Carbohydrate Polymers 100 (2014) 107–115

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

A method for the heterogeneous modification of nanofibrillar cellulose in aqueous media

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ARTICLE INFO

Article history:
Received 16 August 2012
Received in revised form 31 October 2012
Accepted 12 November 2012
Available online 29 November 2012

Keywords:
Click chemistry
Surface functionalization
Nanofibrillar cellulose
Carboxymethyl cellulose
Adsorption
Functional materials

ABSTRACT

Cellulosic substrates were modified by using sequential adsorption of functionalized carboxymethyl cellulose (CMC) and "click" chemistry in aqueous media. First, the effect of degree of substitution (DS), and level of functionalization as well as ionic strength of the medium were systematically investigated in situ by using quartz crystal microbalance with dissipation (QCM-D) in terms of the extent of adsorption of propargyl and azido functionalized CMC. It was found that the functionalization of CMC did not prevent its adsorption on cellulose. However, it was only effective in the presence of electrolytes. Moreover, the adsorption was found to be more efficient for the functionalized CMCs with low initial DS. Next, "click" chemistry, copper (I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC), was carried out for covalent attachment of different molecules on the pre-functionalized ultrathin cellulose films. The modified cellulosic surfaces were further characterized using AFM imaging and XPS. Finally, the method was successfully used in modification of nanofibrillar cellulose (NFC) in aqueous media.

1. Introduction

The copper (I)-catalyzed 1,3-dipolar cycloaddition of alkynes and azides (CuAAC) has sparked an increased attention because it is one of the few reactions that occurs between chemically stable functional groups (Scheme 1, step 2) (Iha et al., 2011; Kolb, Finn, & Sharpless, 2001; Rostovtsev, Green, Fokin, & Sharpless, 2002; Tornøe, Christensen, & Meldal, 2002). Moreover, it is known that the CuAAC reaction tolerates both water and oxygen which allows the use of mild reaction conditions with a large variety of functionalized molecules. This is encouraging especially in terms of biomaterials which are usually highly sensitive toward harsh reaction conditions. In fact, cellulose modifications using CuAAC "click" reaction have already been demonstrated (Filpponen & Argyropoulos, 2010; Liebert, Hänsch, & Heinze, 2006; Pahimanolis et al., 2011).

Cellulose is a biopolymer with a tendency for strong hydrogen bonding which gives it extremely good strength properties (Eichorn et al., 2010). Direct chemical modification, such as etherification and esterification of cellulose is often challenging because of its high stability and hydrophilicity (Carter Fox, Li, Xu, & Edgar, 2011). In addition, to preserve the native cellulose structure, the chemical reactions cannot be made in the dissolved homogeneous state. Moreover, harsh chemical modifications of cellulose usually result in lower degree of polymerization and weaker inter- and intramolecular hydrogen bonding of the cellulose chains. In dry cellulosic materials, such as films or paper, this often means substantially lower mechanical properties of the material (Henriksson, Berglund, Isaksson, Lindström & Nishino, 2008). Therefore, alternative methods to modify cellulosic objects exclusively on the surface by using functionalized polysaccharides have been developed (Filpponen et al., 2012; Xu, Spadiut, Araújo, Nakhai, & Brumer, 2012; Zhou, Rutland, Teeri, & Brumer, 2007). These methods combine chemical or enzymatic modification of water-soluble polysaccharides and their tendency to adsorb irreversibly on cellulose. It is well known that certain water-soluble and linear polysaccharides have an ability to increase the adhesion and decrease the friction between cellulosic fibers as well as increase the tensile strength of paper in traditional papermaking applications (Leech, 1954; Lindström, Wågberg, & Larsson, 2005; Schönberg, Oksanen, Suurnäkki, Kettunen, & Buchert, 2001). In general, the use of polysaccharides for introducing diverse functional groups on the surface of cellulosic materials has a potential to replace some of the traditional synthetic methods and may even provide new modification routes.

The polymer chains of cellulose are assembled in the form of fibrils which are approximately 3–20 nm (Klemm, Heublein, Fink, & Bohn, 2005) in width and are called elementary fibrils. In plant

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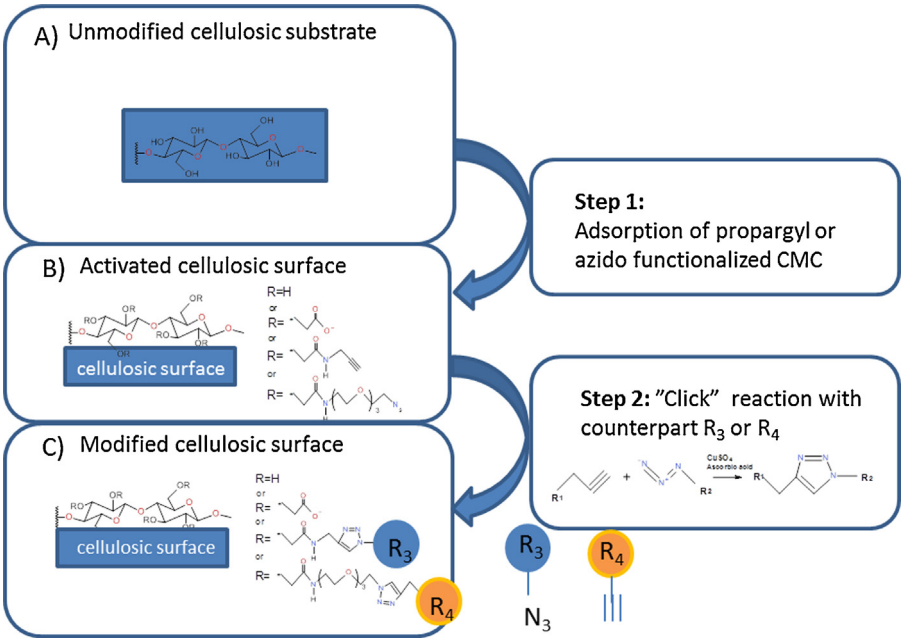
http://dx.doi.org/10.1016/j.carbpol.2012.11.063



Scheme 1. Schematic illustration of the generic method to modify cellulosic surfaces by adsorption of an azido or propargyl functionalized CMC (step 1) followed by CuAAC reaction with various probes containing either an alkyne or azide group (step 2).

cell wall these aggregate even into larger macroscopic cellulosic fibers. The preparation of micro- or nanofibrillar cellulose (NFC) from plants and wood consist on the deconstruction of their hierarchical structure. The intrinsic features of NFC include excellent strength properties, low weight, high aspect ratio, biodegradability and renewability (Klemm et al., 2011).

In the present work we use a generic method to modify cellulosic surfaces (Filpponen et al., 2012) via sequential adsorption of functionalized (azido or propargyl) carboxymethyl cellulose (CMC) and a "click" reaction (Scheme 1). The proof-of-principle of this method has already been established (Filpponen et al., 2012) but here, the objective was to probe further the parameters which determine the adsorption and the "click" reaction. Two different degrees of substitution (DS) and levels of functionalization of CMCs were systematically investigated. The adsorption of pre-functionalized CMCs was used to "activate" ultrathin cellulose model films and the effect of ionic strength on the adsorption was followed by using quartz crystal microbalance with dissipation (QCM-D). Activated cellulose surfaces were further modified in situ by using CuAAC reaction with various clickable molecules. The modified cellulose model surfaces were characterized by using atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS). Finally, we demonstrate that the current methodology can be applied to the modifications of NFC gel – an important piece of information considering the huge potential of NFC in modern materials science.

2. Materials and methods

2.1. Chemicals and solutions

All chemicals used were of analytical grade. Ultrapure MilliQ-water (Millipore Synergy UV unit; Millipore S.A.S. Molsheim, France) was used for dilutions. Two different sodium carboxymethyl celluloses (Na-CMCs, 250 kDa), CMC1 and CMC2 of nominal DS of 0.7 and 1.2, respectively were supplied by Sigma–Aldrich and purified prior to use by dialysis followed by freeze-drying. All the CMC solutions were prepared by dissolving the powder in ultrapure MilliQ-water by stirring the solution overnight. The samples were further diluted to a given concentration, and the ionic strengths were adjusted with NaCl. The DS of the purified CMC samples were calculated based on their charge density measured with polyelectrolyte titration (Hiroshi, 1952). 1-Ethyl-3-(3-dimethylaminopropyld) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Fluka and Thermo Scientific, respectively. Methoxy polyethylene glycol azide (OMe-PEG azide, 20 kDa), Bovine Serum Albumin (BSA, 66 kDa) and $CuSO_4 \cdot 5H_2O$ (98+%) were purchased from Sigma–Aldrich. L-Ascorbic acid (99%) was purchased from Acros Organics. Trimethylsilyl cellulose (TMSC) was synthesized from cellulose powder (spruce, Fluka), according to a previously published method (Kontturi, Thüne, & Niemantsverdriet, 2003). The fully bleached (bleaching sequence Acid wash-Ozone/Chlorine Dioxide-Alkaline Extraction with oxygen and peroxide-Chlorine Dioxide-Peroxide, A-Z/D-EOP-D-P) birch pulp was used as a raw material for the preparation of nanofibrillar cellulose (NFC) These pulps were supplied by UPM Kymmene kraft mill (Pietarsaari, Finland). The Kappa number (KN) of precursor birch pulp was <1, degree of polymerization (DP) of 2100 and hemicellulose content up to 25% (Solala et al., 2011; Ferrer et al., 2012). Prior to fluidization, it was fractionated (200 mesh, SCAN-M 6:69) and washed to sodium form (Swerin, Ödberg, & Lindström, 1990). The molecular structures of cellulose, CMC, propargyl functionalized CMC and azido functionalized CMC, as well as the counterpart molecules, i.e., dansyl-alkyne, OMe-PEG azide and BSA-alkyne are shown in Fig. 1.

2.2. CMC functionalization

The carbodiimide activation of CMC was done according to Filpponen et al. (2012). Two different CMCs (CMC1 and CMC2) were activated to two different levels. 100 mg of CMC was dissolved in distilled water and mixed overnight. The CMC solution was diluted to 2 g dm^{-3} with NaAc/HAC buffer (pH 4.5, I = 10 mM) and the ionic strength was adjusted to 50 mM with NaCl. Next, 1 mmol (CMC1) or 1.5 mmol (CMC2) of EDC, 4 mmol (CMC1) or 6 mmol (CMC2) of NHS and either 1.5 mmol (higher amount, CMC2), 1 mmol (higher amount, CMC1) or 0.8 mmol (lower amount, CMC2 and CMC1) of the amine-group containing molecule (propargylamine for

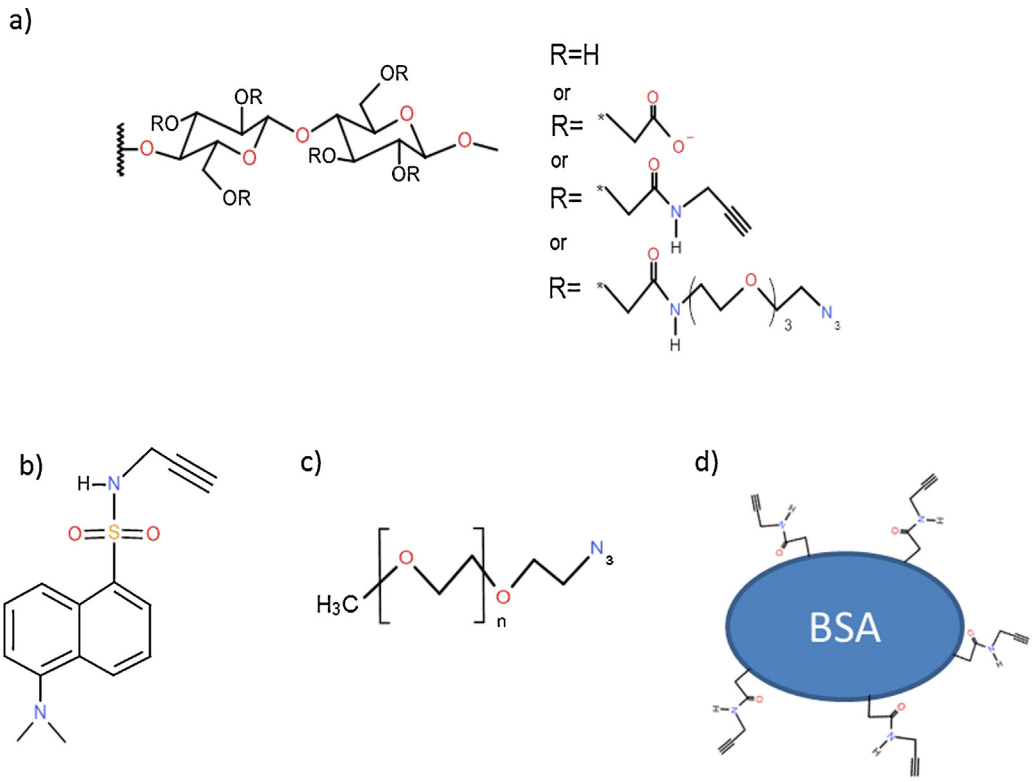


Fig. 1. The molecular structures of (a) cellulose, CMC, propargyl functionalized CMC, and azido functionalized CMC, (b) dansyl-alkyne, (c) OMe-PEG azide and (d) BSA-alkyne.

propargyl functionalization and 11-azido-3,6,9-trioxaundecan-1-amine for the azido functionalization) were added. The solution was mixed in a beaker with a magnetic stirrer overnight protected from light at room temperature. Finally, the solution was dialyzed against deionized water for 2 days prior to freeze-drying.

2.3. Adsorption studies of pre-functionalized CMC on cellulose

The cellulose thin films for quartz crystal microbalance with dissipation (QCM-D) experiments were prepared by spin-coating of TMSC on SiO_2-coated sensor crystals (Q-Sense AB, Västra Frölunda, Sweden) and regenerating it to cellulose by exposure to HCl vapor (Kontturi et al., 2003). It has been reported that these surfaces are amorphous cellulose with approximately 0.5 nm roughness and 20 nm of thickness (Kontturi et al., 2011). The adsorption of CMC and pre-functionalized CMC on cellulose and the following "click" reaction were performed by using an E-4 instrument (Q-Sense AB, Västra Frölunda, Sweden) with controlled flow (Rodahl, Höök, Krozer, Brzezinski, & Kasemo, 1995). The flow rate used in these experiments was 0.1 mL min^{-1}. The CMC solutions were prepared with a constant concentration of 0.2 g dm^{-3}. QCM-D is an electro-acoustic measurement method that utilizes the piezoelectric properties of quartz. The resonance frequency (f) of the oscillating crystal depends on the total oscillating mass, including water and other adsorbed molecules. When the dissipation values are low, the frequency decreases linearly with the mass according to the Sauerbrey Eq. (1) (Sauerbrey, 1959):

$$\Delta m = -\frac{C \cdot \Delta f}{n} \quad (1)$$

where Δm represents the change in mass (mg m^{-2}), C is mass sensitivity (0.177 mg Hz^{-1} m^{-2} for a 5 MHz quartz crystal), n is the overtone number and Δf is the change in frequency (Hz). In the case of CMC adsorption on cellulosic materials the Sauerbrey relation underestimates the adsorbed mass because of the hydration contributions. Soft adsorbed layers dampen the oscillations of the crystal, which translates into energy dissipation (D). The definition of D in Eq. (2) is dependent on the dissipated (lost) energy per oscillation cycle, E_{lost} (J) and E_{stored} (J) is the energy stored in the oscillation.

$$D = \frac{E_{lost}}{2\pi E_{stored}} \quad (2)$$

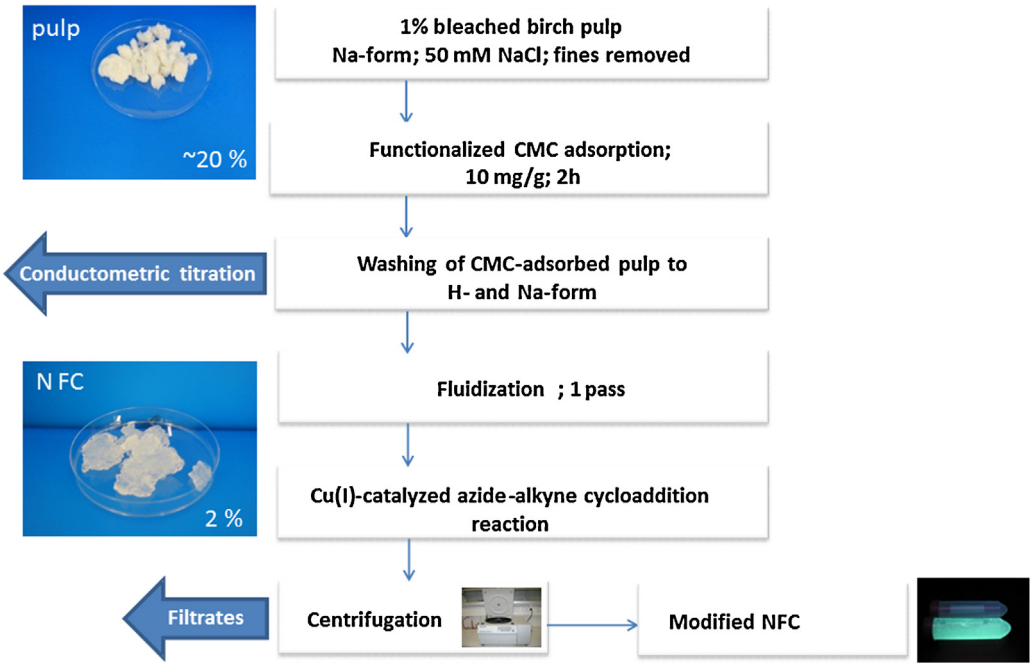
Both the cellulose film and the adsorbing CMC samples bind water. Hence, the changes in Δf and ΔD are due not only to changes in adsorbed mass but also to bound water. It has been observed that up to 80% of the mass increase for polysaccharide adsorption on cellulosic surfaces may arise from coupled water (Ahola, Myllytie, Österberg, Teerinen, & Laine, 2008).

2.4. CuAAC reactions

The functionalized surfaces prepared in situ in QCM-D were further modified by covalent attachment of either OMe-PEG azide or BSA-alkyne by using CuAAC reaction. The reaction was initiated soon after CMC adsorption reached a plateau value in water. Either a solution of 0.5 g dm^{-3} of OMe-PEG azide or 0.1 g dm^{-3} of BSA-alkyne dissolved in 0.8 g dm^{-3} of $CuSO_4$ and 2.8 g dm^{-3} of L-ascorbic acid was added in the QCM-D chamber. Reaction was continued for 1 h with a constant flow of 0.1 mL min^{-1} after which it was stopped by rinsing the system with MilliQ-water until the frequency value reached a plateau. All experiments were at least duplicated.

2.5. NFC modification

Azido functionalized nanofibrillar cellulose (NFC) was prepared by high pressure fluidization (Microfluidics, M-Y) of a birch pulp originating from a Finnish pulp mill (fines removed) which had been pre-treated by azido functionalized CMC. The azido functionalized CMC1 (10 mg g^{-1}) was dissolved in water overnight, and



Scheme 2. Schematic illustration of the procedure used to modify NFC by using functionalized CMC adsorption and "click" chemistry.

added slowly to the 1% fiber suspension (pH 4.5, $I = 50$ mM) while stirring. The suspension was mixed for 2 h and washed in a Büchner funnel for several times in order to remove any unbound polysaccharide. Finally, the suspension was washed to Na-form (Swerin et al., 1990). In order to verify that CMC was irreversibly attached on the fiber surfaces, the charge density of the pulp was measured with conductometric titration (Katz, Beatson, & Scallan, 1984) prior to fluidization. After the functionalized pulp had been fluidized, it was diluted to a 1 g dm^{-3} concentration with a total volume of 50 mL. 175 mg of L-ascorbic acid, 75 mg of $CuSO_4$ and 10 mg of dansyl-alkyne (pre-dissolved in a mixture of 0.5 mL of acetone and 0.5 mL of water) was added to the azido functionalized NFC dispersion. The solution was mixed overnight in a sealed beaker protected from light. In order to remove the unbound counterpart (dansyl-alkyne), the modified NFC was washed for several cycles with centrifugation (10,000 rpm); first with water (three additions of water, 30 min), then with acetone/water (15 min) and finally with water (two additions of water; 75 min) until the filtrate did not have any fluorescence (all the unattached molecules had been removed). Three reference tests for the NFC modifications were performed identically by using unmodified fibers, CMC-modified fibers or the CuAAC reaction was performed in the absence of Cu(I) catalyst; this was done in order to rule out the contribution of interactions between dansyl-alkyne and the substrate different than covalent bonding. Finally, the attachment of the fluorescent probe on NFC was analyzed with UV-light and the sample was freeze-dried for further XPS and FTIR measurements. The procedure to modify NFC by using functionalized CMC adsorption and "click" chemistry is presented in Scheme 2.

2.6. Atomic force microscopy (AFM)

Atomic force microscopy was used for characterizing the morphology of the surfaces after modifications. The instrument used in the measurements was NanoScope IIIa multimode (Digital Instruments Inc., Santa Barbara, CA, USA). The images were scanned from several different locations using two different scan sizes (1 μm^2 and 25 μm^2) with tapping mode in air using silicon cantilevers (NSC15/AIBS, Ultrasharp μmasch, Tallinn, Estonia). No post-image processing was used except flattening.

2.7. X-ray photoelectron spectroscopy (XPS)

The chemical composition of the topmost 10 nanometers of the modified samples was analyzed with X-ray photoelectron spectroscopy, using an AXIS 165 electron spectrometer (by Kratos Analytical) with monochromatic A1 Kα X-ray irradiation at 100 W. Dry samples were pre-evacuated overnight prior to the XPS measurements, in order to stabilize the water content. Spectra were collected at three locations, using an electron take-off angle of 90° and from sample areas less than 1 mm in diameter. Elemental surface compositions were determined from low-resolution survey measurements (80 eV pass energy and 1 eV step), carbon surface chemistry was probed with high resolution regional scans (20 eV pass energy and 0.1 eV step) and for the analysis of trace amounts of nitrogen and sulphur, N 1s and S 2p regions were recorded using low resolution and long measurement times. The carbon C 1s high-resolution spectra were curve fitted using parameters defined for cellulosic materials (Johansson & Campbell, 2004), and all binding energies were referenced to the aliphatic carbon component of the C 1s signal at 285.0 eV (Beamson & Briggs, 1992). According to the in situ reference (100% cellulose ash free filter paper), the conditions in UHV remained satisfactory during the XPS experiments (Johansson & Campbell, 2004).

2.8. Elemental analysis

A Perkin Elmer 2400 Series II CHNS instrument was used for elemental analysis in order to characterize the content (%) of carbon (C), hydrogen (H) and nitrogen (N) in the samples.

2.9. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the samples were measured using Bio-Rad FTS 6000 spectrometer (Cambridge, MA) with a MTEC 300 photoacoustic detector (Ames, IA) using a constant mirror velocity of 5 kHz, 8 cm^{-1} resolution and 1.2 kHz filter.

Table 1
Functionalized CMC samples as a function of dosage levels and degree of substitution of CMC (a and b indicate the two different dosage levels used, i.e., different levels of functionalization). The amount of unreacted carboxyl groups and the observed DS has been calculated based on elemental analysis.

	m_{CMC} (mg)	n_{EDC} (mmol)	n_{NHS} (mmol)	n_{amine} (mmol)	$DS_{theoretical}$	$DS_{observed}$ [a]	$DS_{carboxyl}$ [a]
Propargyl-							
CMC1a	100	1	4	1	0.70	0.62	0.08
CMC1b	100	1	4	0.8	0.59	0.59	0.11
CMC2a	100	1.5	6	1.5	1.20	0.99	0.21
CMC2b	100	1.5	6	0.8	0.73	0.73	0.47
Azido-							
CMC1a	100	1	4	1	0.70	0.30	0.40
CMC1b	100	1	4	0.8	0.59	0.25	0.45
CMC2a	100	1.5	6	1.5	1.20	0.36	0.84
CMC2b	100	1.5	6	0.8	0.73	0.30	0.90

[a] Calculated based on elemental analysis.

3. Results and discussion

3.1. CMC functionalization

Eight different CMC samples bearing either azido or propargyl groups were synthesized. FTIR results showed characteristic stretching bands for azide and amide functionalities which indicates that the modifications took place (Supporting information, Fig. S1). The degrees of substitution (DS) of functionalized samples were calculated based on the elemental analysis (Supporting information, Table S1) and the results are presented in Table 1. It was found that compared to the azido functionalization the propargyl functionalization of CMC occurs more efficiently, i.e., approximately 85% of the available carboxyl groups were converted to the corresponding propargyl amides whereas the level of azido functionalization was found to be approximately 30–40%. However, the use of lower amine dosages (CMC1b and CMC2b) yielded slightly higher conversion rates when compared to the corresponding theoretical values.

It is not expected that the EDC/NHS activation step would result in any differences between the modification efficiencies because the activation procedures were kept constant throughout the azido and propargyl functionalizations. Thus, the most likely explanation for the observed variances comes from the different reactivities of propargylamine and the 11-azido-3,6,9-trioxaundecan-1-amine molecules with the EDC/NHS activated carboxyl groups in CMC.

In addition, we aimed to modify two different CMCs with two different functionalization levels, a and b, by simply adjusting the chemical dosages. A constant concentration of buffer, NaAc/HAC, was used in the modifications. Thus, the amount of carboxyl groups in the buffer in addition to the carboxyl groups in the CMC have been taken into account in the chemical dosages shown in Table 1.

3.2. Adsorption of pre-functionalized CMCs on cellulose thin films

The adsorption of the functionalized CMC samples on cellulose model surfaces was studied by using in situ QCM-D monitoring. We systematically studied how the functionalization type (either azido or propargyl), functionalization level (a and b), initial DS of CMC (1.2 and 0.7) as well as the ionic strength (0 mM and 50 mM) effected CMC adsorption on cellulose. Moreover, the effect of water rinsing on the adsorbed CMC layer was investigated. The results of the adsorption and rinsing experiments for the unmodified and azido functionalized CMC on cellulose surfaces at two different ionic strengths can be seen in Fig. 2.

Azido functionalized and unmodified CMCs were found to adsorb on the cellulose model surface in the presence of salt (I = 50 mM) which is clearly distinguished as a clear negative frequency shift in the QCM-D (Fig. 2). As expected, in the absence of salt neither the functionalized nor the unmodified CMC samples possessed any affinity on cellulose. In addition, the adsorption behavior of the propargyl functionalized CMC (Supporting information, Fig. S2) was found to be similar than that of azido functionalized CMC. The results shown in Fig. 2a are in agreement with previous studies that indicated that CMC adsorbs on cellulose when the electrostatic double layer repulsion between cellulose (negative charge mainly originating from hemicelluloses) and negatively charged CMC is screened by using sufficient electrolyte concentration (Laine, Lindström, Glad Nordmark, & Risinger, 2000; Liu, Choi, Gatenholm, & Esker, 2011). It should be mentioned that the elemental analyses revealed the presence of unreacted carboxyl groups in the functionalized CMCs (see Table 1 for the observed DS values), which explains the need of salt to reduce the electrostatic repulsion.

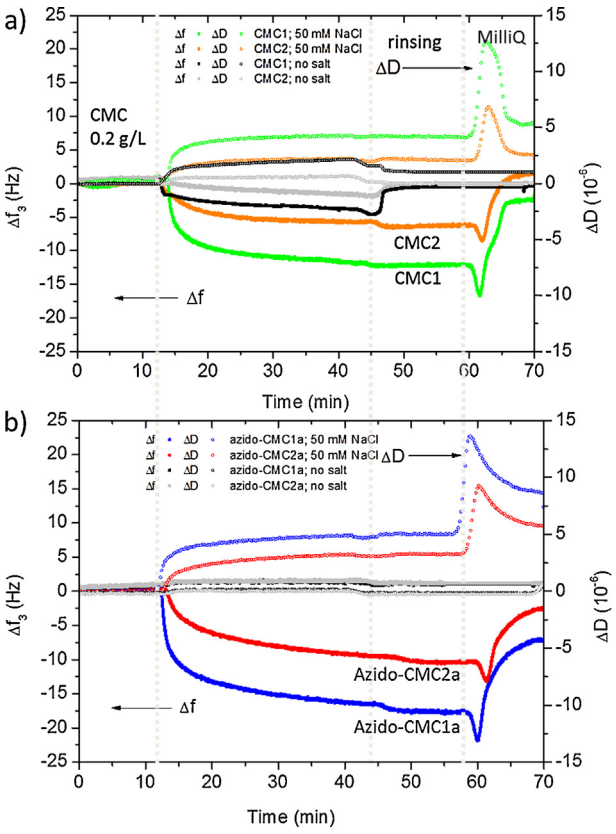


Fig. 2. QCM-D frequency and energy dissipation profiles indicating the effect of solution ionic strength (0 mM and 50 mM) on the adsorption of (a) unmodified (DS 0.7 and DS 1.2) and (b) azido functionalized (initial DS 0.7 and 1.2) CMC on cellulose. The adsorption of CMC was allowed for 30 min after which the system was rinsed for 15 min with buffer followed by rinsing with MilliQ-water until reaching a plateau frequency.

Table 2
Changes in frequency (normalized 3rd overtone) due to adsorption of functionalized CMC on cellulose. CMC1 (DS of 0.7) and CMC2 (DS of 1.2) were used in these experiments where symbols "a" and "b" stand for a high or low level of functionalization, respectively. The adsorbed mass was calculated by using the Sauerbrey equation (Eq. (1)).

Sample	$-\Delta f_3$ (Hz) in 50 mM NaCl	Δm (mg m^{-2}) in 50 mM NaCl
Propargyl-CMC1a	19 ± 4	3.4 ± 0.7
Propargyl-CMC1b	16 ± 3	2.9 ± 0.5
Azido-CMC1a	18 ± 4	3.1 ± 0.7
Azido-CMC1b	19 ± 4	3.3 ± 0.7
Propargyl-CMC2a	6 ± 3	1.0 ± 0.5
Propargyl-CMC2b	10 ± 3	1.9 ± 0.5
Azido-CMC2a	10 ± 3	1.9 ± 0.5
Azido-CMC2b	12 ± 3	2.1 ± 0.5

When comparing the QCM-D results of different samples with different levels and types of functionalization it is evident that the functionalization itself does not affect the interaction between CMC and cellulose (Table 2). However, the initial DS of the CMC does have an effect on the adsorption of functionalized CMC on cellulose: the higher the initial DS the lower the adsorbed amount. This is in good agreement with the previous findings on the adsorption of unmodified CMC on cellulose (Laine et al., 2000; Liu et al., 2011). One possible explanation is that the unsubstituted regions of the CMC backbone are still able to interact and attach irreversibly on cellulose, since the functionalization affects only to the regions that are already substituted with carboxymethyl groups. This suggests that the functionalization neither impairs nor enhances the adsorption process because these substituted regions in CMC do not have any affinity to the cellulose substrate. Similar behavior has also been observed with some other polysaccharides, such as galactomannans (Hannuksela, Tenkanen, & Holmbom, 2002). It was found that the substitution pattern is highly important in terms of the polysaccharide adsorption on cellulose, suggesting that only the unsubstituted mannose chains can interact with cellulose.

A clear positive change in the oscillation frequency of the system was observed when the buffer was changed from 50 mM NaCl to MilliQ-water (Fig. 2). This indicates a decrease of mass adsorbed onto the oscillating crystal. However, the dissipation energy of the system is increased simultaneously which may be explained by a more extended conformation of the attached CMC on the surface toward the solvent in the absence of salt. In addition, also the cellulose model film itself responds to the change in ionic strength. Thus, this swelling of the adsorbed layer can only be detected from the dissipation energy data.

The oscillation frequency of the crystal seems to be markedly affected by the viscosity change between the water and the salt solution which results in an increase in the frequency of the system after rinsing with water. In fact, similar behavior has been previously observed with the adsorption of unmodified CMC on cellulose model surfaces (Liu et al., 2011). However, it should be noted that water rinsing does not remove the adsorbed CMC layer: when the original ionic strength was restored, the frequency and dissipation rose to nearly same initial levels observed before rinsing with water (data not shown).

3.3. CuAAC reactions with modified cellulose films

After modifying the cellulose surfaces with the functionalized CMCs, the surfaces were subjected to CuAAC reaction with different clickable molecules. This was conducted in situ using QCM-D with

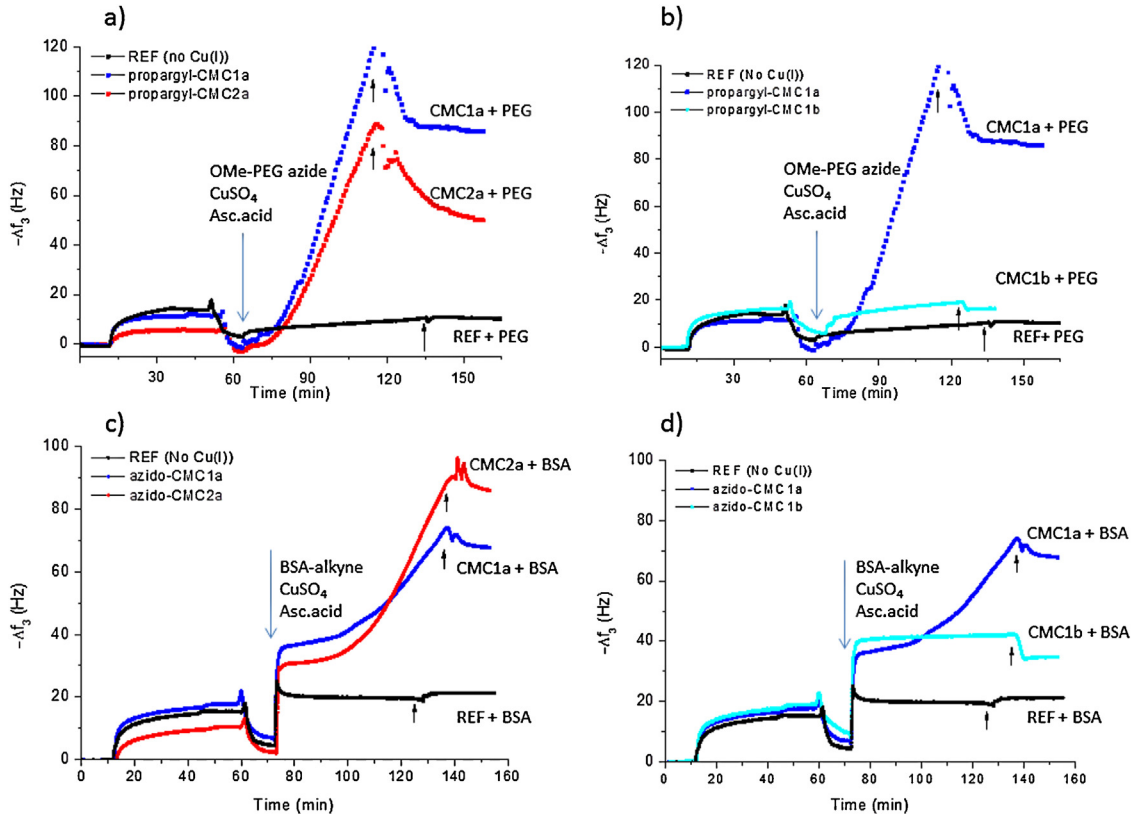
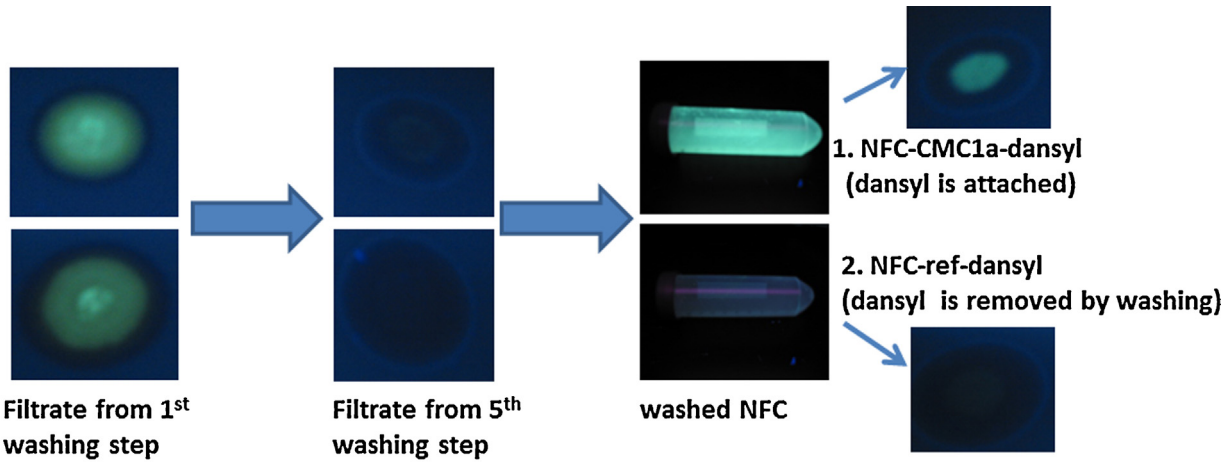


Fig. 3. QCM-D frequency shift as a function of time indicating the adsorption behavior of functionalized CMC on cellulose, and the sequential "click" reaction with two different counterpart molecules. The effect of the amount of adsorption of propargyl functionalized CMC on the "click" reaction with OMe-PEG azide is shown in (a) and the effect of the level of functionalization is displayed in (b). The effect of the amount of adsorption of azido modified CMC on the "click" reaction with BSA-alkyne is shown in (c) and the effect of the level of functionalization is shown in (d). The starting point of water rinsing in each system is indicated with a black arrow.



sample	O 1s	C 1s	N 1s	S 2p
Cellulose reference	42.1%	57.9%		
NFC-ref-dansyl	42.0%	58.0%	0.05%	0.03%
NFC-CMC1a-dansyl	40.6%	58.9%	0.46%	0.14%
sample	**C-C**	**C-O**	**C=O**	**COO**
Cellulose reference	3.5%	76.9%	18.5%	1.1%
NFC-ref-dansyl	5.1%	75.7%	18.4%	0.8%
NFC-CMC1a-dansyl	10.1%	71.8%	17.3%	0.8%

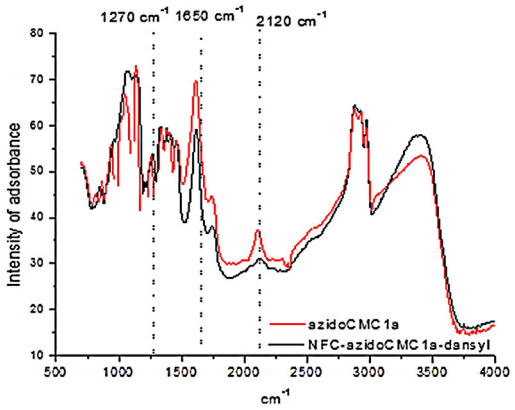


Fig. 4. Fluorescent labeling of NFC. The modified NFC was characterized using UV-light (λ = 254 nm), XPS and FTIR (red line is the azido-CMC1a and black line is NFC-CMC1a-dansyl). Filtrates from 1st washing step and the last washing step (5th) were characterized using UV-light. The NFC-ref-dansyl stands for the reference test where no copper (I) catalyst was used during the reaction. Characteristic bands for amides (1270 and 1650 cm^{-1}) and azide (2120 cm^{-1}) are shown as dotted lines in the FTIR spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clickable counterparts (OMe-PEG azide and BSA-alkyne) which were used as weight markers easily detectable in QCM-D. As can be observed in Fig. 3, the reference trials in the absence of copper (I) catalyst did not reveal attachment of counterparts (no change in oscillation frequency). Likewise, when unmodified CMC was used as an additional reference no reaction with the weight markers (data not shown) was observed. Most importantly, the QCM-D data of the azido or propargyl functionalized surfaces clearly shows that the "click" reaction took place, and the counterpart was not removed even after extensive washing (Fig. 3).

Both the initial degree of substitution and the level of functionalization of the activated cellulosic surface were found to have an important role in the degree of attachment of counterpart molecules. For example, the highest amount of attached OMe-PEG azide counterpart was observed when the cellulose surface was pre-treated with CMC1a (Fig. 3a). The reason might be the higher amount of available propargyl groups due to the higher initial adsorption of CMC1a when compared to that of CMC2a. Interestingly, the attached amount of BSA-alkyne was slightly higher for cellulose carrying CMC2a (initial DS 1.2) when compared to that of pre-treated with CMC1a (initial DS 0.7). It should be noted that the cellulose surface pre-treated with CMC2a should contain a lower amount of available azide groups due to the lower initial adsorbed amount of CMC2a.

The reason for the different adsorption behavior of OMe-PEG-azide and BSA-alkyne might be the larger size of BSA which in turn may promote the formation of interlocked structures when CMC is very loosely bound and thus extended outwards from the cellulose surface as in the case of CMC2. Moreover, the level of CMC functionalization (a and b) seems to have a clear effect on the reaction efficiency as can be observed in Fig. 3b and d. When the level of functionalization is high (CMC1a and CMC2a) it seems that the "click" reaction does not level off before the reaction is stopped by rinsing with water. One possible explanation might be that when the grafting density is high, e.g., the level of functionalization is high, the conformation of the counterpart molecules on the surface is more extended and "brush"-like when compared to that of a low grafting density system (b). Thus, swelling (by bound water) occurs when the reaction takes place which in turn explains the clear difference in results for counterpart molecules.

For further comparison the modified surfaces were characterized by using AFM (Supporting information, Fig. S3). AFM images of OMe-PEG decorated cellulose model surface did not reveal any topographical changes. This is probably due to its similarity with the underlying soft cellulose and CMC layers. However, the attached BSA can be clearly observed in the AFM images and in the subsequent roughness values (Supporting information, Fig. S3). The differences between the different BSA-modified cellulose surfaces are rather small, probably due to the fact that the surfaces were dried before AFM imaging.

XPS was used to characterize the chemical compositions of the surfaces after the modification (Supporting information, Fig. S3). The amount of nitrogen present on the dry surfaces, taken as a fingerprint for a protein, was found to clearly increase with the BSA modification. It should be noted that XPS analysis depth is less than 10 nm and the dimensions of the globular BSA are 14 nm × 4 nm × 4 nm. The results point out thus far that the counterparts (OME-PEG-azide and BSA-alkyne) were covalently attached on the cellulose surface and the attached amount depends on the initial DS (amount of CMC on the surface) as well as the

amount of propargyl or azido functional groups present on the surface.

3.4. NFC modification

After the concept was demonstrated on smooth cellulose model surfaces, we set our focus on the more relevant but also more complex modification of NFC in suspension or as a hydrogel. First, the azido functionalized CMC was adsorbed on precursor cellulosic fibers as described in Section 2. In order to evaluate the amount of functionalized CMC on the fibers, the charge density was measured via conductometric titration. Data shows that the functionalized CMC was attached to the fiber surface as the charge of the pulp increased substantially, from 38 $\mu mol\,g^{-1}$ to 79 $\mu mol\,g^{-1}$. In comparison, the charge density of the adsorbed unmodified CMC1 was 102 $\mu mol\,g^{-1}$ due to the higher amount of available carboxyl groups in the unmodified CMC.

Next, the fibers were fluidized to produce NFC. The fluorescent probe, dansyl-alkyne, was attached on the CMC-modified NFC by using "click" reaction. The procedure details are described in Section 2. As can be observed from Fig. 4, the fluorescence was detected only after the copper (I) catalyzed reaction between the azido-CMC1a modified NFC and the dansyl-alkyne. Again, in case of all the reference trials (see Section 2) fluorescence tests resulted in negative response, i.e., the dansyl-alkyne was removed by washing. This indicates that the probe did not have any other type of affinity with NFC but the covalent bonding upon functionalization via azido-CMC1.

FTIR spectrum (Fig. 4) indicates that the CuAAC reaction took place between the azido CMC1a-NFC and dansyl-alkyne since the characteristic azide stretching band at 2120 cm^{-1} has substantially decreased. In addition, XPS was used to investigate the changes in the elemental composition of the NFC surface (Supporting information, Fig. S4). While the carbon and oxygen signals remained essentially the same in all samples, we observed slight increases in sulphur and nitrogen contents of the NFC sample treated with dansyl-alkyne in the presence of copper (I) (in trace analysis, 0.14 at% and 0.46 at%, respectively). Albeit the increase in the amounts of sulphur and nitrogen were rather low it is evident that the fluorescent probe was attached to NFC. Moreover, the chemical composition of the NFC reference sample was similar to that of pure cellulose, which further indicated that the fluorescent probe was removed after extensive washing.

Conceptually, the approach to prepare "activated" NFC that bears readily clickable azido- or propargyl-groups on the fibril surface is intriguing. NFC is seen as a future renewable nanomaterial with high potential for numerous conventional as well as high-end applications (Klemm et al., 2011); yet its modification is often laborious and requires substantial synthetic expertise. The introduction of a generic method for addition of nearly any kind of functionality via click reactions in aqueous medium is considerable progress in the modification of NFC.

4. Conclusions

We used sequential adsorption of functionalized CMC and the CuAAC reaction for the modifications of ultrathin cellulose films and NFC. The initial degree of substitution of CMC and ionic strength significantly affected the adsorption of functionalized CMC on cellulose. Higher adsorbed amounts were observed for the functionalized CMCs with low initial DS. Moreover, the absence of electrolytes prevented CMC adsorption. Importantly, the functionalization (propargyl or azido) did not disturb the adsorption of CMC on cellulose. In fact, the adsorbed amounts were found to be at the same magnitude than those for the unmodified CMC. Finally, the "click"-activated cellulosic substrates were subjected to versatile modifications (protein decoration, fluorescent labeling, and PEGylation) in order to demonstrate the generic nature of this method. Overall, it is expected that the universal and robust nature of the method, coupled with its mild reaction conditions, will open new venues for the heterogeneous modification of cellulosic materials.

Acknowledgements

This work was funded by Future Biorefinery (FuBio JR2) and Naseva2 projects. Graduate School for Biomass Refining (BIOREGS) is thanked for personal financial support (KJ). Ms. Ritva Kivelä, Ms. Anu Anttila, Dr. Joe Campbell, Ms. Mirja Reinikainen and Ms. Johanna Mareta are thanked for laboratory assistance. M.Sc., doctoral student Nikolaos Pahimanolis is thanked for providing the dansyl-alkyne.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.11.063.

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