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Introduction of aldehyde vs. carboxylic groups to cellulose nanofibers using laccase/TEMPO mediated oxidation

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ABSTRACT

The chemo-enzymatic modification of cellulose nanofibers (CNFs) using laccase as biocatalysts and TEMPO or 4-Amino-TEMPO as mediators under mild aqueous conditions (pH 5, 30 °C) has been investigated to introduce surface active aldehyde groups. 4-Amino TEMPO turned out to be kinetically 0.5-times (50%) more active mediator, resulting to oxoammonium cation intermediacy generated and its in situ regeneration during the modification of CNFs. Accordingly, beside of around 750 mmol/kg terminally-located aldehydes, originated during CNFs isolation, the reaction resulted to about 140% increase of C6-located aldehydes at optimal conditions, without reducing CNFs crystallinity. While only the C6-aldehydes were wholly transformed into the carboxyls after additional post-treatment using NaOH according to the Cannizzaro reaction, the post-oxidation with air-oxygen in EtOH/water medium or $NaClO_2$ resulted to no- or very small amounts of carboxyls created, respectively, at a simultaneous loss of all C6- and some terminal-aldehydes in the latter due to the formation of highly-resistant hemiacetal covalent linkages with available cellulose hydroxyls. The results indicated a new way of preparing and stabilizing highly reactive C6-aldehydes on cellulose, and their exploitation in the development of new nanocellulose-based materials.

1. Introduction

Highly crystalline nanofibrillated cellulose or nanocellulose, being produced from native wood or cotton cellulose fibers, have been growing in importance over the last decade due to their light weight, high aspect ratios (Eichorn et al., 2010; Isogai, Kado, & Goi, 2010b), high elastic moduli and low thermal expansion coefficients (Fukuzumi, Saito, Iwata, Kumamoto, & Isogai, 2009), thus displaying potential applications as bio-based nanomaterials in the development of new nanocomposites, hybrid, nanosensing and nanofiltration materials, etc. Moreover, the chemical character of the cellulose molecule, particularly the presence of three reactive hydroxyl groups on each of the β-1,4-glycosidically linked anhydroglucose units enables their further modification and functionalization, thus introducing new functional groups or even molecules, either for improving their nano-dispersibility or even giving them higher added-value for specific application (Eichorn et al., 2010; Isogai, Kado, & Goi, 2010a; Isogai et al., 2010b; Moon, Martini, Simonsen, & Youngblood, 2011). Amongst all the functional groups where hydroxyls are transformed into oxidized functionalities, carbonyl (mainly aldehydes, CHO, and ketones, C=O) and carboxyl (COOH) groups are one of the prime factors for determining macroscopic properties and chemical behavior of cellulosic materials (Potthast, Rosenau, & Kosma, 2006). The formation of these groups (corresponding however mainly to the number of reducing end-groups) may already occur naturally during the isolation and purification procedures, in particular to cellulose isolated from wood, being the results of a number of processing steps. Anyhow, the introduction of more such functionalities using various oxidants that may simultaneously attack one or more hydroxyls in a cellulose chain without reducing its crystallinity, are still under investigation by many researchers. The transformation of hydroxyl groups into oxidized functionalities on a nano-scale cellulose surface is another particular challenge.

Relatively selective chemical oxidation of primary (C6) hydroxyl groups of cellulose using stable nitroxyl radicals such as TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical)- (Coseri et al., 2013; Fujisawa, Okita, Fukuzumi, Saito, & Isogai, 2011; Fukuzami, Saito, & Isogai, 2013; Saito & Isogai, 2006; Saito, Shibata, Isogai, Suguri, & Sumikawa, 2005; Saito, Kimura, Nishymama, & Isogai, 2007; Saito et al., 2009; Shimizu, Fukuzumi, Saito, & Isogai, 2013; Tanaka, Saito, & Isogai, 2012) or its derivate (Iwamoto et al., 2010) as catalysts, mostly in the presence of NaBr/NaClO or $NaClO/NaClO_2$ reagents as primarily oxidants, and under alkali conditions, was found to be

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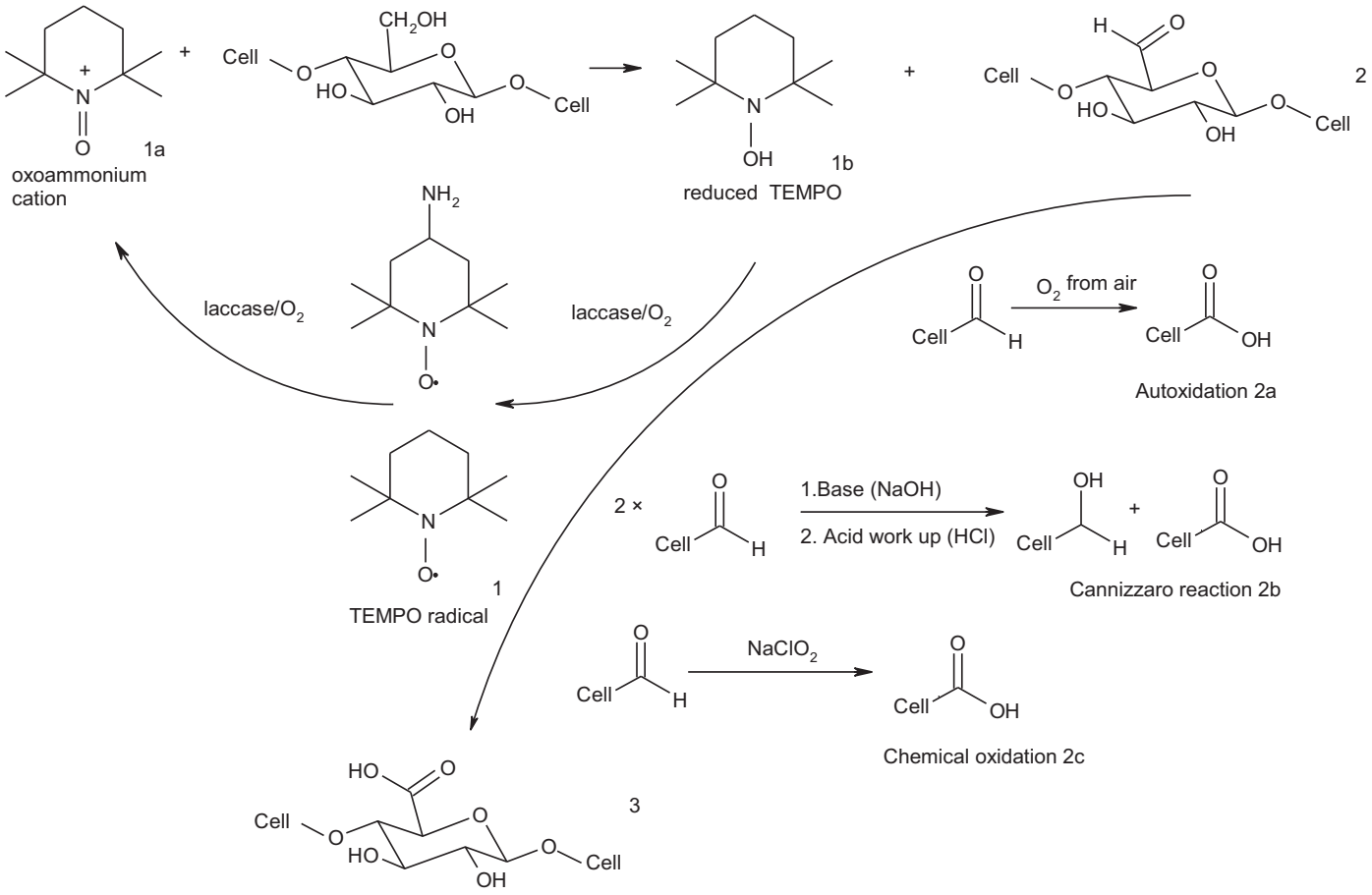

Scheme 1. Tentative mechanisms for the laccase catalyzed regeneration of TEMPO during the oxidation of cellulosic substrate, and its further oxidation using different approaches.

the simplest and efficient procedure for cellulose oxidation. During this process (Scheme 1, reaction 1), the oxoammonium ion ($TEMPO^+$, **no. 1a**) is continuously regenerated in situ by a primary oxidant, the amount of which determines the yield of the oxidation reaction, being also variable within reaction conditions, reaction medium and pH used. The nature of the products obtained is also highly dependent on the starting materials (i.e. the crystal structure, crystal size, and microfibril morphology of the cellulose) bringing different but still relatively high degree of carboxylation (—COOH being between 500–1700 mmol/kg), however with a proportional decrease in the cellulose polymerization degree (Habibi, Chanzy, & Vignon, 2006; Isogai, Saito, & Isogai, 2010; Saito & Isogai, 2006; Saito et al., 2007, 2009). Although more than 90% of the oxidized groups using TEMPO system are usually carboxylates, some amounts of the aldehyde groups (—CHO, 100–300 mmol/kg) formed were also reported to be present on the cellulose after the reaction, being however also dependent on the accessibility and crystallinity of the starting material as well as the oxidation conditions, mainly the pH value. In addition, keto groups can also be introduced at C2 or C3 or even both as 2,3-diketo structures under alkaline conditions, as well as to some extent also hydrates, hemiacetals or hemiketals, but being hardly detected by spectroscopic techniques because of the variety of structures and microenvironments encountered (Potthast, Rosenau, Kosma, Saariaho, & Vurinen, 2005). In any case, all these carbonyl groups induce β-elimination that leads to chain cleavage of the cellulose polymer under alkaline conditions (Potthast et al., 2006).

In order to avoid undesirable side reactions, above all the cellulose depolymerization or discoloration of the oxidized cellulose due to the presence of aldehyde group residuals (Saito et al., 2009), chemo-enzymatic TEMPO-mediated oxidation using oxidative enzymes (Scheme 1, reaction 1) (preferably laccases with 10–100 fold higher oxidation rates than alternative peroxides) at near-neutral or slightly acidic medium, and under mild conditions, has also been studied recently as an ecologically and economically-friendly alternative (Aracri & Vidal, 2012; Aracri, Vidal, & Ragauskas, 2011; Aracri, Valls, & Vidal, 2012; Patel, Rosenau, Ludwig, Haltrich, & Potthast, 2011). This then resulted in the formation of approximately three- to five-times more aldehyde groups in comparison with the carboxyls, where however the latter being mainly a result of aldehyde subsequent auto-oxidation (Scheme 1, **no. 2a**) and/or additionally performed reactions (**no. 2b–2c**), and not a result of the chemical action of the presented reagents. However, TEMPO-mediated oxidation of nano-sized cellulose has being studied only by chemical modifications, leading to insignificant changes in cellulose morphology and crystallinity only when a low carboxylation degree (up to 0.1) was obtained (Fujisawa et al., 2011; Fukuzami et al., 2013; Habibi et al., 2006; Iwamoto et al., 2010). Therefore, no data are available on the contents of the aldehyde groups being created.

The presented paper is thus the first study on the laccase-mediated oxidation of cellulose nanosubstrate (i.e. nanofibres) using TEMPO and 4-amino TEMPO derivatives as mediators and laccase as biocatalyst, being evaluated relative to its efficacy and selectivity of CNFs carboxylation vs. aldehyde groups formation, as well as its influence on the CNFs crystallinity and hydrogen bonding intensity. In addition, the increasing degree of carboxylation during different post-oxidations steps is quantified.

2. Experimental

2.1. Materials

Cellulose nanofibres (CNFs) with diameters ranging between 10 and 70 nm and lengths on few micrometer scale were provided by Lulea University of Technology, Sweden; the CNFs were isolated from cellulose sludge of 95% purity and without any pre-treatment according to (Jonoobi, Mathew, & Oksman, 2012). 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO, T), 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-Amino TEMPO, AminoT), laccase from *Trametes Versicolor* (E.C. 1.10.3.2), ethyl alcohol, sodium hydroxide, sodium chlorite, 2,3,5-triphenyltetrazolium chloride (TTC), 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), citric acid, and sodium citrate were purchased from Sigma Aldrich. All the chemicals used were of analytical grade.

2.2. Laccase activity assays

The laccase activity assay was determined using 2,2-azobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate. The assay reaction mixture contained a 200 μl of enzyme solution, 100 μl of 1 mM ABTS, and a 700 μl of 100 mM citrate buffer at pH 4,5. The oxidation process of ABTS substrate was determined by monitoring the increase in absorbance at 436 nm ($\varepsilon_{436} = 2.9 \times 10^4\ M^{-1}\ cm^{-1}$) according to (Niku-Paavola, Karhunen, Salola, & Raunio, 1988). The enzyme optimal activity was determined within a pH range from 4.5 to 7.5, temperature range from 20° to 70 °C, and expressed in Units (U) defined as μl of ABTS oxidized per min. UV–vis spectrophotometric measurements were performed using a Cary 50 UV VIS spectrophotometer in a 1 cm quartz cuvette.

2.3. CNFs treatment by the laccase/TEMPO system

The treatment of CNFs by the laccase/TEMPO system was performed under mild conditions using TEMPO or 4-Amino TEMPO, respectively. 0.5% (w/v) of CNFs dispersed in a 50 mM sodium citrate buffer at pH 5 was treated with different concentrations of TEMPO (0.1 mM, and 1 mM) and 4-Amino TEMPO (0.01 mM, 0.1 mM, 10 mM, and 30 mM), respectively, in the addition of 0.1 U, 1 U, and 2 U of laccase per 1 mL of reaction mixture in a water bath shaker at 100 rpm and 30 °C for 2, 24 and 48 h. The reaction was stopped and the samples further extensively rinsed by three different procedures to constant conductivity in order to ensure further oxidation of aldehyde to carboxyl groups, according to reactions **no. 2a–2c** presented on Scheme 1, i.e.: (**no. 2a**) by treating the samples at boiling temperature for 10 min following an extensive rinsing with EtOH/water (in ratio 70/30) three times, and a final rinsing with distilled water, enabling auto-oxidation by air-oxygen; (**no. 2b**) by treating the samples with 1 M NaOH (pH 12) at boiling temperature for 10 min performing Cannizzaro reactions (Patel et al., 2011), following by extensive rinsing with distilled water, and further treatment with 0.1 M HCl for 15 min at room temperature for FTIR analysis; or (**no. 2c**) by treating the samples (previously rinsed with EtOH) with 0.2 M $NaClO_2$ for 24 h at pH 6 and room temperature, followed by extensive rinsing with distilled water. After each final rinsing the samples were centrifuged at 7000 rpm for ≈7 min and freeze-dried prior to FTIR analysis, meanwhile the ζ-potential measurements and potentiometric titration were carried out from CNF suspension.

2.4. Spectrophotometric and cyclic voltammetry (CV) monitoring of TEMPO (re)oxidation using laccase

Laccase-mediated TEMPO (and 4-Amino TEMPO) oxidation in the absence and presence of CNFs was monitored spectrophotometrically as well as by cyclic voltammetry performing the electrochemical measurements.

Spectrophotometrical analyzes were performed in 50 mM Na-citrate buffer (pH 5) containing 0.5 mM TEMPO and 0.1 U, 0.5 U, 1 U or 3 U of laccase per 1 mL of reaction mixture, and different pH intervals (pH = 4–7) at 30 ± 0.2° C. The initial rate of the reaction was estimated from the absorbance evaluated at 245 nm and 300 nm (Kulys & Vidziunaite, 2005), corresponding to the TEMPO and TEMPO oxoammonium ion formation. The influence of TEMPO concentration on V_{max} and K_m was calculated using the Michaelis–Menten equation (Michaelis & Menten, 1913).

Cyclic voltammetry (CV) measurements were performed using a PGSTAT101 Autolab controlled by an Autolab Nova Software version 1.8. All experiments were carried out in a 100 mL Metrohm cell using a glassy carbon electrode (GCE) with a surface diameter of 3 mm. A counter (Pt) electrode with a KCl saturated reference electrode (Metrohm) was used throughout. Prior to each experiment the GCE electrode was freshly polished with aluminum oxide (Micropolish, Buehler) on a micro cloth-polishing pad (Buehler). The electrochemical measurements were performed in a thermostated cell (40 mL) containing a 50 mM Na-citrate buffer (pH 5), 0.5 mM TEMPO, and 1 U of laccase per mL of reaction mixture at 30 °C. The voltammetric curves were recorded every 5 min for 30 min at a scan rate of 50 mV/s.

2.5. Characterization of laccase/TEMPO oxidized CNFs

ζ-Potential and particle average size analyses of untreated and differently treated samples were carried out by Zeta-sizer Malvern Nano ZS at 25 °C using a ZEN1010 cell. A field of 40 V was applied across the nominal electrode spacing of 16 mm. The CNFs were prepared at concentrations of 0.05 g/L in MQ-water and measured over a pH range from 10 to 2 which was adjusted using 0.1 M NaOH and 0.1 M HCl. The average value as well as the standard deviation of the mean was calculated from at least three individual measurements.

FTIR-ATR spectroscopy of the freeze-dried CNF samples was carried out using a Perkin Elmer spectrophotometer with a Golden Gate attenuated total reflection (ATR) attachment having a diamond crystal. The spectra were recorded over a range from 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans per samples, followed by baseline correction and smoothing prior to further analysis.

The contents of the aldehyde groups of the CNF samples were determined using a slightly modified spectrophotometric method as published by (Szabolcs, 1961). For that purpose, ≈10 mg of lyophilized CNFs samples were placed into 2 mL microcentrifuge tubes following by the addition of 0.5 mL KOH (0.3 M) and 0.5 mL TTC (0.01 M), and then constantly shaken for 6 min at 80 °C in an Eppendorf thermomixer. The reaction mixture (1 mL) was mixed with MeOH (9 mL) to extract produced *reddish-crystals of* TTF (triphenyltetrazolium formazan) from the CNF suspension, and recorded it spectrophotometrically at 482 nm. The amount of aldehyde groups was calculated from the calibration curve line prepared with anhydrous α-D-glucose as a substrate. The mean values were calculated from at least three individual measurements.

Potentiometric titrations of the CNF samples (0.15 g) were carried out in order to determine the contents of carboxyl groups. Titrations were performed using a Mettler Toledo T70 two-burette instrument within an inert atmosphere (N_2 gas). The burettes were filled with 0.1 M HCl and 0.1 M KOH. All the solutions were prepared in MQ water with low carbonate content (<10^{-5} M) achieved

by boiling and cooling under a nitrogen atmosphere. The samples were titrated in forwards and backward manners between the initial pH 2.5 to the present pH 11. The titration experiments were carried out at 0.1 M ionic strength, set at its appropriate value with KCl. The titrants were added dynamically within a present interval of 0.001–0.25 mL. The equilibrium criteria for the timed addition were set to $dE/dt = 0.1/60$ s, where 60 s was the minimum time to reach equilibrium conditions between two additions of the titrant, and the maximum time was set at 1200 s. The pH value was measured using a Mettler Toledo DG-111-SC combined glass electrode. A blank HCl–KOH titration was carried out under the same conditions as above.

3. Results and discussion

3.1. Kinetics of TEMPO vs. 4-Amino TEMPO (re)oxidation within different reaction systems

Although the mechanistic details of laccase/mediator catalyzed aerobic oxidations of substrates are still a matter of conjecture, it is generally believed that for involving one-electron oxidation ($1e^-_{ox}$) of the mediator by the oxidized (cupric) form of the laccase followed by the reaction of an oxidized mediator with the substrate, either via electron transfer or via hydrogen atom transfer as e.g. with N-hydroxy compounds which form N-oxy radicals yielding a variety of products via a second one-electron ($1e^-_{ox}$) transfer (Arends, Li, Ausan, & Sheldon, 2006); the oxidation process is coupled within the reduction of dioxygen to water by a four-electron transfer.

As presented in Fig. 1a, the laccase-mediated oxidation of TEMPO, being monitored spectrophotometrically, led to a decrease in absorbance at 245 nm and to an increase in absorbance at 300 nm, the latter denoting the formation of oxoammonium cation based on the mechanism presented in Scheme 1 (**no. 1a**) (Fish, Swarts, Sevilla, & Malinski, 1988; Kulys & Vidziunaite, 2005). As can be observed, the fastest TEMPO oxidation rate was obtained within the first 10 min following by slower oxidation rate for the next 30 min when the plateau was reached. The TEMPO oxidation rate is also dependent on laccase concentration (Fig. 1b) where the plateau was reached after approximately 15 min for the concentrations of 0.5 U, 1 U, and 3 U per mL of substrate, meanwhile the laccase concentration of 0.1 U failed to ensure sufficient oxidation of 0.5 mM TEMPO during the applied incubation period. As the maximum oxidation rate of TEMPO was observed to be at pH 5 (Fig. 1c), being related to the laccase pH optimal activity as confirmed by ABTS as a standard substrate (data not shown), this treatment condition were chosen for further laccase/TEMPO and laccase/4-Amino TEMPO-mediated oxidation of the CNFs. The initial rate of the TEMPO oxidation was shown to also be dependent on TEMPO concentration (Fig. 1d) from which the Michaelis–Menten constants were calculated, yielding a V_{max} of 0.028 ± 0001 mM/min and K_m of 1.314 ± 0.07 mM for the laccase/TEMPO system, indicating a relatively low affinity of laccase toward TEMPO, which additionally decreased to V_{max} of 0.1035 ± 0.01 mM/min and K_m of 5.3003 ± 0.4 mM in the presence of CNFs denoting approximately five-times lower affinity of laccase to the substrate (TEMPO) in the presence of CNF, being probably related to the diffusion limitations and high swelling ability of CNFs.

The spectrophotometric analysis correlated well with the electrochemical (electron transfer) analysis of TEMPO oxidation, as investigated by CV, also confirming TEMPO regeneration in situ (i.e. its re-oxidation), the efficacy of which was however shown to be dependent on both the studied systems and the timing period (Fig. 2). While no redox peaks for pure CNFs within the potential window were observed, a well-defined quasi-reversible ($I_{pc}/I_{pa} \leq 1$) and timely-decreased redox couple intensity with an apparent anodic peak (I_{pa}) of 2–4.5 μA at about 550–570 mV and 750 mV, respectively, and cathodic peak (I_{pc}) of (−2)–(−6) μA at about 500 mV and 650–700 mV, respectively, in the reverse scan was obtained for all TEMPO and 4-Amino TEMPO included systems. This indicated a one-electron ($1e^-$) exchange (Scheme 1) between TEMPO radical (**no. 1**) and reduced TEMPO (**no. 1b**) as well as its oxidized (**no. 1a**) state, being formed during the analysis, and also confirmed by the other authors (Kulys & Vidziunaite, 2005; Israeli et al., 2005). As one electron oxidation ($1e^-_{ox}$) of TEMPO or 4-Amino TEMPO by laccase to oxoammonium cation had been well defined already by spectrophotometric analysis, hydroxylamine re-oxidation ($1e^-_{red}$) back to TEMPO and 4-Amino TEMPO, respectively, could be performed by oxygen, oxygen/laccase or the oxoammonium cation generated (Patel et al., 2011; Arends et al., 2006). However, although the electron-transfer in the presence of CNFs was reduced, due diffusion limiting conditions caused by the swelling abilities of the CNFs, its capacity in case of 4-Amino TEMPO was higher for approximately one half (Fig. 2b).

3.2. Characterization of CNFs after different laccase/TEMPO mediated treatment

The oxidation of cellulosic substrate using laccase-catalyzed regeneration of TEMPO or 4-Amino TEMPO, respectively (Scheme 1) is performed by generated oxoammonium cation (**1a**) during the TEMPO laccase-oxidation, which oxidizes the C6 hydroxyl groups of cellulose via a heterolytic pathway in a two-electron oxidation ($2e^-_{ox}$) and giving mainly the carbonyl product (**no. 2**). The further oxidation efficacy of the aldehyde groups produced at CNFs to carboxyl groups (**no. 3**) was then studied using different approaches: (**no. 2a**) by auto-oxidation using air-oxygen, (**no. 2b**) by Cannizzaro reaction using NaOH where redox disproportionation of two aldehydes led to the alcohol ($1e^-_{red}$) and carbonyl ($1e^-_{ox}$) content (Patel et al., 2011), or (**no. 2c**) by chemical oxidation using $NaClO_2$ (Browning, 1967), and the samples then analyzed using different analytical techniques and methods.

The ζ-potential analysis of CNF samples treated according to the procedure **no. 2b** where the reaction was finished with the addition of NaOH according to Cannizzaro reaction (Fig. 3a) showed a negative ζ-potential of around -40 ± 0.9 mV at pH 10 for non-modified CNFs which was additionally lowered to -50 ± 3.47 mV and -39 ± 3.34 mV after being treated with the laccase/4-Amino TEMPO and laccase/4-Amino TEMPO system, respectively, thus indicating changes in the surface charges of the CNFs, being in a correlation with the particle size evaluation as well as the surface carboxyl groups analysis, which is presented and discussed in next paragraphs. The results also indicated that the untreated CNFs already possessed negatively-charged surface groups above acidic pH, being the results of the sample's preparation.

However, negative ζ-potential for all the untreated and laccase/(4-Amino)TEMPO treated CNFs slightly increased up to pH 6 (reaching a value between -27.13 ± 0.49 mV and -40.45 ± 0.77 mV), followed by a faster increase of up to pH 2 and the ip reached at around pH 1. A more negative ζ-potential was obtained for the laccase/4-Amino TEMPO-treated samples following by the laccase/TEMPO treated ones. The changes in ζ-potential between the untreated and laccase/4-Amino TEMPO treated CNFs as a function of CNFs concentration (left down corner in Fig. 3a) showed obviously lower negative ζ-potential for modified CNFs (-55 ± 4.7 mV) at lower CNFs concentration (0.01% w/v) in comparison with the untreated sample (-28 ± 4.2 mV) which increased proportionally with the CNFs concentration.

The ζ-potential values of CNFs having been post-treated according to procedure **no. 1** also showed (figure not shown) a slightly more negative response for laccase/4-Amino TEMPO-treated samples at pH 10 (-43 ± 2.72 mV) in comparison

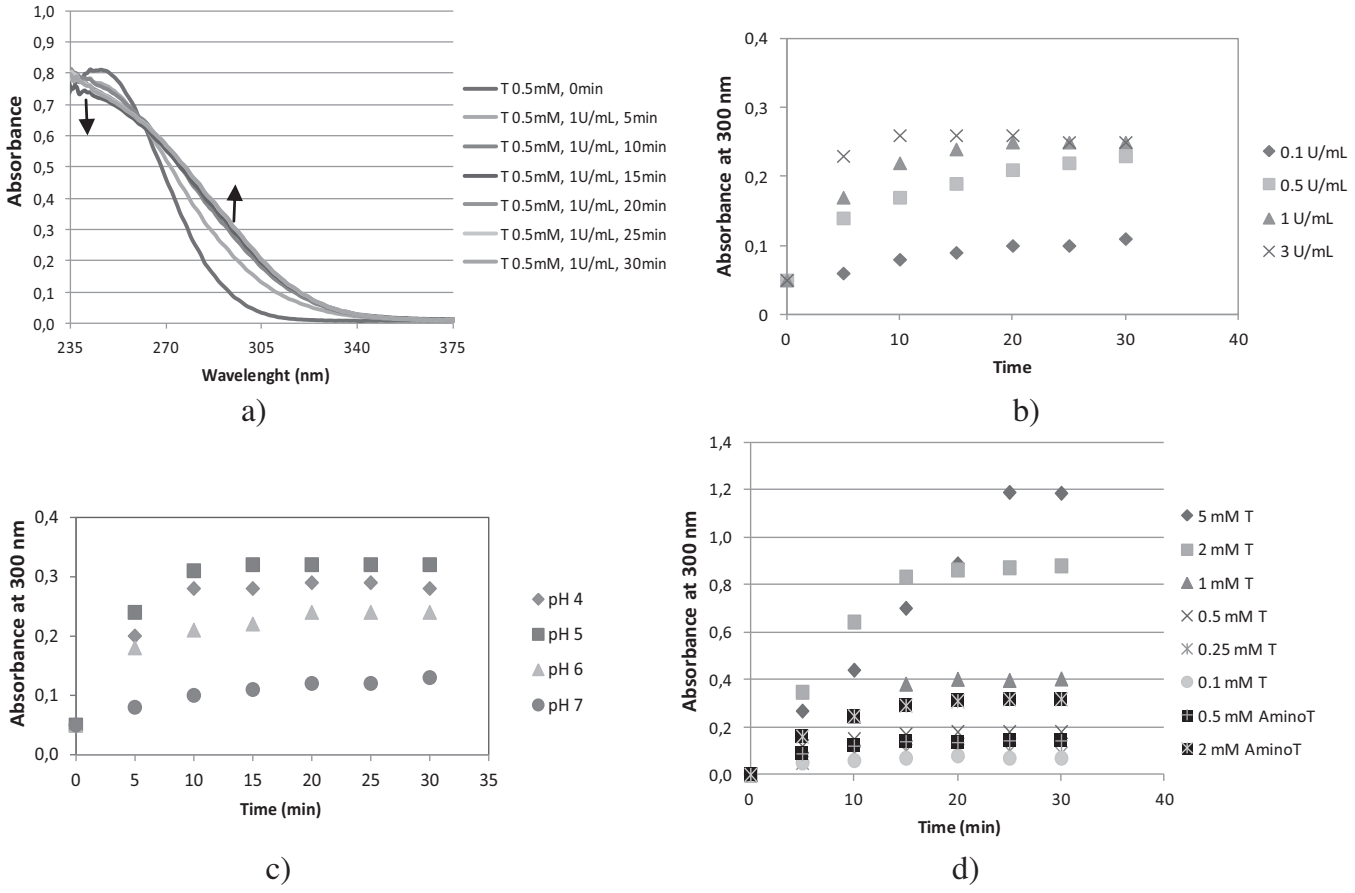

Fig. 1. UV–vis spectrophotometrical time-dependence monitoring of (a) TEMPO (T) oxidation to oxoammonium ion as a function of (b) different concentrations of laccase (0.1, 0.5, 1, and 3 U/mL) performed in 50 mM Na-citrate buffer at pH 5 and using 0.5 mM of TEMPO, (c) different pH (4–7) mediums using 1 U/mL laccase and 0.5 mM of TEMPO, and (d) different TEMPO and 4-Amino TEMPO (AminoT) concentrations (0.1–5 mM) performed at pH 5 and using 1 U/mL of laccase. The spectra were recorded every 5 min for 30 min at 30 °C.

with the laccase/TEMPO treated one (-37 ± 5.44 mV) and the reference but, in general, less negative for all samples as in the case when the reaction was finished according to the **no. 2b** protocol. Even the ζ-potential of CNFs being post-treated with $NaClO_2$ (**no. 2c** protocol) were in general less negative over the whole pH range compared to the **no. 2b** treated CNFs, but were again more efficient using laccase/4-Amino TEMPO than the laccase/TEMPO (data not shown) system.

Such a difference in ζ-potential analysis was definitely related to the final post-treatment step as the TEMPO oxoammonium cation produced by laccase was highly affective for introducing only aldehyde groups but much less the carboxyl's (Aracri et al., 2012; Patel et al., 2011). Hence, the more negative ζ-potential for laccase/4-Amino TEMPO modified CNFs being finished with NaOH (**no. 2b**) probably corresponds to the speculated redox disproportion regarding the two aldehydes to alcohol (reduction) and carboxyl (oxidation) according to the Cannizzaro reaction, and thus a higher amount of carboxyl group formation. The results were also in correlations with the evaluated average sizes of the CNFs after different treatment protocols, indicating either on even more effective distribution (or diminished tendency of aggregation) of CNFs with more negative interfaces within a highly alkaline buffer solution, or eventually on partial degradation of its polymeric structures (Fig. 4).

The FTIR spectroscopy analysis of all samples was performed in order to identify the created carboxyl groups. As presented in Fig. 5a, the CNFs treated according to the procedure where the reaction was finished with NaOH (**no. 2b**) leads to the appearance of a new band at approximately 1585 cm^{-1} and an increased to a band of 1638 cm^{-1} for both the laccase/TEMPO and the laccase/4-Amino TEMPO pre-treated samples. These two bands were unrecorded for the reference CNFs and when the CNFs were treated only with laccase, indicating that the appearance of these two bands was unrelated to the remainder (unwashed) enzyme proteins (amides I and II), and the band at around 1585 cm^{-1} likely to correspond to the carboxylate ions (COO^-). Furthermore, the formation of carboxylic groups after the laccase/TEMPO and laccase/4-Amino TEMPO treatments of CNFs was proven by additional acidic treatment of the lyophilized CNF samples in 0.1 M HCl for 2 min, where the protonation of carboxylate ions led to the formation of a new band at around 1740 cm^{-1} corresponding to the carboxylic acid groups created (C=O stretching, Fig. 5b). It was also noted that the new, but less intense band appeared in the case when the reference sample was only treated with TEMPO and/or 4-Amino TEMPO, meanwhile the new band did not appeared when the reference samples were only treated with buffer and/or laccase, as shown in Fig. 5b. According to the literature (Patel et al., 2011), the TEMPO derived oxoammonium ion in the absence of laccase could cause an introduction of aldehyde groups (but not carboxylic groups) and the new band at 1740 cm^{-1} could be only related to the additional oxidation of aldehyde groups in the presence of NaOH, leading to an increase in the carboxyl group's content. This conclusion was in agreement with the FTIR spectra (Fig. 5c) of e.g. laccase/TEMPO pre-treated samples, where the reaction was finished by rinsing in EtOH/water (**no. 2a**) to remove the enzyme proteins from the reaction mixture and using air oxygen for additional oxidation, and

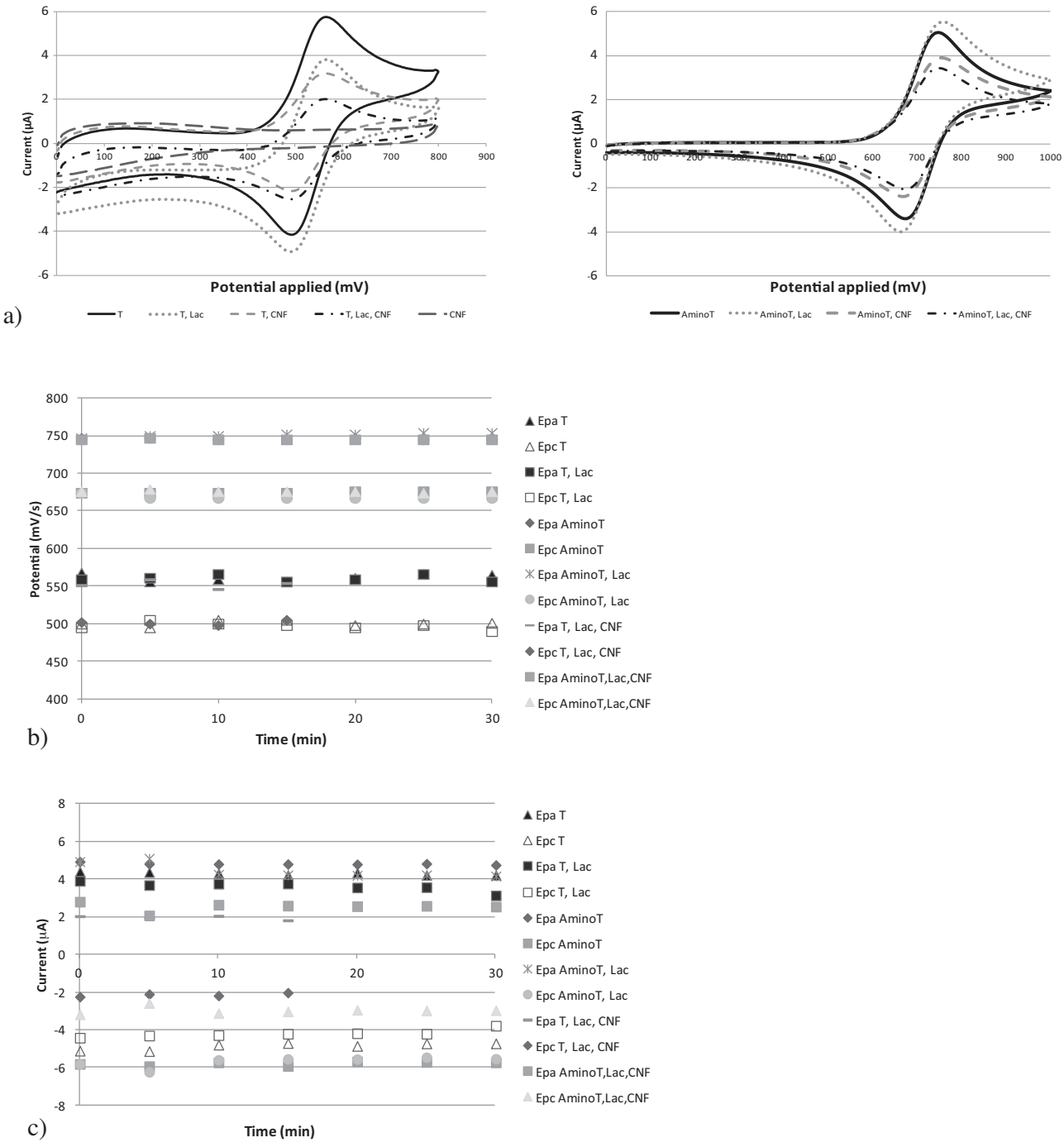

Fig. 2. Cyclic voltammograms (a) and time-dependence of anodic and cathodic peak currents (b) and peak potentials (c) for 0.5 mM of TEMPO (T) and 4-Amino TEMPO (AminoT) recorded in aerobic conditions in pH 5 at 30 °C, and in the absence and presence of 1 U/mL of laccase and/or 0.25% (w/v) of CNFs.

where the intensity of the band at about 1740 cm^{-1} was much lower in comparison with the samples being post-treated either with NaOH (**no. 2b**) or $NaClO_2$ (**no. 2c**, Fig. 5d), where a less intensive band corresponding to the carboxyl groups was also identified.

It is well-known that TEMPO-mediated oxidation can also result in cellulose depolymerization (Mishra, Thirree, Manent, Chabot, & Daneault, 2011). Beside, the incorporation of newly-charged functional groups, as well as any changes in amorphous and crystalline regions of the cellulose structure would disrupt or rearrange the crystallinity (Široky, Blackburn, Bechtold, Taylor, & White, 2010). On the other hand, hemiacetal linkages can be also created between introduced aldehydes and remaining hydroxyls on glucopiranose ring, resulting in additional rearrangement of a cellulose chains in the structure (Fan, Lewis, & Tapley, 2001; Kim, Wada, & Kuga, 2004; Saito & Isogai, 2006). Thus, differently pre-modified and, according to different procedures (Scheme 1, **no. 2a–2c**), additionally post-treated CNFs samples were evaluated regarding the crystallinity changing and hydrogen bonding intensity (HBI) being disrupted, based on the intensities of certain IR bands. The degree of crystallinity was expressed using different crystallinity-index (CrI) values having been quantified from the proportions of intensity of the absorption bands $\alpha_{1429}\ cm^{-1}/\alpha_{893}\ cm^{-1}$ and $\alpha_{1372}\ cm^{-1}/\alpha_{2900}\ cm^{-1}$ (Nelson & O'Connor, 1964a, 1964b), $\alpha_{1280}\ cm^{-1}/\alpha_{1200}\ cm^{-1}$

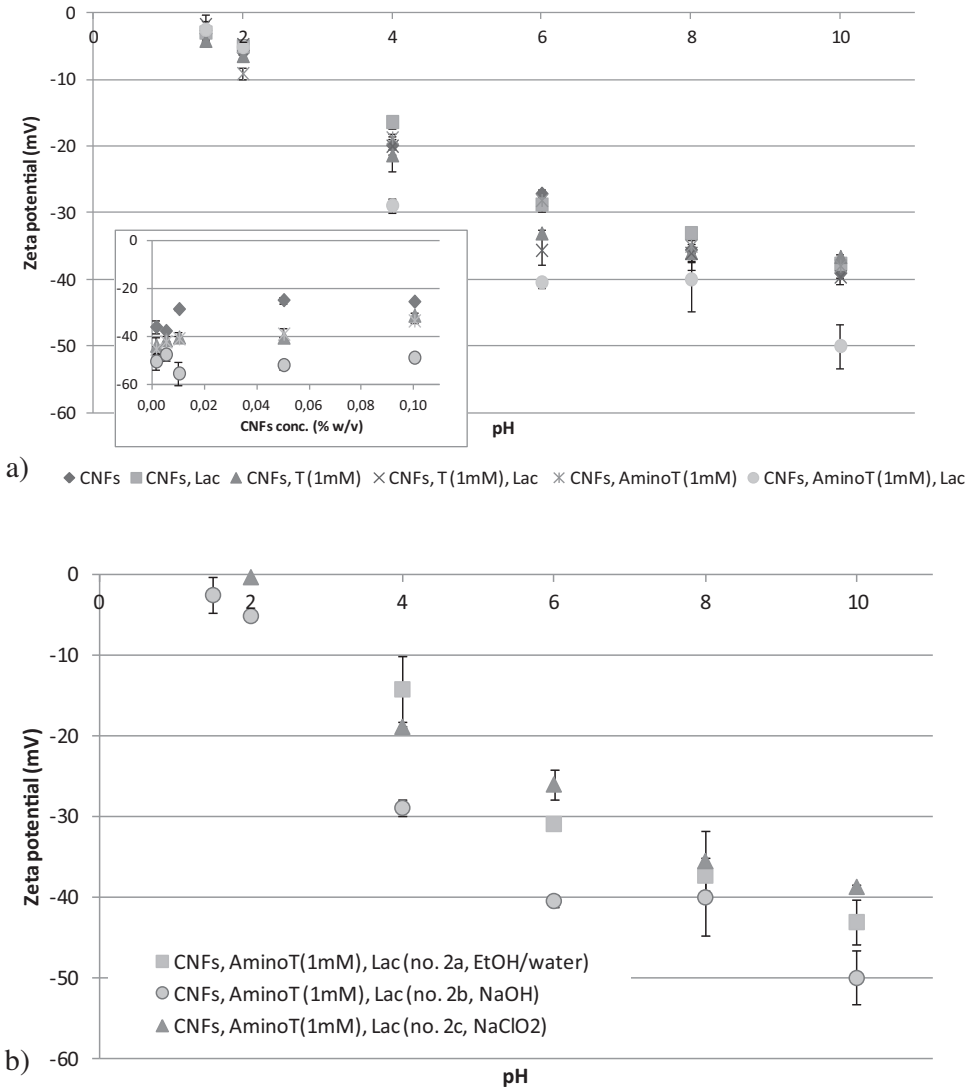

Fig. 3. ζ-Potential analysis of the reference as well as with laccase/TEMPO (T) and laccase/4-Amino TEMPO (AminoT) treated CNFs (0.5 wt.% CNFs, 1 U/mL laccase, pH 5, 48 h, 30 °C) followed by different post-treatment protocols according to Scheme 1: (a) the protocol **no. 2b**, also performing the treatments with the laccase/4-Amino TEMPO system as a function of CNFs concentration (the inserted graph), and (b) the protocols **no. 2a–2c** using laccase/4-Amino TEMPO system.

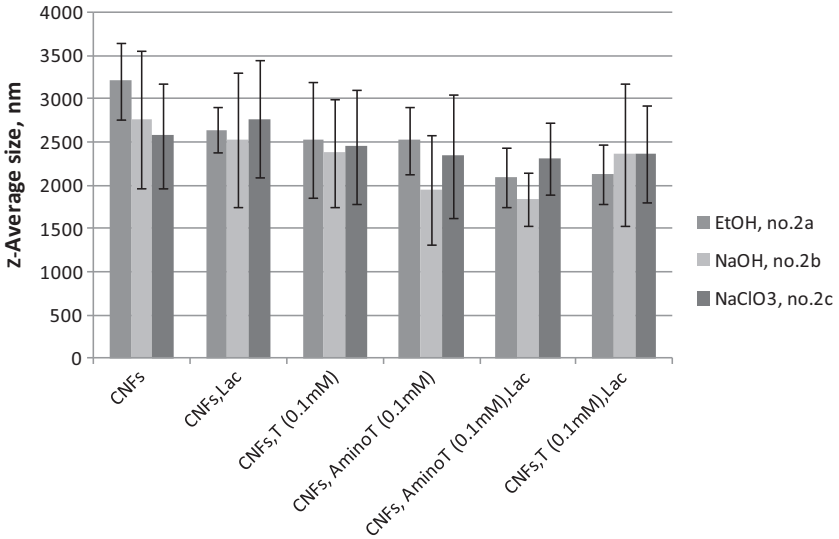

Fig. 4. Z-average size values (determined at pH 10) of the reference as well as with laccase/TEMPO (T) and laccase/4-Amino TEMPO (AminoT) treated CNFs (0.5 wt.% CNFs, 1 U/mL laccase, pH 5, 48 h, 30 °C) followed by different post-treatment protocols according to Scheme 1.

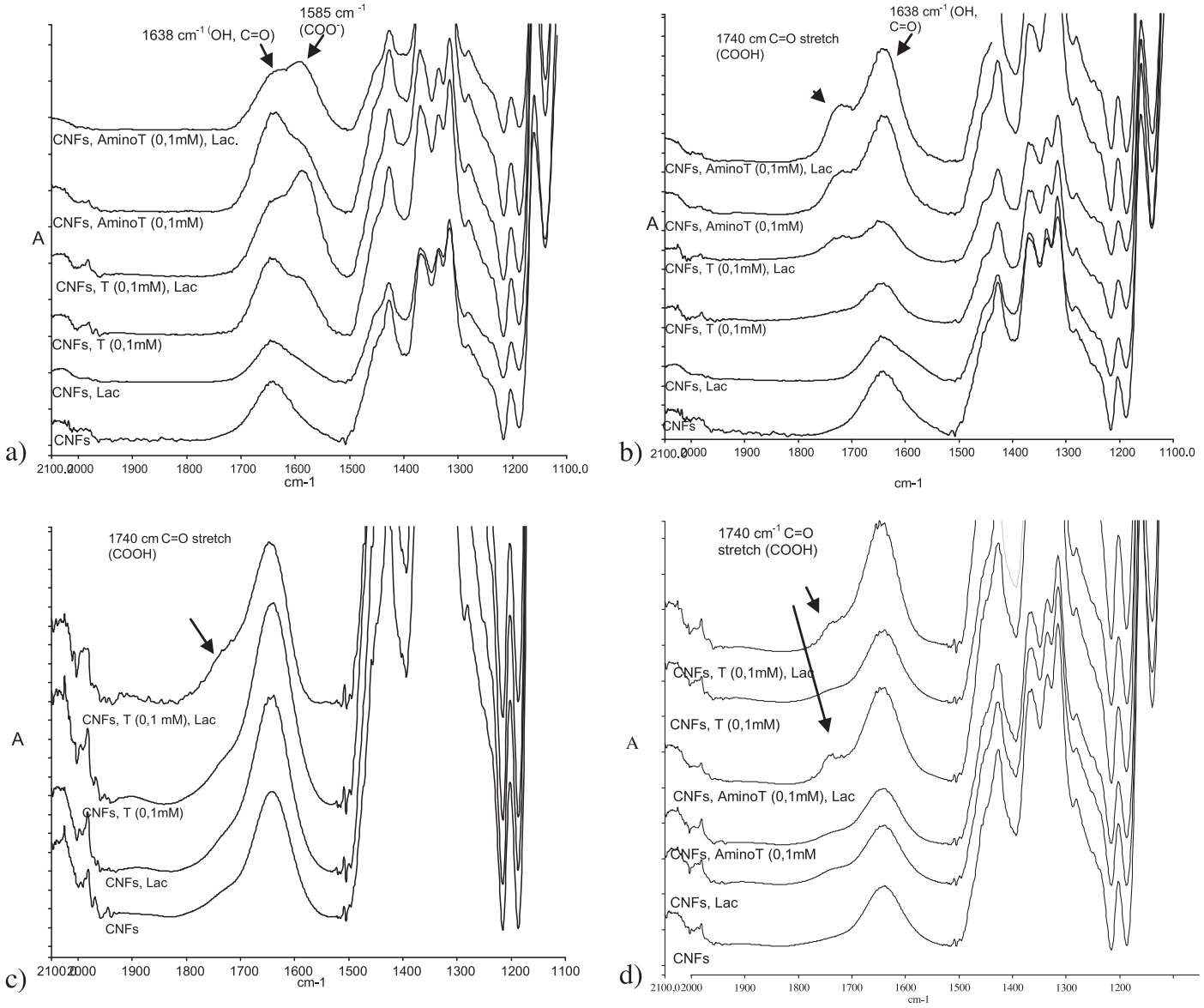

Fig. 5. FTIR spectra of untreated and with laccase/TEMPO (T) and laccase/4-AminoTEMPO (AminoT) system treated CNFs (0.5 wt.% CNFs, 1 U/mL laccase, pH 5, 48 h, 30 °C) followed by different post-treatment protocols according to the Scheme 1: (a) the protocol **no. 2b** (a) before and (b) after additional treatment with 0.1 M HCl, and the protocols (c) **no. 2a** and (d) **no. 2c**.

(Hulleman, Hazendonk, & Dam, 1994), and $\alpha_{1278}\,cm^{-1}/\alpha_{1263}\,cm^{-1}$ (Colom & Carrillo, 2002), whilst the HBI was calculated from the ratio of $\alpha_{3336}\,cm^{-1}/\alpha_{1336}\,cm^{-1}$ (Nada, Kamel, & El-Sakhawy, 2000). As presented in Fig. 6, a small increase in the CrI being related to $\alpha_{1429}\,cm^{-1}/\alpha_{893}\,cm^{-1}$ (i.e. CH_2 banding/C—O stretching) the more relevant bands ratios describing the crystallinity vs. amorphous of cellulose were identified only at CNFs, having been pre-modified with laccase/TEMPO and laccase/4-Amino TEMPO systems and post-treated according to the **no. 2c** procedure. On the other hand, an obvious variation in CrI being related to the second more relevant bands ratio correlating with the total crystallinity (i.e. $\alpha_{1372}\,cm^{-1}/\alpha_{2900}\,cm^{-1}$ being related to the C—H group vibration of cellulose ring vs. C—H stretching) can be seen of being dependent on both treatment steps. As band at $\alpha_{1372}\,cm^{-1}$ is also in the region yielding a symmetric COO— mode, the relatively high CrI values of all CNFs being post-treated with NaOH (**no. 2b**) can be explained by the carboxylic groups deprotonation not being quantitatively dependent, however, thus resulting in the same values at all the differently pre-treated samples. On the other hand, its low band intensity appearing at CNFs post-treated according to **no. 2a**, and being additionally reduced at laccase/TEMPO and laccase/4-Amino TEMPO pretreated samples, can be related to the pure surface interactions of CNFs within EtOH/water medium. In addition, the intensity of the band at $\alpha_{2900}\,cm^{-1}$ being also related to the changing/reduction of the amorphous regions, contributing to relatively lower CrI values for all CNFs post-treated according to the **no. 2c** procedure, were additionally reduced at samples being post-treated according to **no. 2a**.

Moreover, a slight increase in CrIs being related to $\alpha_{1280}\,cm^{-1}/\alpha_{1200}\,cm^{-1}$ and $\alpha_{1278}\,cm^{-1}/\alpha_{1263}\,cm^{-1}$ ratios representing the cellulose fingerprint region due to C—H vs. O—H bending and C—C vs. C—O stretching vibrations within the glucosidic ring of laccase/4-Amino TEMPO pre-modified CNFs and post-treated according to the **no. 2c** procedure, also indicated an lastly increase in the crystallined cellulose structure or its restructuration into amorphous forms. This behavior can be explained by the introduction of new functional groups into the cellulose where the less-ordered amorphous domains of the cellulose were

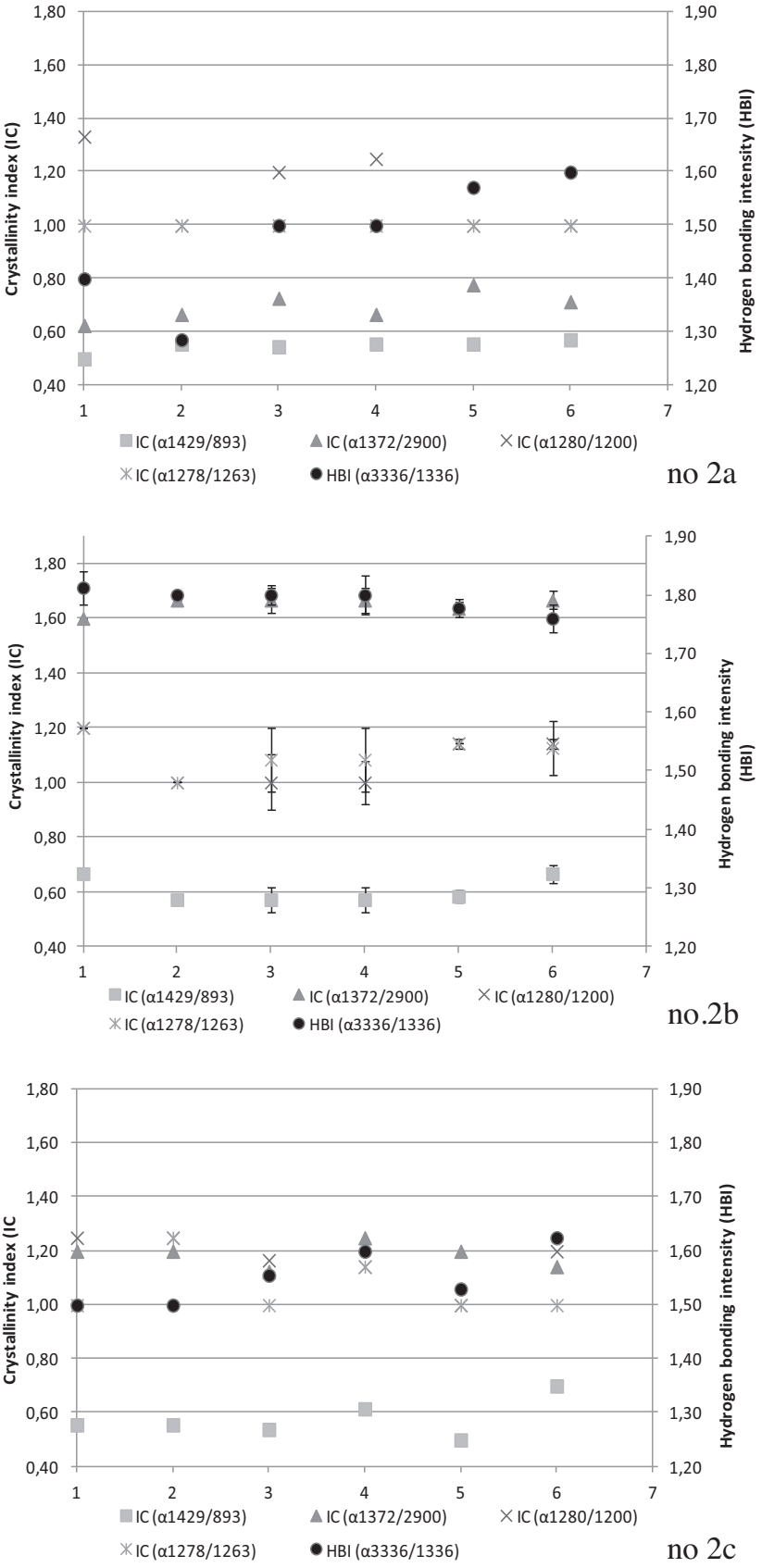

Fig. 6. Crystallinity index (IC) and hydrogen bonding intensity (HBI) of (1) untreated CNFs, and CNFs treated with (2) laccase, (3) TEMPO (0.1 mM), (4) laccase/TEMPO, (5) 4-Amino TEMPO (0.1 mM), and (6) laccase/4-Amino TEMPO, followed by different post-treatments according to protocols **no. 2a**–**2c** presented in Scheme 1. The IC and HBI values were calculated from the ratio of the height intensities of the FTIR bands at selected wavenumbers.

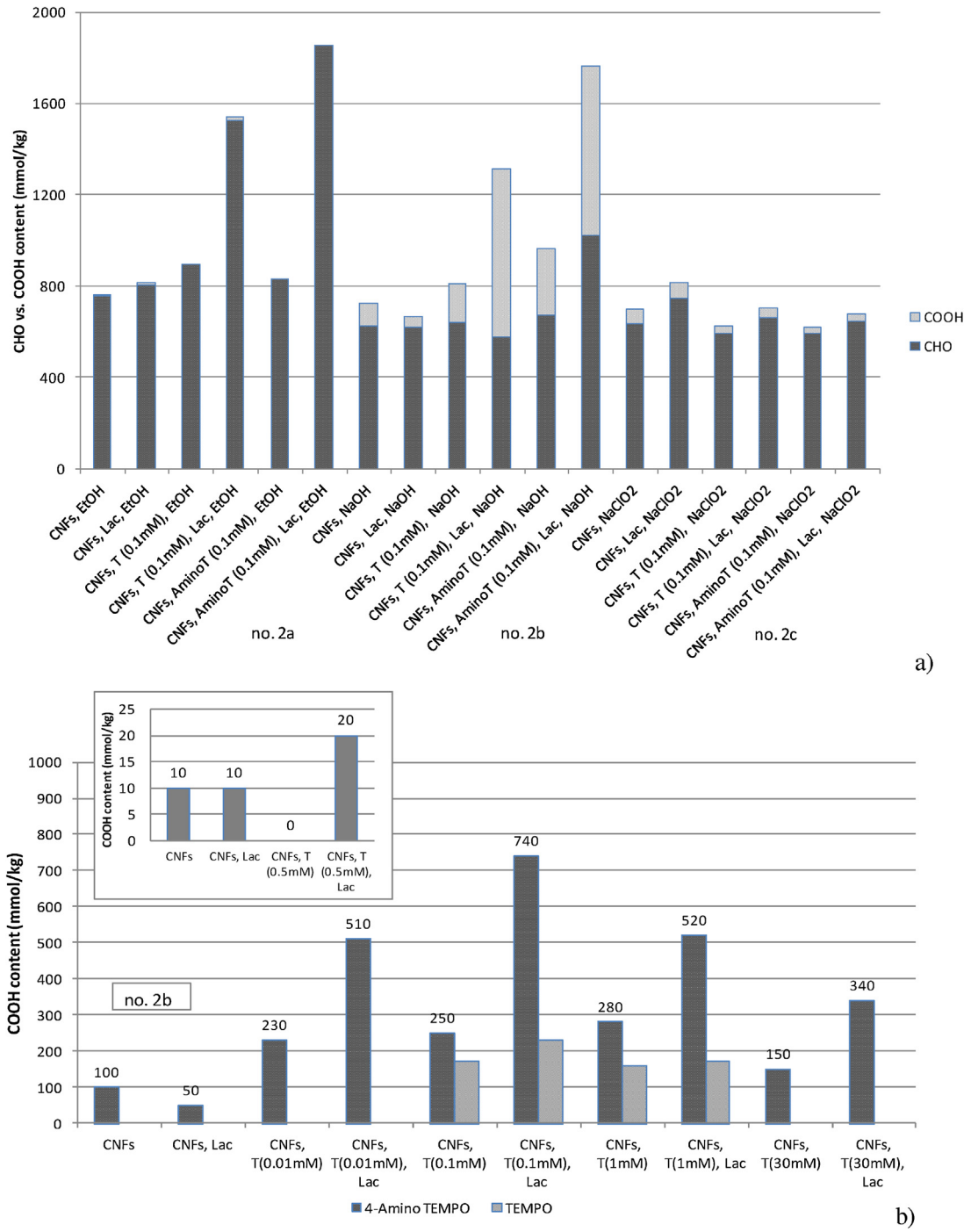

Fig. 7. Aldehyde (—CHO) and/or carboxyl (—COOH) groups content of (a) CNFs pre-treated with laccase/TEMPO and laccase/4-Amino TEMPO followed by post-treatment with EtOH/water (**no. 2a**), NaOH (**no. 2b**) or $NaClO_2$ (**no. 2c**); and (b) CNFs pre-modified with different TEMPO concentrations using 1 U/mL laccase without (inserted graph) or with post-treatment using NaOH (**no. 2b**).

more accessible to the reaction than the high-ordered crystalline segments, resulting in new and/or shorter hydrogen bond formations being generally stronger, thus increasing the crystallinity degree.

The HBI values were related to the OH stretching and flexing vibration frequencies, above all the intra-molecular hydrogen bonding intensity related to the band at above 3336 cm^{-1} (Ciolacu, Kovac, & Kokol, 2010; Široky et al., 2010), were well correlated with the above discussed CrI value's variations, showing a relative decrease in that values for samples being post-treated with EtOH (**no. 2a**) bearing the highest amount of aldehyde groups, compared to the samples post-treated in water using $NaClO_2$ (**no. 2c**) or even NaOH (**no. 2b**) where electron-richer carboxylic functional groups were introduced contributing to a high tendency for hydrogen bonding. This effect was slightly more exposed for samples being pre-modified with laccase/TEMPO and laccase/4-Amino TEMPO systems, further indicating that the different surfaces of pre-modified and post-treated CNFs introduced aldehydes or negatively-charged carboxylic groups, and thus their rearrangement and forming of hydrogen bonding.

3.3. Quantification of aldehyde (—CHO) and carboxyl (—COOH) groups at CNFs after different laccase/TEMPO mediated treatments

In accordance with the results of ζ-potential and FTIR measurements, the spectrophotometrical and potentiometric titration analysis also confirmed that the amount of aldehyde groups (Fig. 7a) more than doubled for the laccase/TEMPO (1523 ± 0.23 mmol/kg) and the laccase/4-Amino TEMPO (1856 ± 24.0 mmol/kg) pre-modified CNFs being post-treated with EtOH/water (**no. 2a**) in comparison with the reference CNFs where about 750 ± 3 mmol/kg of aldehydes were also detected, being however probably only end-located terminal aldehydes that originated during CNFs isolation procedures. When the samples were post-treated with NaOH (**no. 2b**), the new aldehyde groups probably being created selectively only at C6 of cellulose primarily hydroxyls as ascertained by (Arends et al., 2006) for both laccase/TEMPO and laccase/4-Amino TEMPO pre-modified CNFs was reduced by about one-half (to 819 ± 2 mmol/kg and 1022 ± 2 mmol/kg, respectively) at simultaneous carboxylic groups increasing from 100 ± 3 mmol/kg at the reference to about 740 ± 5 mmol/kg using the same quantity of TEMPO or 4-Amino TEMPO, respectively. The results confirmed that further oxidation of aldehyde to carboxylic groups in the presence of NaOH, where two aldehydes led to alcohol and carboxyl content only appearing on C6-created aldehydes. On the other hand, when the laccase/TEMPO and laccase/4-Amino TEMPO pre-modified CNFs were further oxidized using $NaClO_2$ (**no. 2c**), besides all the surface C6-aldehyde groups created in the first step a small amounts of terminal-aldehydes were also reduced, being however accompanied by smaller amounts of additional carboxyl's creation. Such a phenomena can be related only to the formation of hemiacetal linkages between aldehydes and cellulose hydroxyl groups in a water medium being highly resistant to further oxidation as already confirmed by other authors (Fan et al., 2001; Saito & Isogai, 2006), which could also be the reason for slight increasing of inter-cellulose hydrogen bonds and CrI values, above all at laccase/TEMPO and laccase/4-Amino TEMPO pre-modified CNFs.

Moreover, the oxidation of CNFs being performed using different concentrations of TEMPO and 4-Amino TEMPO in the pre-treatment (Fig. 7b), denoted a higher affinity of 4-Amino TEMPO toward CNFs substrate with an optimal concentration being around 0.1 mM for the used laccase concentration and tested samples. It could also be observed that the modification of CNFs with TEMPO or 4-Amino TEMPO in the absence of laccase (and additionally treatment with NaOH, **no. 2b**) also led to some increase of the carboxylic groups (to 170 ± 2 mmol/kg or 290 ± 2 mmol/kg, respectively) in regard to the reference (100 ± 3 mmol/kg). According to (Patel et al., 2011) already the TEMPO itself can form some aldehyde groups which could be further oxidized at alkaline pH in the presence of NaOH. Besides, the amount of carboxyl groups (Fig. 7a) for the samples rinsed in EtOH/water (**no. 2a**) was much lower in comparison with the samples post-treated with NaOH (**no. 2b**) or even $NaClO_2$ (**no. 2c**), and comparable with the untreated reference CNFs (Fig. 7b, inserted graph). The obtained results are in agreement with both ζ-potential measurements where most negative ζ-potential values were obtained as well as FTIR analysis where much stronger bands were observed at about 1740 cm^{-1} corresponding to the carboxylic groups for samples treated with laccase/4-Amino TEMPO followed by NaOH according to Cannizzaro reaction in comparison with the samples post-treated with EtOH/water or $NaClO_2$.

These results are however in opposition to the literature (Iwamoto et al., 2010) where the effect of chemical structures of different TEMPO derivates on the chemical oxidation of wood-based cellulose using the NaClO/NaBr under alkaline conditions (pH 10) resulted in approximately twice the lower amount of carboxylate content and eight times lower nanofibril yields for 4-Amino TEMPO in comparison with the TEMPO modified cellulose. Furthermore, in the laccase/TEMPO and its derivates, mediated oxidation of alcohols (Arends et al., 2006) resulted in a lower conversion of alcohols to aldehydes for 4-Amino TEMPO derivate in comparison with TEMPO. In that aspect, the higher reactivity of TEMPO in comparison with its derivate was attributed to higher stability of their oxoammonium ions under used conditions. According to (Limoges & Degrand, 1997) the 4-Amino TEMPO showed strong pH-dependence, as under the acid conditions the amino groups can react easily with nitroxyl radicals. By take in a consideration all of these parameters, it is also obvious from the results of the presented research that cellulose crystalline morphology, size and shape also influence the oxidation efficiency of TEMPO and its derivate toward nanocellulose as substrate.

Conclusions

In the presented paper laccase/TEMPO and laccase/4-Amino TEMPO oxidation systems were used as ecologically-friendly, non-aggressive and site-specific alternative to chemical systems in order to introduce and tune the content of aldehyde (CHO) vs. carboxyl (COOH) groups on the cellulose nanofiber (CNF), as well as the aldehyde end-groups already originating from the isolation procedure, bringing an additional functionality to the CNFs surface. It was defined that both oxidation systems led to highly reactive oxoammonium ion intermediates, resulting to the formation of aldehyde groups at C6 of cellulose hydroxyls, being efficacy higher using 4-Amino TEMPO mediator and the used laccase, while the carboxylic groups were formed only after additional post-treatment. Amongst three different post-treatment routes investigated (auto-oxidation, Cannizzaro reaction and chemical oxidation using $NaClO_2$), only the C6-aldehydes were transformed to the carboxylic groups when CNFs being additionally treated with NaOH according to Cannizzaro reaction. Meanwhile, the auto-oxidation using the air-oxygen was inefficient for that purpose, although the C6-aldehydes remained stable even after two months in EtOH/water medium, the chemical oxidation using $NaClO_2$ was inefficient due to the precedent forming of highly-stable covalent hemiacetal linkages, between primarily C6-aldehydes (and also small amounts of terminal-ones) and cellulose surrounding hydroxyls. The investigation indicated a novel way of preparing nanocellulose with site-specific and quantitatively-variable surface chemistry, yielding a product with self-assembled and/or simultaneous in situ covalently-coupled properties, a strategy that could be exploited in the development of new nanocellulose-based materials.

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