Carboxymethylation of Bacterial Cellulose

Kerstin Schlufter,[1,2] *Thomas Heinze**[1]

Summary: The carboxymethylation of bacterial cellulose (BC) was studied under typical heterogeneous reaction conditions. It was found that the BC possesses a significantly lower reactivity compared to wood cellulose converted under comparable conditions. Moreover, water-solubility of carboxymethyl cellulose (CMC) obtained from BC appears at rather high degree of substitution of about 1.5 although a nearly statistical functionalization pattern was analyzed by HPLC. Obviously, the nano-structure of BC is important for the reactivity and the properties of the synthesized CMC like water-solubility.

Keywords: bacterial cellulose; carboxymethylation; heterogeneous reaction; structure characterization

Introduction

At present, not only commercially large-scale production but also lab scale synthesis of cellulose derivatives uses the biopolymer isolated from wood or one-year plants as starting material.[1] In addition, it is well known that cellulose is produced by some strains of acetic acid bacteria. The synthesis of cellulose in *Gluconacetobacter xylinum* occurs between the outer membrane and the cytoplasma membrane by a cellulose synthesizing complex, which is in association with pores at the surface of the bacterium. The cellulose produced leaves the pores as fibrils and forms a ribbon of crystalline cellulose. The process of formation of bacterial cellulose is usually mentioned to occur extracellularly.[2] The bacterial cellulose (BC) is very different to cellulose from plants. Although the molecular structure of being a β-(1 → 4)-linked polyglucan is identical, the degree of polymerization (DP) is significantly higher (DP values of up to 10,000 are found for BC).[3] BC consists of extremely fine fibrils (100 nm) compared to cotton with a diameter of 10 μm, which forms a network structure. Cellulose from bacteria is extremely pure and contains no other biopolymers like different types of hemicelluloses and lignin, which are present in plant cellulose. Moreover, BC possesses a higher amount of cellulose I_α crystalline structure compared to cellulose isolated from wood.[4] Therefore, it is very difficult to carry out chemical modification reaction with BC. Very recently, it was shown that ionic liquids (ILs), in particular 1-butyl-3-methylimidazolium chloride ($[C_4mim]^+Cl^-$) dissolve efficiently bacterial cellulose. Cellulose with a DP of about 6,500 can be simple dissolved by mixing the polymer with the IL and stirring at a temperature 10 °C above the melting point (for $[C_4mim]^+Cl^-$ at about 85 °C).[5] The dissolution occurs without the formation of covalent bonds between the cellulose and the solvent; therefore, the IL is considered to be a non-derivatizing solvent. Depending on the IL used, a degradation of the polymer may appear that is, however, not in contrast to the definition of a non-derivatizing solvent as misrepresented by Laus et al.[6] The homogeneous acylation and carbanilation of bacterial cellulose in $[C_4mim]^+Cl^-$ was studied leading to

[1] Kompetenzzentrum Polysaccharidforschung, Friedrich-Schiller-Universität Jena, Humboldtstraße 10, D-07743 Jena, Germany
E-mail: thomas.heinze@uni-jena.de
[2] Permanent address: fzmb GmbH, Forschungszentrum für Medizintechnik und Biotechnologie, Geranienweg 7, D-99947 Bad Langensalza, Germany

products of high degree of substitution (DS) under mild conditions, short reaction time, and low excess of reagent. It could be shown that during the dissolution and reaction no significant degradation of the polymer chain appears.[5] Moreover, up to date little is known about the reactivity of BC under typical conditions carried out commercially, i.e., under heterogeneous conditions applying alcoholic slurries in the presence of aqueous sodium hydroxide. We have chosen to study the carboxymethylation of BC because the carboxymethyl cellulose (CMC) obtained is widely used in food applications as a thickener, water binder, extrusion aid and film former, among others.[7,8]

Experimental Part

Materials

Sodium monochloroacetate, sodium hydroxide and isopropanol were used as obtained from Merck. The bacterial cellulose **1** was produced by bacteria of the strain *Gluconacetobacter xylinum* (strain ATCC 10245). The microorganism was cultivated in glass vessels containing 3.5 l of Schramm-Hestrin medium[9] in static culture at 30 °C. 10 ml of a bacterial suspension (turbidity by McFarland 3–4) was used as inoculation medium. After a cultivation time of about 30 days the cellulose layers (3–4 cm thickness) were taken from the culture medium, cut into small pieces and washed twice with boiling 0.1 N aqueous NaOH for 30 min. After washing with distilled water to neutral reaction of the rinsing agent, the material was freeze dried, milled and dried again at 60 °C in vacuum.[10] The milling was carried out carefully with a centrifugal mill in order to prevent structure changes. The degree of polymerization of the BC was determined to be 6,500.

Carboxymethylation of BC

Carboxymethylation was carried out by a standard slurry method. For a typical reaction, 5 g (30.9 mmol) of bacterial cellulose (**1**) was suspended in 200 ml isopropanol and stirred vigorously while 9.3 ml 30% aqueous NaOH (see Table 1) was added drop wise during 30 min at room temperature. Stirring was continued for 1 h and 10.8 g (3 mol mol^{-1} anhydroglucose unit, AGU) of sodium monochloroacetate was added. The mixture was placed on a water bath at 55 °C for 5 h with stirring. After cooling, the mixture was filtrated, suspended in 300 ml of 80% (v/v) methanol, and neutralized with acetic acid. The product (**2c**) was washed three times with 350 ml 80% (v/v) ethanol and subsequently with 350 ml ethanol and dried at 60 °C.

Yield: 7.3 g (80%); DS_{CM} values are given in Table 1.

IR (KBr): 1607, 1421 cm^{-1} (C=O, carboxylate group).

Table 1.
Degree of substitution of carboxymethylated bacterial cellulose (**2a-h**) in dependence on the reaction time and on the concentration of aqueous NaOH solution (samples obtained by heterogeneous carboxymethylation of 5 g bacterial cellulose (**1**) with sodium monochloroacetate (MCA) in isopropanol at 55 °C.

Sample	Molar ratio	Molar ratio	Concentration	Reaction	Degree of substitution				
	MCA/AGU	$NaOH_{aq}$/MCA	$NaOH_{aq}$(%)	time (h)	HPLC[a)]	^{1}H-NMR[a)]			
						O-2	O-3	O-6	$\sum$
2a	3/1	1/1	30	3	1.53	0.54	0.28	0.65	1.47
2b	3/1	3/1	30	3	0.54	0.22	0.07	0.25	0.54
2c	3/1	1/1	30	5	1.68	0.58	0.36	0.71	1.65
2d	4/1	1/1	30	5	1.60	-	-	-	-
2e	4/1	1/1	15	5	1.24	0.49	0.22	0.55	1.27
2f	4/1	2/1	15	5	0.48	-	-	-	-
2g	4/1	0.5/1	30	5	1.05	0.38	0.15	0.47	1.00
2h	4/1	1/1	30	6	1.84	0.73	0.37	0.90	2.00

[a)]Degree of substitution determined by means of HPLC and ^{1}H NMR spectroscopy, respectively (for details see text)

Measurements

HPLC-analysis

The degree of substitution (DS) of carboxymethyl groups was determined after complete hydrolysis by HPLC according to reference.[11] The samples were hydrolyzed with perchloric acid. 0.1 g of CMC was dispersed in 2 ml of $HClO_4$ (70%) and, after 10 min at room temperature, diluted with 18 ml of deionized water. The mixture was kept at 100 °C for 16 h. The solution obtained was carefully neutralized with 2 M aqueous KOH and kept at 4 °C for 1 h to guarantee complete precipitation of $KClO_4$. The salt was filtrated off and washed tree times with 5 ml deionized water. The volume of the obtained solution was reduced to approximately 3 ml and diluted with deionized water to give exactly 5 ml sample. The HPLC measurement was carried out using a Knauer system with refractive index detector and two Bio-Rad Aminex HPX-87H columns, 0.01 N H_2SO_4 as eluent at a flow rate of 0.5 ml/min at 65 °C.

Spectroscopy

For ^{1}H-NMR spectroscopy the samples were hydrolyzed with a mixture of D_2SO_4/D_2O (25%, v/v) within 5 h at 90 °C. The analysis of the hydrolysates were carried out with a Bruker Avance 250 MHz (16 scans were accumulated). The ^{13}C-NMR spectra were taken in D_2O on a Bruker Avance 400 MHz spectrometer and up to 12,000 scans were accumulated.

The FT-IR spectra were acquired with a NICOLET AVATAR 370 DTGS spectrometer in KBr.

Viscosimetry

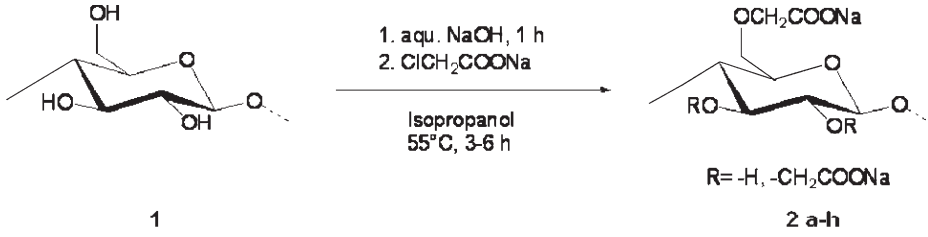
The degree of polymerisation (DP) of the BC was determined by capillary viscometry according to DIN 54270 applying copper-(II)ethylenediamine (Cuen) as solvent (for details see reference[12]).

The DP of the carboxymethylated BC was obtained by measuring the relative viscosities in 6% (w/v) aqueous sodium hydroxide solution using an Ubbelohde viscometer at 20 °C. The concentration of CMC in aqueous NaOH was 0.2%.[13] The relative viscosity $[\eta_{rel}]$.was determined and the degree of polymerization (DP) could be calculated with the following equations (1):

$$[\eta] = \frac{8}{c}\sqrt[8]{\eta_{rel}} - 1$$
$$DP = \frac{[\eta]}{0.0066} \quad (1)$$

Results and Discussion

In our experiments, bacterial cellulose (BC, **1**) with a degree of polymerization (DP) of 6,500 slurried in isopropanol was converted with sodium monochloroacetate (MCA) after activation of the polymer with aqueous NaOH solution using different reaction times from 3 to 6 h at 55 °C. To activate the BC prior the carboxymethylation, aqueous sodium hydroxide solution of concentrations of 15 and 30% was used. In dependence on the lye concentration, the polymer swells to certain extend and decrystallization occurs. Consequently, an even accessibility of any reactive hydroxyl group is guaranteed (Scheme 1).



Scheme 1.
Reaction scheme for the carboxymethylation of bacterial cellulose.

Regarding the results (Table 1), it has to be underlined that under any reaction conditions applied carboxymethylated BC samples were obtained. The IR spectra of the samples synthesized show the typical absorptions of the cellulose backbone as well as peaks at about 1607 and 1421 cm^{-1}, indicating the presence of the carboxymethyl ether groups (Figure 1).

The total degree of carboxymethylation (DS_{CM}) reached a maximum value of 1.84 at a concentration of 30% aqueous NaOH and a molar excess of 4 mol sodium monochloroacetate (MCA) per mol anhydroglucose unit (AGU) after a reaction time of 6 h. With respect to the molar ratio of cellulose/MCA, a conversion of 46% of the MCA took place, which is less compared to the reaction of wood pulp.[14] Any other NaOH concentration and/or reaction time gave products of lower DS_{CM}.

From the results summarized in Table 1, it is obvious that the lye concentration has a strong influence on the DS_{CM} achieved. Applying 15% aqueous sodium hydroxide, a DS_{CM} of 1.24 resulted, while the reaction in the presence of 30% lye yields a product with a DS_{CM} of 1.60. Already a reaction time of 3 h and a molar ratio of 3 mol MCA per mol AGU results in a product with a DS_{CM} of 1.53. A prolongation of the reaction time leads to higher DS_{CM} values (compare sample **2a** and **2c**, Table 1).

Moreover, the ratio of NaOH/MCA influences the DS_{CM} of the carboxymethylated bacterial cellulose; at a ratio of 0.5/1 a DS_{CM} of 1.05 is reached while at a ratio of 1/1 the DS_{CM} is 1.84 provided a molar ratio of MCA/AGU of 4/1 is used. At a ratio of MCA/AGU of 3/1 the DS_{CM} values are 1.53 (NaOH/MCA = 1/1) and 0.54 (NaOH/MCA = 3/1).

Different methods were used in order to get information about the functionalization pattern of the novel CMC samples based on BC. ^{1}H-NMR spectroscopy on depolymerized samples is a rapid method to gain the partial DS values at the different positions 2, 3, and 6 within the AGU. A representative spectrum including the assignment of signals is shown in Figure 2. The x_i values were calculated according to the partial DS

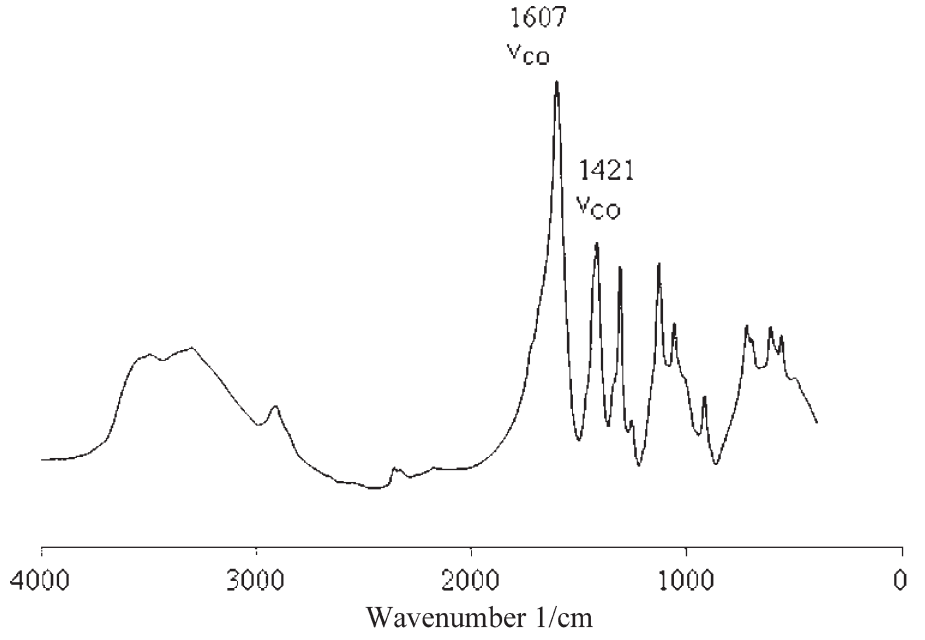


Figure 1.
FT-IR spectrum of carboxymethylated bacterial cellulose (**2h**) with a degree of substitution $DS_{CM} = 1.84$ (determined by HPLC).

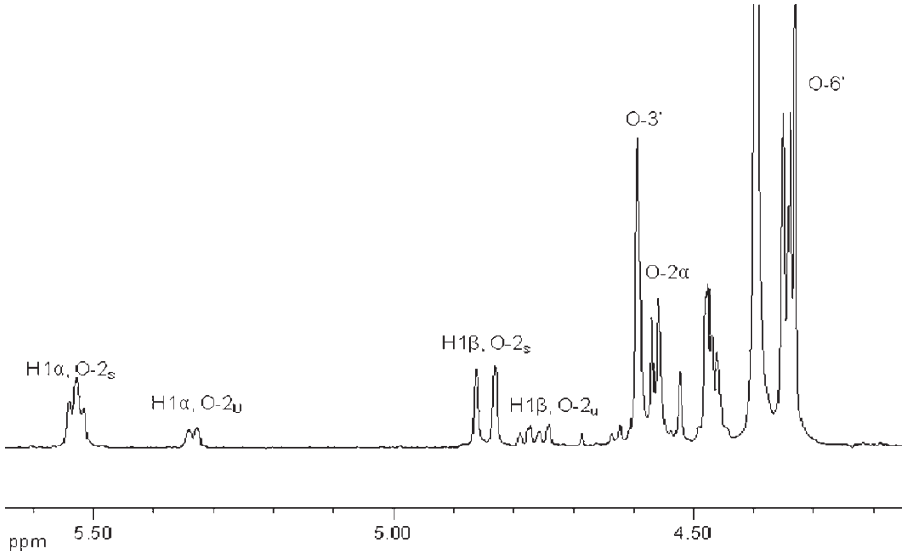


Figure 2.
^{1}H NMR spectrum of carboxymethylated bacterial cellulose (sample **2h**) after complete acidic depolymerization ($DS_{CM} = 1.84$, determined by HPLC).

at the representative position 2, 3 and 6, Equation (2). A represents the peak area, O the oxygen atom at the position i (i = 2, 3, and 6), H-1 the hydrogen atom at the anomeric C, α and β the configuration of glucose, s stand for substituted, u for unsubstituted.

functionalization pattern on the reaction conditions, mainly on the concentration of the aqueous NaOH solution.[14] The question about the structural feature controlling the distribution of the carboxymethyl groups cannot be answered at the present point of studies.

$$x_i = \frac{\frac{1}{2}A\,(methylene\ protons\ at\ position\ O-i)}{A(H^1_\alpha, O-2_s) + A(H^1_\alpha, O-2_u) + A\left(H^1_\beta, O-2_s\right) + A\left(H^1_\beta, O-2_u\right)}$$

$$DS = \sum\nolimits_i x_i \qquad (2)$$

$$x_i = \frac{A(H^1_\alpha, O-2_s) + A\left(H^1_\beta, O-2_s\right)}{A(H^1_\alpha, O-2_s) + A(H^1_\alpha, O-2_u) + A\left(H^1_\beta, O-2_s\right) + A(H^1_\beta, O-2_u)}$$

In any case, the OH groups at C-3 possess the lowest reactivity, i.e., the partial DS at this position shows the lowest value (Table 1). The partial DS values for O-2 and O-6 are almost equal, although position 6 is mostly preferred. It is important to note that in case of carboxymethylation of plant cellulose position 2 is mostly preferred showing a slight dependence of the

Complete depolymerization of the polymer chains is absolutely necessary for the determination of the repeating units by means of HPLC. This was achieved by a treatment of the polymers with concentrated $HClO_4$ for 10 minutes at room temperature and after dilution with water for 16 h at 100 °C. After removal of the perchlorate anions by precipitation with a

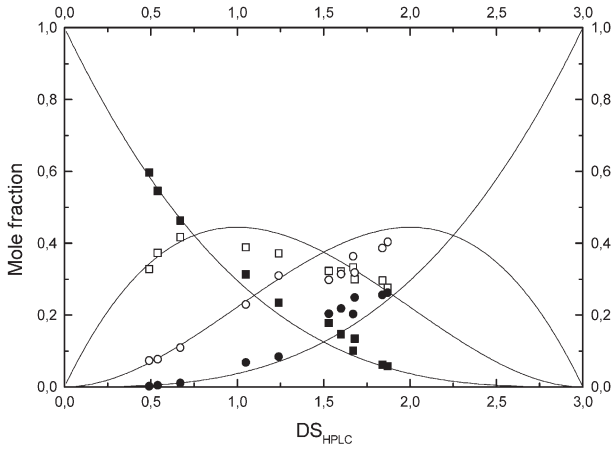


Figure 3.
The mole fractions of (■) glucose (c_u), (□) 2-; 3- and 6-mono-O-carboxymethyl glucoses (c_m), (○) 2,3-; 2,6- and 3,6-di-O-carboxymethyl glucoses (c_d), (●) 2,3,6-tri-O-carboxymethyl glucose (c_t) in hydrolyzed CMC samples plotted as function of DS_{CM} determined by HPLC. The curves are calculated as described in the text.

solution of KOH, a direct separation on a strong cation exchange column is possible. The separation into glucose (glc, c_u), mono-O-CM-glc (c_m), di-O-CM-glc (c_d), and tri-O-CM-glc (c_t) is achieved (Figure 3), however, a separation of the three different mono- and di-O-CM-glc repeating units was neglected, which is in principle also possible by using different chromatographic conditions.[15,16] For the present studies it is more suitable to use the four main repeating units only.

The mole fractions determined are summarized in Table 2. The total DS values calculated from equation $DS = c_m + 2c_d + 3c_t$ agree very well with those obtained by means of ^{1}H-NMR spectroscopy except for sample **2h** where a difference of 0.16 DS units appears (see Table 1).

In Figure 3 the mole fractions obtained are graphically displayed as a function of the total DS_{CM}. On the basis of a statistical model for the arrangement of functional groups in cellulose derivatives – first proposed by Spurlin[17] – the shown curves were calculated. The model assumes that no preference of any hydroxyl group (position 2, 3, and 6) exists and that the relative reactivities of the three hydroxyl groups in the AGU are constant throughout the reaction and independent of the DS

Table 2.
The mole fractions of glucose (c_u), 2-; 3- and 6-mono-O-carboxymethyl glucoses (c_m), 2,3-; 2,6- and 3,6-di-O-carboxymethyl glucoses (c_d) and 2,3,6-tri-O-carboxymethyl glucose (c_t) in hydrolyzed CMC samples (**2a-h**) determined by means of HPLC.

Sample	Mole fraction				DS[b]
No.[a]	c_u	c_m	c_d	c_t	
2b	0.5454	0.3731	0.1542	0.0132	0.54
2c	0.1339	0.2991	0.6363	0.7466	1.68
2d	0.1466	0.3213	0.6278	0.6548	1.60
2e	0.2342	0.3720	0.6193	0.2526	1.24
2f	0.5970	0.3282	0.1462	0.0050	0.48
2g	0.3138	0.3888	0.4589	0.2039	1.05
2h	0.0613	0.2963	0.7725	0.7683	1.84

[a] see Table 1.
[b] DS: degree of substitution calculated according to $DS = c_m + 2c_d + 3c_t$.

of the cellulose chain or the state of functionalization at another position within the same AGU. Summarizing these assumptions, the following binominal distribution results (Equation 3):

$$c_i = \binom{3}{k}\left(\frac{DS}{3}\right)^k (1 - DS/3)^{3-k} \qquad (3)$$

Were c_i are the mole fractions of unsubstituted, mono-, di-, and tri-O-carboxymethylated glucose units, respectively, k is the number of functional groups per AGU (k = 0, 1, 2, 3), and DS is the average degree of substitution. Up to a DS value of 0.7, the determined mole fractions are in good agreement with the statistical model, independent of the concentration of the aqueous NaOH and of the reaction time.

At higher DS values different behavior was found. Samples of DS of 1.68 and 1.84 (highest DS values obtained) possess a statistic content of glc while the mole fractions of mono- and tri-O-CM-glc units are slightly higher than statistically expected. The di-O-CM-glc units are present with a higher content. At medium DS values, mono- and di-O-CM-glc units are formed to a lower amount while the tri-O-CM-glc is formed with a higher amount. Obviously, the CMC based on BC does not show a clear correlation between the repeating units formed and statistic calculation. For CMC made from plant cellulose, the samples possess a statistic functionalization pattern provided a heterogeneous reaction after sufficient mercerization was carried out. On the contrary, CMC from plant cellulose synthesized starting with the dissolved polymer (e.g., dissolved in N,N-dimethylacetamide/LiCl) and activation with solid sodium hydroxide yields products with a non-statistic content of the different repeating units.[18] The non-statistically functionalized CMC dissolve in water at a DS as high as 1.5 to 1.6. Thus, the CMC from BC show a comparable DS limit for water solubility although the deviation from the statistics is rather small. As discussed in ref.[19] CMC with DS values in the range from 0.75 to 0.90 synthesized from BC are water insoluble due to the fact that long sequences of unsubstituted anhydroglucose units appear forming chain aggregates. Regarding our HPLC results, there appears no significant amount of glucose in the CMC; thus the aggregation discussed in ref.[19] is somehow questionable. More likely, at rather low DS the polymer consists of highly functionalized chains at the outside of the nanofibrils while the core is not or only insufficiently functionalized. A mixture of highly functionalized chains and non-substituted ones might indicate a ''statistic'' functionalization as well. The heterogeneous structure of the CMC samples at low DS corresponds with the well-known nanostructure of the BC-fibrils, as shown by SEM (Figure 4).

By increasing the DS up to 1.5, a water soluble product was obtained. It may be assumed that a peeling occurs and a sufficient functionalization was reached. The slight deviation of the functionalization

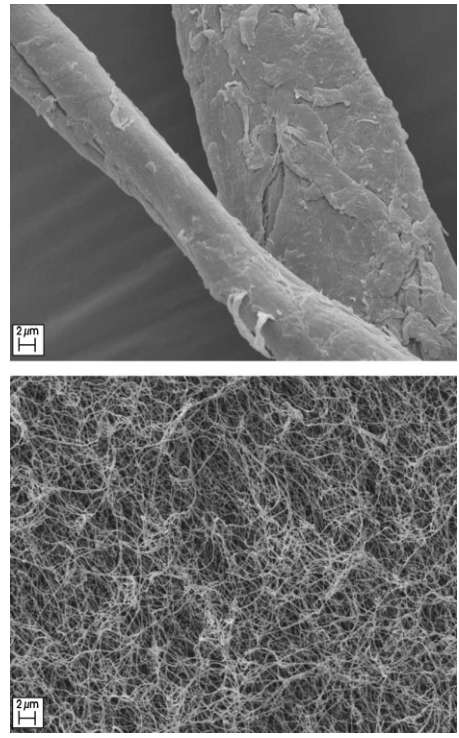


Figure 4.
SEM-images of cotton linters and bacterial cellulose.

Table 3.
Intrinsic viscosity [η] and degree of polymerization (DP) of CMC samples (**2a-h**) dissolved in 6% (w/v) aq. NaOH

Sample	Intrinsic viscosity [η]	DP
2a	5.467	828
2b	6.700	1,015
2c	7.342	1,112
2d	6.466	980
2e	5.912	896
2f	3.518	533
2g	6.446	978
2h	4.900	742

pattern from a statistic one is obviously not important regarding water solubility at thus high DS values.

Viscometric studies of the CMC samples applying a method proposed by Crössmann et al. (0.2% (w/v) CMC in 6% (w/v) aqueous NaOH)[13] indicate a considerable degradation of the BC samples by heterogeneous carboxymethylation (Table 3). The DP values of the CMC are much lower than that of the original BC (DP of 6,500), revealing strong degradation of the polymer during reaction due to the alkaline conditions (oxidative degradation). The reason for the different DP values of the samples **2a–h** cannot be satisfactorily explained with the present results. Detailed studies would be necessary. However, degradation contradicts our goal to synthesize CMC of high molecular weight and hence forming high viscous aqueous solution.

Conclusion

It is possible to obtain carboxymethylated cellulose from bacterial cellulose (BC) by conventional heterogeneous reaction. The degree of substitution (DS) obtained is lower compared to conversion of wood cellulose under comparable conditions showing the low reactivity of BC. A high DS of about 1.5 is needed to get water soluble products. It is obvious the nanostructure of BC is the important structural feature (compared to wood cellulose with micofibrils) determining not only the overall properties but also the chemical reactivity. Moreover, polymer severe degradation was observed. Consequently, the heterogeneous conversion of BC is no appropriate path for the structure design of cellulose derivatives based on BC. On the contrary, homogeneous phase chemistry allows the chemical modification of BC satisfactorily as recently shown applying ionic liquids as reaction medium.[5,20]

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[1] Th. Heinze, *Chemical Functionalization of Cellulose*, In: *Polysaccharide: Structural Diversity and functional versatility*, 2nd edition, S. Dumitriu, Ed., Marcel Dekker, New York, Basel, Hong Kong **2004**, pp. 551.
[2] R. Jonas, L. F. Farah, *Polym. Deg. Stab.* **1998**, *59*, 101.
[3] F. Yoshinaga, N. Tonouchi, K. Watanabe, *Biosci. Biotech. Biochem.* **1997**, *61*, 219.
[4] D. Klemm, *Progr. Polym. Sci.* **2001**, *26*, 1561.
[5] K. Schlufter, H.-P. Schmauder, S. Dorn, Th. Heinze, *Macromol. Rapid Commun.* **2006**, *27*, 1670.
[6] G. Laus, G. Bentivoglio, H. Schottenberger, V. Kahlenberg, H. Kopacka, Th. Röder, H. Sixta, *Lenzinger Ber.* **2005**, *84*, 71.
[7] R. L. Feddersen, S. N. Thorp, in "*Industrial Gums*", 3rd Ed.; R. L., Whistler, J. N. BeMiller, Eds., Academic Press, San Diego **1993**, 537.
[8] Hercules brochure, "Aqualon® Sodium Carboxymethylcellulose. Physical and Chemical Properties" Number 250-10G **1997**.
[9] M. Schramm, S. Hestrin, *J. Gen. Microbiol.* **1954**, *11*, 123.
[10] M. Hornung, M. Ludwig, A. M. Gerrard, H.-P. Schmauder, *Eng. Life Sci.*, **2006**, *6*, 537.
[11] Th. Heinze, U. Erler, I. Nehls, D. Klemm, *Angew. Macromol. Chem.* **1994**, *215*, 93.
[12] S. Barthel, Th. Heinze, *Green Chem.* **2006**, *8*, 301.
[13] F. Crössmann, W. Klaus, F. Mergenthaler, S. W. Souci, *Z. Lebensmitt. Unters.* **1964**, *125*, 413.
[14] Th. Heinze, K. Pfeiffer, *Angew. Macromol. Chem.* **1999**, *266*, 37.
[15] E. A. Kragten, J. P. Kamerling, J. F. G. Vliegenthart, *J. Chromatography* **1992**, *623*, 49.
[16] P. Käuper, W.-M. Kulicke, S. Horner, B. Saake, J. Puls, J. Kunze, H.-P. Fink, U. Heinze, Th. Heinze, E.-A. Klohr, H. Thielking, W. Koch, *Angew. Makromol. Chem.* **1998**, *260*, 53.
[17] H. M. Spurlin, *J. Am. Chem. Soc.* **1939**, *61*, 2222.
[18] Th. Heinze, *Macromol. Chem. Phys.* **1998**, *199*, 2341.
[19] H. N. Cheng, M. Takai, E. A. Ekong, *Macromol. Symp.* **1999**, *140*, 145.
[20] S. Dorn, A. Pfeifer, K. Schlufter, Th. Heinze, *Polym. Bull.* **2009**, in press, 10.1007/s00289-009-0172-6.

