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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