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ORIGINAL RESEARCH

Development of hydrogels based on oxidized cellulose sulfates and carboxymethyl chitosan

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Abstract Cellulose or chitosan represent highly abundant biopolymers possessing excellent biocompatibility that is required in tissue engineering. Both, cellulose and chitosan can be used to form hydrogels that can replace soft human tissues like cartilage. Hence, we developed here an approach to oxidize cellulose after sulfation, which was then crosslinked with carboxymethyl chitosan (CMCh). Sulfation was performed either by direct or acetosulfation reaching different sulfation degrees of $DS_{\mathrm{Sulf}} = 0.8$–2.0. Subsequently oxidation of cellulose sulfate (CS) was performed with sodium periodate, which yielded aldehyde contents of $DS_{\mathrm{Ald}} = 0.1$–0.3. Since oxidation requires the presence of vicinal hydroxyl groups in the anhydroglucose unit (AGU) of CS, higher sulfation degree as obtained by direct sulfation including hydroxyl groups at C2 and C3-position yielded lower aldehyde contents. On the contrary, regioselective sulfation at C6-position by acetosulfation was more suitable to achieve higher oxidation degrees of CS. Consequently, hydrogel formation obtained by chemical crosslinking of oxidized cellulose sulfate (oCS) with CMCh was fast within seconds when oxidation degree was high, but sulfation degree low. Moreover gel formation lasted almost 24 h when sulfation degree was high. It could also be shown that hydrogels based on oCS with a DS_{Ald} of 0.28 or higher were stable for 25 days when incubated in phosphate-buffered saline (PBS) or Dulbeccos modified Eagle medium (DMEM). Studies with pH dependent fluorescent tracer molecules could show that the intrinsic pH value in hydrogels was slightly acidic ($\sim$ pH 6.4) when they were incubated in PBS at pH 7.4. Mass transfer and homogeneity of the gel network was studied by NMR finding that diffusion of water molecules was not hindered inside the hydrogels.

Keywords Hydrogel · Tissue engineering · Oxidized cellulose sulfate · Carboxymethyl chitosan · Imine bond formation

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Introduction

1960, Wichterle and Lím (1960) described the first hydrogel based on hydroxyethyl methacrylate, which was the beginning of a new research era. Hydrogels are polymeric networks which can absorb and swell in water multiple times of their own dry weight and volume, respectively. They can be classified on the base of their cross-linking properties into physical and chemical hydrogels (Hoffman 2012). The connection of physical gels is reversible and consists of physical interactions such as formation of hydrogen bonds, ionic interactions or polymer chain entanglements (Caló and Khutoryanskiy 2015). On the contrary, the chemical junction is permanent and formed by covalent bonds. Today, hydrogels have numerous application possibilities ranging from wastewater treatment (Kumari and Chauhan 2011) over agriculture (Guilherme et al. 2010) to tissue engineering (Drury and Mooney 2003). Likewise the polymers used for gel formation are diverse and can be both of natural and synthetic origin (Wang et al. 2016). With regard to tissue engineering, hydrogels have to meet certain requirements like being non-toxic, biocompatible and bioactive (Shen et al. 2016), hence natural materials such as cellulose or chitosan have been already applied in recent studies (LogithKumar et al. 2016; Svensson et al. 2005).

Cellulose is the most abundant renewable polymer on earth and therefore low prized. The three hydroxy groups per AGU can be functionalized in manifold manner. However cellulose itself is not water soluble and only marginally swellable. The transformation into a water soluble derivate would facilitate the hydrogel synthesis. One such possibility is the implementation of sulfate groups (Wagenknecht et al. 1992). Not only the solubility increases this way, but also the bioactivity as it has been reported by Zhang et al. (2009) showing that cellulose sulfates can bind growth factors like fibroblast growth factor (b-FGF) and hereby promote the proliferation of cells.

Besides cellulose chitosan is another attractive raw material for hydrogel preparation because of its antimicrobial properties (Johney et al. 2017) and biodegradability (Muzzarelli 1997). Moreover chitosan as a polycation has, aside from hydroxy groups, an amino group per AGU which offers further synthetic possibilities. Chen and Fan (2008) used the polycationic nature of chitosan and synthesized a thermoset hydrogel system to form the gel in situ with the potential to serve as targeted drug carriers.

To crosslink chitosan the amino group can be used in a click reaction with an aldehyde or ketone to form an imine bond (Millan et al. 2015). The advantages of click reactions are their entire conversion to the product, their velocity and application to polysaccharides (Yang et al. 2016).

In this report we would like to present a synthesis to oxidize cellulose after sulfation, which was then crosslinked with CMCh to form a hydrogel via Schiff base. The gel components were characterized through Raman spectroscopy, NMR and size exclusion chromatography. The gel itself was characterized with rheological measurements as well as through stability studies in PBS and DMEM. Intrinsic pH during gel formation and diffusion of water inside the hydrogels were investigated with pH sensitive fluorescence probes and Benchtop NMR, respectively.

Experimental

Chemicals and reagents

Sulfite pulp ($DP_W = 1300$) was received from Lenzing (Lenzing, Austria). Carboxymethyl chitosan ($DS = 1$, degree of deacetylation = 94.2%), chitosan lactate and chitosan acetate were purchased from Heppe Medical Chitosan GmbH (Halle/Saale, Germany). Ethanol 99.5% denatured from Grüssing (Filsum, Germany), chlorosulfuric acid for synthesis and PBS from Merck (Darmstadt, Germany), dimethylformamide (DMF) 99% and sodium periodate $\geq 99.0\%$ were purchased from Sigma Aldrich (Munich, Germany), ethylene glycol $\geq 99.5\%$ p.a., sodium hydroxide and sodium acetate trihydrate were obtained from Carl Roth (Karlsruhe, Germany). The fluorescence dye SNARF-4F 5-(and-6)-carboxylic acid (SNARF) was purchased from Invitrogen (Darmstadt, Germany). DMEM was purchased from Lonza (Verviers, Belgium). DMF was dried over molecular sieve with a pore size of 3 Å. All other reagents were used without further purification. All aqueous solutions were prepared using deionized water. Dialysis membranes from Spectra/Por® had a molecular weight cut off of 100 Da to 500 Da and of 3500 Da.

Sulfation of cellulose

Sulfation of cellulose was conducted by two methods referring to Zhang et al. (2011) with slight modifications. For both variations pretreatment was the following: Cellulose (2.50 g; 0.0154 mol) was soaked in 125 ml DMF at ambient temperature for 24 h. Dialysis was carried out using a standard regenerated cellulose membrane with a molecular weight cut off of 3.5 kDa.

Sulfation of cellulose The reaction agent consisting of DMF (25 ml) and chlorosulfuric acid (0.0462 mol, 3.0 eq.; 0.0693 mol, 4.5 eq.; 0.0924 mol, 6.0 eq.) was prepared while cooling in an ice bath. The sulfating agent was added dropwise to the soaked cellulose at room temperature (RT). After 3 h of vigorous stirring the now clear solution was poured in an ethanolic solution (500 ml), which contained NaOH (14.3 g, 0.358 mol), NaOAc·3 H_2O (11.05 g, 0.0812 mol) and H_2O (26.5 ml) filled up to 500 ml with ethanol (99%). The precipitate had to rest at least 12 h before the supernatant was removed by centrifugation. Afterwards the sediment was washed two times with 4% ethanolic/aqueous (v:v; 1:1) sodium acetate solution (100 ml). The product was dissolved in a minimum of H_2O and the pH was adjusted to 8.0 with glacial acetic acid. Next, the cellulose sulfate was precipitated and dissolved two more times, then dialyzed and lyophilized.

Acetosulfation The reaction agent consisting of DMF (25 ml), chlorosulfuric acid (0.0462 mol, 3.0 eq.) and acetic anhydride (0.123 mol, 8.0 eq.) was prepared while cooling in an ice bath. The sulfating agent was added dropwise to the soaked cellulose and heated to 50°C, followed by 5 h of intense stirring at 50°C. To quench the reaction the solution was poured in an ethanolic solution (500 ml) which contained NaOH (14.3 g, 0.358 mol), NaOAc·3 H_2O (11.05 g, 0.0812 mol) and H_2O (26.5 ml) filled up to 500 ml with ethanol (99%). The precipitate had to rest at least 12 h before the supernatant was removed by centrifugation. The sediment was washed two times with 4% ethanolic/aqueous (v:v; 1:1) sodium acetate solution (100 ml). The product was then dissolved and stirred for 20 h at ambient temperature in NaOH (50 ml, 1 m/l) for deacetylation, thereafter the pH was adjusted to 8.0 using glacial acetic acid. The cellulose sulfate was precipitated and dissolved two more times, then dialyzed and lyophilized.

Oxidation of cellulose sulfate

The oxidation method is based on Han et al. (2010). Cellulose sulfate (1.5 g) was dissolved in water (60 ml) and sodium periodate (1.5 g; 0.007 mol; 1 weight equivalent) was added. The solution was stirred at ambient temperature under exclusion of light for 6 h. By adding ethylene glycol (1.5 ml; 0.027 mol) and stirring for another hour the reaction was quenched. The oxidized cellulose sulfate was precipitated in ethanol (300 ml), dialyzed using a cellulose ester membrane with a molecular weight cut off of 100 Da to 500 Da and lyophilized to obtain a white solid product.

Cross-linking of oxidized cellulose sulfate and carboxymethyl chitosan

Oxidized CS (20 mg) and CMCh (20 mg) were each dissolved in 1 ml PBS (KCl (0.2 g), NaCl (8.0 g), KH_2PO_4 (0.19 g) and $Na_2HPO_4 \cdot 2H_2O$ (0.765 g) filled up to 1 l with deionized water, afterwards the pH was adjusted with NaOH to 7.4). Then both solutions were mixed together 1:1 and stirred at ambient temperature with a stirring rod until a colorless gel was obtained.

Hydrogel stability

The oCS and CMCh were dissolved in PBS at a concentration of 20 mg/ml. The hydrogels were created by mixing oCS and CMCh solution 1:1 to a final volume of 100 µl. The hydrogels were incubated 1 h at 37°C for crosslinking. Than the hydrogels were immersed, either with PBS or cell culture medium DMEM for up to 25 days. At each time point the solution was aspirated, remaining solution was eliminated with filter paper and the wet weight was determined.

Measurements

Fourier-transform (FT) Raman spectra were recorded on a Bruker MultiRam spectrometer with a liquid nitrogen cooled Ge diode as detector. All spectra were

recorded over a range from 3500 to 150 cm^{-1} with a resolution of 4 cm^{-1}.

The ^{13}C-NMR spectra of CS in D_2O were recorded at RT on Bruker Avance III 500 MHz (Bruker, Etlingen, Germany) with a frequency of 125.76 MHz, pulse length of 12.05 µs, acq. time of 0.35 s and a relaxation delay of 3 s. The ^{1}H-NMR spectra of oxidized CS in D_2O were recorded at RT on the same device with a frequency of 500.13 MHz, pulse length of 12.1 µs, acq. time of 3.3 s and a relaxation delay of 1 s.

Size-exclusion chromatography (SEC) was performed on a SEC-7 (Jasco, Germany) equipped with UV detector (UV-975) and a RI detector (RI-930) under following conditions: columns: Suprema precolumn, Suprema 1000, Suprema 30; column temperature: 30.00°C; eluent: 0.1 mol/l $NaNO_3$/0.05% NaN_3; flowrate: 1.000 ml/min; standards: pullulan. All samples were filtered through a syringe filter (nylon, pore size 0.45 µm) ahead the injection.

Sulfur content was measured using the sulfur analyzer CS-580 by ELTRA. The degree of substitution (*DS*) was calculated with Eq. 1 based on the determined sulfur content.

$$DS_{\mathrm{Sulf}} = \frac{162.1 \cdot S(\%)}{3207 - 102.1 \cdot S(\%)} \tag{1}$$

The frequency sweep was conducted using an Ares-G2 (TA Instruments) under following conditions: 15 mm parallel plate; temperature: 25°C; strain: 3.0×10^{-3}; logarithmic sweep; angular frequency: 0.1 rad/s to 100 rad/s.

Determination of aldehyde content A blank consisting of hydroxylammonium chloride (0.4 mol/l; 20 ml) and deionized water (25 ml) was made and the pH was measured. Dialyzed oCS (60.0 mg) was dissolved in deionized water (25 ml) and the pH was adjusted to 7.0 using sodium hydroxide (0.01 mol/l). After adding hydroxylammonium chloride (0.4 mol/l; 20 ml), the reaction mixture was stirred for a minimum of three hours at RT. The released HCl was titrated with NaOH (0.01 mol/l) until the pH of the blank was reached. The amount of aldehyde given as DS_{Ald} was calculated by the following equation:

$$DS_{\mathrm{Ald}} = \frac{6 \cdot M_{\mathrm{C}} \cdot 0.01 \cdot t_{\mathrm{NaOH}} \cdot V_{\mathrm{NaOH}}}{C(\%) \cdot m} \tag{2}$$

M_{C}: molar masse of carbon atom (12.01 g/mol), t_{NaOH}: titer of 0.01 mol/l NaOH, V_{NaOH}: volume of consumed 0.01 mol/l NaOH solution in ml, $C(\%)$: carbon content of sample in %, m: mass of sample in mg.

Detection of pH The pH was measured by fluorescence microscopy with the pH-sensitive dye SNARF. This method has the advantage to detect a local pH inside the hydrogel. However, the pH range is limited by the dyes sensitivity range from pH 5 to 7 (Schädlich et al. 2014). The dye was incorporated into the oCS (200 µg/ml) or CMCh (100 µg/ml) solution. The pH of the hydrogels prepared in PBS was measured once with the dye mixed in the cellulose solution and the once mixed in the CMCh solution to exclude a bias towards more neutral pH values in CMCh or towards more acidic values in the CS solutions. 10 µl of the two solutions each were pipetted and stirred in a 100 µl insert inside a glass vial. The vial was placed under the fluorescence microscope consisting of a light source (PhotoFluorII NIR), a microscope (Leica DM4000B) with Nuance EX fluorescence detector and Nuance Software. An image cube was detected with green (515–560 nm) and red (620–660 nm) excitation light. The two different excitations make it possible to compensate varying intensity and spectral broadening (Schädlich et al. 2014). The measurement was repeated without addition of SNARF to measure the background and auto-fluorescence of the hydrogels. The fluorescence spectrum was extracted from a region of interest in the image cube. The spectrum of the blank sample was subtracted. The ratio of the intensities is calculated with Eq. 3 from (Schädlich et al. 2014).

$$ratio = \frac{I^{gr}_{609\mathrm{nm}} - I^{red}_{679\mathrm{nm}}}{I^{gr}_{625\mathrm{nm}}} \tag{3}$$

From the *ratio* the pH can be calculated with the help of a calibration curve. The calibration was made with PBS with 20 µg/ml SNARF and variation of pH with NaOH and HCl.

Homogeneity and permeability of the hydrogels The transverse relaxation time distribution was characterized with a low-field benchtop Maran Ultra ^{1}H-NMR spectrometer (Oxford Instruments, UK) including an air flow temperature control and a 3D imaging unit. Water, CMCh solution and the hydrogels were measured with a CPMG sequence consisting of a single 90° radio frequency (rf) pulse followed by a

series of 180° rf pulses with 20480 Echos, $2\tau = 135$ µs and 25 s recycle delay. The Windows Distributed EXPonential analysis software (WinDXP, Oxford Instruments, UK) was used to calculate T_2 distributions with 256 points in the relaxation time range from 10×10^{-6} s to 20 s.

The diffusion coefficients of the water molecules were measured with pulsed field gradient stimulated echo with gradient pulses of variable length. The diffusion time was 400 ms. The maximal gradient strength of 0.922 T/m was used.

Results and discussion

Synthesis of cellulose sulfates

The direct sulfation of cellulose is a quasihomogenous reaction. During the process of sulfation the cellulose becomes more and more soluble in DMF. The introduced sulfate group can be detected easily by FT-Raman spectroscopy based on the sulfate band at 1070 cm^{-1} (Fig. 1).

In contrast to direct sulfation, acetosulfation is a regioselective derivatization process. The acetyl groups work as protecting groups at C2 and C3-position so that the sulfation proceeds only at the C6-position of the AGU. In the ^{13}C-NMR (Fig. 2) is the sulfation at C6 marked through the shift of the peak from 59.76 ppm (unsubstituted, C6) to 66.09 ppm

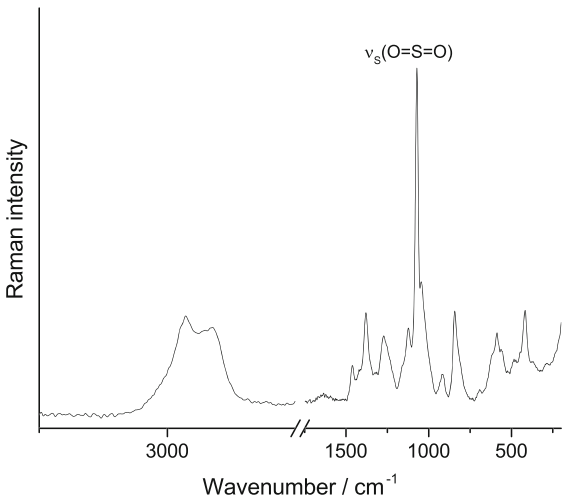


Fig. 1 Raman spectrum of cellulose sulfate with marked sulfate band at 1070 cm^{-1}

(substituted. C6-S) (Nehls et al. 1994). Since the peaks for the substituted and unsubstituted C6 are visible, only a partial sulfation happened. This is confirmed by the *DS* that is below 1 (Table 1).

The DS_{Sulf} ranges as a function of the used amount of chlorosulfuric acid from 0.8 to 2.0, specific values are given in Table 1.

Oxidation of cellulose sulfates

The introduction of aldehyde groups with sodium periodate is common in cellulose chemistry (Klemm et al. 1998). In order to perform the reaction, vicinal hydroxy groups are required. Due to the prior inserted sulfate groups, the oxidation is restricted to AGUs without sulfate groups at C2 or C3-position; otherwise the reaction cannot take place. That explains the low aldehyde content with a DS_{Ald} of approximately 0.1 to 0.3 (Table 3), especially since the distribution of substituents using direct sulfation is not limited to the C6-position as compared to acetosulfation. The low aldehyde content is demonstrated in the ^{1}H-NMR spectrum (Fig. 3); the aldehyde peak at 9.27 ppm is very weak.

To increase the aldehyde content synthesis parameters can only be varied in a small range. The synthesis was carried out at RT in water as solvent. Raising the temperature would lead to a serious degradation of the oCS (Liu et al. 2012) whereas a decrease in temperature is limited because the reaction rates would slow down considerably (Varma and Kulkarni 2002) and water freezes at 0°C. Also the approach to increase the quantity of used sodium periodate from 1 mass-eq. up to 3 mass-eq. of $NaIO_4$ was not successful. Another possibility would be to increase the reaction time, however the amount of decomposed periodate would increase (Varma and Kulkarni 2002) and would not be available for oxidation.

Although sulfation and oxidation were carried out under mild conditions chain degradation was observed after sulfation and oxidation. This is due to the use of chlorosulfuric acid and sodium periodate, since both cause hydrolysis of the glycosidic bond. While the M_W decreased only after direct sulfation with 3.0 mol-eq. HSO_3Cl, it increased for all other sulfation reactions because the influence of the substitution of hydrogen atoms with sodium sulfate groups (Table 2) was higher than the chain degradation. After oxidation

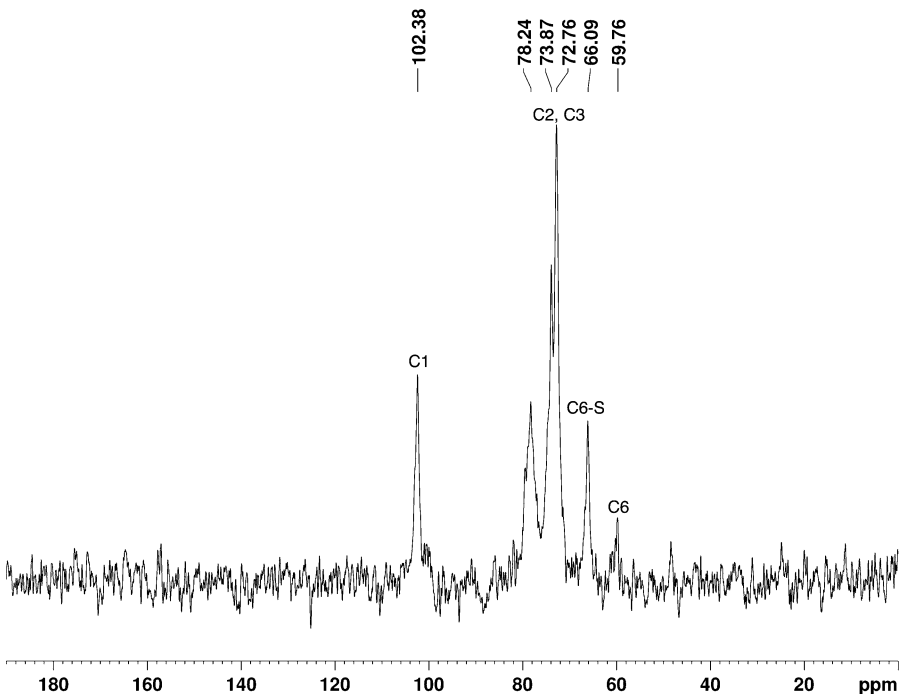


Fig. 2 ^{13}C-NMR of cellulose sulfate synthesized via acetosulfation; key: CX—unsubstituted C-atom at position X in the AGU, CX-S—substituted C-atom at position X in the AGU

Table 1 *DS* values for synthesized cellulose sulfates starting from sulfite pulp in dependency on the molar ratio (mol-eq.) per AGU of chlorosulfuric acid and acetic acid anhydride (Ac_2O); in the first line the result of the acetosulfation is presented, below the results for direct sulfation

Mol-eq. HSO_3Cl/Ac_2O	Temperature/ °C	Time / h	DS_{Sulf}
3.0:8.0	50	5	0.8
3.0:0	RT	3	1.1
4.5:0	RT	3	1.6
6.0:0	RT	3	2.0

chain degradation was observed for all products. Concerning the occurrence of chain degradation sulfite pulp was used as starting material instead of microcrystalline cellulose to obtain higher molecular weights after functionalization, since the viscosity of hydrogels increases with increasing molecular weight (Zhou et al. 2008).

Preparation of hydrogels

First, for the formation of hydrogels by mixing oCS with a chitosan derivative three different derivatives were investigated: CMCh, chitosan lactate and chitosan acetate. These derivatives were chosen because they are soluble under physiological conditions at pH 7.4, which is necessary for any application in tissue engineering. The hydrogel formation was carried out in the same manner analogous for all derivatives. The application of CMCh led to colorless, stable hydrogels (Fig. 4). In contrast oCS mixed with the other two derivatives under conditions of hydrogel formation no hydrogel was obtained. Instead a white solid of irregular shape was formed within 10 s. Therefore CMCh was used in subsequent experimental work as a second gel component.

The cutout of the theoretical structure of the hydrogel in Fig. 5 shows oCS which is connected to CMCh by the formation of imine bonds. The quantity of possible cross-linkings is dependent on the number of aldehyde groups in oCS because they are much lower than the number of amine groups in chitosan. However, one should note that just a part of the aldehyde groups will be involved in cross-linking owing to the steric hindrance of the polymer chains. The sulfate groups have only an indirect influence on the network structure since they are not participating in cross-linking, yet the sulfate groups affect the aldehyde content.

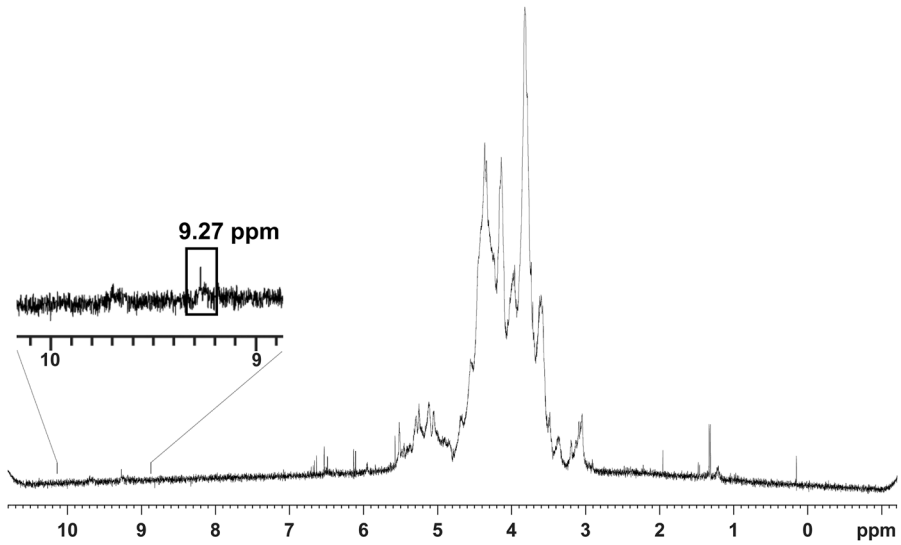


Fig. 3 ^{1}H-NMR spectrum of oxidized cellulose sulfate, the aldehyde peak at 9.27 ppm is marked

Table 2 Change in M_W after sulfation and oxidation, starting with a M_W of 2.1×10^5 g/mol for the cellulose of sulfite pulp

Direct sulfation	M_W (sulfation) g/mol	M_W (oxidation) g/mol
3.0 mol-eq. HSO_3Cl	1.7×10^5	1.2×10^5
6.0 mol-eq. HSO_3Cl	2.3×10^5	1.1×10^5

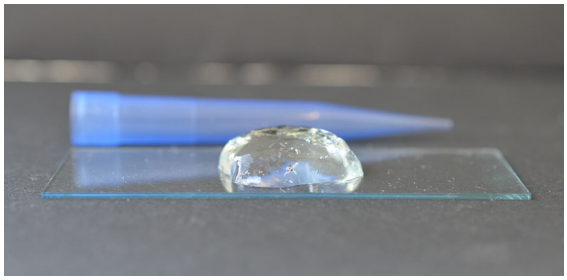


Fig. 4 The colorless hydrogel consists of oxidized cellulose sulfate ($DS_{Sulf} = 1.1$, $DS_{Ald} = 0.33$) and carboxymethyl chitosan

The kinetics of gel formation illustrated clearly its dependence on the aldehyde content. Was the DS_{Ald} greater than 0.20 the gel was formed within five to ten seconds. In contrast to that, it took up to 24 h to develop a gel structure when the DS_{Ald} was less than 0.20. The gelation time was determined as the time required to form a coherent gel. Table 3 shows the relationship between DS_{Sulf} and DS_{Ald} and duration of gel formation.

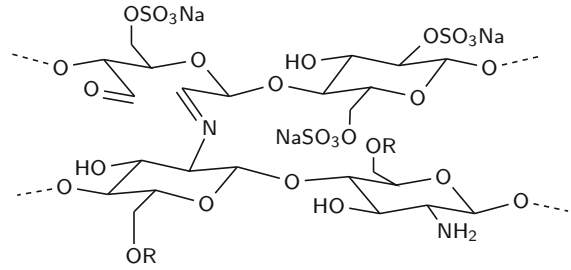


Fig. 5 Cutout of the theoretical structure of the synthesized hydrogel: an oxidized cellulose sulfate chain connected through an imine bond with a carboxymethyl chitosan chain; R: CH_2COOH

Table 3 Relationship between DS_{Sulf} and DS_{Ald} and the dependency on the time needed to form the hydrogel

DS_{Sulf}	DS_{Ald}	Time for gel formation
0.8	0.34	5 s
1.0	0.30	10 s
1.6	0.18	3 h
2.0	0.09	24 h

Besides the kinetics of gel formation, mechanical properties are of interest for the application of hydrogels. The reason is that cartilage and other soft tissues have to endure compressive strains and high stresses (Spiller et al. 2011). We used the frequency sweep to get information on the strength of the gel (Fig. 6). The gel shown in the diagram has a $DS_{\text{Sulf}} = 1.4$ and $DS_{\text{Ald}} = 0.11$ for the oCS and was prepared as described. Because of its low DS_{Ald}, it represents the minimum values which can be reached by all hydrogels. We observed an increase in the strength up to two days. The storage modulus at $\omega = 10^{-1}$ rad/s doubles from 1.4 kPa after one day of storage at RT to 2.8 kPa after two days. In both cases is $G' > G''$, which indicates that the gels represent viscoelastic solids and is a typical behavior for hydrogels. Moreover the value for tan δ is between 0.2 and 0.3 which means the gels are stable against syneresis. So the gels will not lose incorporated liquids and instead of shrinking they will held their shape.

The use of two different routes of sulfation (acetosulfation and direct sulfation) yielded a large number of products that are summarized in Table 4 with information about DS_{Sulf}, DS_{Ald} and route of sulfation either acetosulfation (A) or direct sulfation (D). These products were then later partly used for studies of gel stability in PBS or DMEM and investigations of intrinsic pH value and water mobility by spectroscopic methods.

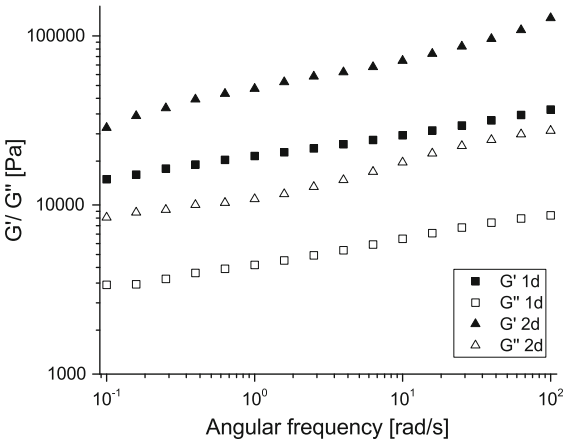


Fig. 6 Storage and loss modulus over angular frequency of a gel with $DS_{\text{Sulf}} = 1.4$ and $DS_{\text{Ald}} = 0.11$ after one and two days of storage at RT

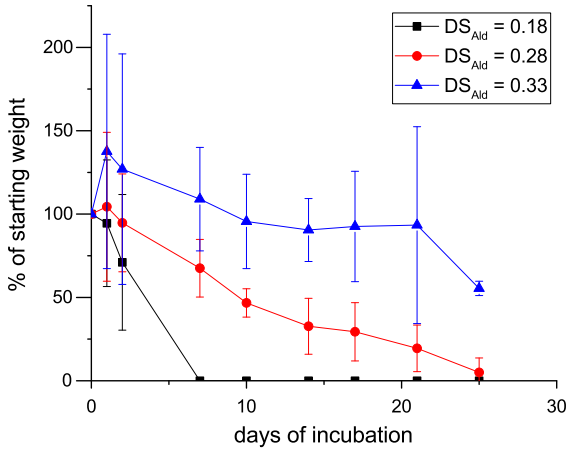
Table 4 Overview of examined samples; route of sulfation either acetosulfation (A) or direct sulfation (D)

Route of sulfation	DS_{Sulf}	DS_{Ald}
A	0.8	0.21
A	0.8	0.34
D	1.1	0.28
D	1.1	0.33
D	1.5	0.20
D	1.6	0.18

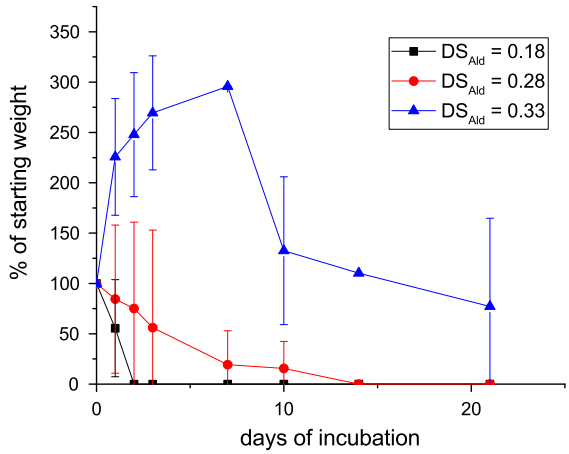
Hydrogel stability

Hydrogels crosslinked via imine bonds are susceptible to hydrolytic cleavage in aqueous conditions (Saito et al. 2007). Therefore, hydrogels made of different oCS were tested towards their stability in aqueous solutions. All hydrogels, which not form a hydrogel within 1 h or dissolve in PBS within 24 h were excluded. Fig. 7a shows hydrogels composed of oCS with different DS_{Ald} incubated for 25 days in PBS. The wet weight of the hydrogels with $DS_{\text{Ald}} = 0.33$ was most stable over time. At the end of the experiment about 55% of the hydrogel weight was remained. The hydrogel with $DS_{\text{Ald}} = 0.18$ lost about 50% of weight within the first 3 days and was completely dissolved after 7 days. The hydrogels with $DS_{\text{Ald}} = 0.28$ lost constantly weight over time and after 25 days only 10% of the hydrogels were left. Higher aldehyde content of oCS in the gels resulted in stable hydrogels for more than 25 days. Lower aldehyde content of the oCS resulted in hydrogels with lower stability in PBS. This is based on less cross-linkings due to the decreased aldehyde content, incomplete cross-linking after 1 h of gelation (Table 3) or both. The increase in stability of hydrogels in PBS due to the increase in oxidation degree was also described by Lü et al. (2010).

For further cell culture studies immersion of hydrogel in cell culture media like DMEM is required. The hydrogels showed different stability in PBS and DMEM (Fig. 7). Hydrogels with lower aldehyde content of oCS dissolved within 2 days ($DS_{\text{Ald}} = 0.18$). Hydrogels of oCS with $DS_{\text{Ald}} = 0.28$ were stable for 14 days, whereas hydrogels with high aldehyde content of oCS ($DS_{\text{Ald}} = 0.33$) lost half of their weight within 14 days. However, more than



(a) PBS immersion



(b) DMEM immersion

Fig. 7 Wet weight of oCS-CMCh-hydrogels composed of oCS with different DS_{Ald} incubated for 25 days in **a** PBS or **b** DMEM at 37°C in humid atmosphere; the oCS and CMCh solutions (20 mg/ml) were mixed 1:1 and rested for 1 h for gelation before immersion in either solvents

80% of the hydrogel remained for a duration longer than 21 days stable.

Cell culture medium contains glucose, different amino acids and other substances, which cells need for metabolism and growth. The amino groups of the amino acids can compete with the amine groups of the CMCh and hence may destabilize existing imine bonds between CMCh and oCS (Elahipanah et al. 2017; Yang et al. 2014). This was probably the reason for the lower stability of hydrogels made of oCS with $DS_{\mathrm{Ald}} = 0.28$ and $DS_{\mathrm{Ald}} = 0.18$. This will eventually result in additional water influx to colloid osmotic swelling and disintegration of hydrogels (Hoffman 2012).

Intrinsic pH of hydrogels after cross-linking

The CS solutions majorly showed pH below the sensitive range of the SNARF, because of their acidic nature due to the presence of sulfate groups despite the presence of the sodium salt form. By contrast, the CMCh solution possessed a pH of 7.4. After mixing of both components as described, changes in the fluorescence intensity of SNARF were measured that permitted the calculation of pH value (see method section for details). In Fig. 8 the pH value is plotted against the sulfate content of oCS. On average the pH of the hydrogels is around 6.4, which means slightly acidic. The higher the sulfate content of the oCS the lower is the pH of the hydrogel. The two acetosulfated oCS hydrogels have nearly the same DS_{Sulf}. The less acidic pH corresponds to the hydrogel with the higher amount of aldehyde. The same observation has been made with the direct sulfated cellulose with a $DS_{\mathrm{Sulf}} = 1.1$.

Homogeneity and permeability of the hydrogels

The transverse relaxation time distribution monitors the mobility of the water molecules in the hydrogels. When their translational or rotational motions are restricted, the relaxation times shift to lower values. The exchange of protons between gelator and water

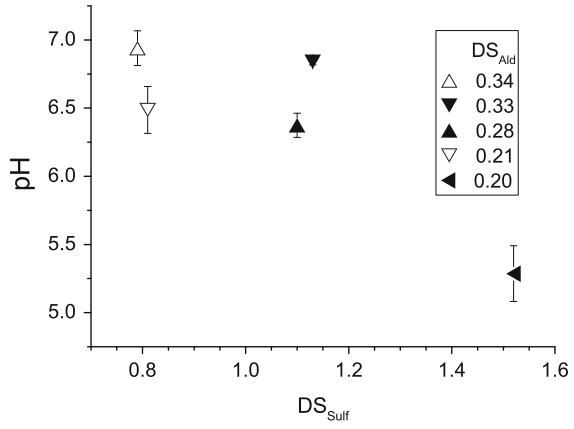


Fig. 8 The pH of the hydrogels prepared in PBS is plotted against the sulfate content; the filled symbols correspond to the direct sulfated cellulose and the open symbols to the acetosulfated cellulose

(Hills et al. 1991) and local magnetic field inhomogenieties formed by gelators (Feng et al. 2014) can also cause slower transverse relaxation times. In inhomogeneous systems a broad distribution is seen. The distribution of T_2 of the gel network is for the majority of the hydrogels as narrow as water, but shifted to lower relaxation times (Fig. 9). From the small distribution follows a high homogeneity of the hydrogels, due to no syneresis (water outside the gel). The CMCh solution had the smallest T_2 distribution width. The peak is shifted to lower T_2 values compared to water due to the higher viscosity of the CMCh solution (Guevara et al. 2018). The two inhomogeneous hydrogel networks ($DS_{Ald} = 0.20$, $DS_{Ald} = 0.28$) showed a broad and bimodal distribution. Their spectra cover two or one order(s) of magnitude, respectively. There was no common feature concerning chemical treatment, DS_{Ald} or DS_{Sulf} between the two inhomogeneous hydrogels.

A small part of the water molecules were hindered in their mobility by the hydrogel forming components. Immobilized water at the glass surface was seen around 300 µs in all samples (not shown), including the pure water sample (Bastrop et al. 2011). The small peaks around 200 ms and at 380 ms in the hydrogel $DS_{Ald} = 0.21$ could origin in attached water molecules or hydrogen of oCS or CMCh.

The homogeneous T_2-distributions of the majority of water molecules were fitted with a log-normal distribution and the mode was used to compare the hydrogels. The transverse relaxation time and its distribution correlated with the mesh size of the hydrogel (Pescosolido et al. 2012). One could expect for higher amounts of aldehyde groups a denser hydrogel with lower T_2 due to more magnetic inhomogeneities or exchange with more rigid gelator protons. There was no correlation between the amount of aldehyde and the mode of the T_2 found, because the T_2 is 1.5 s for two of three homogeneous hydrogels. DS_{Ald} seems to influence the speed of hydrogel formation, but the density of the network varies not enough to see a clear impact on the T_2.

The diffusion coefficients of water in the gels are the same as in pure water in the range of the measurement accuracy. The diffusion of water molecules is not hindered by the hydrogels. Also in peptide based hydrogels there was no impact found on the water diffusion but on the T_2 (Feng et al. 2014).

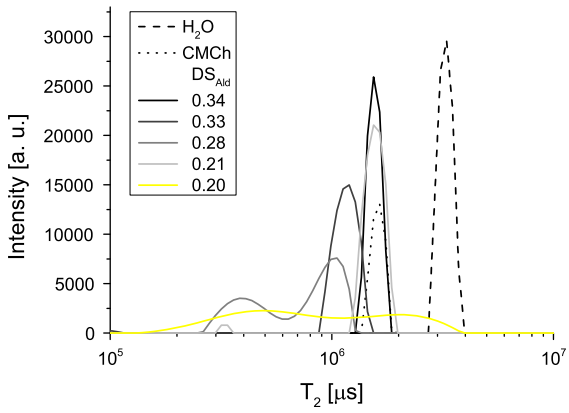


Fig. 9 The distribution of the T_2 shows the homogeneity of the gel network; the water and CMCh solution is also plotted for comparison

Conclusions

The synthesis of oCS with a DS_{Sulf} ranging from 0.8 to 2.0 and a DS_{Ald} up to 0.34 was shown. The successful crosslinking of oCS and CMCh was demonstrated by obtaining colorless hydrogels and the relation between sulfation degree and time of gelation was discussed. The stability of the hydrogels in PBS and DMEM increased with the DS_{Ald}. We could also illustrate the homogeneity of the gel network by the T_2 distribution and proof that the diffusion of water molecules is not hindered by the hydrogel. An obvious problem was the lower pH value inside the hydrogels that is probably related to the presence of sulfate groups. Overall, the hydrogels developed here will undergo further studies to test their applicability to accommodate cells for future use as *in situ* gelling, injectable hydrogels.

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