Carbohydrate Polymers 82 (2010) 92–99

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Synthesis of carboxyl cellulose sulfate with various contents of regioselectively introduced sulfate and carboxyl groups

Kai Zhang[a], Dieter Peschel[b], Thomas Klinger[c], Kathrin Gebauer[d], Thomas Groth[b], Steffen Fischer[a,*]

[a] Institute of Wood and Plant Chemistry, Dresden University of Technology, Pienner Str. 19, D-01737 Tharandt, Germany
[b] Biomedical Materials Group, Department Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Heinrich-Damerow-Straße 4, D-06120 Halle (Saale), Germany
[c] Institute of Soil Science and Site Ecology, Pienner Str. 19, D-01737 Tharandt, Germany
[d] Institute of Power Engineering, Helmholtzstr.10, Dresden University of Technology, D-01069 Dresden, Germany

ARTICLE INFO

*Article history:*
Received 28 February 2010
Received in revised form 5 April 2010
Accepted 13 April 2010
Available online 20 April 2010

*Keywords:*
Cellulose sulfate
Carboxyl cellulose
Carboxyl cellulose sulfate
FT Raman spectroscopy
$^{13}$C NMR spectroscopy

ABSTRACT

Both sulfate and carboxyl groups are found in many glycosaminoglycans exhibiting diverse biological activities, such as heparin. Present study reports on the preparation of cellulose derivatives containing both sulfate and carboxyl groups that were regioselectively introduced into anhydroglucose units (AGU) of cellulose. The products – carboxyl cellulose sulfates (COCS) – with various contents of both functional groups were obtained by two synthesis routes. One way started with sulfation of cellulose yielding cellulose sulfate (CS) and was followed by TEMPO-mediated oxidation of CS. In another way, cellulose at first underwent TEMPO-mediated oxidation yielding carboxyl cellulose (COC). Subsequently, acetosulfation of the COC was carried out. The products were characterized by diverse analysis methods, and the amounts of both functional groups in CS, COC and COCS were determined. Finally, the biological activity of COCS was examined.



## 1. Introduction

Glycosaminoglycans, such as heparin and heparan sulfate, demonstrate important functions in regulation of cellular proliferation and differentiation (Carroll & Koch, 2003). In this regard, heparin and heparan sulfate can bond a large variety of growth factors and cytokines (Capila & Linhardt, 2002; Darnell, Lodish, & Baltimore, 1990; Rabenstein, 2002). Although both heparin and heparan sulfate contain carboxyl, sulfate and acetyl groups in their repeating units, but naturally occurring glycosaminoglycans exhibit various molecular compositions and therefore biological activities (Bourin & Lindahl, 1993; Mulloy, Mourão, & Gray, 2000; Rabenstein, 2002). To overcome this limitation, diverse glycosaminoglycan–analogues have been realized by sulfating other natural polysaccharides.

Cellulose, chitosan, pullulan, colominic acid and others, which can be obtained in huge quantities with specific molecular weights, have been used as starting materials for the synthesis of such glycosaminoglycan-analogues (Alban, Schauerte, & Franz, 2002; Groth & Wagenknecht, 2001; Kunou, Koizumi, Shimizu, Kawase, & Hatanaka, 2000; Xing et al., 2004). For example, cellulose as a renewable resource has been applied for years for the synthesis of many derivatives, such as cellulose acetate, carboxymethyl cellulose, and cellulose sulfate (CS) (Fischer et al., 2008; Heinze & Koschella, 2005; Hettrich, Wagenknecht, Volkert, & Fischer, 2008; Philipp & Wagenknecht, 1983).

As a water-soluble derivative, CS exhibited diverse biological effects, such as inhibition against human immune deficiency virus (HIV) infection and anticoagulant activity (Groth & Wagenknecht, 2001; Yamamoto et al., 1991). CS with high degree of substitution (DS) could bond b-FGF in the same way as natural heparin and could strongly promote FGF-induced proliferation (Peschel et al., in press; Zhang, Peschel, Brendler, Groth, & Fischer, 2009).

In order to prepare CS with different substitution patterns, various sulfation strategies have been developed. CS can be synthesized through either heterogeneous or homogeneous sulfation of cellulose or some cellulose derivatives. Possible solvents can be *N*,*N*-dimethylformamide (DMF), pyridine, dimethyl sulfoxide, $N_2O_4$·DMF system as well as ionic liquids. Also various sulfating agents have been applied for the sulfation, such as $SO_3$, chlorosulfuric acid and $SO_3$·DMF complex (Baumann, Richter, Klemm, & Faust, 2000; Philipp, Nehls, & Wagenknecht, 1987; Wang, Li, Zheng, Normakhamatov, & Guo, 2007). Recently, procedures for sulfation of cellulose as acetosulfation or as homogeneous sulfation in ionic liquids have been developed (Gericke, Liebert, & Heinze, 2009; Hettrich et al., 2008; Wang, Li, Xiao, & Wu, 2009; Zhang et al., 2009).

Another interesting cellulose derivative, regioselectively oxidized cellulose, also attracts more attention now. The selec-

* Corresponding author. Tel.: +49 0 35203 38 31239; fax: +49 0 35203 38 31201.
*E-mail address:* sfischer@forst.tu-dresden.de (S. Fischer).

0144-8617/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2010.04.027

tively oxidized cellulose can be obtained through oxidation by the $NaNO_2/H_3PO_4/NaBH_4$ system or by the 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)/NaBr/NaClO system (Besemer, de Nooy, & van Bekkum, 1998; Chang & Robyt, 1996). The TEMPO-mediated oxidation was normally carried out in aqueous systems at pH 10–11 at room temperature (RT) or 0 °C (Besemer et al., 1998; Chang & Robyt, 1996; Isogai & Kato, 1998). TEMPO-oxidized cellulose was found to be able to form nanofibers and can be used as ion absorber (Saito & Isogai, 2005; Saito, Kimura, Nishiyama, & Isogai, 2007).

The product after a TEMPO-mediated oxidation was predominantly not soluble in water and the recovery of this water-insoluble fraction could be higher than 80% (Saito & Isogai, 2004). The TEMPO-mediated oxidation occurred selectively on the primary hydroxyl groups. Both carboxylate and aldehyde groups were introduced into the surfaces and disordered regions of cellulose I crystallites, while no oxidation was found inside the cellulose I crystallites (Saito & Isogai, 2004, 2005). Even after prolonged oxidation duration up to 24 h at pH 10–11, the native cellulose did not become soluble in aqueous media (Isogai & Kato, 1998). But if the TEMPO-mediated oxidation was applied to regenerated, mercerized or ball-milled cellulose, quantitative cellulose could be converted into cellouronic acid (Isogai & Kato, 1998; Isogai, Yanagisawa, & Isogai, 2009). Additionally, it was evidenced that a decrease of crystal size due to the degradation in the amorphous areas occurred during the TEMPO-mediated oxidation (Montanari, Roumani, Heux, & Vignon, 2005).

Although many groups have devoted themselves to the monofunctionalisation of cellulose, little efforts have been made towards the cellulose derivatives with both sulfate and carboxyl groups. Cellulose derivatives bearing both functional groups may possess new properties and have new potential applications. In this research, two different synthesis routes resulting in COCS containing both sulfate and carboxyl groups were developed and COCS in diverse patterns were prepared as well as characterized. Finally, chosen COCS were investigated for their biological activity with regard to mitogenic activity on 3T3 mouse fibroblasts.

## 2. Experimental

### 2.1. Materials

Microcrystalline cellulose (MCC) with an average degree of polymerisation (DP) of 276 was provided by J. Rettenmaier & Söhne GmbH (Rosenberg, Germany). Pulp V-81 (AC, with 97.0% alpha cellulose) with an average DP of 1180 was purchased from Buckeye Technologies Inc. (Memphis, USA). *N*,*N*-dimethylformamide (DMF) was freshly distilled before use and deionized water was applied in all experiments. Applied chemicals were all of analysis grade and used as received.

### 2.2. Synthesis

#### 2.2.1. Preparation of CS and oxidation of CS

The sulfation of cellulose was carried out as described in Zhang et al. (2009). For a typical acetosulfation, 2.5 g cellulose were suspended in 125 ml anhydrous DMF at RT for over 14 h. The reaction agent consisting of chlorosulfuric acid and 12 ml acetic anhydride in DMF was dropped into the cellulose suspension under vigorous stirring within 15 min. After that, the temperature was raised to 50 °C and the mixture was kept at 50 °C for 5 h. Then, the solution was cooled down to RT and poured into a saturated solution of anhydrous sodium acetate in ethanol. The precipitate was gained through centrifugation and washed with a 4% sodium acetate solution in ethanol. After a deacetylation with 1 M ethanolic solution of sodium hydroxide for 15 h, the pH value was adjusted to 7.5–8.0 with acetic acid/ethanol (50/50, w/w). The product was then washed with ethanol, dissolved in water, filtered, dialyzed in deionized water and lyophilized.

In order to introduce carboxyl groups in CS, a TEMPO-mediated oxidation of CS was executed. 0.5 g dried CS was dissolved in 30 ml water and the oxidation agent consisting of TEMPO, NaBr and 12% aqueous NaOCl (0.05 ml/mg TEMPO) in water (1 ml/mg TEMPO) was prepared under continuous stirring, according to the molar ratios between them and residual primary hydroxyl groups. The oxidation was started after slowly adding the oxidation agent to the solution of CS under stirring. The remaining NaOCl was dropped into the solution to maintain the pH at $10.5 \pm 0.1$. After the addition of the rest of NaOCl, the pH was maintained constant at $10.5 \pm 0.1$ for a designated duration using 0.5 M aqueous NaOH solution. Thereafter, 5 ml methanol were added to stop the oxidation and the pH was adjusted to 7.5 with 0.5 M aqueous HCl solution. Then, the system was poured into 5 volumes of ethanol and centrifuged after stirring for 0.5 h. The precipitate was washed three times with ethanol/water (80/20, v/v), dissolved in water, filtered, dialysed against deionized water and lyophilized.

#### 2.2.2. Preparation of COC and acetosulfation of COC

5 g MCC in 200 ml water were mixed for 3 min with a high speed Ultra-Turrax homogenizer and then stirred with a magnetic stirrer. The oxidation agent consisting of 0.077 g TEMPO, 1.557 g NaBr and 4 ml 12% aqueous NaOCl solution in 25 ml water was prepared under continuous stirring until complete dissolution. The oxidation was started after slowly adding the oxidation agent to the cellulose suspension or to the solution of CS under stirring. The other 35 ml NaOCl solution were dropped into the solution to maintain the pH at $10.5 \pm 0.1$. After adding the NaOCl solution, the pH was maintained constant for 0.5 (yielding product COC1) and 4 (yielding product COC2) hours using 0.5 M aqueous NaOH solution. Thereafter 7.5 ml of methanol were added to stop the oxidation and the pH was then adjusted to 7.5 with 0.5 M aqueous HCl solution. The insoluble fraction and the supernatant were separated by centrifugation. The insoluble fraction was then washed three times with 150 ml ethanol/water (50/50, v/v) and then lyophilized. The product was lowly oxidized, water-insoluble 6-carboxyl cellulose (COC). The supernatant was precipitated in 800 ml ethanol and the precipitate was washed twice with ethanol/water (95/5, v/v), twice with ethanol and then lyophilized. The obtained product was β-1,4-linked polyglucuronic acid in form of sodium salt and the primary hydroxyl groups were fully converted into carboxylate groups.

In order to introduce the sulfate groups in COC, acetosulfation was carried out as described in Section 2.2.1. Briefly, 1.5 g COC in 50 ml DMF were converted by reaction mixture consisting of chlorosulfuric acid or sulfuric acid and 7.05 ml acetic anhydride in DMF. After sulfation, the products were then prepared as in Section 2.2.1.

### 2.3. Characterization of products

#### 2.3.1. FT Raman and $^{13}C$ NMR spectroscopy

FT Raman spectra were recorded on a Bruker MultiRam spectrometer (Bruker Optik GmbH, 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 3500–150 $cm^{-1}$ using an operating spectral resolution of 3 $cm^{-1}$ and a laser power output of 100 mW.

The $^{13}C$ NMR spectra were obtained at RT using a Bruker DPX 400 spectrometer (Bruker Optik GmbH, Etlingen, Germany) at a frequency of 100.13 MHz and with 30° pulse width, 0.3 s acquisition time and a relaxation delay of 3 s. Solutions of the samples with con-

centrations of 5% (wt.%) were prepared in $D_2O$ and scans between 5000 and 20,000 were accumulated.

*2.3.2. Optical emission spectroscopy with inductively coupled plasma (ICP-OES spectroscopy)*

The total contents of the elements – sodium and sulphur – can be measured with ICP-OES spectroscopy. Up to 10 mg of samples were dissolved in 10 ml deionized water and 250 μl 32% aqueous $HNO_3$ were added. Then, the solutions of the samples were analyzed with the atomic emission spectrometer (ICP-OES) Spectro Ciros$^{CCD}$ (Spectro, Germany). For the purpose of determining the total contents of sodium and sulphur, wavelengths of 589.592 and 182.034 nm were used for the detection, respectively.

*2.3.3. Elemental analysis*

The contents of carbon, hydrogen and nitrogen were determined with Elemental Analyser vario El from Elementar (Hanau, Germany). The sulphur contents were measured with Elemental Analyser Eltra CS 500 from Eltra (Neuss, Germany). The total DS ascribed to sulfate groups (DSs) of the products ascribed to sulfation can be calculated according to the following equation:

$$\text{Total DSs} = (S\%/32)/(C\%/72) \quad \text{(I)}$$

*2.3.4. Determination of contents of carboxyl groups and $DS_{COO}$*

The contents of carboxyl groups in COC1 and COC2 were determined according to ASTM standard D1439-03(2008)e1 (American Society for Testing and Materials, 2008).

The DS derived from carboxyl groups ($DS_{COO}$) in COCS could be calculated according to the following equations that are based on the change of the amounts of primary hydroxyl groups for the COCS (Eq. (II)) or the sodium and sulphur contents of COCS with sulfate groups at 3-O-position (Eq. (III)):

$$DS_{COO} = DS_{C6OH\,before\,oxidation} - DS_{C6OH\,after\,oxidation} = DS_{C6OH\,before\,oxidation} - DS_{S6} \times \frac{A_{60\ ppm}}{A_{67\ ppm}} \quad \text{(II)}$$

$$DS_{COO} = [(Na\%/23)/(S\%/32) - 1] \times (\text{total } DS_S) \quad \text{(III)}$$

$DS_{C6OH\ before\ oxidation}$, $DS_{C6OH\ after\ oxidation}$: DS of unsubstituted primary hydroxyl groups before oxidation and after oxidation, determined by elemental analysis and $^{13}C$ NMR spectroscopy; $DS_{S6}$: $DS_S$ at C6 after oxidation; $A_{60\,ppm}$, $A_{67\,ppm}$: the area under the peaks at 60 and 67 ppm after integration of $^{13}C$ NMR spectra of the COCS.

*2.3.5. Determination of number-average degrees of polymerisation ($DP_n$)*

The $DP_n$ were determined by size exclusion chromatography (SEC) with PSS Suprema 3000 and 100 Å columns (Polymer Standards Service GmbH, Mainz, Germany). The detection was carried out with a Waters 410 reflective index (RI) detector (Waters Corporation, Milford, MA) and 0.1 mol/l NaCl aqueous buffer was used as mobile phase. The columns have been calibrated with pullulan standards (Sigma–Aldrich, Buchs, Switzerland). Empower Pro software (Waters Corporation, MA) was used for the analysis.

*2.4. Determination of the biological activity*

The biological activity of chosen COCS was investigated with cultures of 3T3-L1 fibroblasts (ATCC, Manassas, USA) in the presence as well as in the absence of the growth factor FGF-2 (Invitrogen, Karlsruhe, Germany). Cells were seeded at a density of 10.000 cells/well in black 96 well plates in Dulbecco's modified Eagle medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom) and 1% penicillin-streptomycin-fungizone (Promocell, Heidelberg, Germany) in a 37 °C humidified atmosphere of 5% $CO_2$ and 95% air with 24 h culture. Then, the cellulose derivatives were applied to the cells in DMEM without FBS at a concentration between 1 and 1000 μg/ml for 48 h in the presence or absence of 10 ng FGF-2/ml. The proliferation of the cells was determined using the Quant-iT$^{TM}$ PicoGreen dsDNA quantification assay (Invitrogen). The fluorescent intensity was measured by a plate reader with excitation and emission wavelength of 485 and 520 nm, respectively (Fluostar Optima BMG Labtech, Offenburg, Germany).

## 3. Results

*3.1. Preparation of CS and COCS*

CS was prepared as described before and the total as well as partial $DS_S$ attributed to sulfation were determined (Zhang et al., 2009). While only total $DS_S$ of CS could be determined with elemental analysis, the partial $DS_S$ could be analyzed with $^{13}C$ NMR spectroscopy (Nehls, Wagenknecht, Philipp, & Stscherbina, 1994). CS with total $DS_S$ between 0.39 and 0.94 according to elemental analysis was obtained as starting material for further oxidation. The primary hydroxyl groups were predominantly sulfated with application of chlorosulfuric acid during the acetosulfation at 50–70 °C (Table 1). The $DS_{S2}$ remained very low with a use of 0.55 or 0.85 mol chlorosulfuric acid per mol AGU, while it increased significantly with utilization of 3 mol chlorosulfuric acid per mol AGU. Thus, it can be proposed that the chlorosulfuric acid preferred a selective sulfation of primary hydroxyl groups during the acetosulfation and a controlled introduction of sulfate groups into 6-O-position could be conducted.

The CS dissolved in water could be oxidized and Fig. 1 illustrates the $^{13}C$ NMR spectra of chosen CS, COCS and β-1,4-linked polyglucuronic acid. Based on the spectra of CS and COCS, the sig-

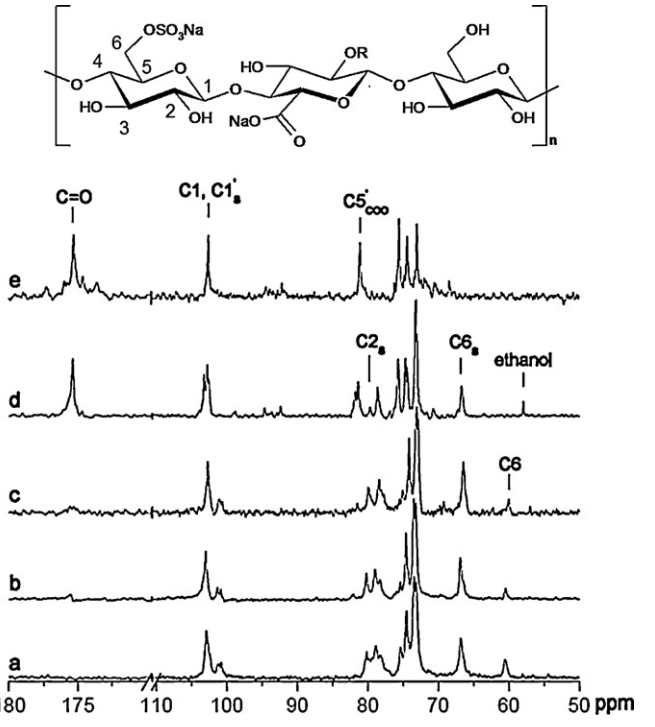


**Fig. 1.** Simplified structure of COCS with R = –H or –$SO_3Na$ (above); $^{13}C$ NMR spectra (180–50 ppm) of (a) CS7, (b) COCS7, (c) COCS8, (d) COCS2 and (e) β-1,4-linked polyglucuronic acid (bottom).

**Table 1**
Preparation of CS with chlorosulfuric acid and 8 mol acetic anhydride/mol AGU for 5 h.

| Samples | Starting materials | Molar ratio[a] | $T$ (°C)[b] | $DS_S$ ($^{13}$C NMR)[c] | | | Total $DS_S$[c] (elemental analysis) |
|---|---|---|---|---|---|---|---|
| | | | | $DS_{S6}$ | $DS_{S2}$ | $DS_{S3}$ | |
| CS1 | AC | 0.85 | 50 | 0.47 | 0.05 | 0 | 0.60 |
| CS2 | AC | 0.85 | 60 | 0.33 | 0.04 | 0 | 0.42 |
| CS3 | AC | 0.55 | 70 | 0.31 | 0.05 | 0 | 0.39 |
| CS4 | AC | 0.85 | 70 | 0.34 | 0.02 | 0 | 0.41 |
| CS5 | MCC | 0.85 | 50 | 0.48 | 0.05 | 0 | 0.59 |
| CS6 | MCC | 0.85 | 70 | 0.38 | 0.03 | 0 | 0.48 |
| CS7 | MCC | 3 | 50 | 0.73 | 0.19 | 0 | 0.94 |

[a] Molar ratio in mol chlorsulfuric acid per mol AGU.
[b] $T$ (°C): reaction temperature in °C.
[c] $DS_{S6,2\text{ or }3}$ represent the partial $DS_S$ at the corresponding 6-, 2- or 3-O-positions, determined by $^{13}$C NMR spectroscopy. Total $DS_S$ were determined by elemental analysis.

nals at 67 ppm reflecting the C6 with sulfate groups are still visible after oxidation and new signals at 175.4 ppm represent the carboxyl groups at C6.

The TEMPO-mediated oxidation results in conversion of the primary hydroxyl groups into carboxyl groups. According to Fig. 1e, the β-1,4-linked polyglucuronic acid with complete oxidation of the remaining primary hydroxyl groups shows no peak at 60 ppm and the new signals around 175.3 ppm indicate the presence of carboxyl groups (Isogai & Kato, 1998). Alike, no peak can be found at 60 ppm in the spectrum of COCS2 and therefore, a conclusion that the residual primary hydroxyl groups in COCS2 were totally oxidised can be drawn (Fig. 1d). However, the remaining primary hydroxyl groups of COCS7 and COCS8 were not entirely converted according to the spectra b and c in Fig. 1. Because although a new small peak at 175.4 ppm attributed to carboxyl groups appeared, a small peak at 60 ppm ascribed to C6 with hydroxyl groups can still be seen.

The $DS_{COO}$ of COCS according to the amounts of carboxyl groups can be determined (Table 2). If the amounts of primary hydroxyl groups before and after the oxidation are simply analysable, the amounts of introduced carboxyl groups can be calculated based on this difference. After TEMPO-mediated oxidation, COCS with $DS_{COO}$ between 0.10 and 0.67 were synthesized (Table 2).

According to Table 2, the COCS from MCC exhibit lower $DS_{COO}$ up to 0.15. Even when the oxidizing agents were applied in doubled amounts, no obvious increase could be obtained (COCS7 and 8). In contrast, the $DS_{COO}$ of COCS from AC could be raised when more oxidizing agents were used (COCS1 and 2). A complete oxidization of residual hydroxyl groups was realized (COCS2 with $DS_{COO}$ of 0.67).

However, during TEMPO-mediated oxidations of CS after various oxidation durations, no significant reduction of total or partial $DS_S$ can be observed (Table 2).

FT Raman spectroscopy provides some other features when applied to characterize the obtained products. Fig. 2 shows the

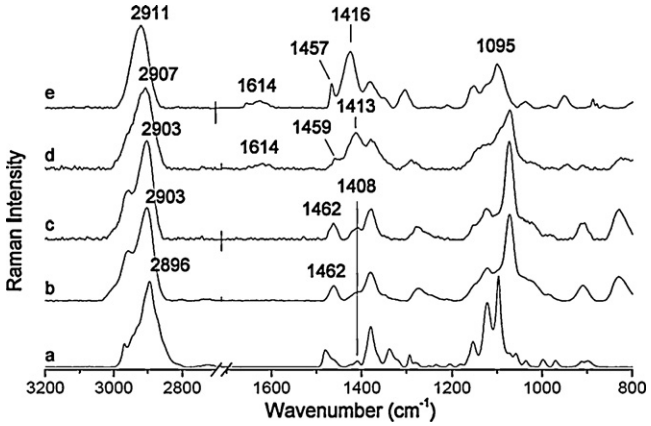


**Fig. 2.** FT Raman spectra (3200–800 $cm^{-1}$) of (a) MCC, (b) CS7, (c) COCS8, (d) COCS2 and (e) β-1,4-linked polyglucuronic acid.

Raman spectra of chosen products between 3200 and 800 $cm^{-1}$. New signals at 1274, 1072 and 830 $cm^{-1}$ can be seen in CS7 in comparison to cellulose and the details about the Raman spectra of CS were already reported (Zhang, Brendler, & Fischer, 2010). After oxidation, COCS8 with a $DS_{COO}$ of 0.15 presents a similar spectrum as that of CS7, except for the appearance of a shoulder near the peak at 1408 $cm^{-1}$. Within the spectrum of COCS2 with higher $DS_{COO}$, this peak was shifted to 1413 $cm^{-1}$ with stronger intensity (Fig. 2d). This peak can be attributed to the symmetric stretching vibrations of $COO^-$-groups (Socrates, 2001). Within the spectrum of β-1,4-linked polyglucuronic acid, this peak was shifted to 1416 $cm^{-1}$ and the intensity was also enhanced due to the presence of exclusive carboxylate groups on C6. Another new signal around 1614 $cm^{-1}$ was also found and can be ascribed to asymmetric stretching vibrations of carboxylate groups (Socrates, 2001).

**Table 2**
Preparation of COCS from CS in water at RT with a pH value of 10.5 ± 0.1.

| Samples | Starting cellulose/CS | Molar ratio[a] | | | $t$ (h)[b] | $DS_{S2}$ ($^{13}$C NMR)[c] | Total $DS_S$[c] | $DS_{S6}$[c] | $DS_{COO}$[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | TEMPO | NaBr | NaClO | | | | | |
| COCS1 | AC/CS2 | 0.05 | 1.5 | 8 | 3 | 0.06 | 0.40 | 0.34 | 0.42 |
| COCS2 | AC/CS2 | 0.1 | 3 | 16 | 3 | 0.04 | 0.37 | 0.33 | 0.67 |
| COCS3 | AC/CS3 | 0.05 | 1.5 | 8 | 3 | 0.06 | 0.30 | 0.24 | 0.38 |
| COCS4 | AC/CS4 | 0.05 | 1.5 | 8 | 3 | 0.04 | 0.36 | 0.32 | 0.25 |
| COCS5 | AC/CS4 | 0.05 | 1.5 | 8 | 20 | 0.02 | 0.37 | 0.35 | 0.10 |
| COCS6 | MCC/CS5 | 0.05 | 1.5 | 8 | 3 | 0.06 | 0.53 | 0.48 | 0.13 |
| COCS7 | MCC/CS7 | 0.05 | 1.5 | 8 | 1 | 0.19 | 0.91 | 0.72 | 0.13 |
| COCS8 | MCC/CS7 | 0.1 | 3 | 16 | 1 | 0.21 | 0.89 | 0.68 | 0.15 |
| COCS9 | MCC/CS7 | 0.05 | 1.5 | 8 | 3 | 0.20 | 0.92 | 0.72 | 0.10 |

[a] Molar ratio in mol oxidizing agents per mol primary hydroxyl groups.
[b] $t$: reaction duration in hours.
[c] $DS_{S2,S6,COO}$ demonstrate the partial DS of sulfate groups at 2- and 6-O-positions and DS of carboxyl groups at C6. Total $DS_S$ were determined by elemental analysis. $DS_{S2}$ was determined by $^{13}$C NMR spectroscopy and $DS_{S6}$ was calculated by the equation: $DS_{S6}$ = total $DS_S$ − $DS_{S2}$.

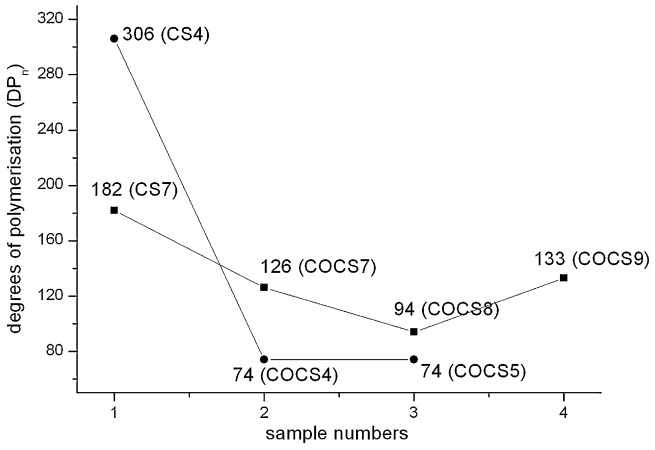


**Fig. 3.** The degrees of polymerisation ($DP_n$) of: ● 1: CS4, 2: COCS4, 3: COCS5; ■ 1: CS7, 2: COCS7, 3: COCS8, 4: COCS9.

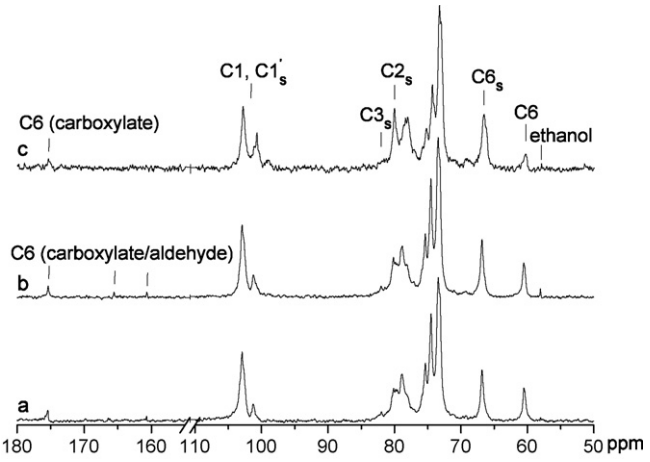


**Fig. 4.** $^{13}C$ NMR spectra (180–50 ppm) of (a) COCS10, (b) COCS11 and (c) COCS12.

Furthermore, the small peak at 2969 $cm^{-1}$ attributed to stretching vibrations of $CH_2$ groups in the spectrum of cellulose disappeared within the spectra of $\beta$-1,4-linked polyglucuronic acid, while it was still visible at 2957 $cm^{-1}$ in the spectrum of CS7 and COCS8. Even within the spectrum of COCS2, there is still a small shoulder with much lower intensity near the peak at 2907 $cm^{-1}$. The disappearance of the $CH_2$-signal is due to the conversion of $CH_2OH$ groups into COONa groups. Together with the reduction of the $CH_2$-signal, the peak ascribed to stretching vibrations of CH groups at 2896 $cm^{-1}$ was shifted to 2907 $cm^{-1}$ in COCS2 and 2911 $cm^{-1}$ in β-1,4-linked polyglucuronic acid.

Besides that, within the spectrum of β-1,4-linked polyglucuronic acid no peak of $CH_2$-bending vibrations can be found at 1462 $cm^{-1}$, which exists in the spectra of cellulose and CS. This is ascribed to the conversion of $CH_2OH$– into $COO^-$-groups and only a signal of 1457 $cm^{-1}$ is observable.

During the TEMPO-mediated oxidation, the molecular weights of the polysaccharide were strongly reduced (De Nooy, Besemer, Van Bekkum, Van Dijk, & Smit, 1996; Isogai & Kato, 1998). The measurement of molecular weights of chosen CS and COCS with SEC demonstrates that a significant decrease of $DP_n$ has taken place during the TEMPO-mediated oxidation. It is visible that all COCS have a lower $DP_n$ than the starting CS (Fig. 3).

The CS4 from AC has a $DP_n$ of 306, but $DP_n$ of the yielded COCS is only 74 (COCS4 and 5). For the CS7 (with a $DP_n$ of 182) from MCC, the decrease of the $DP_n$ after oxidation is also significant ($DP_n$ of 126 and 133 for COCS7 and 9), especially when more oxidizing agents were applied ($DP_n$ of 94 for COCS8).

### 3.2. Preparation of COC and COCS

The primary hydroxyl groups of cellulose can be oxidized by a TEMPO-mediated oxidation and two fractions were gained after oxidation. The yield of water-insoluble fraction was more than 90% and the contents of carboxyl groups in COC1 and COC2 were determined as 0.74 and 0.69 mmol/g (Table 3). The water-soluble fraction was found to be totally oxidized β-1,4-linked polyglucuronic acid, because in its $^{13}C$ NMR spectrum no signal at 60 ppm can be found and the new signals appear around 175 ppm, which represent the signals of carbonyl groups on C6 (Fig. 1e).

COC can be acetosulfated by chlorosulfuric acid or sulfuric acid with acetic anhydride. In Fig. 4, the $^{13}C$ NMR spectra of COCS from COC were presented. The signal at 175.4 ppm is ascribed to the carboxyl groups and the signal at 67 ppm derives from C6 with sulfate groups at 6-O-position. Other peaks at 80 and 82 ppm can be seen with sulfation at 2-O- and 3-O-positions (Zhang et al., 2009). In addition, differences between the spectra of prepared COCS in the range of 160 and 180 ppm can be observed. In spectra of COCS10 and 11, two small peaks beside the peak at 175.4 ppm are existent, which are due to C6 of aldehyde groups, while the COCS12 obviously possessed no aldehyde groups due to the absence of both small peaks (Kato, Matsuo, & Isogai, 2003). Using $^{13}C$ NMR spectroscopy, ICP-OES spectroscopy and elemental analysis, $DS_{S2}$, $DS_{S6}$, total $DS_S$ and $DS_{COO}$ can be determined (Table 4). The total $DS_S$ up to 1.07 was obtained and the $DS_{COO}$ was maximal 0.1.

According to the results in Table 4, the total $DS_S$ increased with rising amount of applied chlorosulfuric acid. Besides, the sulfuric acid led to a much higher $DS_S$ than chlorosulfuric acid during the acetosulfation under other equal reaction conditions. However, a reduction of the amounts of carboxyl groups in the cellulose derivatives can be found after the sulfation comparing the contents of carboxyl groups before and after the sulfation (Tables 3 and 4). Thus, COCS with high contents of sulfate groups and relatively low contents of carboxyl groups could be prepared with another synthesis route.

### 3.3. Effects of chosen COCS on the proliferation of 3T3-L1 fibroblasts

Two products COCS2 and COCS9 were further examined regarding their effects on the proliferation of 3T3-L1 fibroblasts in the presence and absence of the growth factor FGF-2. With 1 mg/ml of the derivatives and 10 ng/ml of FGF-2, a significant difference could be seen between the two samples, in that COCS2 significantly suppressed the proliferation and COCS9 showed an increase (Fig. 5). Therefore, only COC9 was used for a subsequent concentration dependent approach. Here, COCS9 increased the proliferation

**Table 3**
Yields, contents of carboxyl groups and $DS_{COO}$ of COC1 and COC2.

| Samples | Starting material | Oxidation duration (h) | Yields | Contents of carboxyl groups | $DS_{COO}$ |
|---|---|---|---|---|---|
| COC1 | MCC | 0.5 | 91.11% | 0.75 mmol/g | 0.124 |
| COC2 | MCC | 4 | 90.46% | 0.69 mmol/g | 0.114 |

**Table 4**
Synthesis of COCS from COC at 50 °C after acetosulfation with sulfating agent and 8 mol acetic anhydride per mol AGU.

| Samples | Starting materials | $t$ (h)[a] | Molar ratio[b] | $DS_{COO}$[c] | $DS_S$ ($^{13}C$ NMR)[c] | | Total $DS_S$[c] |
|---|---|---|---|---|---|---|---|
| | | | | | $DS_{S2}$ | $DS_{S6}$ | |
| COCS10 | COC1 | 5 | 1.5 | 0.1 | 0.12 | 0.53 | 0.70 |
| COCS11 | COC2 | 6 | 2.1 | 0.08 | 0.15 | 0.58 | 0.80 |
| COCS12 | COC1 | 5 | 1.5 | 0.06 | 0.28 | 0.78 | 1.07 |

[a] t: reaction duration in hours.
[b] Molar ratio in mol sulfuric acid per mol AGU for COCS12 or mol chlorosulfuric acid per mol AGU for COCS10 and 11.
[c] $DS_{S2,S6}$ are the partial $DS_S$ at the corresponding positions which were determined by $^{13}C$ NMR spectroscopy. Total $DS_S$ was determined by elemental analysis. The $DS_{COO}$ was calculated according to ICP-OES spectroscopy.

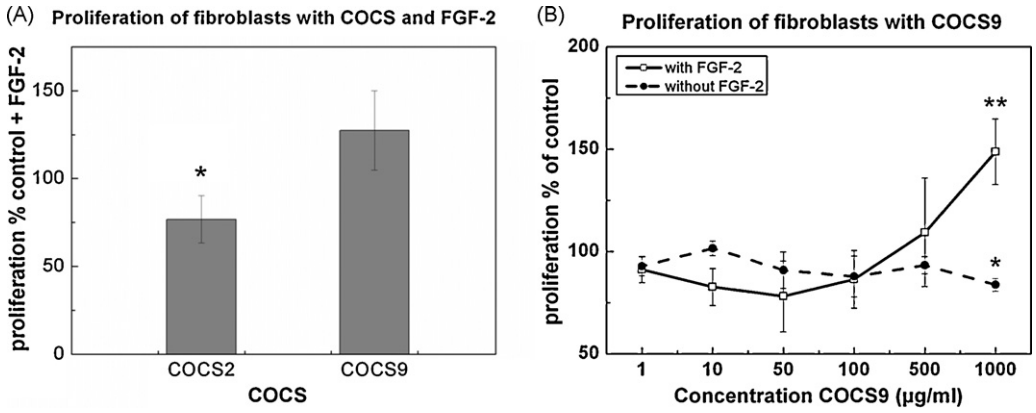


**Fig. 5.** Proliferation of fibroblasts with COCS. Proliferation was measured by incubation of the 3T3-L1 fibroblast cells with different concentrations of COCS with or without FGF-2 for 48 h. (A) 1 mg of COCS2 or COCS9 was incubated together with 10 ng FGF-2/ml. (B) 1 μg/ml up to 1 mg/ml of COCS9 was incubated with or without 10 ng FGF2/ml. Data are expressed as percentage of the control with or without 10 ng FGF-2/ml, respectively. Data represent means of 6 wells ± S.E., $^{*}p < 0.02$; $^{**}p > 0.0001$ compared to the control.

in the presence of FGF-2 at a concentration of 1 mg/ml significantly, lower concentrations than 500 μg/ml provoked a slight decrease (Fig. 5). Without the addition of FGF-2, COCS9 showed only a slight inhibition, with 1 mg/ml a significant suppression of proliferation could be seen (Fig. 5).

## 4. Discussion

According to the data of various CS in Table 1, the primary hydroxyl groups of cellulose were preferred to be sulfated and the total $DS_S$ increased with rising amount of sulfating agent, while higher reaction temperature resulted in smaller total $DS_S$, comparing CS1, 2 and 3 or CS5, 6 and 7. It can be assumed that an intensive desulfation took place under high temperature in comparison to a low temperature (Zhou et al., 2009).

Besides, CS (CS5 and 6) from MCC and CS (CS1 and 4) from AC prepared under equal reaction conditions show analogue total $DS_S$, although their starting materials demonstrate different DP. In this study, cellulose AC and MCC have an average DP of 1180 and 276, respectively, but the total $DS_S$ after acetosulfation seem not to be affected by the application of cellulose with various DP.

After oxidization of CS, COCS exhibit $DS_{COO}$ between 0.1 and 0.67 (Table 2). Based on their $^{13}C$ NMR spectra, no other peaks are found in the region of 140 and 200 ppm within the spectra of COCS and therefore, the primary hydroxyl groups in CS were only converted into carboxylate groups and no aldehyde groups were formed (Isogai & Kato, 1998; Kato et al., 2003). Regenerated cellulose (amorphous cellulose and cellulose II) and cellulose III could be highly oxidised and almost completely soluble polyglucuronic products were obtained (Da Silva Perez, Montanari, & Vignon, 2003; Tahiri & Vignon, 2000). CS presents a cellulose II-analogue structure and this structure could be confirmed by Raman analysis (Fig. 2) (Zhang et al., 2010). CS with this cellulose II-analogue structure could exhibit a higher reactivity and accessibility of the primary hydroxyl groups than native cellulose. Otherwise, it was suggested that the TEMPO-mediated oxidation under homogeneous conditions led to conversion of primary hydroxyl groups into carboxylate groups completely in comparison to oxidation under heterogeneous conditions (Saito & Isogai, 2004). In consistence with this, CS in this investigation was readier to be oxidized because of the raised reactivity and higher $DS_{COO}$ were obtained in comparison to oxidation of MCC. Moreover, only a very slight decrease of the total $DS_S$ because of decomposition reaction can be observed after oxidation of CS (Tables 1 and 2).

It was suggested that the oxidation duration and the amounts of oxidizing agents had great influences on the DP of the products (De Nooy et al., 1996; Isogai & Kato, 1998). The depolymerisation during the TEMPO-mediated oxidation was turned to be inevitable (De Nooy et al., 1996; Kato et al., 2003; Saito & Isogai, 2004). A remarkable decrease of DP was found after oxidation of viscose rayon or cellulose II and ball-milled native celluloses (Isogai et al., 2009; Shibata, Yanagisawa, & Isogai, 2006). Some reasons, such as β-elimination, have been proposed, but a remarkable decrease in $DP_W$ was observed with combination of TEMPO/NaBr/NaClO at pH 11 and hydroxyl radicals formed could be the primary reason for this depolymerisation (Shibata & Isogai, 2003).

Based on the results presented in Table 2, double amount of TEMPO/NaBr/NaClO resulted in an increase of $DS_{COO}$, but the $DP_n$ of the products becomes much lower (COCS7 and 8 in Fig. 3). On the other hand, the prolonged oxidation from 1 to 3 h (COCS7 and 9) or from 3 h to even 20 h (COCS4 and 5) under other equal conditions caused no significant reduction in $DP_n$ of the products with various total $DS_S$ (Fig. 3), but the $DS_{COO}$ was lowered (Table 2). Thus, both oxidation duration and the amounts of oxidizing agents could affect the oxidation of CS and therefore, the characteristics of COCS.

On account of the fact that the $DP_n$ stays constant even after long time oxidation, whereas the $DS_{COO}$ decreases, it can be concluded that a subsequent complete depolymerisation of the once-formed oxidized cellulose chains or a split of the already oxidized anhydroglucose units (AGU) took place (Isogai et al., 2009).

The depolymerisation of CS during the oxidation could result in diverse by-products with much smaller molecular weights or even glucuronic and hexenuronic acid and these small molecules could be afterwards eliminated in the washing process (Saito, Yanagisawa, & Isogai, 2005).

In contrast to the oxidation of CS, the TEMPO-mediated oxidation of cellulose resulted at least in a water-soluble and a water-insoluble fraction (Da Silva Perez et al., 2003). The water-insoluble fraction forms the much larger one and can be separated by centrifugation. The water-soluble fraction was found to be completely oxidized cellulose-β-1,4-linked polyglucuronic acid (Figs. 1e and 2e), while the water-insoluble fraction consisted of COC with a low content of carboxyl groups. This COC could be acetosulfated and a reduction in the amounts of carboxyl groups can be found after the sulfation. A possible explanation is the hydrolysis of cellulose chains in small molecules with followed separation as discussed above. The primary hydroxyl groups were primarily sulfated in comparison to secondary hydroxyl groups (Table 3). The utilization of sulfuric acid as sulfating agent resulted in a higher total $DS_S$ and no signals attributed to the aldehyde groups can be observed within the $^{13}C$ NMR spectrum of corresponding COCS (Fig. 5). These results indicate the stronger sulfation and depolymerisation effects of the applied sulfuric acid in comparison to chlorosulfuric acid.

The effects of sulfated polysaccharides and polymers in relationship to growth factors on the proliferation and differentiation of cells have been investigated previously by other groups (Hatanaka, Ohtsuki, & Kunou, 1994; Leali et al., 2001; Liekens et al., 1999). In the present examination, COCS with sulfate and carboxyl groups at 6-O- and C6-position were applied. For COCS2 which demonstrates an overall DS of 1.00 at C6-position with a $DS_{S6}$ of 0.33 and a $DS_{COO}$ of 0.67, no increase of the effect of FGF-2 was observed. In contrast, COCS9 with an overall DS at C6-position of only 0.82 but a much higher $DS_S$ at C6- and C2-positions caused a significant increase at a concentration of the derivative of 1 mg/ml, but not at lower concentrations. It can therefore be presumed that the content of sulfate groups is more important for the mitogenic activity on 3T3 fibroblasts in the presence of FGF-2 than carboxyl groups. Similar conclusions have been drawn from a strong increase of the effect of FGF-2 at lower concentrations with other highly sulfated compounds (Kunou & Hatanaka, 1995). Possibly, a higher $DS_{COO}$ of COCS might be required to show a substantial mitogenic activity. Investigations with other growth factors than FGF-2 that show a cooperative activity with heparin or heparan sulfate have to be performed to find out if COCS has other biological effects with respect to regulation of proliferation, differentiation and function of cells.

## 5. Conclusion

Sulfate and carboxyl groups were regioselectively introduced into the AGU of cellulose yielding various products: CS, COC, β-1,4-linked polyglucuronic acid and COCS. Diverse substitution patterns and DS were achieved during two synthesis routes.

In one way, various CS with predominant sulfation at 6-O-position were prepared and the TEMPO-mediated oxidation was executed on them to introduce carboxyl groups. The DP values of used cellulose seemed not to affect the $DS_S$ and the residual primary hydroxyl groups in CS are ready to be oxidized. The measurement of molecular weights of chosen CS and COCS demonstrated a significant decrease of $DP_n$ after TEMPO-mediated oxidation. The utilization of more oxidizing agents reduced the $DP_n$ more intensively than the oxidization duration.

Another way started with TEMPO-mediated oxidation of cellulose leading to COC with low content of carboxyl groups and β-1,4-linked polyglucuronic acid with complete oxidation of primary hydroxyl groups. An acetosulfation of COC could be conducted to synthesize COCS. The use of sulfuric acid led to higher total $DS_S$ and a stronger reduction of $DS_{COO}$ in comparison to chlorosulfuric acid.

Finally, COCS with higher contents of sulfate groups instead of carboxyl groups was found to be able to promote significantly the proliferation in the presence of FGF-2.

## Acknowledgements

The financial support from German Research Foundation (Deutsche Forschungsgemeinschaft, Germany, grants: FI755/4-1 and FI755/4-2, GR1290/7-1 and GR1290/7-2) is gratefully acknowledged and the authors wish to thank Dr. Bern Volkert from IAP (Golm, Germany) for the determination of carboxyl groups.

## References

Alban, S., Schauerte, A., & Franz, G. (2002). Anticoagulant sulphated polysaccharides: Part I. Synthesis and structure-activity relationships of new pullulan sulphates. *Carbohydrate Polymers*, *47*, 267–276.

American Society for Testing and Materials. (2008). Standard test methods for sodium carboxymethylcellulose. ASTM D1439-03(2008)e1. ASTM International, West Conshohocken, PA, doi:10.1520/D1439-03R08E01.

Baumann, H., Richter, A., Klemm, D., & Faust, V. (2000). Concepts for preparation of novel regioselective modified cellulose derivatives sulphated, aminated, carboxylated and acetylated for homocompatible ultrathin coating on biomaterials. *Macromolecular Chemistry and Physics*, *201*, 1950–1962.

Besemer, A. C., de Nooy, A. E. J., & van Bekkum, H. (1998). Methods for the selective oxidation of cellulose: Preparation of 2,3-dicarboxycellulose and 6-carboxycellulose. In T. Heinze, & W. G. Glasser (Eds.), *Cellulose derivatives: Modification, characterization, and nanostructures: ACS Symposium Series 688* (pp. 73–82). Washington, DC: American Chemical Society.

Bourin, M. C., & Lindahl, U. (1993). Glycosaminoglycans and the regulation of blood coagulation. *Biochemical Journal*, *289*, 313–330.

Capila, I., & Linhardt, R. J. (2002). Heparin-Protein Wechselwirkungen. *Angewandte Chemie*, *114*, 426–450.

Carroll, L. A., & Koch, R. J. (2003). Heparin stimulates production of bFGF and TGF-beta 1 by human normal, keloid, and fetal dermal fibroblasts. *Medical Science Monitor*, *9*(3), BR97–BR108.

Chang, P. S., & Robyt, J. F. (1996). Oxidation of primary alcohol groups of naturally occurring polysaccharides with 2,2,6,6-tetramethyl-1-piperidine oxoammonium ion. *Journal of Carbohydrate Chemistry*, *15*(7), 819–830.

Da Silva Perez, D., Montanari, S., & Vignon, M. R. (2003). TEMPO-mediated oxidation of cellulose III. *Biomacromolecules*, *4*, 1417–1425.

Darnell, J., Lodish, H., & Baltimore, D. (1990). *Molecular cell biology* (2nd edition). New York: De Gruyter.

De Nooy, A. E. J., Besemer, A. C., Van Bekkum, H., Van Dijk, J. A. P. P., & Smit, J. A. M. (1996). TEMPO-mediated oxidation of pullulan and influence of ionic strength and linear charge density on the dimensions of the obtained polyelectrolyte chains. *Macromolecules*, *29*, 6541–6547.

Fischer, S., Thümmler, K., Volkert, B., Hettrich, K., Schmidt, I., & Fischer, K. (2008). Properties and applications of cellulose acetate. *Macromolecular Symposia*, *262*, 89–96.

Gericke, M., Liebert, T., & Heinze, T. (2009). Interaction of ionic liquids with polysaccharides, 8 – Synthesis of cellulose sulphates suitable for polyelectrolyte complex formation. *Macromolecular Bioscience*, *9*, 343–353.

Groth, T., & Wagenknecht, W. (2001). Anticoagulant potential of regioselective derivatized cellulose. *Biomaterials*, *22*, 2719–2729.

Hatanaka, K., Ohtsuki, T., & Kunou, M. (1994). Effects of synthetic polyanions on 3T3-L1 fibroblast proliferation stimulated by fibroblast growth factors. *Chemical Letters*, *8*, 1407–1410.

Heinze, T., & Koschella, A. (2005). Carboxymethyl ethers of cellulose and starch – a review. *Macromolecular Symposia*, *223*, 13–29.

Hettrich, K., Wagenknecht, W., Volkert, B., & Fischer, S. (2008). New possibilities of the acetosulfation of cellulose. *Macromolecular Symposia*, *262*, 162–169.

Isogai, A., & Kato, Y. (1998). Preparation of polyuronic acid from cellulose by TEMPO-mediated oxidation. *Cellulose*, *5*, 153–164.

Isogai, T., Yanagisawa, M., & Isogai, A. (2009). Degrees of polymerization (DP) and DP distribution of cellouronic acids prepared from alkali-treated celluloses and ball-milled native celluloses by TEMPO-mediated oxidation. *Cellulose*, *16*, 117–127.

Kato, Y., Matsuo, R., & Isogai, A. (2003). Oxidation process of water-soluble starch in TEMPO-mediated system. *Carbohydrate Polymers*, *51*, 69–75.

Kunou, M., & Hatanaka, K. (1995). Effects of heparin, dextran sulphate, and synthetic (16)-α-D-mannopyranan sulphate and acidic fibroblast growth factor on 3T3-L1 fibroblasts. *Carbohydrate Polymers*, *28*, 107–112.

Kunou, M., Koizumi, M., Shimizu, K., Kawase, M., & Hatanaka, K. (2000). Synthesis of sulphated colominic acids and their interaction with fibroblast growth factors. *Biomacromolecules*, *1*, 451–458.

Leali, D., Belleri, M., Urbinati, C., Coltrini, D., Oreste, P., Zoppetti, G., et al. (2001). Fibroblast growth factor-2 antagonist activity and angiostatic capacity of sulphated Escherichia coli K5 polysaccharide derivatives. *The Journal of Biological Chemistry*, *276*, 37900–37908.

Liekens, S., Leali, D., Neyts, J., Esnouf, R., Rusnati, M., Dell'Era, P., et al. (1999). Modulation of fibroblast growth factor-2 receptor binding, signaling, and mitogenic activity by heparin-mimicking polysulfonated compounds. *Molecular Pharmacology*, *56*, 204–213.

Montanari, S., Roumani, M., Heux, L., & Vignon, M. R. (2005). Topochemistry of carboxylated cellulose nanocrystals resulting from TEMPO-mediated oxidation. *Macromolecules*, *38*, 1665–1671.

Mulloy, B., Mourão, P. A. S., & Gray, E. (2000). Structure/function studies of anticoagulant sulphated polysaccharides using NMR. *Journal of Biotechnology*, *77*, 123–135.

Nehls, I., Wagenknecht, W., Philipp, B., & Stscherbina, D. (1994). Characterization of cellulose and cellulose derivatives in solution by high resolution $^{13}$C-NMR spectroscopy. *Progress in Polymer Science*, *19*, 29–78.

Peschel, D., Zhang, K., Aggarwal, N., Brendler, E., Fischer, S., & Groth, T. (in press). Synthesis of novel celluloses derivatives and investigation of their mitogenic activity in the presence and absence of FGF2. *Acta Biomaterialia*, doi:10.1016/j.actbio.2009.12.032.

Philipp, B., Nehls, I., & Wagenknecht, W. (1987). $^{13}$C-NMR spectroscopy study of the homogeneous sulphation of cellulose and xylan in the $N_2O_4$-DMF system. *Carbohydrate Research*, *164*, 107–116.

Philipp, B., & Wagenknecht, W. (1983). Cellulose sulphate half-ester. Synthesis, structure and properties. *Cellulose Chemistry & Technology*, *17*, 443–459.

Rabenstein, D. L. (2002). Heparin and heparin sulphate: Structure and function. *Natural Product Reports*, *19*, 312–331.

Saito, T., & Isogai, A. (2004). TEMPO-mediated oxidation of native cellulose. The effect of oxidation conditions on chemical and crystal structures of the water-insoluble fractions. *Biomacromolecules*, *5*, 1983–1989.

Saito, T., & Isogai, A. (2005). Ion-exchange behaviour of carboxylate groups in fibrous cellulose oxidized by the TEMPO-mediated system. *Carbohydrate Polymers*, *61*, 183–190.

Saito, T., Kimura, S., Nishiyama, Y., & Isogai, A. (2007). Cellulose nanofibers prepared by TEMPO-mediated oxidation of native cellulose. *Biomacromolecules*, *8*, 2485–2491.

Saito, T., Yanagisawa, M., & Isogai, A. (2005). TEMPO-mediated oxidation of native cellulose: SEC-MALLS analysis of water-soluble and–insoluble fractions in the oxidized products. *Cellulose*, *12*, 305–315.

Shibata, I., & Isogai, A. (2003). Depolymerisation of cellouronic acid during TEMPO-mediated oxidation. *Cellulose*, *10*, 151–158.

Shibata, I., Yanagisawa, M., & Isogai, A. (2006). SEC-MALLS analysis of cellouronic acid prepared from regenerated cellulose by TEMPO-mediated oxidation. *Cellulose*, *12*, 305–315.

Socrates, G. (2001). *Infrared and Raman characteristic group frequencies* (3rd edition). England: John Wiley & Sons Ltd.

Tahiri, C., & Vignon, M. R. (2000). TEMPO-oxidation of cellulose: synthesis and characterisation of polyglucuronans. *Cellulose*, *7*, 177–188.

Wang, Z. M., Li, L., Xiao, K. J., & Wu, J. Y. (2009). Homogeneous sulfation of bagasse cellulose in an ionic liquid and anticoagulant activity. *Bioresource Technology*, *100*, 1687–1690.

Wang, Z. M., Li, L., Zheng, B. S., Normakhamatov, N., & Guo, S. Y. (2007). Preparation and anticoagulation activity of sodium cellulose sulphate. *International Journal of Biological Macromolecules*, *41*, 376–382.

Xing, R., Liu, S., Yu, H., Zhang, Q., Li, Z., & Li, P. (2004). Preparation of low-molecular-weight and high-sulphate-content chitosans under microwave radiation and their potential antioxidant activity in vitro. *Carbohydrate Polymers*, *339*, 2515–2519.

Yamamoto, I., Takayama, K., Matsuzaki, K., Hatanaka, K., Uryu, T., Yamamoto, N., et al. (1991). Synthesis, structure and antiviral activity of sulphates of cellulose and its branched derivatives. *Carbohydrate Polymers*, *14*, 53–63.

Zhang, K., Brendler, E., & Fischer, S. (2010). FT Raman investigation of sodium cellulose sulfate. *Cellulose*, *17*, 427–435.

Zhang, K., Peschel, D., Brendler, E., Groth, T., & Fischer, S. (2009). Bioactivity of cellulose derivatives. *Macromolecular Symposia*, *280*, 28–35.

Zhou, H., Qian, J., Wang, J., Yao, W., Liu, C., Chen, J., et al. (2009). Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-*N*, 6-*O*-sulfated chitosan *in vitro* and *in vivo*. *Biomaterials*, *30*, 1715–1724.