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FT Raman investigation of novel chitosan sulfates exhibiting osteogenic capacity

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ABSTRACT

Chitosan sulfates (CHS) exhibiting various total degrees of substitution ascribed to sulfate groups (DS_S) were synthesized. The sulfation could be under homogeneous or non-homogeneous conditions. The obtained CHS were characterized and total DS_S of up to 1.73 were determined. Using chlorosulfonic acid as sulfating agent, CS with total DS_S between 0.86 and 1.67 were obtained and the total DS_S can be regulated by varying the sulfation parameters. Using other sulfating agents, CS with distinct total DS_S of up to 1.73 were prepared. By means of FT Raman spectroscopy, marker bands at 1070 cm^{-1} or 1014 cm^{-1} attributed to vibrations of sulfation groups can be applied for quantifying the total DS_S of CHS. Calibration curves with correlation coefficients of more than 0.95 were established, suggesting the feasibility of Raman spectroscopy for quantifying the total DS_S of CHS. Finally, the capacity of CHS to improve the osteogenic activity of bone morphogenetic protein-2 (BMP-2) was presented.

1. Introduction

Chitosan is the deacetylated form of naturally occurred chitin with a degree of acetylation below 0.4. Chitosan consists of 2-amino- and 2-acetamino-2-deoxy-β-D-glucopyranose (Muzzarelli & Muzzarelli, 2005; Rinaudo, 2006). In order to prepare products with desired properties based on this biopolymer, chemical modifications of chitosan including carboxymethylation and sulfation have been frequently carried out and investigated (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli & Muzzarelli, 2005; Muzzarelli et al., 1984; Rinaudo, 2006; Zhou et al., 2009).

Chitosan sulfate (CHS), a half-ester of chitosan, is proved to be anticoagulant, antiviral, antimicrobial, and antioxidant (Huang, Du, Zheng, Liu, & Fan, 2004; Nishimura et al., 1998; Vikhoreva et al., 2005; Xing et al., 2004, 2005). CHS could be synthesised heterogeneously or quasi-homogeneously in aprotic organic solvents, such as *N*,*N*-dimethylformamide (DMF) (Huang et al., 2004; Vikhoreva et al., 2005). CHS could also be obtained after homogeneous sulfation of chitosan. For this purpose, chitosan was dissolved in dichloroacetic acid or formic acid. Then, the solution was diluted with an aprotic organic solvent before adding sulfating agents. Usually applied sulfating agents are SO_3-DMF complex and chlorosulfonic acid (Gamzazade et al., 1997; Xing et al., 2005).

The determination of the amounts of sulfate groups in CHS was normally realised via elemental analysis. Other analysis methods including IR or NMR spectroscopy can also be applied to analyse CHS (Huang et al., 2004; Xing et al., 2005; Zhou et al., 2009). Raman spectroscopy is a rapid and non-destructive analysis method with beneficial properties, such as ultra-sensitive characterization and no requirement of sample preparation. It has been applied to characterise biological systems and polymer derivatives (Li et al., 2010; Schenzel & Fischer, 2001; Yuen, Choi, Phillips, & Ma, 2009; Zhang, Brendler, & Fischer, 2010). Raman spectroscopy can not only qualify but also quantify the polymer derivatives, such as carboxymethyl cellulose and cellulose sulfate. Characteristic vibrations derived from substituents could be used to determine the total DS attributed to these substituents (Yuen et al., 2009; Zhang et al., 2010).

In this report, diverse novel CHS were prepared with various sulfating agents and their total DS_S were determined. Then, FT Raman analysis of CHS was carried out and strong linear correlations between Raman analysis parameters and the total DS_S were observed, suggesting that FT Raman can be another alternative for determining the total DS_S of CHS. Finally, the feasibility of CHS for stimulating the biological activity of BMP-2 was examined with selected CHS.

2. Experimental

2.1. Materials

Chitosan with a degree of deacetylation of >95.5% and viscosity of 145 mPa s or 7 mPa s (1% in 1% acetic acid at 20 °C) was obtained from Heppe Medical Chitosan GmbH (Halle, Germany). SO_3-DMF and pyridine complex were purchased from Sigma–Aldrich Chemie

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Table 1
Synthesis of CHS using chlorosulfonic acid with their total DS_S and the intensities of band at 1070 cm^{-1} or band intensity ratios between the marker bands and the band at 1384 cm^{-1}.

CHS	Sulfation methods	Molar ratio[a]	T (°C)/t (h)[b]	Total DS_S [c]	I_{1070}	I_{1070}/I_{1384}	I_{1012}/I_{1384}	I_{822}/I_{1384}
Chitosan	–	–	–	0[d]	0	0	0	0
CHS1	II	4	50/3	0.86	0.248	2.157	0.652	0.426
CHS2[e]	I	15	70/24	1.12	0.291	2.798	0.731	0.538
CHS3	I	6	50/5	1.13	0.366	3.297	0.793	0.486
CHS4	II	6	70/3	1.21	0.302	3.512	0.837	0.686
CHS5	I	6	RT/3	1.23	0.322	3.389	0.779	0.621
CHS6	I	6	50/3	1.25	0.426	3.109	0.788	0.606
CHS7	II	6	40/5	1.33	0.357	3.839	0.946	0.720
CHS8	I	13	50/3	1.35	0.402	4.232	0.989	0.632
CHS9	I	6	50/1	1.48	0.486	3.827	0.874	0.669
CHS10	I	6	RT/5	1.58	0.581	4.882	1.017	0.714
CHS11	I	6	50/5	1.59	0.549	4.067	0.911	0.815
CHS12	II	10	50/3	1.61	0.538	4.936	0.972	0.817
CHS13	II	13	50/3	1.67	0.642	4.686	1.066	0.883
CHS14	I	6	RT/7.5	1.67	0.521	4.453	0.940	0.838

[a] Molar ratio in mol sulfating agent per mol GlcN units. 10 ml formic acid was used for CHS10, 11 and 14, 20 ml for CHS2, 3, 5, 8 and 9, and 30 ml for CHS6.
[b] T (°C)/t (h): reaction temperature in °C and reaction duration in hours.
[c] Total DS_S of CHS were determined with elemental analysis.
[d] The total DS_S and analysis parameters were taken as 0 for chitosan.
[e] CHS2 was prepared with sulfamic acid.

GmbH (Steinheim, Germany). Chlorosulfonic acid was received from Merck Schuchardt OHG (Hohenbrunn, Germany) and sulfamic acid from Carl-Roth GmbH (Karlsruhe, Germany). DMF was freshly distilled before use and demineralised water was applied in all experiments. Other chemicals are all of analysis grade and used as received. Dialysis membrane from Spectrum Laboratories Inc. (Rancho Dominquez, USA) has an approximate molecular weight cut off of up to 500 Da.

2.2. Sulfation of chitosan

During a typical homogeneous sulfation (Method I), 1 g chitosan was dissolved in formic acid at room temperature (RT) and 156 DMF were added under stirring. For the dissolving, 10 ml formic acid was used for CHS10, 11 and 14, 20 ml for CHS2, 3, 5, 8, 9 and 21 and 30 ml for CHS6 (Tables 1 and 4). Then, chlorosulfonic acid in DMF was dropped slowly into the chitosan solution within 30 min and the mixture was kept at RT for 5 h. Next to the reaction, the 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) and the pH of this solution was adjusted to 7.5. Finally, the product was dialyzed against water and lyophilised.

For the non-homogeneous sulfation (Method II), chitosan has to be activated before sulfation. 1 g chitosan was dissolved in 100 ml 1% aqueous acetic acid. 100 ml of methanol and 4% sodium hydrogen carbonate in water were added afterwards. After centrifugation and washing with methanol and DMF, the activated chitosan was dispersed in 50 ml DMF for the subsequent sulfation. Next to the activation, the sulfating agent was added and the mixture was kept at 50 °C for 3 h. After reaction, products were obtained after precipitating in 250 ml alkaline ethanolic solution of anhydrous sodium acetate. After washing with ethanol–water-mixture (8/2, v/v), CHS was obtained after being dissolved in water, pH-adjustment to 7.5, dialysis against water and lyophilising.

2.3. Measurements

The sulfur content 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).

^{13}C NMR spectroscopy was conducted at RT using a Bruker DPX 400 spectrometer (Bruker Biospin, Etlingen, Germany) 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.

FT Raman spectra of CHS in small metallic discs were recorded on a Bruker MultiRam spectrometer (Bruker Optics) over a range of 3500–150 cm^{-1}. A liquid-nitrogen cooled Ge diode was used as detector and a cw-Nd:YAG-laser with an exciting line of 1064 nm was applied as light source for the excitation of Raman scattering. An operating spectral resolution of 3 cm^{-1} and a laser power output of 100 mW were used. Double analysis per 400 scans was carried out for each sample and an average Raman spectrum was formed afterwards. The spectrum was vector normalised and the band intensities were acquired from the spectra using the operating spectroscopy software OPUS Ver. 6.5 (Bruker Optics).

The analysis of the data was executed with OriginPro 7.0 (OriginLab Corporation, MA, USA).

2.4. Determination of biological activity of CHS

2.4.1. Cell culture

For investigations on the biological activity we used the mouse myoblast cell line C2C12 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) with osteogenic potential under the effect of bone morphogenetic protein-2 (BMP-2, Peprotech, London, UK). For maintenance and proliferation, the cells were cultured in 75 cm^3 culture flasks with Dulbecco's modified Eagle medium (DMEM, Biochrom AG, Berlin, Germany) containing 10% fetal bovine serum (Biochrom AG), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and 2 mM L-glutamine (Biochrom AG) at 37 °C in humidified atmosphere consisting of 5% CO_2 and 95% air. When confluence was reached, the cells were detached with 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) and the reaction was stopped after 5 min by growth medium. Then the cell number was calculated and cells were seeded at desired densities for further experiments.

2.4.2. Quantification of alkaline phosphatase (ALP) activity

For these experiments, the C2C12 cells were seeded in 96-well plates at a density of 2×10^4/96-well in a normal growth medium. After 18 h, the cells were washed with phosphate-buffered saline

and refreshed with DMEM containing 2% fetal bovine serum as well as 100 ng/ml BMP-2 with or without CHS. The ALP activity was measured after 3 d. For this purpose, the cells were lysed by adding 40 μl of 0.5% Triton 100 in distilled water at RT with subsequent agitation on an orbital shaker (IKA, Staufen, Germany) at 300 rpm for 30 min. From that lysate, 20 μl were transferred to a new 96-well plate and 40 μl of 1 mg/ml *p*-nitrophenylphosphate (Carl-Roth GmbH) in 0.5 M 2-amino-2-methyl-1-propanol buffer (AMP) with pH value of 10.3 was added for determining the ALP activity. After 15 min incubation at 37 °C the absorbance at 405 nm was measured in a microplate reader (BMG Labtech, Offenburg, Germany). The measurements were done in quadruplicates and mean ± standard deviations were calculated.

2.4.3. Determination of the cellular protein content

To determine the influence of the modified polysaccharides on the viability of the cells, the protein content was measured. For this aim, the residual 20 μl of the cell lysate from the ALP activity measurement were used and 80 μl of bicinchoninic acid (Pierce, Rockford, USA) containing 0.08% $Cu(II)SO_4$ were added to the lysate with following 20 min incubation at RT. The amount of the protein was quantified as the absorbency shift of the dye to 550 nm and was measured in a microplate reader (BMG Labtech).

3. Results and discussion

3.1. Preparation of CHS

Fig. 1 depicts the ^{13}C NMR spectra of chitosan and two prepared CHS. A new signal at 66.7 ppm is visible within the spectrum of CHS, which is ascribed to sulfation of primary hydroxyl groups ($C6_S$), while C6 without sulfate groups at 6-*O*-position shows a peak at 60.4 ppm. Because both signals are derived from C6, the partial DS_S due to sulfate groups at 6-*O*-position (DS_{S6}) can be calculated based on the integrals of both peaks. Without the peak at 60.4 ppm, the DS_{S6} can be regarded as 1 (Fig. 1). Thus, the DS_S at other positions can be estimated according to the difference between the total DS_S and DS_{S6}.

C1 and C2 within repeating units of CHS present similar chemical shifts as those of chitosan. With sulfation at 3-*O*-position, $C3/C3_S$ have signals at 79 ppm. Other carbons within CHS, i.e. C4/5, show different chemical shifts compared to chitosan, which is due to the introduction of sulfate groups into chitosan chains.

The total DS_S of CHS in the range of 0.86–1.67 were determined via elemental analysis as can be seen in Table 1. By varying the amounts of chlorosulfonic acid, reaction temperature or duration, the total DS_S can be regulated. Generally, it rises with higher amounts of sulfating agents, while higher reaction temperature and longer reaction duration reduced the total DS_S. Besides chlorosulfonic acid, sulfamic acid can also be used to sulfate chitosan homogeneously (Table 1, CHS2).

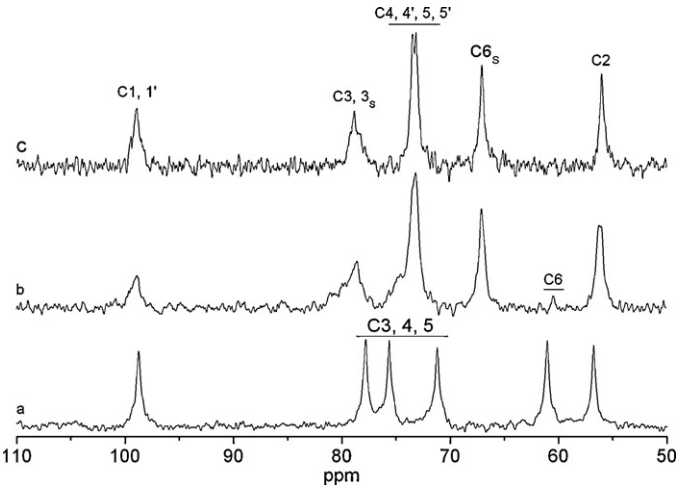

Fig. 1. ^{13}C NMR spectra (110–50 ppm) of (a) chitosan in 1% acetic acid/D_2O, (b) CHS11 (total DS_S = 1.59) and (c) CHS13 (total DS_S = 1.67) in D_2O at RT.

Appling other sulfating agents including SO_3-DMF/pyridine complex and sulfuric acid, CHS could be prepared non-homogeneously (with Method II). Various total DS_S could be received according to Table 2.

3.2. FT Raman spectroscopy

Fig. 2 depicts the FT Raman spectra of chitosan and CHS exhibiting distinct total DS_S. Comparing the spectra, some changes due to the sulfation of chitosan are visible.

First, new bands emerged at 1070, 1014, between 823 cm^{-1} and 834 cm^{-1}, around 588 cm^{-1} as well as at 417 cm^{-1}. The signal at 1070 cm^{-1} is attributed to stretching vibrations υ(O=S=O), while the band around 588 cm^{-1} is ascribed to deformation vibrations δ(O=S=O). The new band between 823 cm^{-1} and 834 cm^{-1} arises from the stretching vibrations υ(C–O–S). The band at 417 can be assigned to the deformation vibrations $\delta(SO_3)$ (Cabassi, Casu, & Perlin, 1978; Socrates, 2001; Zhang et al., 2010). The presence of these new bands suggests a successful introduction of sulfate groups into chitosan chains. Moreover, the intensities of these new peaks increase with rising total DS_S, which means that these intensities should correlate positively with the total DS_S. In addition, the

Table 2
Synthesis of CHS using other sulfating agents with sulfation method II with total DS_S and the intensities of band at 1070 cm^{-1} or band intensity ratios between the marker bands and the band at 1384 cm^{-1}.

CHS	Molar ratio[a]	T (°C)/t (h)[b]	Total DS_S [c]	I_{1070}	I_{1070}/I_{1384}	I_{1012}/I_{1384}	I_{822}/I_{1384}
Sulfation with SO_3-DMF complex							
CHS15	4	50/3	0.82	0.217	2.260	0.656	0.489
CHS16	4	70/3	1.17	–	–	–	–
Sulfation with SO_3-pyridine complex							
CHS17	3	50/5	1.09	0.340	3.063	0.721	0.541
CHS18[d]	6	70/26	1.73	0.636	4.969	1.047	0.688
Sulfation with sulfuric acid							
CHS19	4	50/1	1.73	–	–	–	–
CHS20	6	50/1	1.72	0.58	5.133	1.266	0.584

[a] Molar ratio in mol sulfating agent per mol GlcN units. For the preparation of CHS19 and 20, 8 mol acetic anhydride per mol GlcN units were added with sulfuric acid together.
[b] T (°C)/t (h): reaction temperature in °C and reaction duration in hours.
[c] Total DS_S of CHS were determined with elemental analysis.
[d] For the preparation of CHS18, chitosan with the viscosity of 7 mPa s was used.

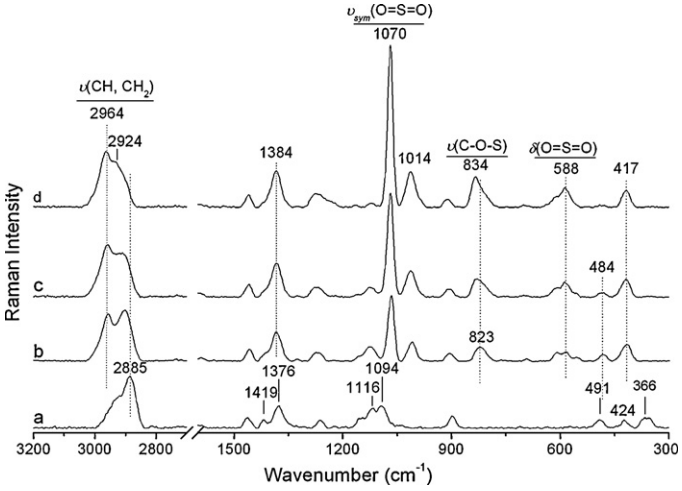

Fig. 2. FT Raman spectra (3200–300 cm^{-1}) of (a) chitosan, (b) CHS15 (total DS_S = 0.82), (c) CHS17 (total DS_S = 1.09) and (d) CHS14 (total DS_S = 1.67) at RT.

peak maximum of the signal due to υ(C–O–S) shifts to higher wave number with elevating total DS_S, while the peak maxima of the other new peaks stay constant.

Second, the band at 2885 cm^{-1} within the spectrum of chitosan shifts to 2924 cm^{-1} after sulfation. A new signal at 2964 cm^{-1} is notable and its intensity rises with increasing total DS_S. Within the spectrum of CHS14, this new peak turns to be the dominant one and the band at 2924 cm^{-1} becomes only a shoulder. In the range of 2800–3000 cm^{-1}, the signals are normally derived from stretching vibrations of CH or CH_2 groups (Atalla, 1976; Schenzel & Fischer, 2001; Socrates, 2001). Thus, the signals at 2885, 2924 and 2964 cm^{-1} can be attributed to these vibrations. The disappearance of the signals at 2885 cm^{-1} should be due to the presence of the sulfate groups, which is the only difference compared to chitosan. Especially, the sulfate groups should have changed the vibration modes of CH and CH_2 groups, so that the peak at 2964 cm^{-1} becomes the dominant one within the spectrum of CHS in comparison to the peak at 2885 cm^{-1} within the spectrum of chitosan.

Third, the bands at 1419, 1116 and 1094 cm^{-1} become weaker or even disappear after sulfation. These three bands are ascribed to vibrations of polysaccharide's backbones as known from the Raman spectrum of cellulose (Atalla, 1976; Schenzel & Fischer, 2001). The bands at 1116 cm^{-1} and 1094 cm^{-1} can be derived from the symmetric vibrations of glycosidic bonds, as shown for cellulose. Within the spectrum of CHS, the peak at 1419 cm^{-1} shows lower intensities and even disappears if total DS_S of CHS is higher than 1 (Fig. 2). The intensity of the band at 1116 cm^{-1} becomes lower with increasing total DS_S and the band at 1094 cm^{-1} is no more visible after sulfation (Fig. 3). The band at 1376 cm^{-1} ascribed to chitosan backbone shifts slightly to higher wave number of 1384 cm^{-1} and its intensity stays constant with rising total DS_S.

Finally, the signal at 491 cm^{-1} shifted to 484 cm^{-1} after sulfation. Within the spectrum of CHS exhibiting high total DS as 1.67, the signal at 484 cm^{-1} is hardly observable. Moreover, the signals at 424 cm^{-1} and 366 cm^{-1} disappear after sulfation.

For the purpose of quantifying the total DS_S of CHS, the signals at 1070, 1014 and around 830 cm^{-1} are chosen as marker bands. The signal at 1384 cm^{-1} can be used as internal standard, because it is ascribed to the vibrations of chitosan backbone (Fig. 2). The intensities of marker bands and internal standard were acquired from the Raman spectra of chitosan and CHS. The band intensity ratios can be calculated and are found in Tables 1 and 2. Both the intensity of band at 1070 cm^{-1} and the band intensity ratios between marker bands and internal standard are used as Raman analysis parameters for the quantification.

The calibration curves are generated after plotting the Raman analysis parameters against the total DS_S. The linear regression parameters are visible in Table 3. Besides using the band intensity ratio between the band at 830 cm^{-1} and internal standard, linear correlations were obtained with high correlation coefficients $r > 0.95$, suggesting positive relationships between these Raman analysis parameters and the total DS_S determined by elemental analysis. The use of the parameter, I_{1070}/I_{1384}, exhibits the highest correlation coefficient of 0.976. The other two parameters, I_{1070} and I_{1070}/I_{1384}, deliver similar correlation coefficients of 0.955 and 0.951. Thus, Raman spectroscopy presents another alternative for determining the total DS_S of CHS and the band intensity ratio as I_{1070}/I_{1384} provides the best quantifying method for the total DS_S between 0 and 1.73.

Table 3
Linear regression parameters for the calibration curves in Fig. 3 [a].

	Raman parameters					
	a	*b*	*r*	*SD*	*p*	*n*
I_{1070}	0.739	0.377	0.955	0.050	19	<0.0001
I_{1070}/I_{1384}	−0.155	2.928	0.976	0.281	19	<0.0001
I_{1014}/I_{1384}	0.093	0.582	0.951	0.081	19	<0.0001
I_{830}/I_{1384}	0.069	0.427	0.911	0.083	19	<0.0001

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

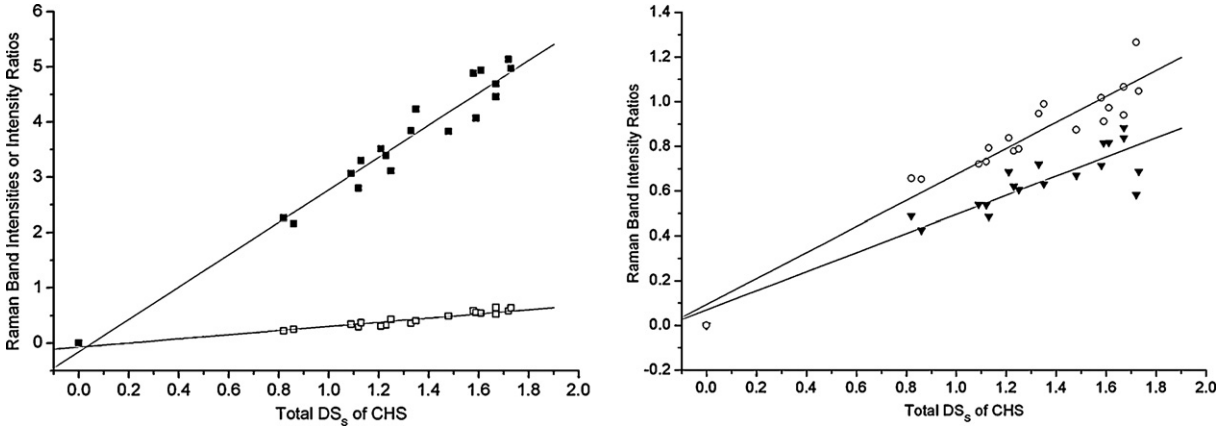

Fig. 3. Calibration curves obtained by plotting the Raman analysis parameters against total DS_S of CHS as: (□) I_{1070}, (■) I_{1070}/I_{1384}, (○) I_{1014}/I_{1384} and (▼) I_{830}/I_{1384}.

Table 4
CHS applied for determination of biological activity.

CHS	Sulfation methods	Molar ratio[a]	T (°C)/t (h)[b]	DS_{S6} [c] (^{13}C NMR)	Total DS_S [c]	DS_{S2+3} [c]
CHS8	I	13	50/3	0.87	1.35	0.48
CHS13	II	13	50/3	1	1.67	0.67
CHS21	I	6	RT/5	1	1.29	0.29

[a] Molar ratio in mol sulfating agent per mol GlcN units. 20 ml formic acid was used for CHS21.
[b] T (°C)/t (h): reaction temperature in °C and reaction duration in hours.
[c] Total DS_S of CHS were determined with elemental analysis. DS_{S6} (partial DS_S at 6-*O*-position) is estimated via ^{13}C NMR.

3.3. Determination of the biological activity of CHS

To determine the biological activity of CHS, we investigated the effects of CHS8, CHS13 and CHS21 with diverse total DS_S and DS_{S6} (Table 4) on the osteogenic differentiation induced by BMP-2. As an early osteogenic marker, the quantity of the alkaline phosphatase (ALP) is frequently used for that purpose, because it is easy to verify with a great number of samples and diverse concentrations. As shown in Fig. 4, all derivatives gave increased ALP activity in C2C12 mouse myoblasts over a concentration range from 0.3 μg/ml to 10 μg/ml. The ALP activity increased from the base level of 0.02 to more than 0.1 depending on the derivative and the concentration. With a rising overall DS_S the ALP activity became higher in the lower concentration range, but CHS13 led to a steady decline in the BMP-2 induced osteogenic activity with concentrations higher than 0.6 μg/ml. On the contrary, the ALP activity increased with higher concentrations with application of CHS21 exhibiting much lower DS_S and gave rise to higher values than CHS13 at 5 and 10 μg/ml (Fig. 4A).

BMP-2 is known to bind heparin via an interaction of positively charged cavities of BMP-2 and negatively charged sulfate and carboxylate groups within heparin (Ruppert, Hoffmann, & Sebald, 1996; Scheufler, Sebald, & Hülsmeyer, 1999). It was also stated that heparin or heparan sulfate play a role in binding of BMP-2 to its BMP receptor (BMPR) and the initiation of the BMP signal transduction (Irie, Habuchi, Kimata, & Sanai, 2003; Kanzaki et al., 2008). As shown previously, highly sulfated chitosans with substitution at 2-*N*- or 6-*O*-position, or at both 2-*N*- and 6-*O*-position led to a strong stimulation of BMP-2 induced ALP activity at 0.6 μg/ml. However, at higher concentrations all derivatives provoked a strong reduction in ALP values down to, or even below the base level at 10 μg/ml (Zhou et al., 2009). In our studies, this strong decline was not observed even for the relatively low sulfated derivative. The reason for that can possibly be the different distribution of the sulfate groups within the repeating units in comparison to the products in Zhou et al. (2009). As known, low concentrations of a highly 2-*N*-/6-*O*-sulfated chitosan enhanced the binding of BMP-2 to its receptor, but 10 μg/ml almost abolished that binding (Zhou et al., 2009). This correlates with our results on ALP activity with CHS13. The lower sulfated derivatives are possibly less effective in mediating the BMP–BMPR binding, though higher amounts can lead to increased ALP activity as could be seen for CHS21.

Parallel to the ALP activity we verified the overall protein content that represents a marker for viability/proliferation. Higher concentrations of the used CHS provoked a decline below the base level for all samples. The influence of the overall DS_S was opposite to the effects on ALP activity and CHS with a higher total DS_S provoked a stronger negative impact on the protein content. At low concentrations, CHS with a lower total DS_S led to a small stimulation of viability, but the highest sulfated CHS13 impaired the viability over the whole concentration range. Probably there is a correlation between the stronger impact on viability and the low ALP activity for CHS13 at higher concentrations. On the other hand, CHS13 reduced the viability slightly at low concentrations, which is contrary to the strongly enhanced ALP activity. An explanation for the observed effects can be an increasing toxicity of CHS with higher total DS_S. Another reason can be attributed to cellular mechanism independent of the activity of BMP-2. Whereas the osteogenic activity is triggered by the initiation of the BMP-2 pathway, the cell proliferation could be directly affected by the binding of CHS to the cells, as shown for heparin (Ghosh, Eis, Mullaney, Ebert, & Gill, 1988; Reilly, Kindy, Brown, Rosenberg, & Sonenshein, 1989). These processes can also strongly rely on the total DS of polysaccharides derivatives (Peschel et al., 2010). Taken together, CHS with a higher total DS_S leads to a stronger BMP-2 induced osteogenic activity, but at the same time, it exhibits a stronger negative impact on viability/proliferation than CHS with a lower total DS_S.

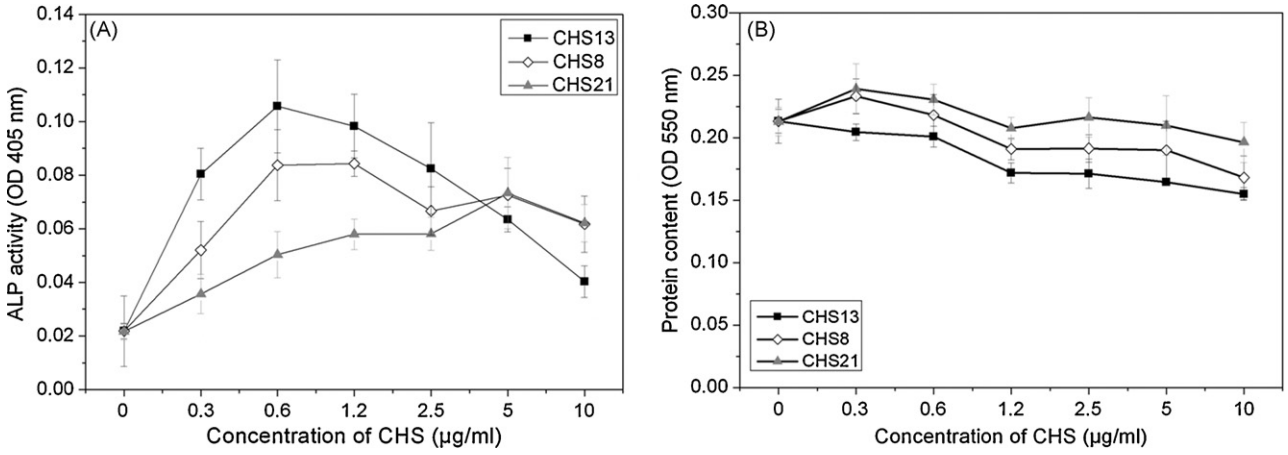

Fig. 4. Effects of CHS on BMP-2 induced osteogenic activity and protein contents in mesenchymal C2C12 mouse myoblasts. Cells were grown in the presence of 100 ng/ml BMP-2 and increasing concentrations of CHS for 3 d. (A) ALP activity. For CHS13 it was significantly higher than CHS21 with the concentration of CHS up to 2.5 μg/ml ($p < 0.02$). (B) Protein contents. CHS13 was significantly lower than CH21 over the range of applied concentrations up to 10 μg/ml ($p < 0.02$).

4. Conclusion

Novel CHS with various total DS_S between 0.86 and 1.73 were prepared homogeneously and non-homogeneously with chlorosulfonic acid or non-homogeneously with SO_3-DMF complex, SO_3-pyridine complex as well as sulfuric acid. By varying the sulfation parameters including the sulfation temperature, duration and the amount of the sulfating agents, the total DS_S can be regulated.

The sulfation of chitosan was confirmed via ^{13}C NMR and FT Raman spectroscopy. Within the FT Raman spectra of CHS, characteristic bands attributed to the sulfate groups are visible in the range of 1200 cm^{-1} and 300 cm^{-1}. Raman band intensity of the band at 1070 cm^{-1} or band intensity ratios between marker band at 1070 cm^{-1} or 1014 cm^{-1} and internal standard at 1384 cm^{-1} can be used as analysis parameters for quantifying the total DS_S. After plotting these Raman analysis parameters against the total DS_S, calibration curves with correlation coefficients of more than 0.95 were obtained. Thus, Raman spectroscopy presents an alternative for determining the total DS_S of CHS.

Finally, CHS have a strong capacity to enhance the osteogenic potential of BMP-2, which make them highly interesting for in vitro and in vivo applications in bone repair or treatment of diseases.

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