Revealing the autofluorescence properties of nanocellulose isolated from different raw materials by different methods

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Abstract. This work explored the autofluorescence properties of nanocellulose which isolated from different raw materials (dissolving pulp, bleaching chemical pulp) using acid hydrolysis, mechanically refining and TEMPO oxidation. Results showed that all samples show a typical emission peak at 574 nm due to glycosidic linkages and aliphatic C=O absorption identified by FTIR and Raman spectroscopy independent of lignin. Increasing the excitation wavelengths (510-530 nm) caused red shift of fluorescence emission peaks (570-582nm) with unchanged fluorescence intensity. Conversely, changing acid/alkaline conditions led to an increase of fluorescence intensity with no shifting of fluorescence emission peak. This study provides new insight in applying nanocellulose with special luminous characteristics in biomedicine area such as multi-color biological imaging.

1. Introduction

Cellulose, as the main skeletal component in plants, is an inexhaustible polysaccharide-based raw material with favorable structure and properties.¹ It is essentially composed of homopolymerized β-D-glucopyranose units with a unique structural grade in its biological origin. However, its properties, functionality, durability and uniformity should not be stayed at traditional cellulosic materials, but need to be developed for the next generation of cellulose based products.² Once cellulose extracted in a nanoscale, most of the defects associated with the hierarchical structure would be removed. The new construction of cellulose-based “building blocks” can be used for the next generation of cellulose-based composites.

Extensive research had been focused on the methods for isolating cellulose nanocrystals (CNCs)/cellulose nanofibrils (CNF) and investigating their functional modifications. Based on their intriguing properties such as low cost and low toxicity, optical clarity, reproducibility, biodegradability, low thermal expansion, nanocellulose’s application area could be extensively expanded when it was functionalized.² In addition, as a reinforcing filler for polymers, CNCs and CNF were also utilized to fabricate a variety of other functional materials, including photonic crystals, barrier films, shape memory polymers, photo-sealable, drug delivery and mechanically compatible nanocomposites. The most important features of nanocellulose for structure applications include size, strength/toughness, modulus, and surface function.³ All of these parameters help us to explicate the nanoscale enhancement mechanism.⁴ For example, surface functionality is crucial because it determines how nanocellulose interacts with itself (nanofiber-nanofiber interactions) or polymers through ionic, hydrogen bonding, and hydrophobic interactions.⁵ Aspect ratios, as well as surface area and functionality, are also key parameters by affecting rheological properties and thus relate to their
processing and manufacturing capabilities. Moreover, although the dimensions and surface functionalization of nanocellulose have been applied to improve the structural and mechanical properties, its relationship to fluorescence properties have rarely been reported, which may inspire a more extensive application. Olmstead et al. had reported that pulp cellulose had unique autofluorescence properties mainly due to the linkage between lignin and cellulose rather than lignin itself. Nonetheless, the fluorescence properties of nanocellulose has rarely been reported, as well as its elaborate characterization, intensity regulation and application. Kalita et al. used saw dust and rice husks to prepare nanocellulose and found its autofluorescence properties, but the mechanism of fluorescence was not further explored.

In this work, we reported on the fluorescence properties of nanocellulose isolated from two raw material sources (dissolving pulp and bleaching chemical pulp) using typical hydrolysis conditions (acid, mechanical and 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO)-mediated oxidation). Regarding the characterization of nanocellulose in various states, a series of modern characterization methods were used to expound the relationship between structural changes and fluorescence performance concerning their dimensions, chemical structures, microstructures, chemical component for better comprehension of the autofluorescence mechanisms. The effect of changes in the external environment (excitation wavelength and pH) on fluorescence properties was further investigated. This will be beneficial for the development and application of cellulose-based fluorescent materials.

2. Material and methods

2.1. Material
The cellulose source of never-dried dissolving pulp (DP) and bleaching chemical pulp (BP) was provided by Tai Yang Company (Shandong, China). Bacterial cellulose (BC) was purchased from Hainan Yide Food Co., Ltd. All other chemicals and reagents were purchased from Qianhui Chemical Reagent Co., Ltd. (Guangzhou, China) and were of analytical grade unless otherwise stated.

2.2. Acid hydrolysis
The preparation of CNCs was carried out according to the previously published acid hydrolysis method. Briefly, the bleached chemical slurry was reacted with 64 wt% sulfuric acid at 45 °C for 40 minutes with mechanical agitation. The suspension was then centrifuged at 10,000 rpm for 15 minutes under low temperature conditions and the collected supernatant was dialyzed to neutral. The CNCs powder was recovered by freeze drying before use.

2.3. Mechanically refined
First, the concentration of bleaching chemical pulp and dissolving pulp were adjusted to about 1%. The two slurries were then ground by Super Masscolloider (MKCA6-2, Japan). The grinding cycle was performed at intervals of -50μm, -80 μm, and -100 μm for 10 times. The three samples obtained by grinding the bleached chemical pulp were respectively referred to as B-CMF-50, B-CMF-80, B-CMF-100. The three samples obtained by grinding the dissolving pulp were respectively referred to as D-CMF-50, D-CMF-80, D-CMF-100.

2.4. TEMPO oxidation
In brief, CNF was defibrillated via TEMPO oxidation employing 2.0/4.0/6.0 mmol/g NaClO/cellulose at pH 10.0. Adjustment of pH at 7.0 with 0.5 M of NaOH, followed by homogenization (Microfluidizer LM20, USA) at 25000PSI for 5 times. The three samples obtained by oxidizing the bleaching chemical pulp by TEMPO were respectively referred to as B-CNF-2, B-CNF-4, B-CNF-6. The samples obtained by oxidizing the dissolving pulp by TEMPO were respectively referred to as D-CNF-6.
2.5. Chemical analysis
Chemical analysis of pulp samples and nanocelluloses was performed to estimate the total cellulose and lignin content present in the samples. The cellulose content in the samples was estimated based on the TAPPI standard T203 CM-09. The total lignin content present in the samples was determined according to the TAPPI standards T222 OM-02 and T222 OM-11.

2.6. Characterization
FTIR spectra were collected on a VERTEX 70 (Bruker, Germany) Fourier transform infrared spectrometer. The test specimens were prepared by a standard KBr pellet method. Spectra were recorded between 600 and 4000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\), and 64 scans were collected for each spectrum. The charge density (CD) of samples were measured using a Particle Charge Detector (Mütek PCD-03, Herrsching, Germany). Dynamic Light Scattering (DLS) (Zetasizer Nano ZS 90, Malvern instrument, UK) was used to measure the Zeta potential (ζ). Fluorescent measurements were performed with a HORIBA Scientific FluoroMax-4 equipped with a 150 W xenon lamp. The widths of both the excitation slit and emission slit were set at 3.0 nm. Scanning Electron Microscope (SEM) and MultiMode 8 SPM (Bruker, USA) Atomic Force Microscope (AFM) was used to image the nanocellulose surface morphology. The further image processing was finished using Nanoscope Analysis software (version 1.5, Bruker Corporation). Fluorescent images of pulp fiber and nanocellulose were measured by Laser Scanning Confocal Microscopy (LSCM) (Leica TCS-SP5, Germany). The excitation wavelength was 405 nm.

3. Results and discussion

3.1. Characterization of nanocellulose
A comprehensive information needs to be obtained including the raw materials, preparation methods, physical and chemical properties to study the autofluorescence properties of nanocellulose. Sulfuric acid hydrolysis is the most common hydrolysis technique to produce CNCs since the negative charge of sulfate ester group, increased the electrostatic revision forces to increase CNCs well dispersed.\(^{[11]}\) TEMPO-mediated oxidation process can modify the surface of the cellulose where the primary hydroxyl groups are oxidized to carboxylate groups. Mechanical refining keeps the surface chemistry of the cellulose unchanged, but brings about irreversible damage in microfibril structure, resulting in the production of MFC. Therefore, different materials and preparation methods can impart nanocellulose with various properties. As shown in Fig 1 the size and morphology of nanocellulose were strongly dependent on the cellulose source and preparation techniques. Without the addition of chemicals, mechanically refining the wood pulp gave the largest nanofibers of B-CMF-100 and D-CMF-100 (Fig 1g, h). As the cycle number increased, the external fibrillation of fibers was gradually peeled off the external cell wall layers. The increase of the degree of fibrillation also led to significantly reduced length and width of cellulose with a large aspect ratio. Sulfuric acid hydrolysis of the bleached chemical pulp produced a short rod-shaped CNCs (Fig 1d). Fig 1a, b, c, e and f demonstrated that TEMPO-mediated oxidation of wood pulp produced very thin (2-6 nm) nanofibers. The size information and other properties of nanocellulose were listed in Table 1. It was noted that the amount of charge did not change for B-CMF-50, B-CMF-80, B-CMF-100 and D-CMF-50, D-CMF-80, D-CMF-100, but the zeta potential (ζ) was larger with increasing the cycle number of refining for both pulp produced by mechanical method. This was occurred because the mechanical methods reduced the size of the fibers to form a more stable suspension. Furthermore, the charge amount of CNF and CNCs were significantly higher than that of the raw material. The chemical treatment increased the charge amount due to the introduction of chemical group (-COOH, -OSO\(_3\)). Smaller sized fibers were more likely to form a stable colloidal suspension with higher charge amount.\(^{[12]}\) In addition, the mechanical treatment exerted little influence on the composition of the raw materials. The chemical method significantly increased the proportion of cellulose and reduced the content of lignin and hemicellulose. This was in conformity to the process of pulping and bleaching.
Figure.1 AFM topography images showing height measurements of (a) B-CNF-2, (b) B-CNF-4, (c) B-CNF-6, (d) CNCs, (e) D-CNF-6, (f) AFM line scan of nanocelluloses image. The height data corresponds to the green bar in the AFM images. SEM images of (g) B-CMF-100, (h) D-CMF-100.

Table.1 Properties comparison and component analysis of nanocellulose and raw pulp.

| Samples      | Length (μm) | Width (nm) | CD (eq/g) | ζ (mV) | Cellulose (%) | Lignin (%) | Hemicellulose (%) |
|--------------|-------------|------------|-----------|--------|---------------|------------|-------------------|
| BP           | 800-1300    | 900-1500   | 1.55*10^-6 | -19.9  | 76.23         | 3.94       | 15.83             |
|              | 950-1500    | 1100-1600  | 0.81*10^-6 | -19.3  | 92.15         | --         | 3.66              |
| DP           |             |            |           |        |               |            |                   |
| B-CMF-50     | 250-550     | 120-180    | 1.58*10^-6 | -23.9  | 76.48         | 3.89       | 15.23             |
| B-CMF-80     | 220-450     | 110-155    | 1.62*10^-6 | -26.7  | 76.31         | 3.75       | 15.64             |
| B-CMF-100    | 150-230     | 80-130     | 1.58*10^-6 | -26.7  | 76.18         | 3.86       | 15.29             |
| D-CMF-50     | 300-550     | 150-240    | 0.83*10^-6 | -23.0  | 92.35         | --         | 3.54              |
| D-CMF-80     | 200-480     | 130-160    | 0.79*10^-6 | -26.1  | 92.64         | --         | 3.65              |
| D-CMF-100    | 180-250     | 110-160    | 0.81*10^-6 | -26.3  | 92.13         | --         | 3.57              |
| B-CNF-2      | 0.35-0.8    | 5-13       | 0.84*10^-4 | -51.5  | 89.62         | --         | 3.52              |
| B-CNF-4      | 0.32-0.7    | 2-10       | 1.02*10^-4 | -51.8  | 87.23         | --         | 2.14              |
| B-CNF-6      | 0.3-0.72    | 2-10       | 1.56*10^-4 | -55.3  | 89.52         | --         | 1.72              |
| D-CNF-6      | 0.3-0.65    | 2-10       | 1.54*10^-4 | -56.9  | 94.37         | --         | --                |
| CNCs         | 0.1-0.3     | 1-8        | 1.55*10^-4 | -40.3  | 95.62         | --         | --                |

3.2. Fluorescence properties of nanocellulose

There are two necessary conditions for a substance to produce fluorescence: (1) The energy absorbed by a photon undergoing multiple invariant transitions is less than the energy required to break its weakest chemical bond. (2) A fluorophore containing an unsaturated bond in the structure of substance.[10] The fluorophore containing an unsaturated bond of the raw materials and nanocellulose was studied using infrared spectroscopy. A schematic diagram of the transition of the ground state electrons to the excited state and the structure of the glucose unit undergoing the pulping and bleaching process were shown in Fig 2(a, b). The order of energy E required for the four transitions is: n→π*< π→π*< n→σ < σ→σ*. The unsaturated chromophoric group contained in cellulose is mainly a carbonyl group (C=O). The lowest energy required for glycosidic linkages and aliphatic C=O absorption (n→π* transition) mean that it was easier to achieve electron transitions to produce fluorescence.[8] The fluorescence spectrum of nanocellulose was shown in Fig 2(c, d, e). Nanocellulose without any treatment exhibited a fluorescence excitation and emission spectrum at 522 and 574 nm. This can conclude that nanocellulose itself has the property of autofluorescence. The emission peaks was assigned to the glycosidic bond and aliphatic C=O absorption (n→π* transition), which were
produced during isolation and purification of the cellulose.\(^{[13]}\) And the position of the fluorescent peak was not affected by the preparation method.

![Diagram of electronic energy level transitions](image)

Figure 2 (a) Schematic diagram of electronic energy level transitions. (b) Various forms of oxidized glucose units. Fluorescence spectrum of (c) CNCs, (d) B-CNF-6, (e) B-CMF-100.

3.3. Effect of physical properties on fluorescence properties

It is well known that lignin has natural autofluorescence properties.\(^{[14, 15]}\) Therefore, the content of lignin in the raw material and nanocellulose suspensions may be one of the important factors determining the fluorescence properties. In addition, the size of nanocellulose is another important parameter affecting the physical properties such as rheological properties, liquid crystal properties, optical properties and mechanical properties.\(^{[16, 17]}\)

The fluorescence emission spectra of samples with different lignin content and different size were shown in Fig. 3 (a, b, c). It could be seen that the intensity of all fluorescence spectra exhibited a significant emission peak at 574 nm. BP and DP with different lignin contents had similar fluorescence spectra. As the circle number of refining increased, their fluorescence intensity gradually decreased (Fig 3b, c). And the LCSM image also showed that the fluorescence signal was gradually weakened as the number of refining increases (Fig 4). Compared to the raw material, the fluorescence intensities of B-CNF-6 and D-CNF-6 were clearly decreased due to the introduction of π-conjugated groups (-COOH) which could alter the electronic structure of original core luminogens.\(^{[18]}\) Surprisingly, BC also exhibited the same emission peak at 574 nm. This indicated that the fluorescence properties of cellulose were not related to lignin content but to their sizes.

As shown in Fig. 3 (d, e, f), all the samples exhibited similar absorption peaks. The broad region 3600-3200 cm\(^{-1}\) was related to -OH vibrations. The wide peak can be assigned to three types of O(2)H-O(6) intramolecular, O(3)H-O(5) intramolecular, and O(6)H-O(3) intermolecular hydrogen bonding.\(^{[19]}\) After refining or TEMPO oxidation, the nanocellulose had a significantly stronger absorption peak compared to BP and DP in the 3600-3200 cm\(^{-1}\) region. This can be attributed to the fact that refining process caused more hydroxyl groups exposed on the surface of the cellulose, and more carboxyl groups after TEMPO oxidation. The peak observed at 2906 cm\(^{-1}\) was due to the aliphatic saturated –CH\(_2\) and –CH\(_2\)OH stretching vibration of polysaccharides.\(^{[20]}\) The small absorption peak at the 1594 cm\(^{-1}\) band was designated as the bound water and carboxylate from the bleaching process. As previously published reports, the acetyl and uronic ester groups of the celluloses were represented by the characteristic peak at 1405-1302 cm\(^{-1}\).\(^{[21]}\) Absorption band at 1030 cm\(^{-1}\), the strongest band across the cellulose spectra, was assigned to CO stretching at the C3 position. The minor signature at 895 cm\(^{-1}\) was attributed to the glycosidic linkages of glucose ring of cellulose. The presence of glycosidic bond and unsaturated groups may be responsible for the autofluorescence property. The absorption peak of 670-550 cm\(^{-1}\) was related to CH deformation and OH out-of-plane bending.\(^{[22]}\)
Figure 3 The effect of chemical composition and size on fluorescence properties. (a) Effect of chemical components on fluorescence properties. (b) Effect of fiber size of BP on fluorescence properties. (c) Effect of the fiber size of the DP on the fluorescence properties. All fluorescence emission spectra were obtained at an excitation wavelength of 350 nm. (d) Comparison of infrared spectra of BP, DP, B-CN F-6, and D-CN F-6. (e) Comparison of infrared spectra of BP, B-CMF-50, B-CMF-80, and B-CMF-100. (f) Comparison of infrared spectra of DP, D-CMF-50, D-CMF-80, and D-CMF-100.

Figure 4 Laser Scanning Confocal Microscopy images of (a) BP, (b) B-CMF-50, (c) B-CMF-80, (d) B-CMF-100, (e) DP, (f) D-CMF-50, (g) D-CMF-80, (h) D-CMF-100.

3.4. Effect of chemical groups on fluorescence properties

Morphologies and properties of nanocelluloses can be obtained by preparation methods. The effect of nanocellulose prepared by different methods on the fluorescence properties was shown in Fig 5 (a, b). As mentioned above, the TEMPO oxidation can reduce the fluorescence intensity of nanocellulose due to the increase of the number of π-conjugated groups (-COOH) on the cellulose surface.\(^{[18]}\) The carboxyl groups content of B-CN F-2, B-CN F-4 and B-CN F-6 were 0.41 mmol/g, 0.79 mmol/g, and 1.18 mmol/g. As shown in Fig 5b, the increased carboxyl groups could negatively affect fluorescence performance, from which the lowest carboxyl groups content of B-CN F-2 was shown a highest fluorescence intensity and the fluorescence intensity gradually decreased after further oxidation. This can be attributed to the carboxyl group acted as an electron acceptor which weakened the flow of π electrons in the system and then limited the fluorescence property.\(^{[23]}\) Similarly, the presence of surface electron acceptor (-OSO\(_3\)) in CNCs resulted in undesirable fluorescence intensity of CNCs in comparison with BP.

Raman spectroscopy was used to collect chemical information on autofluorescent particles in an attempt to identify (based on the presence of chemical functions) compositions or to further
differentiate (based on unique spectral characteristics) these nanoparticles deriving from cellulose. Cellulose as a polysaccharide had a characteristic Raman band from 1000 to 1200 cm\(^{-1}\) corresponding to \(\nu(C-O-C)\) asymmetric stretching, \(\nu(C-C)\) stretching and Raman bands from 1300 to 1500 cm\(^{-1}\) corresponding to \(\delta(CH_2)\) and \(\delta(CH_2OH)\) deformations.\(^{[24]}\) The four cellulose samples had comparable changes in the peak shape of the Raman shift which was related to the fraction of light scattering crystals and the cellulose phase/crystallization effect.\(^{[25]}\) The fluorescent particles in the CNCs sample were so hardly photobleached that Raman spectrum was dominated by the fluorescent background (Fig. 4c). The Raman peak of CNCs at 1607 cm\(^{-1}\) was distinguishable and different from the BP Raman spectrum. The stretching vibration of aromatic \(\nu(CC)\) in CNCs may be resulted in an excellent fluorescence property. The peaks at 795, 1385 and 1709 cm\(^{-1}\) can be designated as \(\nu(C=O)\) stretching, \(\nu(C=C)\) and \(\nu(C=O)\) stretching. The information on glycosidic bonds and unsaturated groups is consistent with the infrared spectrum.

Figure 5 The effect of different substituent groups on the fluorescence properties. (a) The effect of different preparation methods of nanocellulose on fluorescence properties. Inset: chemical structure of nanocellulose surface. (b) The effect of different carboxyl groups on the fluorescence properties. (c) Raman spectra of nanocellulose samples.

3.5. Effect of different excitation wavelengths on fluorescence properties.
As discussed above, the raw materials and preparation methods can influence their fluorescence intensity. However, the fluorescence properties of nanocellulose can be affected by many factors such as raw materials, preparation methods, temperature, pH, excitation wavelength, etc. In Fig. 5, the emission peaks of both CNCs and B-CN6 showed a significant red shift as increasing the excitation wavelength (510-530nm). This excitation-dependent behavior can be attributed to the \(\nu(C=C)\) and \(\nu(C=O)\) stretching (Fig 5c)\(^{[26]}\) These defects produced by unsaturated groups as capture centers of excitons give rise to the surface-state-related fluorescence.\(^{[27]}\) They can modulate the emission wavelength at different excitation wavelengths, which is important for some practical applications, such as photoluminescent material.

Figure 6 3D synchronous fluorescence spectrum of nanocellulose. The excitation wavelength ranges from 510 to 530 nm. The emission wavelength ranges from 550 to 600 nm. (a) 3D synchronous fluorescence spectrum of CNCs. (b) 3D synchronous fluorescence spectrum of B-CN6.

3.6. Effect of different pH on fluorescence properties.
The pH of the system was one of the important factors affecting the luminescent properties of fluorescent substances. The fluorescence spectra of nanocellulose at different pH was shown in Fig 8.
Both CNCs and B-CNF-6 exhibited excellent fluorescence properties regardless of acidic or basic conditions. And the position of the emission peak did not change. This was very advantageous for the application of nanocellulose in different environments. The fluorescence intensities of both CNCs and B-CNF-6 were increased with acid/basic enhancement. This can be attributed to the introduction of electron-donating groups (H^+, OH^-) which can increase the mobility of π electrons in the molecule.[10] And the increase in the polarity of the suspension also facilitated the generation of fluorescence.[28]

![Figure 7](image-url)  
Figure 7: The fluorescence intensity of nanocellulose at different pH. (a), (b) Fluorescence intensity of CNCs at different pH. (c), (d) Fluorescence intensity of B-CNF-6 at different pH.

4. Conclusion
In conclusion, this work investigated the effects of the physical and chemical structure of nanocellulose on its autofluorescence properties. We concluded that nanocellulose autofluorescence came from unsaturated aromatic groups in the system. And the fluorescence intensity could be controlled dramatically by the size and amount of introduction of chemical groups. The smaller size and the introduction of electron acceptors groups (-OSO_3, -COOH) resulted in a significant decrease in the fluorescence intensity of nanocellulose. The essential ingredient of cellulose and lignin has no significant effect on the fluorescence intensity of nanocellulose. Moreover, the fluorescence emission peak of nanocellulose could undergo blue/red shift and high/low regulation with different excitation wavelengths and pH values. Besides, the sensitive wavenumber position of nanocellulose produce by external disturbance conditions and the interaction between each group were thoroughly studied by two-dimensional correlation fluorescence spectroscopy.

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