Preparation of Unmodified Cellulose Nanocrystals from *Phyllostachys heterocycla* and their Biocompatibility Evaluation

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Cellulose nanocrystals (CNCs), also called cellulose nanowhiskers, cellulose nanorods, or nanocrystalline cellulose, were prepared from *Phyllostachys heterocycla* using a commercial cellulase for hydrolysis. The enzymatic hydrolysis process and application performance as well as the biocompatibility of the CNCs were investigated. Here, the cellulase hydrolysis conditions were optimized at a cellulase dosage of 0.01 mL/g dried fibers, a hydrolysis temperature of 60 °C, a hydrolysis time of 3 h, and a bamboo fiber concentration of 2 wt%. Under these conditions, the resultant CNCs retained more similarities to the original bamboo fibers than those fabricated by sulfuric acid hydrolysis. The product also demonstrated potential biocompatibility, which expands its applicability in the biopharmaceutical and biomedical fields.

Keywords: Cellulose nanocrystals; Phyllostachys heterocycla; Biocompatibility; Preparation; Characterization

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INTRODUCTION

As a renewable material, cellulose and its graft copolymers have been widely studied, focusing on their biological, chemical, and mechanical properties (Tehrani and Neysi 2013; Liang *et al*. 2013). Materials based on cellulose and its derivatives have been used for more than 150 years in a wide variety of applications such as food (Pérez *et al.* 2013), paper production (Shi *et al*. 2013; Yang and Wu 2013), biomaterials, and pharmaceuticals (Sievens-Figueroa *et al.* 2012; Dash and Ragauskas 2012).

Cellulose nanocrystals (CNCs), also called cellulose nanowhiskers, cellulose nanorods or nanocrystalline cellulose, obtained from hydrolysis of cellulose have been identified as a class of nano-materials (Dash *et al.* 2012; Xu *et al.* 2013). Compared to cellulose fibers, CNCs possess many advantages, such as having a nanoscale dimension, high specific strength and modulus, high surface area, and unique optical properties. These amazing physicochemical properties and wide application prospects have attracted significant interest from both research scientists and industry.

During the traditional CNCs preparation process, the hydrolyzing agent, sulfuric acid, introduces bulky ester groups onto hydroxyl groups and stabilizes CNCs in solution by preventing agglomeration (Visakh *et al*. 2012). However, the use of sulfuric acid has a number of important drawbacks, such as corrosivity, cellulose surface modification, and environmental incompatibility (Zhu *et al*. 2011). Apart from use in composites, CNCs

find applications in personal hygiene products, biomedicines, cosmetics, *etc.* (Park *et al.* 2013; Male *et al*. 2012). Biocompatibility is an important parameter for materials applied in the biomedicine field. A series of works related to biocompatibility evaluation have been reported in recent years, including inorganic and organic materials used in cardiovascular stents (Zhou *et al*. 2013), drug delivery (Li *et al*. 2012), food packaging (Zhuang *et al*. 2012), *etc*. CNCs in their pure form are safe and biocompatible. However, the traditional sulfuric acid hydrolysis process inserts sulfate groups on the surface of CNCs, which may affect their biocompatibility compared to the cellulase hydrolysis process (Satyamurthy *et al*. 2011). Enzyme-degraded cellulose retains its original chemical properties (Kou *et al*. 2013). Cellulase produced by various microbes, with its proven biotechnological advances in various fields, may be of immense use in the production of CNCs.

Essentially, *Phyllostachys heterocycla* is classified as a long-fibered fibrous material, as the length of its fibers is comparable to that of softwood fibers. With the advantages of a short growth period, suitable fiber morphology, and similar chemical composition to that of softwood, *P. heterocycla* has attracted increasing attention in many countries and is being used as feedstock for high-value added products such as cellulose esters, cellulose ethers, and textile fibers (Lam *et al*. 2012).

Here we report a biological method without any chemical modification to prepare CNCs from *P. heterocycla* with the intention of developing a novel biocompatible nano-material for potential biomedical applications. The preparation of CNCs by controlled hydrolysis of *P. heterocycla* fibers using extracellular cellulase was investigated, and the properties of the product, such as morphology, crystallization, and colloid stability, were characterized. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazoluim bromide (MTT) assay and a fluorescence microscope were also employed to quantitatively evaluate the biocompatibility of the resultant CNCs.

EXPERIMENTAL

Materials

P. heterocycla was purchased from a bamboo farm in Anji, China. Commercial cellulase (Giant A) and Tylose-C600 sodium-carboxymethyl-cellulose (CMC) were supplied by the Shanghai Tianye Biotechnology Co., Ltd, China. Human osteosarcoma MG-63 cells (MG-63) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Beyotime Institute of Biotechnology, China. Analytical grades of hydrochloric acid, sulfuric acid, sodium hydroxide, acetic acid, and sodium hypochlorite were purchased from the Hangzhou Mike Chemical Agents Company, China. All solutions were prepared with distilled water.

Preparation of Bamboo Fibers

P. heterocycla was cut up, crushed, and screened to collect the bamboo particles between 40 and 60 mesh (0.250 to 0.425 mm). The powders were treated with a 4 wt% NaOH solution at 80 °C for 90 min, repeating this process 3 times. After each treatment, the powders were filtered and washed with distilled water until the alkali was completely eliminated. Then, a chlorine bleaching was carried out with a solution consisting of equal parts of acetate buffer, 1.7 wt% aqueous chlorite, and distilled water. This process was performed at 80 °C for 180 min and repeated 4 times. The powders were filtered and

washed with distilled water after each repetition. Figure 2a presents the main chemical components of *P. heterocycla* and the prepared bamboo fibers.

Preparation of CNCs

The bamboo fibers were dispersed in distilled water (2 wt%) mixed with cellulase (0.01 mL/g, based on dried fibers) and the mixture was incubated at 60 °C. Subsequently, the product was incubated at 100 °C for 10 min to halt the reaction and then washed by successive centrifugations at 10,000 rpm and 10 °C for 10 min to remove particles larger than 1 μm. The resultant supernatant was filtered through a 100-kDa ultrafiltration membrane, and the CNCs retained on the membrane were collected and freeze-dried for further analyses. For comparison, CNCs were prepared by a conventional process using 60 wt% sulfuric acid hydrolysis of bamboo fibers at 45 °C for 1 h. The acid-hydrolyzed sample was washed with distilled water by repeating the centrifugation and dilution process until its pH was neutral. The pH value of the centrifugate was monitored.

Transmission Electron Microscope (TEM) Observation

CNW samples were examined with a Philips CM 200 transmission electron microscope using an acceleration voltage of 80 kV. A droplet of diluted suspension was deposited on a carbon microgrid (400 mesh) and allowed to dry. The grid was stained with a 1.5 wt% solution of uranyl acetate and dried at room temperature.

X-ray Diffraction (XRD) and Zeta Potential Analyses

Diffractograms were recorded on a Rigaku diffractometer operating at 50 kV and 100 mA with Cu Kα radiation. The samples were scanned in 2θ ranges varying from 5 to 40° (2° min^{-1}). The extent of crystallinity was estimated on the basis of areas under the crystalline and amorphous peaks after appropriate baseline correction. Potential charges were measured with a Malvern 3000 Zetasizer. A CNW suspension (0.05 wt%), previously sonicated for 5 min, was prepared and analyzed to determine zeta potential.

Cell Proliferation

Each of the bamboo fibers and as-prepared CNCs were placed in a 24-well plate and sterilized by 70 wt% ethanol for 5 min. They were then washed 2 times with phosphate buffer saline (PBS, pH=7.2) solution and then with fresh culture medium. Prior to cell seeding, 500 μL of fresh culture medium was pipetted into each well. Human osteosarcoma MG-63 cells (MG-63) proliferation was studied at days 1, 3, and 5, and 7. 3×10^4 of the cells were seeded and allowed to mix with sample for 20 h. The cells were later starved with serum-free medium (SFM) (DMEM containing 100 U/mL penicillin and 100 μg/mL streptomycin but without FBS) 2 times. The number of cells was determined with an MTT assay. MG-63 stained with a fluorescent dye was observed using a fluorescence microscope (Olympus IX71) after being cultured for 1, 3, 5, and 7 days.

MTT Assay

Samples were incubated with 0.5 mg/mL MTT solution in DMEM without phenol red (250 μL per well). After incubation at 37 °C for 1 h, the MTT solution was removed. A solution containing 900 μL of dimethylsulfoxide (DMSO) and 125 μL of glycine buffer (pH=10) was added to the 24-well plate to dissolve the formazan dye. Solutions were then transferred into a cuvette, and a visible-light spectrophotometer (Thermo-

spectronic Genesis 10 UV) was used to measure the absorbance at 570 nm. The intensity of the absorbance is proportional to the number of living cells. Figure 1 exhibits the experimental procedure of the fabrication, characterization, and biocompatibility evaluation of CNCs.

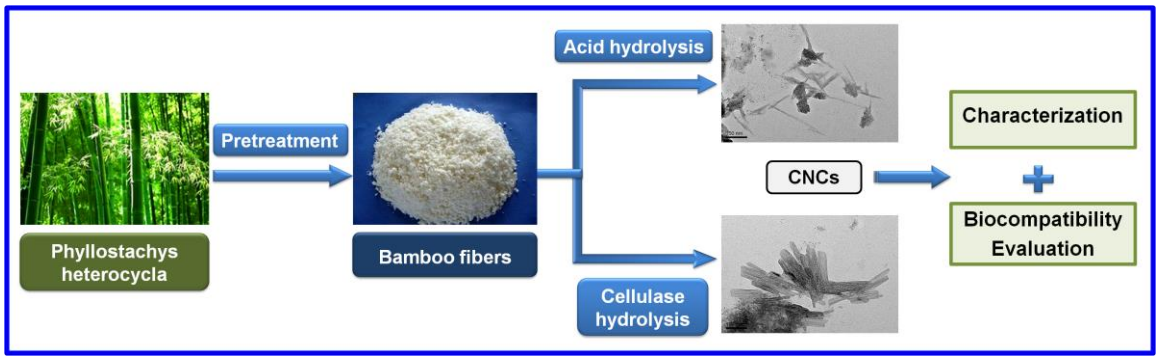

Fig. 1. Schematic of the fabrication, characterization, and biocompatibility evaluation of CNCs

RESULTS AND DISCUSSION

CNW Yield at Different Hydrolysis Times

The enzymatic hydrolysis of cellulose, particularly the hydrogen-bonded and ordered crystalline regions of it, is a complex process. The influences of pH and temperature on the cellulase activity of Giant A have been investigated in a previous study by the authors, which showed that the highest CMC activity of Giant A appeared at 60 °C and pH 5 as 2614 IU/mL (Zhang *et al*. 2012).

According to the optimal conditions for cellulase activity, the bamboo fibers were hydrolyzed by Giant A at 60 °C and pH 5. The cellulose analysis by a chemical method was used for the estimation of the CNW yield at different hydrolysis times (Updegraff 1969).

The yield of CNCs is given in Fig. 2b. Until 1.5 h of hydrolysis, the CNW yield was very low; afterwards, exponential growth occurred. The optimal yield of the CNCs was 28.4% after 3 h of hydrolysis. After 3 h, the growth trend slowed, showing that it was not necessary to hydrolyze for longer than 3 h.

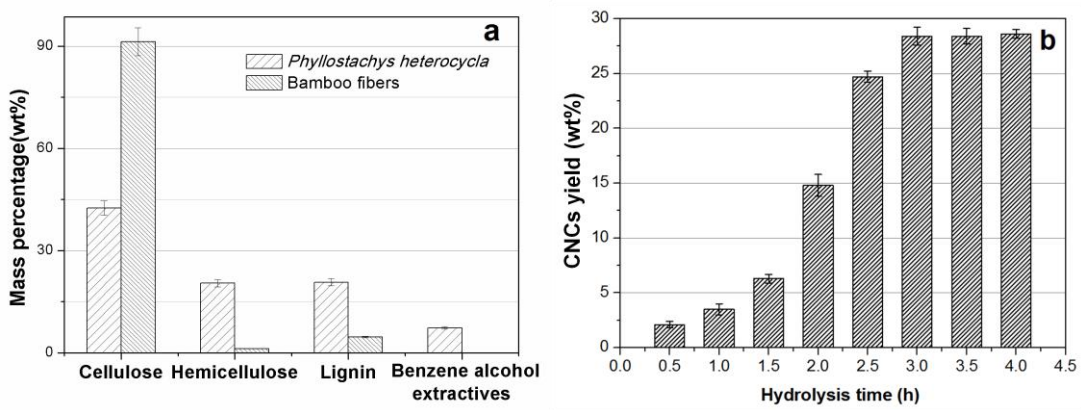

Fig. 2. (a) Main chemical composition of *P. heterocycla* and the treated bamboo fibers; (b) Yield of the CNCs during the cellulase hydrolysis process

Characterization of CNCs

Figure 3a–b shows TEM images of the CNCs prepared by cellulase hydrolysis (a) and acid hydrolysis (b). In both cases, the CNW particles had similar rod-like morphologies. However, the dispersion of the CNCs prepared by acid hydrolysis had better stability than those prepared by enzymatic hydrolysis, due to the fact that acid hydrolysis resulted in the CNCs having greater anionic charge (Satyamurthy *et al*. 2011). The length and diameter of the CNCs were analyzed using the UTHSCSA Image Tool and are shown in Table 1. CNCs prepared by cellulase hydrolysis presented an average diameter of 28.4 nm and length of 343 nm, with an aspect ratio of 1:12. The CNCs prepared by acid hydrolysis were narrower and sharper, leading to a higher aspect ratio of 1:15. The resultant shape could have been due to the differences in the mode of action by acid and enzyme.

Because acid hydrolysis is completely a surface phenomenon, its action is influenced purely by crystallite size and shaking conditions. With cellulase hydrolysis, two other parameters that influence the shape of CNCs are the effect of the cellulase binding domain secreted by the enzyme on the crystalline structure and the penetration of cellulase into cellulose.

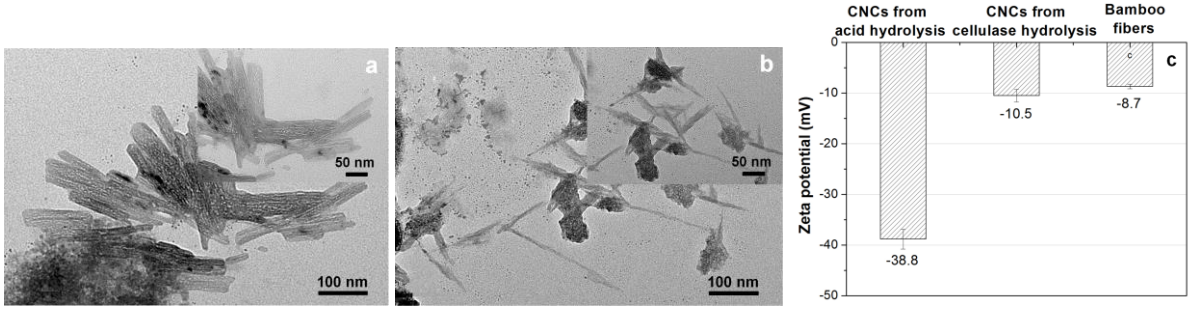

Fig. 3. (a-b) TEM images of the CNCs from cellulase hydrolysis and acid hydrolysis, respectively; (c) Zeta potential of the different CNCs and bamboo fiber (control) suspensions

The XRD analysis of the bamboo fibers and CNCs was done to calculate crystallinity, which is an important aspect for understanding moisture absorption, swelling ability, and the accessibility to cellulose.

Table 1 shows the crystallinity changes of the resultant CNCs. There was a marginal increase in the crystallinity of CNCs prepared by cellulase hydrolysis, while a significant increase (6.7%) was noticed in the case of the acid hydrolysis-treated CNCs; this may be caused by the penetration of cellulase into fibers and the hydrolysis of the crystalline regions of cellulose.

Table 1. Crystallinity, Morphology, and Dimensional Analysis of the CNCs

Samples	Crystallinity (%)	Morphology	Dimension		
			Length (nm)	Diameter (nm)	Aspect ratio (L/D)
Bamboo fibers (control)	72.3	-	1.86 ± 0.13 μm	20.2±0.16 μm	92
CNCs from acid hydrolysis	79.0	Rod-like	274±32	18.2±1.9	15
CNCs from cellulase hydrolysis	74.2	Rod-like	343±27	28.4±2.1	12

Zeta potential can be evaluated by measuring the mobility distribution of charged particles as they are subjected to an electric field. As shown in Fig. 3c, the average zeta potentials were -38.8 mV and -10.5 mV, respectively, for the CNCs prepared by acid hydrolysis and cellulase hydrolysis. The higher negative charge density of the acid hydrolysis-treated CNCs is caused by the attachment of sulfate groups to the surface of CNCs. In addition to their effect on zeta potential, these sulfate groups on CNCs may have negative effects on their biocompatibility. On the other hand, the CNCs prepared by cellulase hydrolysis gained biocompatibility, but flocculation appeared frequently.

Biocompatibility Evaluation

The MTT assay is based on the reduction of yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of the purple formazan crystals formed is proportional to the number of viable cells. The proliferation of viable MG-63 cells after they were cultured with bamboo fibers and different CNCs was studied at days 1, 3, 5, and 7. The cells were allowed to mix with the bamboo fibers and CNCs for 20 h. Figure 4a shows the absorbance intensity obtained after 1, 3, 5, and 7 days in culture. According to the results, the proliferation of MG-63 with bamboo fibers was better than that with both types of CNCs. After 1 day in culture, the proliferation of the cells with the acid hydrolysis-treated CNCs were comparable to that of the cellulase hydrolysis-treated CNCs. On the contrary, after 3 and 5 days in culture, the proliferation of the cells with the cellulase hydrolysis-treated CNCs was better than that with the acid hydrolysis-treated CNCs. After 7 days, the proliferation of MG-63 with both the bamboo fibers and cellulase hydrolysis-treated CNCs increased linearly. A fluorescence microscope was used to observe the MG-63 proliferation after culturing with cellulase hydrolysis-treated CNCs for different times. As shown in Fig. 4b–e, the MG-63 proliferated significantly with the CNCs with increasing culture time, which demonstrated proof of potential biocompatibility of the cellulase hydrolysis-treated CNCs. Both the proliferation and fluorescence microscope results suggest the possibility that cellulase hydrolysis-treated CNCs could be applied as a drug delivery carrier or a tissue scaffolding enhancer.

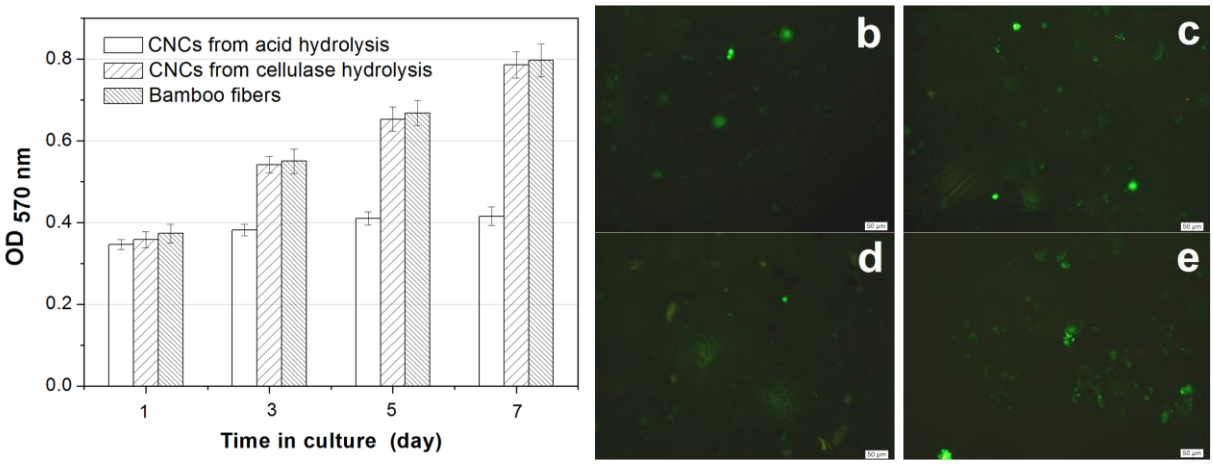

Fig. 4. (a) Proliferation of MG-63 cells mixed with the CNCs from acid hydrolysis and cellulase hydrolysis as well as bamboo fibers (control) after days 1, 3, 5, and 7 of cell culture; (b–e) Fluorescence microscope photographs of MG-63 cells cultured with the CNCs from cellulase hydrolysis for 1, 3, 5, and 7 days, respectively

CONCLUSIONS

1. CNCs were successfully prepared from *P. heterocycla* by enzymatic hydrolysis using a commercial cellulase. The cellulase hydrolysis process without any chemical modification resulted in CNCs with potential biocompatibility, which suggests several applications of the CNCs in the biopharmaceutical and biomedical fields.
2. During the cellulase hydrolysis process, after 3 h, the CNW yield reached 28.4 wt% at optimal cellulase hydrolysis conditions of cellulase dosage of 0.01 mL/g dried fibers, hydrolysis temperature of 60 °C, hydrolysis time of 3 h, and bamboo fiber concentration of 2 wt%. The prepared CNCs had sleeker morphology and lower crystallinity and zeta potential compared with those from sulfuric acid hydrolysis, which indicated that the cellulase hydrolysis-treated CNCs retained properties more similar to those of the original bamboo fibers.

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