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Labelling of N-hydroxysuccinimide-modified Rhodamine B on cellulose nanofibrils by the amidation reaction

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We have labelled cellulose nanofibrils with Rhodamine B and characterized the grafting and the fluorescent properties by infrared and UV/Visible spectroscopy together with fluorescence spectroscopy and confocal laser scanning microscopy. The cellulose nanofibrils were surface modified with amine groups using 4-(Boc-aminomethyl)phenyl isothiocyanate, that allows grafting of a wide number of commercially available molecules that e.g. contain carboxylic acid groups. The amine-modified cellulose nanofibrils were reacted with N-Hydroxysuccinimide-modified Rhodamine B ester through the amidation reaction. The labelling of the dye onto CNF results in a red-shift of the absorption and emission bands and despite some quenching of the fluorescent intensity of the surface-bound chromophore, it was possible to image the luminescent CNF at both low and high magnification using a confocal laser scanning microscope.

Introduction

Natural polymers extracted from biological entities, e.g. chitosan, lignin, starch and cellulose, are attractive biodegradable alternatives to fossil-carbon based polymers. Cellulose is one of the most abundant biopolymers on earth and the ability to extract and utilize nanocellulose from wood and other sources has attracted a significant interest to use this novel material for a wide range of applications, ranging from composites to barriers and biological scaffolds[1]. Nanocellulose can come in different forms, e.g. as short and stiff cellulose nanocrystals (CNC) and as long and flexible cellulose nanofibrils (CNF), depending on the extraction method. CNC have the ability to form chiral nematic phases in aqueous dispersions and has been successfully used as a template for materials synthesis[2,3] while CNF are extensively used as a scaffold and reinforcement for composite films and foams[4]. Many applications require that nanocellulose is surface modified, e.g. by grafting of various types of organic groups [5–7]. Surface modification of nanocellulose has been performed by e.g. oxidation[8], esterification[9], and etherification[10] reactions followed by subsequent post-modifications involving e.g. peptidic coupling[11], polymerization[12], silylation[13], photo-cyclisation[14], thiourea[15], and also click chemistry[16].

Labelling nanocellulose with luminescent molecules is of interest for sensor applications, and also for the study of nanocellulose using widely available fluorescent techniques. Luminescent CNF are also of interest in biomedical applications for the study of e.g. cell uptake and cell viability[17,18]. Previously, Nielsen *et al.*[19] labelled CNC with either isothiocyanate dyes or succinimidyl ester dyes through the formation of a thiocarbamate or an ester bond with the surface hydroxyl group, respectively. More recently, Abitbol *et al.*[20] introduced 5-(4, 6-dichlorotriazinyl) aminofluorescein on CNC using the Williamson reaction and showed that the dye grafting density was related to the CNC surface charge density.

In this paper, we show that Rhodamine B, a widely used fluorescent dye, can be grafted onto CNF through the amidation reaction between an amine-modified CNF and a N-Hydroxysuccinimide(NHS)-modified Rhodamine B. The amide bond is chemically stable and thus offer a labelling route with p a better chemical stability, compared e.g. to an ester bond [21]. We graft an isothiocyanate BOC protected amine molecule onto the CNF immediately followed by the BOC deprotection, yielding CNF with amine groups. We demonstrate how the N-Hydroxysuccinimide Rhodamine B ester was synthesized and subsequently reacted with the amine-modified CNF. The luminescent CNF was characterized by infrared and UV-Vis spectroscopy, and the fluorescence properties were characterized by fluorescence spectroscopy and confocal laser scanning microscopy.

Materials and methods

Materials

4-(Boc-aminomethyl)phenyl isothiocyanate, Hydrochloric acid, Rhodamine B, N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), chloroform, dimethyl sulfoxide, acetonitrile were purchased from Sigma-Aldrich and used as received. All the synthesis and surface modification were performed in milliQ water. Infrared spectroscopy was performed using a Varian 610-IR spectrometer, equipped with an attenuated total reflection (ATR) accessory (Specac) and a single-reflection diamond ATR

element. Measurements were normally performed by accumulating 64 scans in the spectral region of 4000–390 cm^{-1} with a spectral resolution of 2 cm^{-1}. Absorption spectra were recorded using a Perkin-Elmer Lambda 19 UV-Vis-NIR spectrometer. Fluorescence spectra were obtained using a Varian Cary Eclipse Fluorescence spectrophotometer with an excitation wavelength of 540 nm. Single nanoparticle fluorescence visualization is performed using a Zeiss LSM5.

Extraction of cellulose nanofibrils (CNF) from wood pulp

The commercial pulp was obtained from Nordic Paper, Sweden with a hemicellulose and lignin contents of 13.8%, and 0.7%, respectively, and a degree of polymerization (DP) of 1200. CNF were extracted according a previously reported procedure[22].In short, the fibers were subjected to an enzymatic treatment in aqueous solution using the enzyme endoglucanase, Novozyme 476 at 50ºC for 2h. The fibers were washed using a Büchner funnel and incubated at 80ºC for 30 min to quench the enzyme activity. After treatments, the fibers were subjected to 8 passes through a M–110EH microfluidizer (Microfluidics Inc., USA) to extract the fibrils from their agglomerated state. The treatment yielded a CNF suspension in water with 1.6 wt% solid content.

Preparation of amine modified CNF

The cellulose nanofibrils (1g, 1.6% w/w) were dispersed in water (2 mL) and the suspension was slowly diluted with the addition of DMSO (30 mL) over a period of 20 min and finally sonicated for 20 min. The solution was then stirred for 4 hours at room temperature and heated up to 70ºC. A solution of 4-(Boc-aminomethyl)phenyl isothiocyanate (70 mg/ 20mL) in DMSO was slowly added drop by drop and the mixture was allowed to stir for 24h at 70ºC. Finally, the cellulose nanofibers were purified by centrifugation (6 000 RPM / 40 min). The supernatant was discarded and replaced with milliQ water. The purification steps were repeated 6 times. The CNF suspension was then diluted with 5 mL of water and added to an HCl solution (1.5 M, 40 mL). The suspension was stirred for 16h. The CNF was purified by centrifugation (6 000 RPM / 40 min / 6 cycles). The amine modified cellulose was characterized by FTIR spectroscopy.

Synthesis of N-Hydroxysuccinimide rhodamine B ester (RB-NHS)

Rhodamine B (500 mg) was dissolved in acetonitrile (30 mL) and a solution of N-Hydroxysuccinimide (170 mg, 10 mL) in acetonitrile was added. The solution was heated to 45 ºC and a solution of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (260 mg) in chloroform (20mL) was added. The solution was allowed to stir for 15h. The solvent was removed under reduced-pressure distillation and the product was used without further purifications.

Labelling of rhodamine B onto CNF (RB@CNF)

The RB-NHS and the amine modified CNF were separately dissolved in DMSO (10 mL). The RB-NHS solution was slowly added to the cellulose suspension under vigorous stirring. The suspension was stirred for 16 hours. The RB@CNF was purified by centrifugation (6 000 RPM / 40 min / 10 cycles) until a clear supernatant was obtained. To ensure that no unbound chromophores were present in solution, each of the supernatants were analyzed in fluorescence spectroscopy and the purification was continued until no fluorescence signal from the solution was detected.

Results and discussions

Scheme 1 illustrates the synthetic pathway to produce luminescent cellulose nanofibrils (CNF): (a) introduction of an amine function on the CNF backbone, (b) modification of the luminescent probe by reaction with the N-Hydroxysuccinimide ester, (c) grafting the activated Rhodamine B onto the amine-modified CNF by an amide bond.

The terminal amine function was introduced by reacting the hydroxyl group on the C-6 position on the cellulose backbone with the isothiocyanate group of the 4-(Boc-aminomethyl)phenyl isothiocyanate molecule, in a DMSO:H_2O mixture. This results in covalent attachment through the formation of a thiocarbamate bond, as shown in Scheme 1A. The amine- modified CNF was purified through several centrifugations steps where the supernatants, containing by- and un-reacted products, were removed, and replaced with fresh milliQ water. The BOC protecting group was then removed by hydrolysis[23], yielding CNF with amine groups. Figure 1 (Figure 1-bottom) shows that surface modified CNF exhibit infrared bands at 1710 (C=O), 1031 (C-N) and 810 cm^{-1} (C-H, aromatic) in addition to the characteristic absorption bands of the nanocellulose. This confirms that the 4-(aminomethyl)phenyl isothiocyanate molecule has been successfully grafted onto the CNF.

The fluorescent probe (Rhodamine B) was converted to its ester form through the reaction of Rhodamine B with N-Hydroxysuccinimide in the presence of the coupling agent N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Scheme 1B). The obtained Rhodamine B - NHS was used without further purification and added to the amine modified nanocellulose dispersion (Scheme 1C). The resulting luminescent CNF (RB@CNF) was separated from the unreacted chromophores through several centrifugations steps. This operation was repeated until a clear supernatant was obtained. All the supernatants were analyzed by fluorescence spectroscopy and the purification was continued until no fluorescence signal was detected in the supernatant (e.g. all unbound chromophores have been removed).

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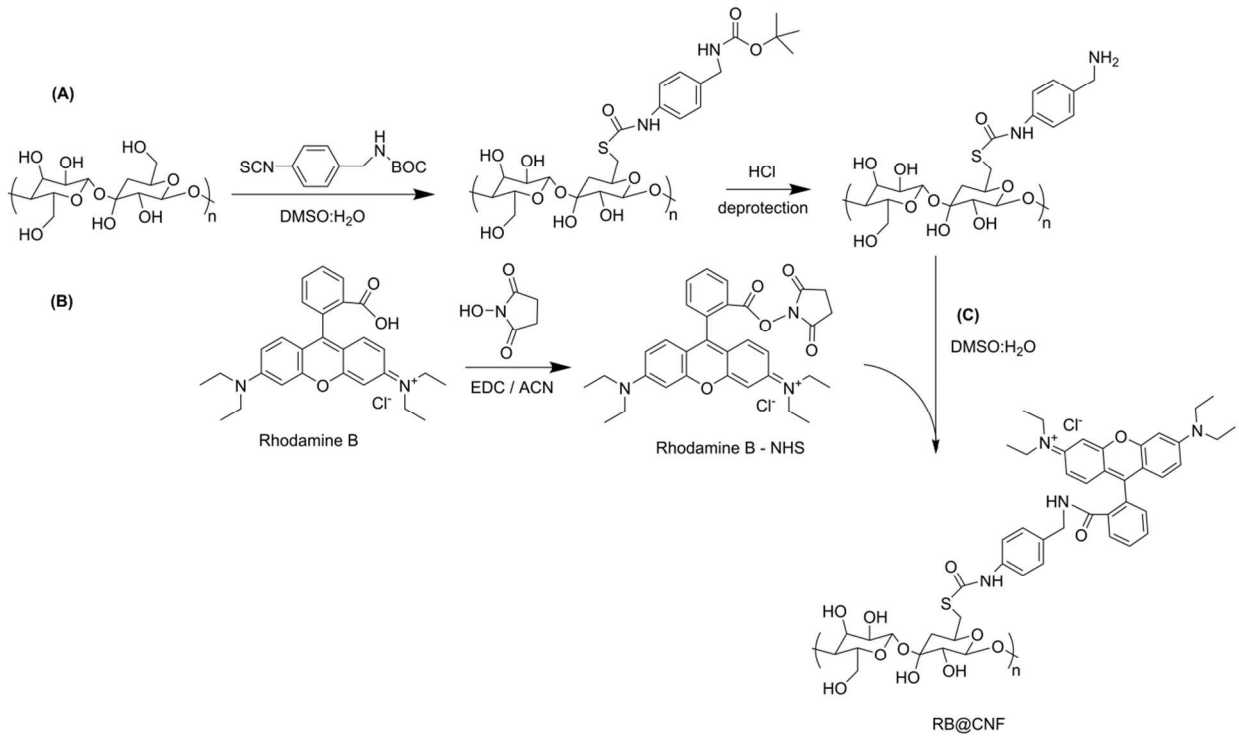

Scheme 1. Fluorescent labeling of cellulose nanofibrils (CNF) with Rhodamine B. (A) Surface modification of the CNF with a terminal amine function; (B) activation of Rhodamine B through the N-Hydroxysuccinimide ester; and (C) fluorescent labeling of the amine-modified CNF by the activated Rhodamine B via the production of an amide bond.

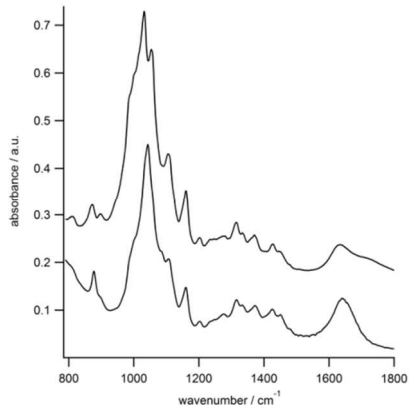

Fig.1 Infrared spectra of the unmodified CNF (bottom) and (top) amine-modified CNF.

The aqueous suspension of the RB@CNF hybrid was characterized by UV-Visible and fluorescence spectroscopy (Figure 2). For comparison, the spectra of the RB@CNF was plotted and compared to the spectrum of a solution of Rhodamine B (Figure 2, left). The CNF labelling results in a red shift of the main absorption band of the chromophore, from 555 nm to 569 nm. The shape of the bands remains unchanged except for a broadening of the absorption band and an increase of the intensity of the absorption band localized at 535 nm for the CNF-grafted chromophores. The red shift of the chromophore absorption band is attributed to the covalent attachment of the dye onto the cellulose nanofibrils. Similar red shift for luminescent probes covalently bond to cellulose have been shown in recent studies[24,18,25]. We also estimated the concentration of the grafted chromophores per cellulose mass from the Uv-vis absorption intensity and the optical density of the grafted chromophores, and obtained a rhodamine B content of 3.75 µmol per g of CNF.

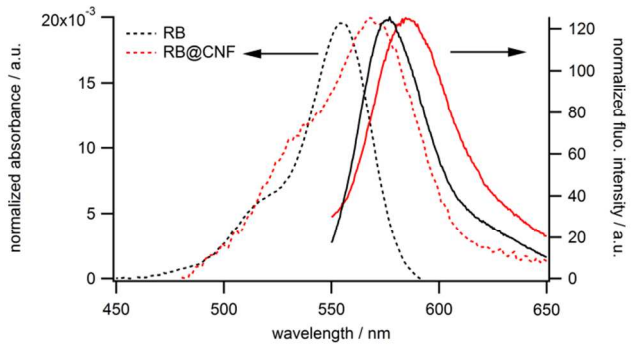

Fig. 2 Fluorescent properties of Rhodamine B@CNF hybrids. Normalized UV-Visible (left y-axis) and normalized fluorescence (right y-axis) spectroscopic data of rhodamine B labelled cellulose nanofibrils (RB@CNF) compared with the free dye (RB). The spectra were recorded in a DMSO: H_2O mixture.

For a better comparison and to highlight the bands shift, both the absorption and the emission spectrum of both RB and RB@CNF in Fig. 2 were rescaled with respect to the maximum intensity. However, the integrated fluorescence intensity of RB@CNF hybrids is smaller when compared to the integrated fluorescence intensity of a Rhodamine B solution (free dye) of similar concentration (not shown). The photophysical properties of molecular probes are strongly influenced by their environment (e.g. the interaction with the solvent and the proximity of the dye molecules) which can either lead to an enhancement or a quenching of the fluorescence signal. Recently, Bhagwat et *al.*[26], in a study on organic chromophores coupled to a helical peptide template, showed that as the chromophores came closer to each other, the emission intensity was reduced and the emission maximum was red-shifted. In the case of CNF, the substrate (CNF) has a relatively low density of reactive sites (the amine-modified c6- hydroxyl groups), which suggest that the CNF should be able to maintain a favorable intramolecular distance and thus avoid strong chromophore-chromophore interactions between molecules grafted onto the same CNF. Hence, the observed quenching is probably dominated by inter- RB@CNF interactions rather than intra- RB@CNF interactions. The flexibility and the organization of the CNF and the possible aggregation in the dispersed state are thus expected to have a major influence on the luminescence intensity of the RB@CNF.

Further investigation on the photophysical properties of the Rhodamine B labelled cellulose nanofibrils was performed using confocal laser scanning microscope. A dispersion of Rhodamine B@CNF was drop cast from a DMSO dispersion onto a glass substrate and dried under vacuum overnight. The material forms the typical coffee-stain pattern with a high density of material close to the edge and significantly less material in the center of the deposit. The fluorescence, bright field and the combined overlay images are shown in Figure 3. The fluorescent images taken after excitation at 543 nm and collected emission at 560 nm show that the fluorescent intensity is higher at the periphery where most of the CNF was deposited. No free luminescent spots were observed outside the Rhodamine B@CNF deposit, showing that the removal of unbound chromophores was successful.

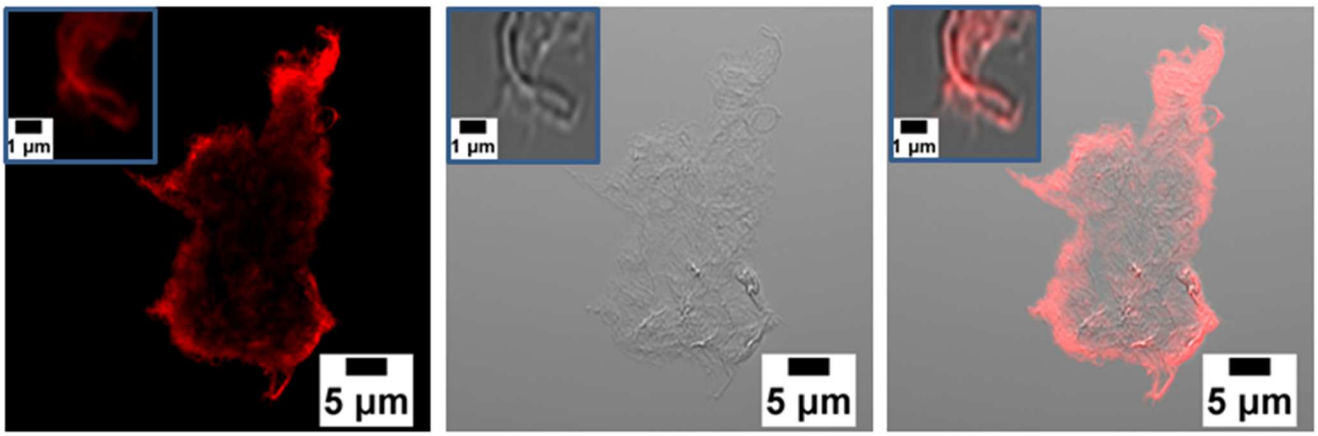

Fig. 3 Fluorescence (left), bright field (middle) and combined overlay confocal laser scanning microscopy images (right) of Rhodamine B@CNF hybrids. The inset shows a high magnification image of a strand of labelled CNF.

Conclusions

We have introduced an amine function on the CNF using 4-(Boc-aminomethyl)phenyl isothiocyanate,. N-Hydroxysuccinimide rhodamine B ester was synthesized and used to fluorescently label the modified CNF. The covalent attachment of the fluorescent probe onto the amine-modified CNF was confirmed by UV-Visible and infrared spectroscopy. The labelling of the dye onto CNF results in a red-shift of the absorption and emission bands. Despite some quenching of the fluorescent intensity of the surface-bound chromophores, it was possible to image the luminescent CNF at both low and high magnification using a confocal laser scanning microscope. The demonstrated grafting route based on amine-modification of nanocellulose could be used for a wide range of chromophores and other organic moieties that e.g. contain carboxylic acid groups.

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Notes and references

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1. R. J. Moon, A. Martini, J. Nairn, J. Simonsen, and J. Youngblood, *Chem. Soc. Rev.*, 2011, **40**, 3941–3994.
2. J. P. F. Lagerwall, C. Schütz, M. Salajkova, J. Noh, J. Hyun Park, G. Scalia, and L. Bergström, *NPG Asia Mater.*, 2014, **6**, e80.
3. K. E. Shopsowitz, H. Qi, W. Y. Hamad, and M. J. Maclachlan, *Nature*, 2010, **468**, 422–425.
4. B. Wicklein and G. Salazar-Alvarez, *J. Mater. Chem. A*, 2013, **1**, 5469–5478.
5. S. Galland, R. L. Andersson, M. Salajková, V. Ström, R. T. Olsson, and L. A. Berglund, *J. Mater. Chem. C*, 2013, **1**, 7963–7972.
6. Y. Okahisa, A. Yoshida, S. Miyaguchi, and H. Yano, *Compos. Sci. Technol.*, 2009, **69**, 1958–1961.
7. M. V Biyani, E. J. Foster, and C. Weder, *ACS Macro Lett.*, 2013, **2**, 236–240.
8. T. Saito, S. Kimura, Y. Nishiyama, and A. Isogai, *Biomacromolecules*, 2007, **8**, 2485–2491.
9. I. Filpponen and D. S. Argyropoulos, *Biomacromolecules*, 2010, **11**, 1060–1066.
10. N. Pahimanolis, U. Hippi, L.-S. Johansson, T. Saarinen, N. Houbenov, J. Ruokolainen, and J. Seppälä, *Cellulose*, 2011, **18**, 1201–1212.
11. I. Filpponen, E. Kontturi, S. Nummelin, H. Rosilo, E. Kolehmainen, O. Ikkala, and J. Laine, *Biomacromolecules*, 2012, **13**, 736–742.
12. Y. Habibi, A.-L. Goffin, N. Schiltz, E. Duquesne, P. Dubois, and A. Dufresne, *J. Mater. Chem.*, 2008, **18**, 5002–5010.
13. C. Goussé, H. Chanzy, M. L. Cerrada, and E. Fleury, *Polymer (Guildf).*, 2004, **45**, 1569–1575.
14. T. Liebert and T. Heinze, *BioResources*, 2008, **3**, 576–601.
15. S. Dong and M. Roman, *J. Am. Chem. Soc.*, 2007, **129**, 13810–13811.
16. G. Mangiante, P. Alcouffe, B. Burdin, M. Gaborieau, E. Zeno, M. Petit-Conil, J. Bernard, A. Charlot, and E. Fleury, *Biomacromolecules*, 2013, **14**, 254–263.
17. J. V. Edwards, N. Prevost, A. French, M. Concha, A. DeLucca, and Q. Wu, *Engineering*, 2013, **05**, 20–28.
18. K. A. Mahmoud, J. A. Mena, K. B. Male, S. Hrapovic, A. Kamen, and J. H. T. Luong, *ACS Appl. Mater. Interfaces*, 2010, **2**, 2924–2932.
19. L. J. Nielsen, S. Eyley, W. Thielemans, and J. W. Aylott, *Chem. Comm.*, 2010, **46**, 8929–8931.
20. T. Abitbol, A. Palermo, J. M. Moran-Mirabal, and E. D. Cranston, *Biomacromolecules*, 2013, **14**, 3278–3284.
21. A. C. M. van Bennekom, P. A. A. T. Willemsen, and R. J. Gaymans, *Polymer (Guildf).*, 1996, **37**, 5447–5459.
22. M. Henriksson, G. Henriksson, L. A. Berglund, and T. Lindström, *Eur. Polym. J.*, 2007, **43**, 3434–3441.
23. D. M. Shendage, R. Fröhlich, and G. Haufe, *Org. Lett.*, 2004, **6**, 3675–3678.
24. L. F. Vieira Ferreira, P. V Cabral, P. Almeida, A. S. Oliveira, M. J. Reis, and A. M. Botelho do Rego, *Macromolecules*, 1998, **31**, 3936–3944.
25. S. Barazzouk and C. Daneault, *Cellulose*, 2011, **19**, 481–493.
26. N. Bhagwat and K. L. Kiick, *J. Mater. Chem. C*, 2013, **1**, 4836–4845.