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ORIGINAL PAPER

Synthesis of carboxyl cellulose sulfates with regioselective sulfation and regiospecific oxidation using cellulose trifluoroacetate as intermediates

Kai Zhang · Steffen Fischer · Andreas Geissler · Erica Brendler · Kathrin Gebauer

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Abstract Synthesis of cellulose sulfates (CSs) and carboxyl cellulose sulfates (COCSs) with regioselectively or regiospecifically distributed functional groups within anhydroglucose units was reported. CS with regioselectively distributed sulfate groups at 2,3-*O*- or 2,6-*O*-position were homogeneously synthesized and cellulose trifluoroacetate (CTFA) was used as intermediates. The trifluoroacetyl groups were detected primarily at 6-*O*-position and their distributions could be altered by changing the amount of trifluoroacetyl anhydride (TFAA). Various sulfating agents were used for further homogeneous sulfation of CTFA. The total degree of sulfation (DS_S) and the distribution of sulfate groups within the repeating units were affected by the amount of TFAA, the type and amount of sulfating agents. Subsequent homogenous 4-acetamide-TEMPO or TEMPO-mediated oxidation of CS led to COCS with carboxyl groups regiospecifically distributed at C6 position, which may be interesting structural mimics for natural occurring heparin.

Keywords Carboxyl cellulose sulfate · Cellulose sulfate · Regioselective · Cellulose trifluoroacetate · TEMPO

K. Zhang (✉) · A. Geissler
Institute of Macromolecular Chemistry and Paper Chemistry, Technische Universität Darmstadt, Petersenstr. 22, 64287 Darmstadt, Germany
e-mail: zhang@cellulose.tu-darmstadt.de

S. Fischer
Institute of Plant and Wood Chemistry, Dresden University of Technology, Pienner Str. 19, 01737 Tharandt, Germany

E. Brendler
Institute of Analytical Chemistry, Freiberg University of Mining and Technology, Leipziger Str. 29, 09599 Freiberg, Germany

K. Gebauer
Institute of Power Engineering, Dresden University of Technology, Helmholtzstr. 10, 01069 Dresden, Germany

Introduction

Naturally occurring glycosaminoglycans, such as heparin and heparan sulfate, have important functions in cell signaling and development, as well as in the regulation of cellular proliferation and differentiation (Capila and Linhardt 2002; Sasisekharan et al. 2002; Hacker et al. 2005). Important functional groups in the repeating units of heparin and heparan sulfate are sulfate and carboxyl groups, which primarily contribute to their biological properties including anticoagulant activity and interactions with growth factors (Sasisekharan et al. 2002; Gandhi and Mancera 2008; Rabenstein 2002). In addition to hexuronic acids, sulfated and acetylated glucosamines are also monosaccharide building blocks of heparin and heparan

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sulfate (Fig. 1b, Gandhi and Mancera 2008; Rabenstein 2002). Despite the pivotal function of heparin and heparan sulfate in cell activities, they exhibit a few limitations, e.g. big difference in their molecular compositions depending on their sources and contamination possibilities (Capila and Linhardt 2002; Laremore et al. 2009; Liu et al. 2009). Furthermore, the dependence of the great structural diversity and micro-heterogeneity of heparin or heparan sulfate on the sources complicates further structural analysis (Jones et al. 2011). A combination of various analytical methods is often required.

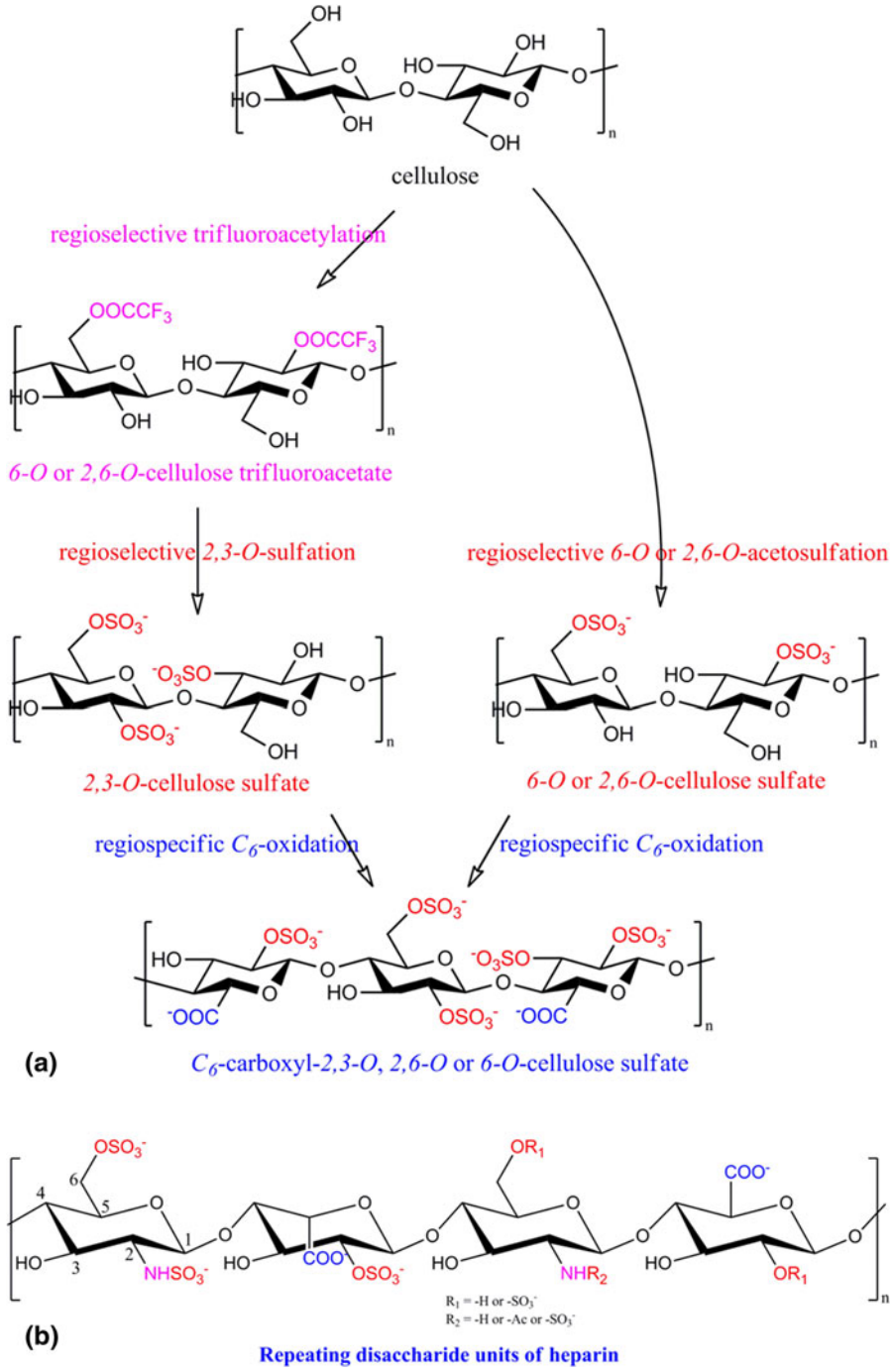

Fig. 1 **a** Schematic representation of the synthesis routes leading to corresponding derivatives (see the text for details); **b** repeating disaccharide units of heparin for comparison according to (Gandhi and Mancera 2008)

Substances with superior or comparable heparinoid activity and predictable structures in purified state are attractive for biomedical and biotechnological applications. It has been reported that natural polysaccharides without sulfate groups have also exhibited biological activities after the introduction of sulfate groups. For example, pullulan, amylose and cellulose sulfate (CS) which are half-esters of these polysaccharides showed both anticoagulant and antiviral functions (Alban et al. 2002; Chaidedgumjorn et al. 2002; Groth and Wagenknecht 2001; Wang et al. 2009). Furthermore, CS was able to promote the activity of fibroblast growth factor 2 (FGF2) and bone morphogenic protein 2 (BMP2) (Peschel et al. 2010, 2012). In particular, the effect of highly sulfated CS was even higher than that of natural heparin. This effect of CS was related to high degrees of sulfation, i.e. high contents of sulfate groups for interacting with growth factors.

Cellulose sulfate containing carboxyl groups (carboxyl cellulose sulfate, COCS) have also been examined regarding their biological effects. However, COCS used in previous studies have low degrees of sulfation and oxidation, so that their enhancing effects on the activity of growth factors were not comparable to that of highly sulfated CS (Zhang et al. 2010). Carboxyl groups can be introduced into polysaccharide chains through regiospecific TEMPO- or TEMPO derivatives-mediated oxidation (de Nooy et al. 1995; Isogai and Kato 1998). After the oxidation, only primary hydroxyl groups are converted into carboxyl groups. Especially COCS which is prepared this way showed a similar structure to that of natural heparin which contains carboxyl groups at C6 position and sulfate groups at 6-*O* as well as 2-*O*-position within its repeating units (Fig. 1b, Gandhi and Mancera 2008; Rabenstein 2002). However, the presence of several different functional groups with distinct distributions within the repeating units of heparin complicates not only their exact characterizations, but also predictions on the structure–function relationship for heparin (Jones et al. 2011). In contrast, the inclusion of pre-known synthetic approaches can provide indications on the exact structures of obtained products. Furthermore, they can be used as references for compounds with similar structures to precisely analyze the structure–function relationship.

Thus, in this report, COCS with high degrees of substitution (DS) ascribed to regioselectively distributed sulfate groups (DS_S) and regiospecifically distributed carboxyl groups (DO) within anhydroglucose units (AGU) of cellulose were prepared. The sulfate groups were regioselectively introduced into 2,3-*O*-, 6-*O*- or 2,6-*O*-positions. The primary hydroxyl groups were regiospecifically oxidized leading to carboxyl groups at C6 position. The structures of obtained COCS were characterized with various methods and the contents of functional groups as well as their distributions within repeating units were determined, respectively.

Experimental

Materials

Microcrystalline cellulose (MCC) from cotton linters with an average degree of polymerisation (DP) of 276 was received from J. Rettenmaier & Söhne GmbH (Rosenberg, Germany). Pulp V-81 (with 97.0 % alpha cellulose, AC) with an average DP of 1180 was purchased from Buckeye Technologies Inc. (Memphis, USA). 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and 4-acetylamino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-acetamide-TEMPO) were obtained from Acros (Geel, Belgium). Trifluoroacetic acid, trifluoroacetic anhydride and chlorosulfonic acid were obtained from Merck Schuchardt OHG (Hohenbrunn, Germany). SO_3·pyridine complex was purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hypochlorite with 14 % active chlorine was received from VWR International S.A.S. (Fontenay-sous-Bois, France). *N*,*N*-dimethylformamide (DMF), sulfamic acid and sodium chlorite ($NaClO_2$, 80 %) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Dialysis membrane from Spectrum Laboratories Inc. (Rancho Dominquez, USA) has an approximate molecular weight cut off of 1,000 Daltons. Demineralised water was used in all experiments.

Synthesis of 2,3-*O*- or 2,6-*O*-sulfated CS by homogeneous sulfation of cellulose trifluoroacetate (CTFA) (Fig. 1a)

Synthesis of regioselectively trifluoroacetylated cellulose

A suspension of 1 g cellulose in 20 ml trifluoroacetic acid (TFA) was stirred under N_2 atmosphere for 15 min and then 7 ml of trifluoroacetic anhydride

(TFAA) was added. The amount of TFAA was changed in order to alter the degree of substitution attributed to trifluoroacetyl groups. After 3 h stirring at room temperature (RT), the solution was precipitated in 90 ml diethyl ether and the precipitate was washed three times with 50 ml diethyl ether. Thereafter, it was dried at 103 °C for 30 min under vacuum, which was realized by continuous suction using a pump. Finally, 1 g of obtained CTFA was dissolved in 50 ml *N*,*N*-dimethylformamide (DMF) for further synthesis.

Synthesis of regioselectively 2,3-O- or 2,6-O-sulfated CS

In a typical case, sulfating agents, e.g. SO_3·pyridine complex, were added into a solution of CTFA in DMF and the solution was kept at 50 °C for 3 h. After the reaction, reaction mixture was precipitated in 5 volumes ethanolic solution of 13.5 g anhydrous sodium acetate and the product was collected through centrifugation. After washing with ethanol/water (8:2, v:v), the product was dissolved in water. The pH value of the solution was adjusted to 7.5 and the solution was filtered. After dialysis of part of the product against water until salt-free, colorless or slightly brown aggregates were obtained by lyophilisation. Dissolved CTFA was totally converted into CS, since no precipitation during the sulfation was observed. The yield for CS4 was 93 % based on 1 g starting cellulose.

Synthesis of 6-*O* or 2,6-*O*-sulfated CS through regioselective quasi-homogeneous acetosulfation (Fig. 1a)

For the comparison, CS with sulfate groups at 6-*O* and 2,6-*O*-positions were prepared through the acetosulfation of cellulose (Zhang et al. 2011a). Briefly, 1 g cellulose was suspended in 50 ml anhydrous DMF at RT for averagely 16 h. Reaction agent consisting of 0.35 ml chlorosulfuric acid and 4.8 ml acetic anhydride in DMF was dropped into the cellulose suspension under vigorous stirring within 15 min and the mixture was kept at 70 °C for 5 h. Then, obtained solution was cooled down to RT and poured into a saturated solution of anhydrous sodium acetate in ethanol. Precipitate was recovered via centrifugation and washed with a 4 % ethanolic sodium acetate solution. After the deacetylation with 50 ml 1 M ethanolic solution of sodium hydroxide for 15 h, the pH value was adjusted to around 7.5 with acetic acid/ethanol (50/50, w/w). The product was dissolved in water and filtered. Finally, products were dialyzed against water and lyophilized. The yield for CS1 was 113 %.

Regiospecific C_6-oxidation of CS

Regiospecific oxidation of CS was carried out through TEMPO or 4-acetamide-TEMPO-mediated oxidation (Fig. 1a).

Before the 4-acetamide-TEMPO-mediated oxidation, 0.5 g CS was dissolved in 50 ml aqueous 2 % sodium acetate solution. The oxidation agent consisting of 4-acetamide-TEMPO (0.05 mol/mol primary OH-groups of corresponding CS) and 12 % NaClO aqueous solution (0.3 mol/mol primary OH-groups of corresponding CS) in 10 ml water was added into the solution. Then, the pH value of the suspension was adjusted to 6 with 50 wt% acetic acid aqueous solution. Subsequently, $NaClO_2$ (3.5 mol/mol primary OH-groups of corresponding CS) was slowly added into the solution under vigorous stirring and the temperature of the mixture was raised to 60 °C. After a reaction at 60 °C for 24 h, the reaction was stopped via addition of 5 ml methanol, the mixture was cooled to RT and the pH value was adjusted to 7.5. The mixture was poured into 5 volumes ethanol to precipitate the product. After the removal of the solvents via centrifugation, the product was dissolved in water. After the filtration, dialysis against water and lyophilization, colorless or slightly brown aggregates were obtained. The yield for COCS6 was 83 % based on 1 g CS4.

For the TEMPO-mediated oxidation, 0.5 g CS was dissolved in 50 ml water. The oxidation agent consisting of TEMPO (0.05 mol/mol primary OH-groups of corresponding CS) and NaBr (1.5 mol/mol primary OH-groups of corresponding CS) in 10 ml water was prepared under continuous stirring until complete dissolution and added to the CS solution. The oxidation was started after slowly adding 12 % NaClO aqueous solution (10 mol/mol primary OH-groups of corresponding CS) to the solution under stirring. The pH value of the mixture was maintained between 10 and 10.5 by slowly adding NaClO drop by drop. After the total addition of the NaClO solution, the pH was

kept constant at RT for 1 h using 0.5 M NaOH aqueous solution. Thereafter, methanol (5 ml) was added to stop the oxidation and the pH value was adjusted to 7.5 with 0.5 M aqueous HCl solution. Finally, the mixture was treated and purified as described above leading to colorless or slightly brown products. The yield for COCS8 was 67 % based on 1 g CS4.

Characterization

FT Raman spectroscopy

FT Raman spectra were recorded on a Bruker Multi-Ram spectrometer (Bruker Optics, Ettlingen, Germany) with Ge diode as detector that is cooled with liquid-nitrogen. A cw-Nd:YAG-laser with an exciting line of 1,064 nm was applied as light source for the excitation of Raman scattering. The spectra were recorded over a range of 3,500–100 cm^{-1} using an operating spectral resolution of 3 cm^{-1} and a laser power output of 100 mW. The spectrum was normalised and analysed using the operating spectroscopy software OPUS Ver. 6.5 (Bruker).

Determination of trifluoroacetyl groups

The determination of trifluoroacetyl groups was conducted after complete saponification. In brief, 0.25 g CTFA was swollen in 10 ml 75 % aqueous ethanol at 60 °C for 3 h. Then, 6 ml 0.5 M aqueous NaOH solution were added and the mixture was kept at 60 °C for another 15 min. After that, the mixture stayed at RT for 48 h. A titration with 0.1 M HCl aqueous solution was carried out and 2 ml more HCl solution was added. After another day at RT, the mixture was titrated back with 0.1 M aqueous NaOH solution in the presence of phenolphthalein as indicator. The DS_{TFAc} were calculated based on the contents of trifluoroacetyl groups using following Eqs. (1–3) according to the method of Deus et al. (1991) with a few modifications:

$$V_{NaOH} = 5 \times V_1 \times f_1 + V_2 \times f_2 - V_3 \times f_3, \quad (1)$$

$$\%_{TFAc} = (97 \times V_{NaOH}/m) \times 100, \quad (2)$$

$$DS_{TFAc} = 3 \times \%_{TFAc}/52.68; \quad (3)$$

where the V_1 is 6 ml 0.5 M aqueous NaOH solution; V_2 is the amount of 0.1 M aqueous NaOH solution for the back-titration; V_3 is the amount of 0.1 M HCl aqueous solution; f_1, f_2 and f_3 are the titers of corresponding solutions; $\%_{TFAc}$ is the determined content of trifluoroacetyl groups in CTFA; m is the dried weight of CTFA for titration; 97 is the molar mass of one trifluoroacetyl group in CTFA and 52.68 is the content of trifluoroacetyl groups in the totally substituted CTFA.

Elemental analysis

The contents of carbon, hydrogen and nitrogen were determined with Elemental Analyser vario EL from Elementar (Hanau, Germany). The content of sulphur was measured with Elemental Analyser Eltra CS 500 (Neuss, Germany). The total DS_S and DO were calculated according to Eqs. (4) and (5), respectively:

$$\text{Total } DS_S = (S\%/32)/(C\%/72), \quad (4)$$

$$H\% \times C\% = 72 \times (10 - DS_S - 3 \times DO)/(162 + 102 \times DS_S + 36 \times DO)^2; \quad (5)$$

where S%, H% and C% are determined contents of the elements sulphur, hydrogen and carbon.

^{13}C NMR spectroscopy

Liquid-state ^{13}C NMR spectra of CTFA in DMSO-d_6 as well as CS and COCS in D_2O were recorded at RT on Bruker DPX 400 spectrometer with a ^{13}C frequency of 100.13 MHz, 30° pulse length, 0.35 s acquisition time and a relaxation delay of 3 s. Scans of 10,000 for CS and of 20,000 for COCS were accumulated. Solid-state CP/MAS ^{13}C NMR was carried out using Bruker Avance 400 WB spectrometer at RT with a ^{13}C frequency of 100.65 MHz, 1 ms contact time, 10 kHz spinning speed, 35 ms acquisition time and a relaxation delay of 3 s. Scans of 7,000 were accumulated.

Results and discussion

Synthesis of CS with regioselective distributions of sulfate groups

As established from the natural glycosaminoglycans containing sulfate and carboxyl groups, e.g. heparin, the distribution of substituents probably plays a very important role for their biological effects (Capila and

Linhardt 2002; Gandhi and Mancera 2008; Rabenstein 2002). Subsequently, the regulation of positions and degrees of functional groups is important for synthetic heparin-analogues. In this report, products containing regioselectively distributed sulfate groups and regiospecifically distributed carboxyl groups within AGU of cellulose were synthesized. At first, various CSs were prepared through homogeneous sulfation of cellulose trifluoroacetate (CTFA) or quasi-homogeneous acetosulfation of cellulose.

The mixture of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) is a derivatizing solvent for cellulose (Liebert et al. 1994; Heinze and Liebert 2001). During the dissolution, primary hydroxyl groups and also part of hydroxyl groups at C2 are trifluoroacetylated depending on amounts of TFA and TFAA. Thus, CTFA with regioselectively distributed trifluoroacetyl groups can be readily prepared by changing the amount of TFA or TFAA. CTFA exhibits advantages for subsequent modification of residual hydroxyl groups in respect of two factors: (1) it is easily soluble in common organic solvents including DMF and DMSO; and (2) the trifluoroacetyl group works as protecting groups and also as leaving groups, since it can be simply removed after the contact with water for just a few minutes (Liebert et al. 1994, 1996). Moreover, trifluoroacetyl groups were stable during the carboxymethylation or sulfation of CTFA (Baumann et al. 2000; Liebert et al. 1996).

The degrees of substitution ascribed to trifluoroacetyl groups (DS_{TFAc}) were tuned by changing the amount of TFAA (Table 1). The total content of trifluoroacetyl groups was determined via back titration. As shown in Table 1, CTFA1 and CTFA2 prepared using 8.25 and 4.125 mol TFAA per mol AGU have diverse DS_{TFAc} of 1.28 and 0.91, respectively. Obtained CTFA were well soluble in DMF or DMSO. As illustrated in Fig. 2, characteristic ^{13}C NMR quartets between 110 and 120 ppm are ascribed to carbons in CF_3-groups. The signals ascribed to C1 and C6 of AGU were shifted from 102.5 to 98 ppm and from 60 to 67.5 ppm, respectively. The shifts of both signals are attributed to the trifluoroacetylation of primary and C2-hydroxyl groups (Liebert et al. 1994). As shown before, the partial DS of cellulose derivatives can be measured using ^{13}C NMR spectroscopy (Nehls et al. 1994). The estimated partial DS_{TFAc6} and DS_{TFAc2} for CTFA1 and CTFA2 are listed in Table 1. It is apparent that primary hydroxyl groups were preferentially trifluoroacetylated. For CTFA1 with higher DS_{TFAc}, also part of secondary hydroxyl groups was esterified.

FT Raman measurement shows a strong signal at 1,791 cm^{-1} attributed to stretching vibrations of C=O bonds (Fig. 2, Zhang et al. 2011b). As demonstrated on cellulose acetate, the intensity of this signal possibly allows a quantitative measurement of DS_{TFAc} based on FT Raman spectroscopy. Several other new signals ascribed to vibrations of trifluoroacetyl groups are visible between 700 and 900 cm^{-1}. Furthermore, the signals derived from vibrations of cellulose backbone at 1,481, 1,153 and 1,121 cm^{-1} shift to 1,456, 1,172 and 1,120 cm^{-1} after the trifluoroacetylation, respectively. Moreover, the intensity of the signal at 1,096 cm^{-1} was strongly reduced, indicating that this signal should be attributed to the vibrations of the bonds containing OH-groups.

Subsequently, sulfations of CTFA with protected primary and partially also secondary hydroxyl groups were carried out. For the comparison, CS with different distributions of sulfate groups were prepared through the regioselective acetosulfation of cellulose (CS1 and 2 in Table 2).

Synthesized CS showed characteristic FT Raman spectra as well as ^{13}C NMR spectra (Figs. 3, 4). Within FT Raman spectra, typical bands attributed to the vibrations of sulfate groups at 1,072 and around 827 cm^{-1} are visible (Fig. 3a, c). These bands are ascribed to symmetric stretching vibrations of O=S=O groups as well as stretching vibrations of C–O–S groups, respectively (Zhang et al. 2011a).

The total DS_S was determined using elemental analysis, while the partial DS_{S6} and DS_{S2} were analyzed via ^{13}C NMR spectroscopy as reported before (Table 1) (Nehls et al. 1994; Zhang et al. 2011a). Typical ^{13}C NMR spectra of CS in D_2O are depicted in Fig. 4. CS2 with a DS_{S6} of 0.38 and almost all sulfate groups at 6-*O*-position shows a significant shift of C6 signal from 60.5 to 66.8 ppm (Fig. 4a). After the homogeneous sulfation of CTFA, obtained CS only showed very minimal or no sulfation at 6-*O*-position based on the fact that no significant chemical shift at 66.8 ppm was visible (Fig. 4b, c) (Chaidedgumjorn et al. 2002). With sulfate groups at 2-*O*-position, $C2_S$ (carbon 2 within the AGU of cellulose) shows a signal at 80.4 ppm. At the same time, the signal of C1 shifts partly from 102.4 to 101.2 ppm, indicating a partial sulfation at 2-*O*-position (Fig. 4).

Table 1 CTFA prepared using different amounts of TFAA from MCC at RT for 3 h

Samples	Amounts of TFAA (mol/mol AGU)	DS_{TFAc} (^{13}C NMR)[a]		Total DS_{TFAc}[b] (titration)
		DS_{TFAc6}	DS_{TFAc2}	
CTFA1	8.25	0.92	0.29	1.28
CTFA2	4.125	0.87	0	0.91

[a] Partial DS_{TFAc6} and DS_{TFAc2} were measured using ^{13}C NMR spectroscopy

[b] Total DS_{TFAc} was determined via back titration after the complete saponification

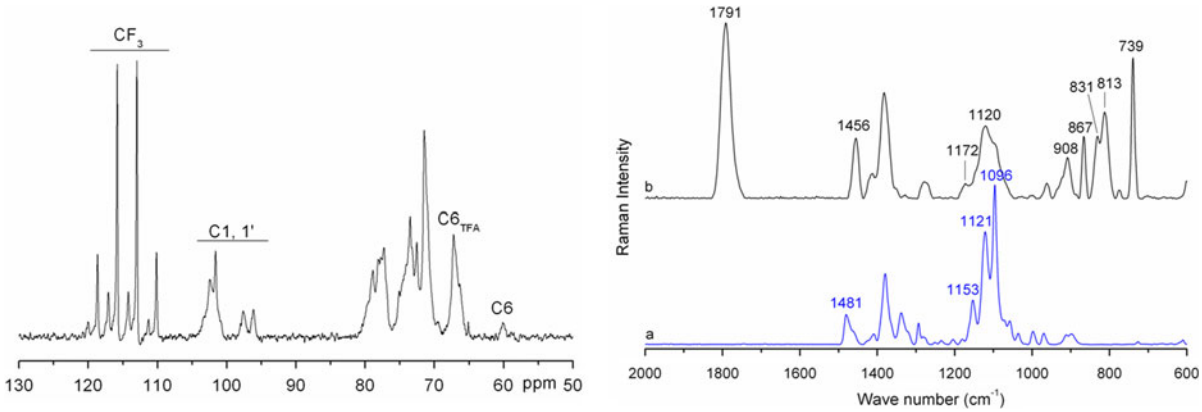

Fig. 2 *Left* ^{13}C NMR spectrum (130–50 ppm) of CTFA1 in DMSO-d_6 at RT. *Right* FT Raman spectrum (2,000–600 cm^{-1}) of **a** cellulose and **b** CTFA1 at RT

Table 2 Preparation of CS with diverse distributions of sulfate groups within repeating units

Samples	TFAA[a]	Sulfating agents[b]	Temperature (°C)/time (h)	Partial DS_S^c		Total DS_S^c	DS_{S3}^c
				DS_{S6}	DS_{S2}		
CS1	0	0.85 ($ClSO_3H$)	70/(0)[e]	0.55	0.13	0.68	0
CS2	0	0.85 ($ClSO_3H$)	70/5	0.38	0.03	0.41	0
CS3	2	7.2 (NH_2SO_3H)	70/24	0.18	0.59	0.97	0.2
CS4	4.125	7.2 (NH_2SO_3H)	70/24	0.16	0.33	0.58	0.09
CS5	4.125	1.5 ($ClSO_3H$)	50/3	0.04	0.45	0.91	0.42
CS6	8.25	1.5 ($ClSO_3H$)	50/3	0	0.32	0.42	0.1
CS7	4.125	3.6 (SO_3·pyridine)	50/3	0.17	0.39	0.62	0.06
CS8	4.125	7.2 (SO_3·pyridine)	50/3	0	0.55	0.67	0.12
CS9	6	3.6 (SO_3·pyridine)	50/3	0.03	0.34	0.53	0.16
CS10[d]	8	3.6 (SO_3·pyridine)	50/3	0	0.12	0.89	0.77
CS11[d]	10	3.6 (SO_3·pyridine)	50/3	0	0.12	0.65	0.53

[a] TFAA: trifluoroactic anhydride in mol per mol AGU of cellulose

[b] Sulfating agents in mol per mol AGU of cellulose

[c] Total DS_S was determined via elemental analysis or the sum of DS_{S6} and DS_{S2} (only for CS1 and 2). Partial DS_{S6} and DS_{S2} were estimated using ^{13}C NMR spectroscopy. DS_{S3} is equal the difference between the total and partial DS_S

[d] CS10 and CS11 were prepared using AC with an average DP of 1180 as starting cellulose

[e] For CS1, the reaction was stopped right after the reaction temperature of 70 °C was reached and no further heating was executed

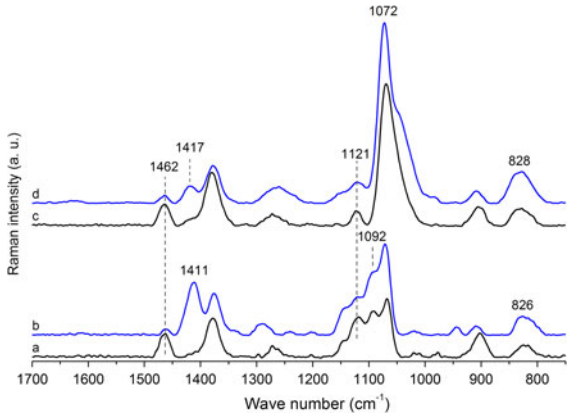

Fig. 3 FT Raman spectra (1,700–750 cm^{-1}) of (*a*) CS2, (*b*) COCS3, (*c*) CS3 and (*d*) COCS5 at RT

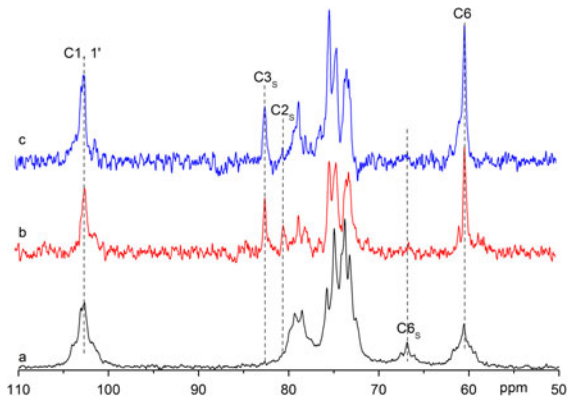

Fig. 4 ^{13}C NMR spectra (110–50 ppm) of (*a*) CS2, (*b*) CS10 and (*c*) CS11 in D_2O at RT

Moreover, $C3_S$ with sulfate groups at 3-*O*-position has a signal at 82.3 ppm (Groth and Wagenknecht 2001; Wang et al. 2009).

As described above, two signals for C6 as well as C1 are visible in the ^{13}C NMR spectrum of CS, which are due to the presence of C6 or C2 with respective hydroxyl groups and sulfate groups. The ratio of the integral of the signal at 66.8 ppm against the sum of the integrals of both signals at 60.5 and 66.8 ppm represents the ratio of sulfated primary hydroxyl groups and thus DS_{S6}. DS_{S2} was calculated indirectly based on the integrals of the signals of C1 and C1′ in the same way as the calculation of DS_{S6} (Zhang et al. 2011a).

The distribution of sulfate groups within repeating units also provides information on the trifluoroacetylation before sulfation. Exact distributions of trifluoroacetyl groups within AGU and especially degrees of 6-*O*-substitution are still not entirely clear, although several studies have been done in respect to the analysis on its distributions (Liebert et al. 1994, Mischnick 2002). By analyzing the distribution of sulfate groups after the homogeneous sulfation of CTFA, it is apparent that secondary hydroxyl groups were preferentially sulfated due to the protection of primary hydroxyl groups by trifluoroacetyl groups (Table 2). Therefore, one can estimate a trifluoroacetylation of cellulose primarily at 6-*O*-position and also partially at 2-*O*-position depending on the amount of used TFAA. However, it is difficult to come to an exact conclusion, since slightly different DS_{S6} were determined even using the same CTFA for subsequent sulfations (Table 1).

Generally, no sulfate group at 6-*O*-position is detectable for CS from CTFA that were prepared using high amounts of TFAA, as shown on CS10 and 11. Thus, the primary hydroxyl groups should be completely and the 2-*O*-psotion also highly esterified by trifluoroacetyl groups before the sulfation. Only if low amounts of TFAA (4.125 or 2 mol per mol AGU) were used, significant minor parts of 6-*O*-position were sulfated during following sulfations. Subsequently, preferentially regioselectively 2,3-*O*- and 2,6-*O*-sulfated CS were synthesized through the homogeneous sulfation of CTFA.

Moreover, the type of the sulfating agents and their amounts also affected the distribution of sulfate groups within the repeating units. Using a strong sulfating agent (e.g. chlorosulfonic acid for CS5) or a high amount of mild sulfating agent (SO_3·pyridine for CS8), almost no 6-*O*-sulfation was detectable, even though low amount of TFAA was used to dissolve cellulose. Furthermore, sulfamic acid that is a mild sulfating agent required a higher reaction temperature and longer reaction time, in order to prepare CS with comparable total DS_S to CS prepared with other sulfating agents. Thus, it is possible to alter the total as well as partial DS_S by changing the sulfation parameters (Table 2).

In addition, CS obtained after the acetosulfation of cellulose preferentially has the sulfate groups at 6-*O*-positions (Table 2), resulting in 6-*O* or 2,6-*O*-sulfation of cellulose. The introduction of sulfate groups through the acetosulfation required only low amounts of sulfating agents, compared to CS which were synthesized via CTFA with comparable total DS_S.

Regiospecific introduction of carboxyl groups

In the following step, selected CS with various distributions of sulfate groups were regiospecifically oxidized through TEMPO- or 4-acetamide-TEMPO-mediated oxidations (Tables 3, 4). These oxidations convert only primary hydroxyl groups into carboxyl groups. However, TEMPO and 4-acetamide-TEMPO-mediated oxidation have different effects on the oxidation of primary hydroxyl groups of cellulose. The 4-acetamide-TEMPO-mediated oxidation was found to be more effective towards converting primary hydroxyl groups of cellulose into carboxyl groups (Shibata and Isogai 2003; Zhang et al. 2012). Due to the good water-solubility of CS, the oxidations proceeded homogeneously in water. An advantage of this homogeneous derivatization is a possibly uniformly distributed carboxyl groups along the backbone chains. Figure 3 shows FT Raman spectra of obtained COCS3 and COCS5. The signals around 1,412 cm^{-1} are attributed to the vibrations of carboxylate groups (Zhang et al. 2012).

Figure 5 depicts liquid-state ^{13}C NMR spectra of COCS. The signal at 60.5 ppm represents the unmodified C6 with primary hydroxyl groups. The signal at 66.8 ppm within the ^{13}C NMR spectrum of COCS4 is attributed to the $C6_S$ with sulfate groups at 6-*O*-position. Moreover, a strong signal at 175.3 ppm is visible within the liquid-state ^{13}C NMR spectra of both COCS. This signal is ascribed to C6 in carboxylate groups (Zhang et al. 2012). Thus, CS containing carboxyl groups at 6-*O*-position were successfully synthesized.

Table 4 Oxidation of CS with TEMPO/NaBr/NaClO at pH 10–10.5 in water at 0 °C for 1 h

Samples	Starting materials	Molar ratio[a]			DS_S[b]	DO[b]
		TEMPO	NaBr	NaClO		
COCS7	CS3	0.05	1.5	10	1.02	0.81
COCS8	CS4	0.05	1.5	10	0.75	0.87
COCS9	CS5	0.05	1.5	14	0.7	0.72

[a] Molar ratio in mol per mol primary hydroxyl groups

[b] DS_S und DO were determined via elemental analysis

Apart from liquid-state ^{13}C NMR, solid-state CP/MAS ^{13}C NMR spectroscopy can also be used to analyze the introduction of carboxyl groups (Fig. 6). The signal at 174.8 ppm represents C6 in carboxylate groups, while the signal at around 62 ppm is ascribed to C6 with primary hydroxyl groups. Furthermore, a small signal at 68.7 ppm is notable, which is due to the sulfation at 6-*O*-position (Zhang et al. 2010, 2011a). The signals between 80 and 92 ppm are ascribed to chemical shifts of C4, whereas the signals between 60 and 68 ppm are due to chemical shifts of C6 (Dick-Perez et al. 2011). The signals at 88 and 63 ppm are attributed to C4 and C6 of cellulose that is present as ordered core (Hult et al. 2002). The signals at 84 and 65 ppm are derived from C4 and C6 of disordered cellulose at surface (Larsson et al. 1999; Ha et al. 1998). However, after the derivatization, only the signals at 83 and 62 ppm are still visible. This fact indicates that the ordered structure of cellulose was destroyed during the derivatization and obtained COCS have only a disordered structure. The presence

Table 3 Oxidation of CS with 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 6 in water at 60 °C

Samples	Starting materials	Molar ratio[a]			Oxidation duration (h)	DS_S[b]	DO[b]
		4-acetamide-TEMPO	NaClO	$NaClO_2$			
COCS1	CS1	0.03	0.2	2.5	24	0.65	0.21
COCS2	CS1	0.05	0.3	3.5	72	0.65	0.11
COCS3	CS2	0.05	0.3	3.5	18	0.29	0.76
COCS4	CS2	0.05	0.3	3.5	24	0.33	0.62
COCS5	CS3	0.05	0.3	3.5	18	1.18	0.52
COCS6	CS4	0.05	0.3	3.5	24	0.84	0.69

COCS2 was prepared at RT

[a] Molar ratio in mol oxidizing agent per mol hydroxyl groups

[b] DS_S und DO were determined via elemental analysis

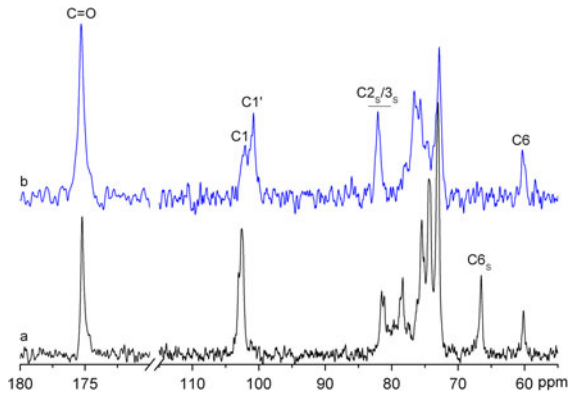

Fig. 5 ^{13}C NMR spectra (180–55 ppm) of (*a*) COCS4 and (*b*) COCS9 in D_2O at RT

of a disordered structure is advantageous for subsequent applications regarding the water solubility of these compounds. Moreover, the presence of C6-signal at 62 ppm suggests the presence of unmodified primary hydroxyl groups.

After the oxidations, the DO of obtained COCS were determined using elemental analysis and distinct DO in the range of 0.11–0.87 were calculated (Tables 3, 4). Generally, using TEMPO as catalyzer, higher DO were obtained within 1 h at 0 °C, while much longer time was required to reach similar DO using the same molar amount 4-acetamide-TEMPO as catalyzer. It was reported previously that 4-acetamide-TEMPO-mediated oxidation of cellulose led to water-soluble cellouronic acid with higher average degree of polymerization within shorter oxidation time than TEMPO (Shibata and Isogai 2003). The oxidation system containing 4-acetamide-TEMPO/NaClO/$NaClO_2$ is probably more effective than TEMPO/NaBr/NaClO towards the oxidation of primary hydroxyl groups of cellulose. However, in this study, the TEMPO-mediated oxidation of CS under homogeneous condition demonstrated a higher conversion rate of primary hydroxyl groups than that of the 4-acetamide-TEMPO-mediated oxidation.

Moreover, the contents of sulfate groups, i.e. total DS_S, maintained during the oxidations, although they were determined to be slightly different from those of starting CS for corresponding COCS. The change of total DS_S may be due to the side reactions during the oxidation, e.g. the depolymerization of cellulose chains resulting in the elimination of repeating units with or without sulfate groups. The depolymerization of cellulose chains during the TEMPO-mediated oxidation has been previously reported (Hirota et al. 2009; Shibata et al. 2006). Furthermore, it is apparent that the DO of COCS decreased with prolonged oxidation time (Table 3), indicating that a depolymerization took place during the oxidation.

In comparison to the previous study (Zhang et al. 2010), high content of carboxyl groups can be achieved and the content is tunable in a widely variable area. Moreover, the regioselective sulfation pattern within obtained COCS was more accurately defined through the synthesis routes. In particular, 2,3-*O*-cellulose sulfate with C_6-carboxyl groups was firstly synthesized. This class of compounds displays more similar chemical structure to heparin than CS and may show interesting heparin-analogue activities.

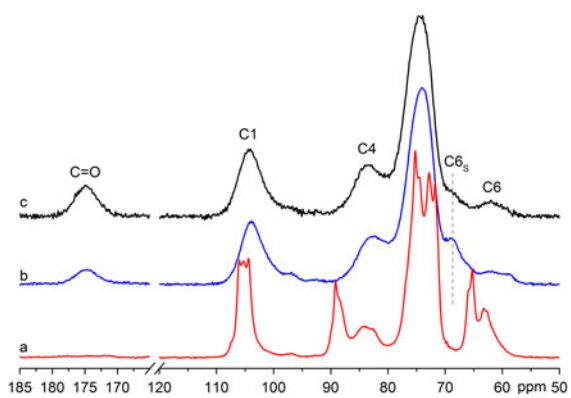

Fig. 6 Solid-state CP/MAS ^{13}C NMR spectra (185–50 ppm) of (*a*) starting microcrystalline cellulose, (*b*) COCS1 and (*c*) COCS7 at RT

Conclusion

In this report, sulfate groups were regioselectively and carboxyl groups regiospecifically introduced into AGU of cellulose, leading to various CSs as 6-*O*, 2,6-*O* or 2,3-*O*-cellulose sulfates and C_6-carboxyl cellulose sulfates (COCS). The synthesis of CS was realized after the homogeneous sulfation of CTFA or after the one-pot acetosulfation of cellulose. Using CTFA as intermediate, CS with sulfate groups primarily at 2,3-*O*- or 2,6-*O*-positions were obtained, while CS prepared through acetosulfation have the sulfate groups preferentially at 6-*O*-position. The introduction of carboxyl groups at C6 position was carried out as homogeneous TEMPO- or 4-acetamide-

TEMPO-catalyzed oxidation of primary hydroxyl groups. The amounts of both functional groups within repeating units were determined and their distributions analyzed. These compounds may be interesting structural mimics for naturally occurring pharmaceutical—heparin.

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