Additionally, the protons on C6 with complete sulfation of primary hydroxyl groups exhibit a signal at 4.28 ppm, while the protons on C6 without sulfate groups display signals between 3.75 and 4 ppm (Fig. 4a). This signal is adjacent to the signals of H5′, that was shifted from 3.59 ppm (signal attributed to H5) to this region because of the sulfation at 6-*O*-position.

The COSY and HSQC of two distinct CHS, CHS8 and CHS15, are displayed in Figs. 5 and 6. Although CHS8 and CHS15 demonstrate analogue spectra, some differences are notable due to diverse distributions of sulfate groups within repeating units and different DS_{S6}. CHS15 with complete sulfation at 6-*O*-position presents only one peak for each proton and carbon. In contrast, CHS 8 with DS_{S6} of 0.79 has more peaks for some protons and carbons, e.g. H4, H5, H6 and C4, C5, C6. This emergence of more peaks can be attributed to different influences of the carbons and protons at C3 and C6 on the chemical environment of sugar rings due to the presence of both hydroxyl and sulfate groups.

The strongly overlapping signals of carbons C3/4/5 exhibiting similar chemical shifts can be distinguished. Based on HSQC of CHS that reflects the correlation of the carbons and the corresponding protons, an accurate assignment of their signals can be carried out and the results are visualized in Fig. 6. The signal of C3 displays a higher chemical shift at 78.3 ppm. The carbons C4′ and C5′ with sulfation at 6-*O*-position have peaks at 73.2 and 72.8 ppm, while the C5 with C6–OH shows a weak signal at 75 ppm (Fig. 6).

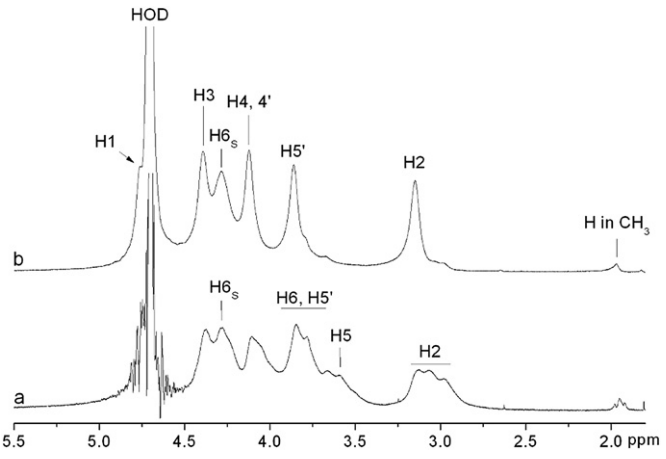

Fig. 4. ^{1}H NMR spectra (5.5–1.8 ppm) of (a) CHS8 ($DS_S = 1.29$) and (b) CHS15 ($DS_S = 1.59$) in D_2O at RT.

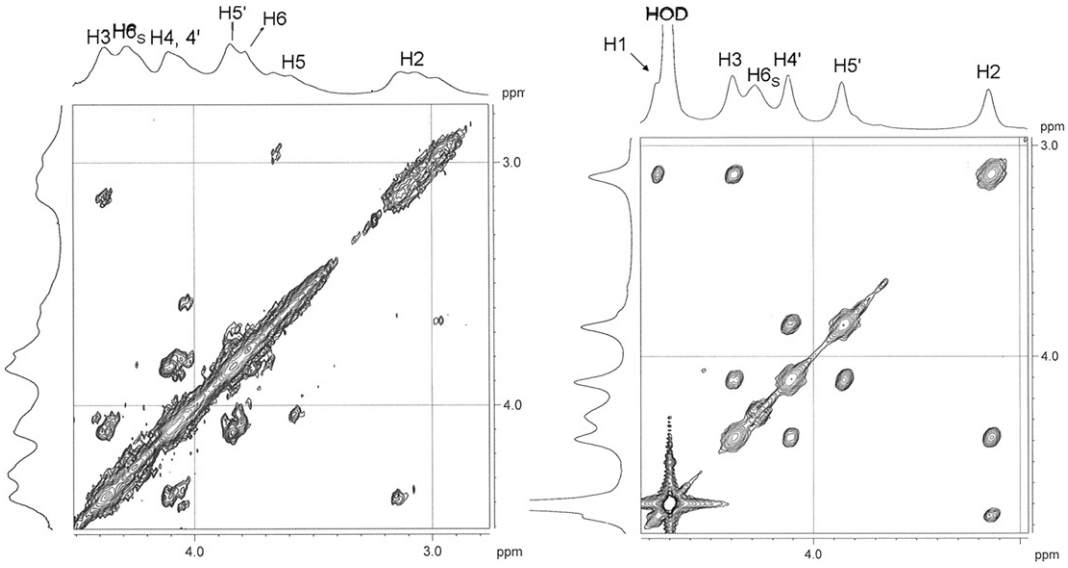

Fig. 5. COSY spectra of CHS8 with $DS_S = 1.29$ and $DS_{S6} = 0.79$ (left) and CHS15 with $DS_S = 1.59$ and $DS_{S6} = 1$ (right) in D_2O at RT.

According to NMR of CHS8, the substitution of primary hydroxyl groups resulted in downfield shifts of H5 and H/C6, while the C5 was shifted upfield (Figs. 5 and 6). However, the signals derived from H/C4 were mingled with those of H/C4′ and a peak with a shoulder is visible for H4/4′. Besides, H2 shows also more than one peak maximum.

In addition, $^1H-^1H$ ROESY was recorded in order to find out the space structure of repeating units within CHS. A characteristic ROESY of CHS is illustrated in Fig. 7 and the signals arise from the protons that are close to each other in space. As shown, H1 within the repeating units of CHS15 dissolved in D_2O is close to all other protons, while $H6_S$ is only adjacent to H1 and H5′. Other protons, i.e. H2/3/4/5′, within the sugar ring are stereoscopically close to each other. However, it should be noted that these signals could not only be ascribed to stereoscopic adjacencies of protons inside the same repeating units, but also to that of protons within different repeating units.

Solid-state ^{15}N NMR spectroscopy has been used to characterize biopolymers, such as DNA and peptide. It has also been applied to analyse chitin and chitosan regarding their degrees of acetylation [45,46]. Fig. 8 depicts ^{15}N NMR spectra of CHS prepared homogenously or non-homogenously and all these CHS show only one peak at −344 ppm representing. This fact suggests that the amino groups in these CHS were not sulfated using both sulfation methods.

As the result, the structure of CHS can be analysed by means of 1D and 2D-NMR techniques and the distributions of sulfation groups within the repeating units of CHS can be estimated.

As described before, sulfated cellulose and chitosan exhibited biological effects. Cellulose sulfate with high total DS_S could promote the FGF2-induced proliferation and high partial DS_{S6} and DS_{S2} were found to be necessary [10]. CHS and cellulose sulfate with sulfate groups at 6-*O*-position presented high AT III affinity and platelet adhesion, while 3-*O*-sulfated chitosan and cellulose demonstrated much lower AT III affinity and platelet adhesion [39,47]. As well, 2-*N* and 3-*O*-disulfated chitosan showed very low anticoagulant activity [25]. Besides, it was reported that 6-*O*-sulfated chitin did not display anticoagulant activity, but 3,6-*O*-disulfated chitin has elevated anticoagulant activity [44]. Moreover, CHS with sulfate groups at 2-*N* or 6-*O*-position, or simultaneously

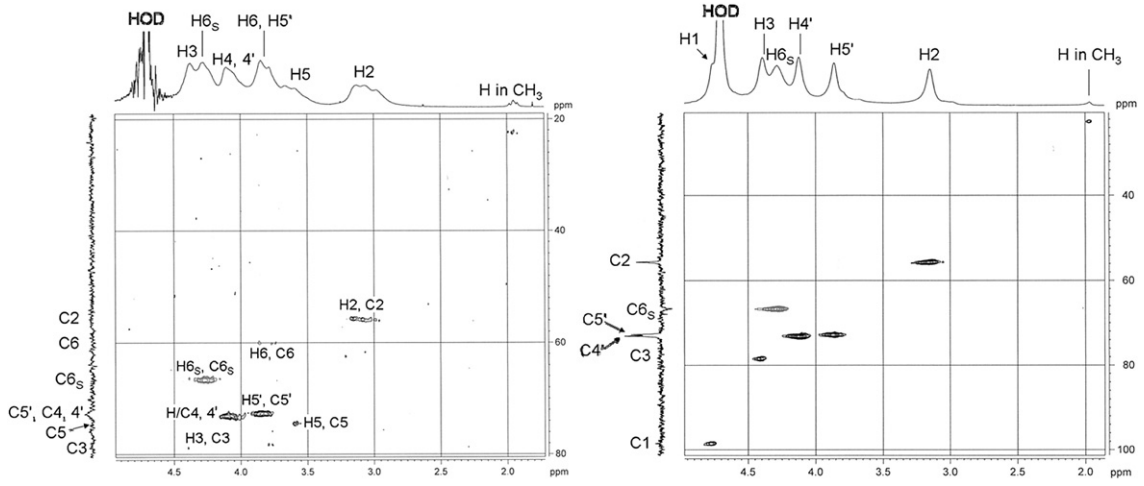

Fig. 6. HSQC spectra of CHS8 with $DS_S = 1.29$ and $DS_{S6} = 0.79$ (left) and CHS15 with $DS_S = 1.59$ and $DS_{S6} = 1$ (right) in D_2O at RT.

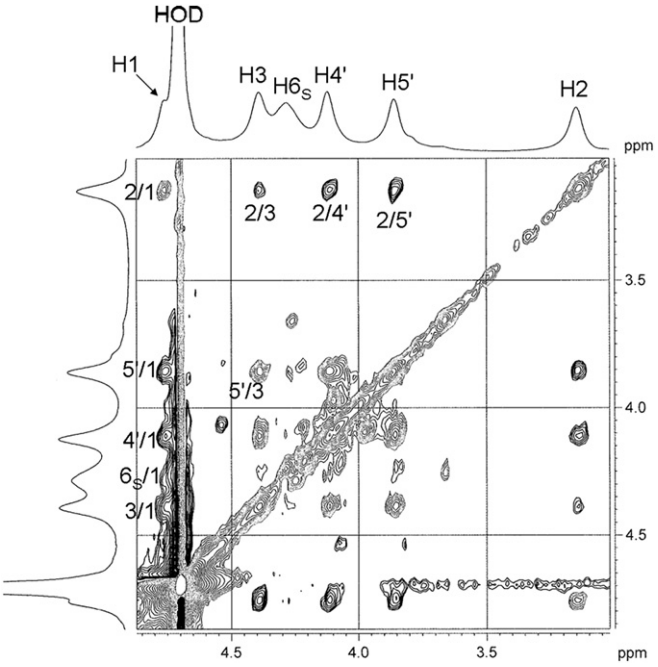

Fig. 7. $^{1}H-^{1}H$ ROESY spectrum of CHS15 with $DS_S = 1.59$ and $DS_{S6} = 1$ in D_2O at RT.

at both positions enhanced the BMP2-induced ALP activity. However, this activity was attributed primarily to the sulfate groups at 6-*O*-position and the sulfate groups at 2-*N*-position had only subsidiary functions [29].

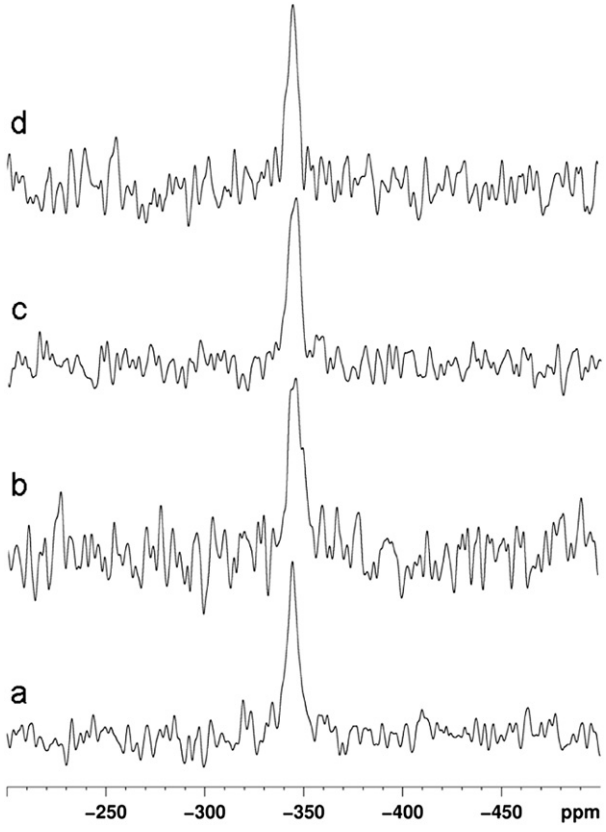

Fig. 8. Solid-state CP/MAS ^{15}N NMR spectra (−200–500 ppm) of (a) CHS1, (b) CHS3, (c) CHS18 and (d) CHS19 at RT.

Hence, the sulfation pattern, i.e. the distribution of the sulfate groups, of sulfated polysaccharides including CHS is essential for their biological activities. The determination of the partial DS_S should be of pivotal importance for finding out the structure property relationship of CHS.

3.3. Effects of reaction conditions

According to Table 2, the total DS_S of homogeneously prepared CHS are higher than 1 and the distribution of sulfate groups are distinct. The 6-*O*-position can be differently substituted and the DS_{S6} lies in the range of 0.65–1. Complete substitution of primary hydroxyl groups could be obtained after sulfation at RT, but higher sulfation temperature of 50 °C lowered the DS_{S6} to maximal 0.93. High reaction temperature reduced the total DS_S as well according to Table 2. Thus, the total and partial DS_S can be regulated by varying the reaction temperature. The decrease of the DS_S at a high reaction temperature was probably ascribed to the desulfuration and degradation reactions leading to formation of low-molecular by-products that were removed afterwards during the washing and dialysis process [29,30].

Besides the reaction temperature, the amount of formic acid and the dissolving duration could also affect the total and partial DS_S. During the dissolving, the formation of the amide or ester groups should be promoted with rising amounts of formic acid or longer dissolving durations. The application of a larger volume of formic acid from 10 to 20 ml and the prolongation of dissolving duration from 0.5 to 1 h resulted in CHS with lower total and partial DS_S. However, if chitosan was dissolved in 20 ml formic acid for 3 h, CHS exhibiting similar total DS_S were obtained despite the use of more sulfating agent. A more intensive sulfation seems to be not possible. This fact indicates the stability of the amide and ester bonds against subsequent sulfation with chlorosulfonic acid and the formyl groups can only partly be displaced by sulfate groups under the applied conditions.

Due to the high reactivity of the primary hydroxyl groups, they are always preferred in comparison to other hydroxyl groups during the sulfation of polymers without any other protecting methods [27,29,48]. In accordance with that, the homogeneous sulfation of chitosan in this report produced CHS with dominant sulfation at 6-*O*-position up to 100%, while the other positions were only partly sulfated. On the other hand, considering the predominant substitution of amino groups by formyl groups during the dissolving (Table 1 and Fig. 1), it can be proposed that the 2-*N*-position may not be sulfated. Results from solid-state ^{15}N NMR of CHS in Fig. 8 confirm the presumption. Another proof is that the C1 within the ^{13}C NMR of CHS shows only one peak [33,41]. Thus, the amino groups have reacted with formic acid yielding chitosan formate and subsequently added chlorosulfonic acid could not displace the formyl groups on amide bonds. The formyl groups were eliminated during the subsequent neutralization with sodium hydroxide and the amino groups were regained.

It is well established that chitosan in solid-state presents a semicrystalline structure consisting of a hydrogen bonds system [15,20]. According to CHS12, chitosan cannot or only under difficulties be non-homogeneously sulfated without activation (Table 3). After the activation, the sample CHS13 possesses a total DS_S of 1.33 even at 40 °C and the total DS_S was raised to 1.49 at a higher temperature of 50 °C, but an increase of the reaction temperature from 50 to 70 °C resulted in a reduction of the total DS_S. Moreover, the use of more sulfating agent caused a higher total DS_S. In addition, reaction duration of 5 h decreased the total DS_S slightly in comparison to 3 h after sulfation with 6 or 10 mol chlorosulfonic acid per mol GlcN units. A sulfation longer than 3 h should have hydrolysed

Table 4
CHS with total DS_S and Raman band areas and calculated area ratios.[a]

Samples	Total DS_S	Band area or area ratios	
		A_{1070}	A_{1070}/A_{1384}
Chitosan	0	0	0
CHS14	0.86	6.964	1.456
CHS6	1.13	10.081	2.599
CHS17	1.21	8.644	2.271
CHS7	1.23	8.950	1.988
CHS11	1.25	11.705	2.121
CHS13	1.33	10.331	2.563
CHS10	1.35	11.096	2.860
CHS3	1.58	15.284	3.267
CHS2	1.59	13.798	2.543
CHS18	1.61	14.001	3.134
CHS20	1.67	17.584	3.063

[a] The Raman band area and band area ratio were taken as 0 for chitosan.

chitosan more strongly and produced more low-molecular by-products. After 5 h reaction the colour of the reaction system was more intensive than after 3 h, which indicates the formation of more low-molecular compounds.

During the non-homogeneous sulfation, the primary hydroxyl groups of chitosan are preferably sulfated. Using 6 or more mol chlorosulfonic acid per mol GlcN units for the sulfation, complete sulfation of primary hydroxyl groups was always achieved. The activation of chitosan should have significantly increased the reactivity of the hydroxyl groups, especially the primary hydroxyl groups.

However, the amino groups within these CHS remain non-sulfated according to the ^{13}C and solid-state ^{15}N NMR (Figs. 3 and 8). During the non-homogeneous sulfation, the sulfation of the amino groups was not possible because (1) the amount of chlorosulfonic acid (up to 13 mol/mol GlcN units) was not adequate; (2) the amino groups in protonated form under acidic conditions cannot be sulfated by chlorosulfonic acid. Because the amino groups could still be more intensively protonated if more chlorosulfonic acid was applied, it can be concluded that the sulfation of the amino groups within chitosan may be very difficult under acidic conditions and only sulfation of hydroxyl groups can be realized. Alkaline surroundings or other sulfating agents are probably necessary for the sulfation of amino groups within chitosan [39,49].

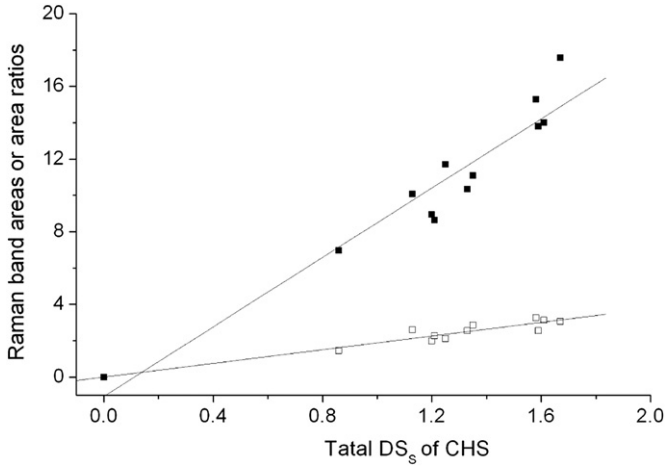

Fig. 9. Calibration curves of Raman band areas or area ratios against total DS_S with ■ for A_{1070}: areas of band at 1070 cm^{-1} and □ for A_{1070}/A_{1384}: area ratios between bands at 1070 and 1384 cm^{-1}.

Table 5
Linear regression parameters from the calibration curves in Fig. 9.[a]

Raman parameters	a	b	r	SD	p	n
A_{1070}	−1.058 ± 1.218	9.530 ± 0.931	0.955	1.404	<0.0001	12
A_{1070}/A_{1384}	0.0005 ± 0.248	1.881 ± 0.190	0.953	0.286	<0.0001	12

[a] $Y = a + bX$; where Y is the Raman parameter, X is the total DS_S, a is the Y-intercept, b is the slope, r is the correlation coefficient, SD is the standard deviation, p is the significance level and n is sample volume.

3.4. Quantification of total DS_S with FT Raman spectroscopy

As described before, Raman spectroscopy can be used to quantify the contents of substituents within polysaccharide derivatives [32,33]. For the purpose of quantifying the total DS_S of CHS in this report, the signal at 1070 cm^{-1} attributed to O═S═O groups is used as marker band and the band at 1384 cm^{-1} as internal standard, because the latter band is derived from chitosan backbone and should remain stable. The band areas were acquired from the spectra (Table 4) and band area ratios can be calculated. Band areas and band area ratios can be applied as analysis parameters for the quantification. The calibration curves are generated after plotting the Raman analyse parameters against total DS_S (Fig. 9). Linear correlations were obtained with high correlation coefficients $r > 0.95$ suggesting strong positive relationships between these Raman analyse parameters and the total DS_S determined by elemental analysis. The parameters of linear regressions are listed in Table 5. According to Table 5, both parameters provide similar correlation coefficients $r > 0.95$. Thus, the application of Raman band area of band at 1070 cm^{-1} and area ratio between the bands at 1070 and 1384 cm^{-1} can be another alternative for determining the total DS_S of CHS. In particular, this method based on Raman spectroscopy is rapid and non-destructive in comparison to other conventional methods.

4. Conclusion

CHS with various total DS_S and diverse distributions of sulfate groups within GlcN units were prepared homogenously or non-homogeneously. A previous dissolution or activation was found to be essential for the sulfation. By varying the reaction conditions, the total and partial DS_S can be regulated.

During the dissolution of chitosan in formic acid, chitosan formate was generated at first. The formyl groups were bound predominantly to amino groups and partly to hydroxyl groups. The subsequent homogeneous sulfation by chlorosulfonic acid resulted in dominant substitution of primary hydroxyl groups. The amount of formic acid, dissolving duration and reaction temperature could affect the total and partial DS_S of obtained CHS. The non-homogeneous sulfation also caused the preferable sulfation of primary hydroxyl groups and the DS_{S6} was determined always to be 1 with application of 6 or more mol chlorosulfonic acid per mol GlcN units. In addition, the amino groups of CHS proved to be not sulfated during both sulfation processes.

FT Raman spectroscopy demonstrates another alternative for determining the total DS_S of CHS with the band at 1070 cm^{-1} attributed to symmetric stretching vibrations of O═S═O groups as marker band and the band at 1384 cm^{-1} as internal standard. Strong linear correlations with $r \geq 0.95$ were obtained.

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