Research Article

Advances in Nanoscience and Nanotechnology

Enzymatic Synthesis, Characterization and Biocompatibility Studies of Cellulose Nanoparticles from Cotton Fibers

Prathima Prabhu Tumkur[1,2], Tejaswini Ronur Praful[1,2], Babu R Lamani[1], Nicole Nazario Bayón[1], Krishnan Prabhakaran[1], Joseph C Hall[1] and Govindarajan T Ramesh[1,2]*

[1]*Molecular Toxicology Laboratory, Center for Biotechnology and Biomedical Sciences, Norfolk State University, 700 Park Avenue, Norfolk, VA, 23504, USA*

[2]*Center for Materials Research, Norfolk State University, 555 Park Avenue, Norfolk, VA, 23504, USA*

***Corresponding author**
Govindarajan T Ramesh, Center for Biotechnology & Biomedical Sciences, Department of Biology, Norfolk State University, Norfolk, VA 23504, Tel : 757 823-8951; Fax: 757 823-2618; E-mail: gtramesh@nsu.edu

Submitted: 03 July 2018; **Accepted**: 10 July 2018; **Published**: 01 Aug 2018

Abstract

Interest in cellulose nanoparticles has been increasing exponentially in the past few decades due to its unique characteristics such as reinforcement properties, high tensile strength, and excellent thermal and electrical properties. Cellulose nanoparticles were produced by an enzymatic method including hydrolysis of cotton fibers by cellulase enzyme and sonication process. Further, cellulose nanoparticles were characterized to determine the morphology and purity of the material. Characterization of cellulose nanoparticles was performed by Scanning Electron Microscope (SEM) with Energy Dispersive X-Ray Spectroscopy (EDX) and Fourier Transform Infrared Spectroscopy (FT-IR). Biocompatibility studies of cellulose nanoparticles were carried out by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay and Live/Dead viability assay. Using SEM, the average size of cellulose nanoparticles was found to be around 100-125nm and the particles were spherical in shape. FTIR spectrum showed the formation of cellulose nanoparticles from cotton fibers without any presence of impurities. MTT assay and Live/Dead viability assay showed no significant induction of cell death even at higher concentrations (100 µg) upon exposure to Rat Lung Epithelial cells. The results revealed that the synthesized cellulose nanoparticles could be used in wide range of emerging applications in the development of new energy storage devices, enzyme immobilization, antimicrobial and medical materials, green catalysis, bio-sensing and controlled drug delivery.

Keywords: Cellulose nanoparticles, Hydrolysis, Sonication, Morphology, Viability.

Introduction

Cellulose is the most ubiquitous and abundant biopolymer in the world with wide industrial use in the present age, but also in the past age for ropes, sails, paper, timber for housing and many other applications [1]. Cellulose is a natural polymer that provides cell structure in plant cells. Cellulose consists of repeating units of glucose connected by β-1,4 glycosidic linkage and thus makes it unique and different from starch, which is also a natural polymer containing repeating units of glucose having α-1,4 and α-1,6 glycosidic linkage [2]. Over the past decades, interest in sustainability and green chemistry has led to the development of novel cellulosic material from the various source where cotton serves as the largest source for cellulose production [3]. Several other sources for cellulose extraction have been previously reported and include waste plant biomass, sugarcane bagasse, commercially available microcrystalline cellulose, waste cotton fibers, commercially available cellulose fibers, fibers from banana, jute and pineapple leaf fibers, milkweed stem and sisal fibers [4-10].

Cellulose nanoparticles can be synthesized by chemical, biological and mechanical methods and their combinations. In recent years, enzyme application has increased significantly in making useful products and developing new processes. Cellulase enzymes act as a biocatalyst for the hydrolysis of the cellulose [8]. Due to depletion of non-renewable energy sources, alternatives for sustainable energy is of prime focus. Nanocellulose has received attention due to its potential properties like aspect ratio, high stiffness, and strength, low weight, and biodegradability. Serving as alternative carbon source, cellulose can be applied for various exceptional applications. The present study involves three main objectives: (i) Synthesize of cellulose nanoparticles by an enzymatic method (ii) Characterization of synthesized cellulose nanoparticles by Scanning Electron Microscope (SEM) with Energy Dispersive X-Ray Spectroscopy (EDX) and Fourier Transform Infrared Spectroscopy (FT-IR) (iii) Biocompatibility studies of cellulose nanoparticles by MTT assay and Live/Dead viability assay.

Experimental Section

Materials

Cotton fibers obtained from Indian farm fields were used as a primary source for the enzymatic synthesize of cellulose nanoparticles. The cotton fibers were separated from cotton linkers and stored. The cellulase enzyme (IRWBE005-20866) used in hydrolysis was

purchased from Innovative Research, Inc. (Peary Court Novi, MI). Also, other chemicals such as acetic acid (99.9% purity, analytical plus grade), anhydrous sodium acetate (99.9% purity, analytical plus grade) and a non-ionic surfactant Bioworld wash buffer – Tween 20 (1 g/L) were purchased from Fisher Scientific (New Jersey). Dulbecco's modified eagle medium (DMEM), Phosphate Buffered Saline (PBS) solution, Fetal Bovine Serum (FBS), penicillin and streptomycin were purchased from Atlanta Biologicals, Inc. (Atlanta, GA). Single wall carbon nanotubes (SWCNTs), 3-(4,5-dimethylthiozol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Live/Dead viability assay kit were purchased from Sigma Aldrich (St. Louis, MO, USA).

Pretreatment

Cotton fibers were washed in non-ionic surfactant Bioworld wash buffer – Tween 20 (1g/L) for an hour at room temperature. The fibers were again washed thrice with distilled water and later, dried in hot air oven at 95 °C overnight.

Enzymatic Hydrolysis

Pretreated cotton fibers were used to carry out enzymatic hydrolysis at 48 °C for 180 h including 0.05 M acetate buffer of pH 4.8, 5 g/L cotton fiber and 2.5% of cellulase enzyme. Later, the enzyme was inactivated and thereby hydrolysis was terminated by raising the temperature to 80 °C for 20min. The obtained hydrolysate was washed thrice with distilled water at 5000rpm for 15min using Sorvall Legend Mach 1.6R Centrifuge from Thermo Electron Corporation.

Sonication

The hydrolyzed cotton suspension was sonicated using Brason 2510 sonicator for 15min and then it was allowed to rest for 15min. The top turbid part containing cellulose nanoparticles was separated and then equal amount of deionized water was added to the bottom solid part. Sonication step was repeated until there was no formation of the top turbid layer. After 2 cycles of sonication, a clear top layer was observed.

Freeze Drying

Cellulose nanoparticle solution was centrifuged to remove the excess water. Obtained cellulose nanoparticles were frozen using liquid nitrogen and subjected to freeze drying in Labconco freeze dry system for 48 h. The freeze-dried cellulose particles were further characterized using SEM, EDX, and FTIR.

Characterization of cellulose nanoparticles

The SEM-EDX and FT-IR characterization analysis were performed to determine the particle size and crystallinity of nano-cellulose. SEM-EDX and FT-IR experiments were performed in Jefferson Lab, Newport News, Virginia.

Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDX)

SEM-EDX was performed using Phenom ProX Table top SEM at 5 kV. Morphology of cellulose nanoparticles and the composition of the elements present in the mixture were also determined.

Fourier Transform Infrared Spectroscopy (FTIR)

The Nicolet Nexus 670 Fourier Transform – Infrared Spectrometer (FT-IR) was used in absorbance mode ranging between 4000-500 cm-1 at a resolution of 2 cm-1 and 100 scans per sample. Obtained FT-IR spectroscopy data of hydrolyzed cotton fibers were analyzed using the Thermo Nicolet OMNIC software.

Biocompatibility studies of cellulose nanoparticles

To perform biocompatibility studies, Rat LE cells were used in MTT assay and Live/Dead viability assay to determine the viability of cells in the presence of cellulose nanoparticles.

Cell culture and treatment

Rat LE cells (RL-65) were purchased from American Type Culture Collection (Manassas, VA). Cell lines were cultured in DMEM with 10% FBS, 100 IU/mL of penicillin and 100 g/mL of streptomycin and incubated in a carbon dioxide (CO_2) incubator with 5% CO_2 and 95% humidified atmosphere. For all studies, cellulose nanoparticles stock was prepared in PBS. In all control experiments, cells treated with equivalent volume of PBS was considered as negative control and SWCNTs dispersed in DMSO was considered as positive control.

MTT Assay

The assay was performed by using a yellow tetrazolium salt, MTT, to assess the cell viability as described earlier [11]. Briefly, 5000 cells/well of RL-65 was seeded in a 96-well plate and cultured overnight in a CO_2 incubator with 5% CO_2. The cells were washed with PBS and different concentration of cellulose nanoparticles stock were added and incubated for 48 hours at 37°C. After 48 hours, the plate was washed with PBS and MTT was added in each well at a final concentration of 125µg/ml and further incubated for another 3 hours at 37°C. Following incubation, the formazon formed inside the cells were dissolved in DMSO and the absorbance was measured at 570 nm.

Live/Dead Viability Assay

The Live/Dead viability assay was performed according to the manufacturer's protocol provided by the Live/Dead assay kit to reconfirm the MTT assay. This assay helps to distinguish the live cells and dead cells using a two-color dye probe with relatively very low background fluorescence. The live cells take up the non-fluorescent cell permeant dye, calcein AM, enzymatically converted to a fluorescent molecule by intracellular esterase and produces a green fluorescence in live cells that can be detected by ex/em at 495 nm/ 515 nm. Whereas the dead cells were determined by a bright red fluorescence of Ethidium Homo dimer (EthD-1) bound to nucleic acid, detected at an ex/em at 495 nm/ 635 nm [12]. Briefly, 5000 cells/well were seeded on 6 well plate and grown overnight at 37 0C, 5% CO_2. Confluency was attained after 48h and the cells were gently washed with PBS and treated with different concentration of cellulose nanoparticles. Following incubation, a dye mixture of 5µM ethidium homodimer and 5µM calcein- AM was added to the cells and allowed to rest for 30 min. The stained cells were then imaged under a fluorescence microscope (Nis Element, Nikon Instruments Inc, Melville).

Results and Discussion

Synthesis of Cellulose Nanoparticles

Spherical cellulose nanoparticles were successfully synthesized from cotton fibers by enzymatic method. The enzymatic hydrolysis of cellulose present in the cotton fibers is a slow process which involves dissociation of glucosidic bonds. The structure of the molecule is not changed as insoluble cellulose doesn't allow the cellulase enzyme to diffuse into it. The hydrolysis is a long process because of the dominance of Iβ structure in cellulose. Later, the obtained cellulose

hydrolyzate is further broken down by sonication method which helps to disrupt the β-D-(1 → 4) glycosidic linkages and produces nano-size cellulose particles.

There are several ways to synthesize cellulose nanoparticles. Few researchers have used different sources for the hydrolysis of cellulose to produce nano-cellulose. It has been previously reported that various size distributions of the cellulose nanoparticles depend on the initial substrate chosen for the study. Lu and Hsieh have recently reported that 10–100 nm spherical cellulose nanocrystals were synthesized from acid hydrolysis and freeze drying of the cotton cellulose [13]. Zhang et al. used an additional pretreatment process with Sodium Hydroxide (NaOH) and DMSO and 8-hour acid hydrolysis in a sonicator to produce spherical cellulose nanoparticles with sizes ranging 60–570 nm from cellulose fibers [8].

Cellulose nanoparticles are commonly produced by chemical mediated acid hydrolysis method. This method has several disadvantages like the acids used are not environment-friendly and difficult to control, handle, transport and maintain. Along with the cellulose nanoparticles, byproducts are also obtained which is not desirable. Therefore, our enzymatic method is preferred over chemical method.

SEM-EDX Analysis

SEM images revealed the presence of spherical cellulose nanoparticles (Fig. 1). The average size of the nanoparticles ranges from 100-125nm. EDX results showed the presence of carbon and oxygen elements of cellulose nanoparticles (Fig. 2). Small peaks seen in the EDX graph is due to the sulfur and nitrogen elements from cellulase enzyme.

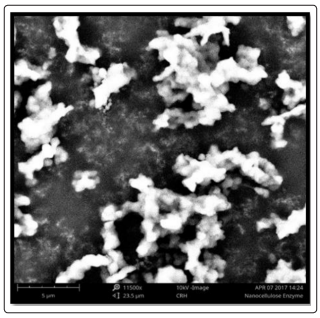

Figure 1: Scanning Electron Microscopy images of synthesized Cellulose nanoparticles

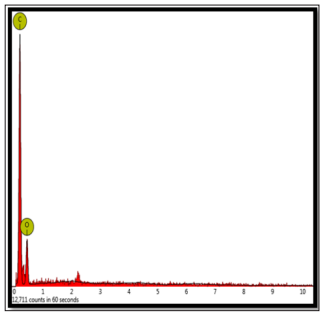

Figure 2: EDX analysis of synthesized cellulose nanoparticles from cotton fibers.

FT-IR Analysis

FT-IR spectrum of synthesized cellulose nanoparticles clearly indicates the characteristic peaks of cellulose: 3272, 2930, 2900-2870, 1640, 1520, 1430-1400, 1340-1300, 1170, 910, 710 (Fig. 3). Accordingly, the peaks from 3270-3000 cm-1 indicate the –OH stretch; 2900-2880 cm-1 are attributed to CH2 asymmetric vibrations; 1640 cm-1 indicate the OH bending; 1430-1400 cm-1 are associated with the HCH and OCH in-plane bending vibrations; the peaks at 900-700 cm-1 show the COC, CCO and CCH deformation modes and stretching vibrations of the C-5 and C-6 atoms [3,14].

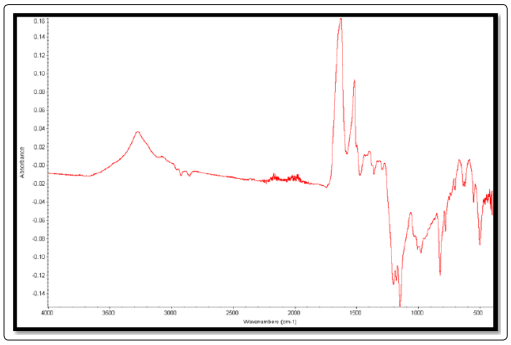

Figure 3: The FT-IR spectrum of synthesized cellulose nanoparticles from cotton fibers.

Effect of cellulose nanoparticles on Cell Viability

MTT assay was performed to assess the cell death caused by the cellulose nanoparticles. Fig. 4 shows the cell viability assay in Rat LE cell line with different concentration of 5, 10, 25, 50, 100µg/mL cellulose nanoparticles respectively. The cells were exposed to the varying concentration of the cellulose nanoparticles for 48 hours. 25µg/mL of SWCNTs were considered as positive control. The MTT assay was consistent with live/dead viability assay showing no decrease in cell viability. Significant decrease in cell viability was observed when cells were treated with SWCNTs. To reconfirm the MTT assay, a Live/Dead assay was performed on Rat LE cells as shown in Fig. 5. The cells were treated with 10, 25, 50, 100µg/ml of cellulose nanoparticles and 25µg/mL of SWCNTs. The image clearly shows no cell death with the cells treated with varying concentration of the cellulose nanoparticles and more cell death is observed with the cells treated with SWCNTs. These results are consistent with the MTT assay.

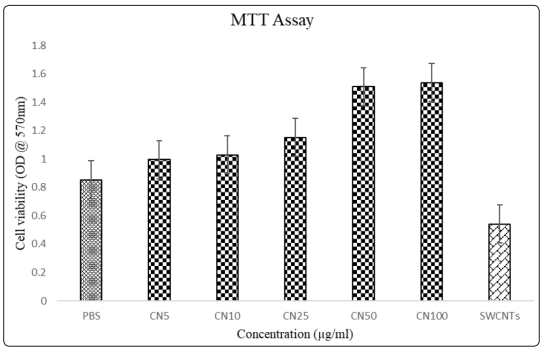

Figure 4: MTT assay. 5,000 cells/well of Rat LE cell was seeded in 96-well plate in DMEM media containing 10% FBS and penicillin (100µg/ml) and streptomycin (100µg/ml), incubated in a CO2 incubator with 5% CO2 overnight. The cells were washed with PBS and treated with varying concentration of cellulose nanoparticles (CN) for 48 hours. The MTT dye uptake was read at 570nm. (Absorbance is mean ± 8 wells, 6 experiments performed independently). Negative Control – PBS, CN5 - 5µg/ml, CN10 - 10µg/ml, CN25 - 25µg/ml, CN- 50µg/ml, CN - 100µg/ml and SWCNTs- 25µg/ml.

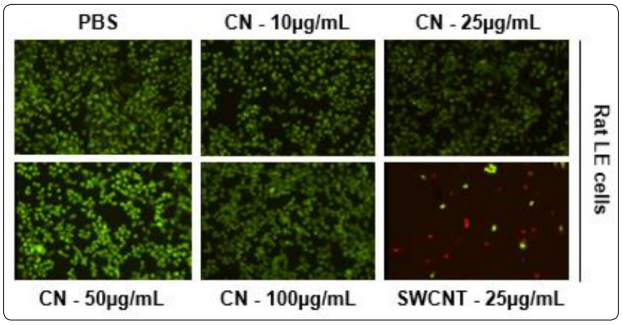

Figure 5: Live/Dead viability assay. 5,000 cells/well of Rat LE cell was seeded in 6 well plate in DMEM media containing 10% FBS and penicillin (100µg/ml) and streptomycin (100µg/ml), incubated in a CO2 incubator with 5% CO2 overnight. The cells were washed with PBS and treated with varying concentration of cellulose nanoparticles (CN) for 48 hours. Following incubation, the cells were stained for 30 min with a mixture of 5µM ethidium homodimer and 5µM calcein- AM. The stained cells were then imaged under fluorescence microscope at ex/em 495nm/ 635nm.

Conclusion

The present work illustrates that the nano-sized spherical cellulose particles could be synthesized from cotton fibers by the enzymatic method including pretreatment of cotton, enzymatic hydrolysis, sonication and freeze-drying technique. Obtained cellulose nanoparticles were studied by various means such as SEM, EDX, and FT-IR. The SEM image showed that the particles are spherical in shape with an average size of 100-125 nm. EDX revealed the cellulose nanoparticles composition by indicating the presence of carbon and oxygen elements. FT-IR results revealed that the nanoparticles retained the cellulose structure as in the fiber. Biocompatibility studies performed using Rat LE cells in MTT assay and Live/Dead viability assay showed that the cellulose nanoparticles are compatible with the cells and there was no cell death even at higher concentrations (100 μg) of the cellulose nanoparticles.

Acknowledgements

This work was supported by NSF-CREST RD-1547771 grant. We also thank Mrs. Olga Trofimova at Jefferson Lab, Newport News, Virginia for SEM, EDX and FT-IR experiments; Dr. Wondwossen Arasho, Department of Chemistry, Norfolk State University for FT-IR experiments.

References

1. Eichhorn SJ, Dufresne A, Aranguren M, Marcovich NE, Capadona JR, et al. (2009)Review: current international research into cellulose nanofibres and nanocomposites. Journal of Materials Science 45: 1.
2. Chen H (2014) Chemical Composition and Structure of Natural Lignocellulose. In:Biotechnology of Lignocellulose: Theory and Practice. Springer, Dordrecht 25-71.
3. Fattahi Meyabadi T, Dadashian F, Mir Mohamad Sadeghi G, Ebrahimi Zanjani Asl H (2014) Spherical cellulose nanoparticles preparation from waste cotton using a green method. Powder Technology 261: 232-240.
4. Kopania E, Wietecha J, Ciechanska D (2012) Studies on Isolation of Cellulose Fibres from Waste Plant Biomass. Fibres and Textiles in Eastern Europe 96: 167-172
5. Mandal A, Chakrabarty D (2011) Isolation of nanocellulose from waste sugarcane bagasse (SCB) and its characterization. Carbohydrate Polymers 86: 1291-1299.
6. Ioelovich M (2012) Optimal Conditions for Isolation of Nanocrystalline Cellulose Particles. Journal of Nanoscience and Nanotechnology 2: 9-13.
7. Han J, Zhou C, French AD, Han G, Wu Q (2013) Characterization of cellulose II nanoparticles regenerated from 1-butyl-3-methylimidazolium chloride. Carbohydrate Polymers 94: 773-781.
8. Zhang J, Elder TJ, Pu Y, Ragauskas AJ (2007) Facile synthesis of spherical cellulose nanoparticles. Carbohydrate Polymers 69: 607-611.
9. Abraham E, Deepa B, Pothan LA, Jacob M, Thomas S, et al. (2011) Extraction of nanocellulose fibrils from lignocellulosic fibres: A novel approach. Carbohydrate Polymers 86: 1468-1475.
10. Reddy N, Yang Y (2009) Extraction and characterization of natural cellulose fibers from common milkweed stems. Polymer Engineering & Science 49: 2212-2217.
11. Sarkar S, Sharma C, Yog R, Periakaruppan A, Jejelowo O, et al. (2007) Analysis of stress responsive genes induced by single-walled carbon nanotubes in BJ Foreskin cells. J Nanosci Nanotechnol 7: 584-592
12. Babu RL, Charanya S, Biradar SK, Goornavar V, Jeffers R, et al. (2017) Synthesis, Characterization and Biocompatibility Studies of Gold Nanoparticles from Zingiber officinal. BioNanoScience 7: 558-564.
13. Lu P, Hsieh Y-L (2010) Preparation and properties of cellulose nanocrystals: Rods, spheres, and network. Carbohydrate Polymers 82: 329-336.
14. Satyamurthy P, Jain P, Balasubramanya RH, Vigneshwaran N (2011) Preparation and characterization of cellulose nanowhiskers from cotton fibres by controlled microbial hydrolysis. Carbohydrate Polymers 83: 122-129.