Cellulose (2019) 26:971–977
https://doi.org/10.1007/s10570-018-2162-z

ORIGINAL RESEARCH

Rapid quantification of cellulose nanocrystals by Calcofluor White fluorescence staining

Roi Peretz · Hadas Mamane · Elizaveta Sterenzon · Yoram Gerchman

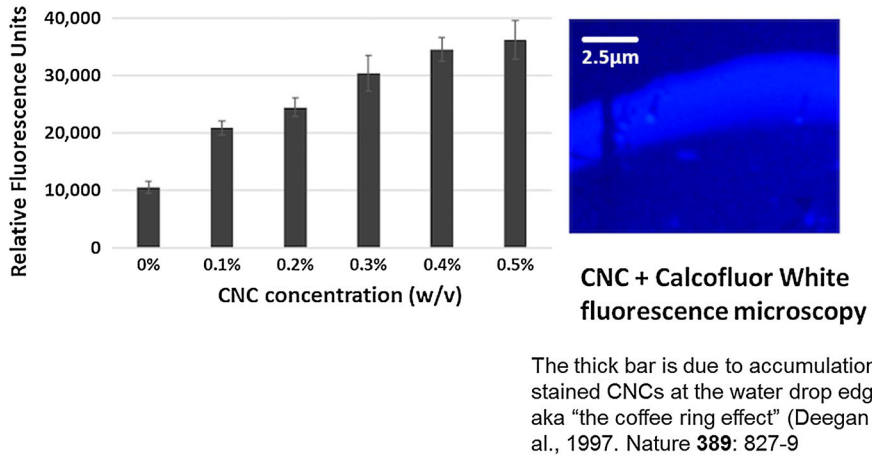
Received: 24 June 2018 / Accepted: 26 November 2018 / Published online: 30 November 2018

Abstract Cellulose nanocrystals (CNCs) have gained increased interest worldwide for their unique properties. CNC production by acid hydrolysis of cellulose-rich biomass is well established, but its' quantification is still complicated. In this study, a rapid method for the determination of CNC concentration using Calcofluor White (CW) fluorescence dye is demonstrated for both purified and homemade CNCs recovered from Whatman filter paper. The method is robust, selective for crystalline cellulose, suitable for routine measurement in CNC production and that the pH must be basic for staining using CW.

R. Peretz · H. Mamane · E. Sterenzon
School of Mechanical Engineering, Faculty of Engineering and Water Research Center, Tel Aviv University, 69978 Tel Aviv, Israel

Y. Gerchman (✉)
Department of Biology and Environment, Faculty of Natural Sciences, University of Haifa-Oranim, 3600600 Tivon, Israel
e-mail: gerchman.yoram@gmail.com

Graphical abstract



Keywords Cellulose nanocrystals (CNC) · quantification · Calcofluor White · Fluorescence · Whatman filter paper · quantification

Introduction

In recent years, cellulose nanocrystals (CNCs) have gained increased interest worldwide due to their unique and interesting properties. These rod-like cellulose whiskers have been applied in many industries including water treatment and purification (Carpenter et al. 2015; Mautner et al. 2015; Voisin et al. 2017), building and reinforcement composites (Lee et al. 2014), paper and packaging (Cowie et al. 2014) and biomedical (George and Sabapathi 2015). CNCs vary in size from 100 to 250 nm in length and 5 to 70 nm in width (Du et al. 2016) and demonstrate high strength, thermal stability, large surface area, unique optical properties, biodegradability, and are considered non-toxic (Song et al. 2014; Xu et al. 2017).

CNCs are produced from cellulosic biomass, mostly wood (Sheltami et al. 2012), although their production from other types of biomass has been reported, e.g. wastepaper (Danial et al. 2015), filter paper (Tang et al. 2013), eucalyptus pulp (Bian et al. 2017), citrus wastes (Mariño et al. 2015), bleached pulp fibers (Wang et al. 2017), rice husks (Johar et al. 2012) and even industrial sludge (Jonoobi et al. 2012).

The production of CNCs is done by degrading the disordered part of the cellulose, most commonly by the use of concentrated sulfuric acid (Capron et al. 2017; Chen et al. 2016), although other, milder treatments are being researched (Bian et al. 2017; Wang et al. 2017; Chen et al. 2016). After the degradation step, the CNCs are washed, and its amount is estimated by separation of the CNCs from the acids, drying and weighing. This process is tedious, time consuming, and does not allow for easy optimization of CNC production process. Chemical Oxygen Demand (COD) analyses was also suggested, however errors might occur due to the presence of lignin or hemicellulose (Bian et al. 2017), and COD measurement is also time consuming.

Calcofluor White (CW) is a fluorescence dye used widely in the paper and textile industries as whitening agents (Harrington and Hageage 2003). This dye (and probably similar dyes, such as Phore-white, Tinopal) bind oriented structural polysaccharides like crystalline cellulose and chitin, resulting in a dramatic increase in the dye fluorescence through an unknown mechanism (Harrington and Hageage 1991; Herth and Schnepf 1980; also see Fig. 1). Previous papers have demonstrated CW's ability to bind oriented cellulose fibrils and suggested that CW is not only adsorbed on the CNC surfaces but does so in an oriented way (Herth and Schnepf 1980). Due to this ability, CW has been used in the study of plant structure (Hughes and McCully 1975), the study of bleaching process in the paper industry (Gray and Olmstead 1993), and rapid detection of clinical fungi infection (Harrington and Hageage 2003), but to date it was not used for detection of CNCs.

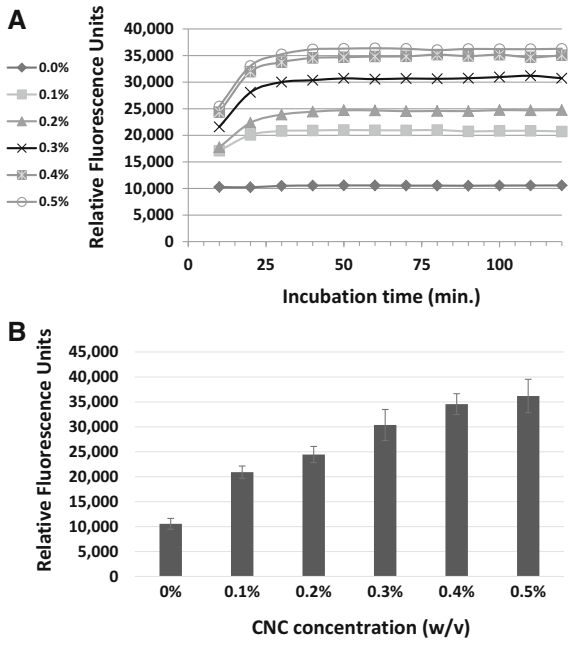


Fig. 1 Fluorescence as a function of CNC concentration: **a** Fluorescence reading kinetics for different CNC concentrations; **b** fluorescence reading after 30 min; average of 5 readings ± SD

In this study, we present a rapid method for CNC quantification based on CW fluorescence. A calibration curve is presented using the model developed from commercial CNCs, and the method was then applied for rapid detection of CNCs produced from filter paper, demonstrating its potential for industrial and academic uses.

Materials and methods

Materials and standards

CNC powder was purchased from Nanografi (Turkey). Calcofluor White (CW) M2R (Cat #18909), CMC (419303; degree of substitution = 0.9) and α-cellulose (C8002) were purchased from Sigma-Aldrich (Israel) and was used in this study as received. Sulfuric acid ACS reagent grade and Potassium hydroxide (KOH) were obtained from Merck (Germany). Working CNC suspensions were prepared by adding the desired amount of commercial CNCs to known volume of deionized water (DIW) (Direct-Q3 UV System, Millipore-France) and continuously stirring.

Fluorescence procedure

Samples (100 µl) were placed in in a flat-bottomed black 96 wells plate (Nunc^{TM}, Denmark) and mixed with CW reagent and 5M KOH solution for total volume of 200 µl. Fluorescence signal was quantified using Spark 10M plate reader (Tecan, Switzerland). This was reported in the instrument's Relative Fluorescence Units (RFU), which was calculated by SparkControl software. Excitation was measured at 355 nm and emission at 433 nm. The plate was continuously shaken to prevent CNC sedimentation. Temperature was kept at 30 °C. Fluorescence was read in kinetic mode every 10 min with maximal incubation time of 120 min. At least five wells were examined for each time interval tested.

In-house CNC production from Whatman filter paper

Cellulose nanocrystals were prepared by acid hydrolysis as previously described (Johar et al. 2012; Sheltami et al. 2012). Whatman filter paper 1 was ground to fibers using a 250W laboratory blade mill (MRC Ltd., Israel), aqueous sulfuric acid (64 wt%) was added to the fibers at 1:20 ratio (w/w), and the mixture incubated at 45 °C for the designated time. Samples were taken after 0and 45 min of hydrolysis and diluted 10-folds with cold water to stop the reaction. The acid was removed from the CNCs by centrifugation (10,000 rpm, 15 min, 10 °C; Jouan B4i), decanting the liquid, and re-suspending the pellet in DIW (three repeating washes). Finally, the CNC pellet was suspended in 30 ml DIW, and dialyzed against deionized water until a constant pH

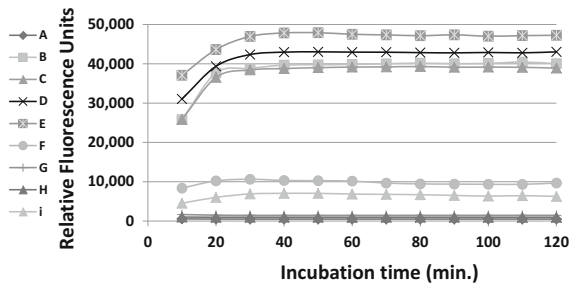


Fig. 2 CNC fluorescence as function of conditions and time

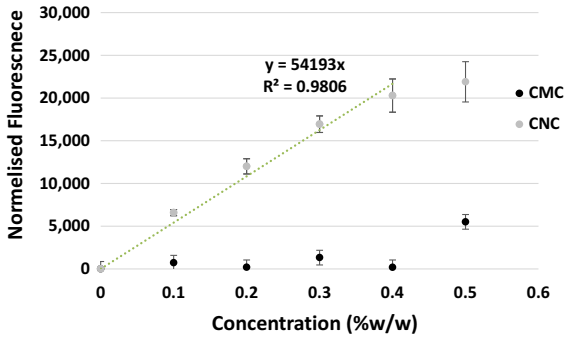


Fig. 3 CNC and CMC solutions fluorescence (average of 5 readings ± SD). The linear fit was passed only through the linear readings (0–0.4%)

was reached. CNC fluorescence procedure was done as mentioned above.

Results and discussion

CNC fluorescence after CW

CW reagent has been used extensively for dyeing oriented structural (i.e. crystalline) cellulose in plant tissues (Hughes and McCully 1975). Since CNCs are highly structurally oriented (and that is where their crystallinity arises from), we assumed that CW can also be used for staining and quantification of such. To examine this approach, commercial CNC suspensions (10 mL) at different concentrations were prepared in DIW (0%, 0.2%, 0.4%, 0.6%, 0.8% and 1% w/v). For each suspension, a volume of 100 µl was mixed with 50 µl CW reagent and 50 µl 5M (10% w/v) Potassium hydroxide (KOH), as suggested for staining plant tissue (Harrington and Hageage 2003), providing a final CNC concentrations of 0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (w/v) respectively. The samples were placed in a black 96 well plate and tested for fluorescence kinetics (Fig. 2).

Figure 1a illustrates the relative fluorescence units (RFU) as a function of CNC concentration from 0 to 0.5% and incubation time up to 120 min and Fig. 1b illustrates fluorescence reading after 30 min for the various CNC concentration. From Fig. 1 it is evident that the fluorescence reading increased in the first 30 min, probably due to diffusion of the CW into the CNC particles and then reached a plateau up to 120 min. Figure 1b shows an increase in CNC concentration for 30 min. incubation time. Equilibrium fluorescence was positively correlated to CNC concentration with near linear correlation up to 0.4% (w/v) CNCs (Fig. 3).

Another set of experiments examined the optimum amounts of CNCs (1%; 0–200 µl), CW (0–200 µl) and 5M KOH (0–200 µl) for maximal RFU signal (Table 1). The different conditions were also plotted as RFU vs. incubation time. Optimum was determined by the highest RFU reading after 30 min and observed for condition 'E' (80 µl of CW reagent and 20 µl of 5 M KOH).

To test whether this fluorescence dyeing is selective for CNCs and not for cellulose in general, a similar experiment was conducted using carboxymethyl cellulose (CMC), a cellulose derivative with very low

Table 1 Different samples mixtures for CNC fluorescence tests

	CNC 1% solution (µl)	CW (µl)	5 M KOH (µl)	Total volume (µl)	Final CW concentration (%v/v)	Final KOH concentration in well (M)	pH	Fluorescence reading (RFU) after 30 min
A	100	0	100	200	0	2.5	14	500.6 ± 6.4
B	100	20	80	200	10	2.0	14	39,668.8 ± 1177.0
C	100	40	60	200	20	1.5	14	38,801.2 ± 1315.2
D	100	60	40	200	30	1.0	13–14	42,971.6 ± 3573.0
E	100	80	20	200	40	0.5	12–13	47,859.4 ± 8608.1
F	100	100	0	200	50	0.0	6	10,321.8 ± 727.9
G	200	0	0	200	0	0.0	N.A.	1445.4 ± 207.0
H	0	0	200	200	0	5.0	N.A.	968.4 ± 11.5
I	0	200	0	200	100	0.0	N.A.	7045.2 ± 149.2

crystallinity (Xiquanq et al. 1990) at high degree of substitute (here 0.9), compared to the much higher 92% for the CNCs used (manufacturer data). Solutions of all materials were prepared as above, where 100 µl of each was mixed with 80 µl CW reagent and 20 µl 5 M KOH, incubated, and read after 30 min in the plate reader as detailed above (Fig. 3). The results demonstrated linearity of the fluorescence signal up to 0.4% (w/v) CNCs (Fig. 3), with specificity of the CW staining, since CMC (a modified cellulose with very low crystallinity; Xiquanq et al. 1990) gave only negligible fluorescence, and even this only at the highest concentration.

KOH effect on CNC fluorescence

Previous work (Harrington and Hageage 1991) and common clinical protocols recommended the addition of KOH to the CW reagent as KOH was demonstrated to be important for softening the fungal chitinous cell wall (Hamer et al. 2006), however still no optimization was evident. Given this, we examined the impact of various KOH additions on CNC fluorescence value. The results presented in Fig. 4 demonstrate that the presence of KOH not only quench background fluorescence (compare 0% CNC and 0% CNC + KOH) but is also mandatory for effective labeling of the CNCs.

To determine the proper KOH conc. for maximal effect, a 5 M KOH solution was used to make various dilutions of KOH. For each KOH solution, the pH was tested using a simple pH stick. A volume of 100 µl of 1% CNC solution was mixed with 20 µl KOH (at various conc.) and 80 µl of CW, in different wells. Fluorescence reading was examined (Fig. 5).

The highest fluorescence was obtained with final conc. of 0.125M KOH and measured pH value of 12–13. There were statistically significant differences between group means as determined by one-way ANOVA ($F\ (5,24) = 198.8$, $p < 0.0001$). Letters above the graph indicate statistically significant different groups. This suggests that there is an optimum pH for CNC fluorescence, and the examined samples should be titrated to pH =12–13 prior to

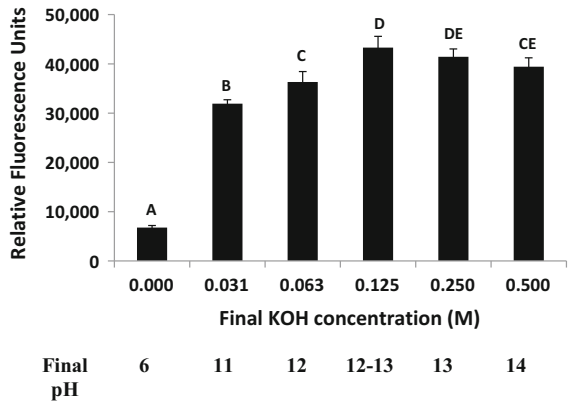


Fig. 5 1% CNC solution fluorescence at 30 min as function of KOH concentrations; average of 5 readings ± SD

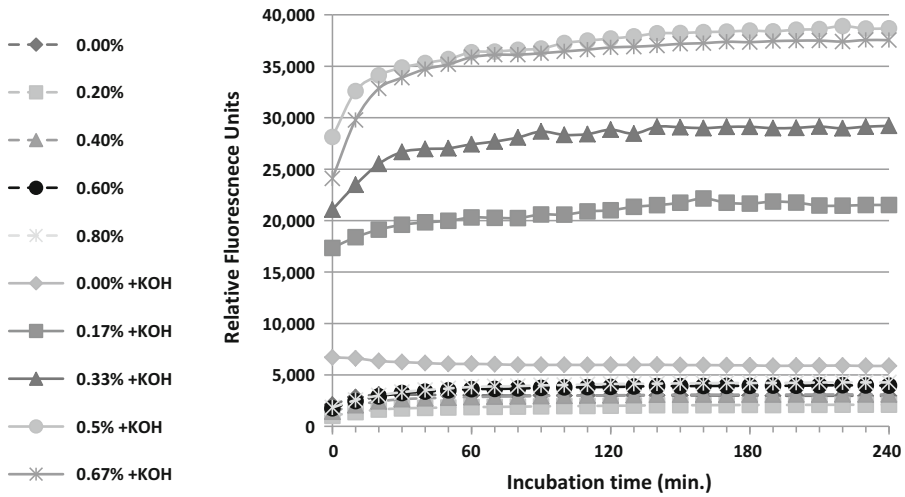


Fig. 4 CNC Fluorescence w/wo KOH

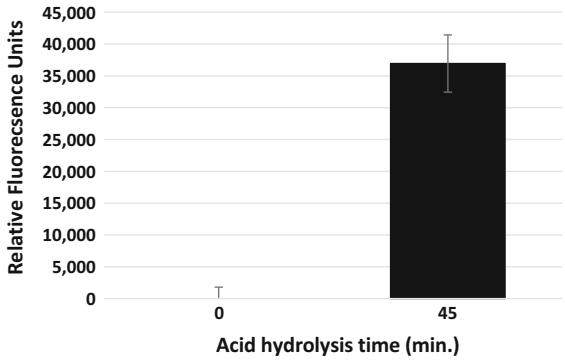


Fig. 6 Whatman filter paper CNC fluorescence as function of different hydrolysis times. Fluorescence was measured after 30 min incubation of the washed CNCs with CW; average of 5 readings ±SD

conducting the tests. This also correlates to previous results that suggested optimum at this pH (Table 1).

CW staining of in-house CNCs

To further test our quantification approach on 'real-life' samples, we used the CW fluorescence method to quantify CNCs prepared from Whatman paper 1 *in-house*. Whatman filter paper was hydrolyzed with 64% sulfuric acid, the crystals separated from the liquid by centrifugation, washed from the acid, re-suspended in DIW, and CW and KOH added—to 100 µl recovered CNCs, 20 µl 5M KOH, and 80 µl of CW. Fluorescence reading was determined (Fig. 6).

No fluorescence was evident at time 0, indicating the lack of solubilized CNCs. Clear and high fluorescence was visible after 45 min of hydrolysis, indicating the presence of soluble CNCs and demonstrating the applicability of the CW method for detection of CNCs without the need for time consuming drying.

Conclusion

In this study, a new, simple and rapid approach for the determination of CNC concentration in solution is presented, based on the fluorescence resulting from the interaction of crystalline cellulose, but much less with disordered cellulose, with CW. Staining of crystalline cellulose resulted in significantly higher fluorescence than staining of disordered cellulose and cellulose derivative, with linear relation between CNC concentration and fluorescence signal. This method is therefore suggested to depend on the cellulose crystallinity index, making this method highly suitable for CNC quantification. pH conditions for effective staining were also established, demonstrating the importance of basic pH (ideally pH =12–13). The method was tested on CNCs produced from Whatman 1 paper, demonstrating its validity. This method could simplify optimization of CNCs production conditions. It should be noted that although CW has been widely used for histological staining of plant tissues, it has not been used to-date for CNCs applications.

Acknowledgments This research was conducted in the framework of the Israeli Ministry of National Infrastructure, Energy and Water Resources Grant No. 214-11-006 and the Israeli Ministry of Environmental Protection Grant No. 132-3-4.

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