

EXPLORING THE POTENTIAL OF PLANT BYPRODUCTS FOR SYNTHESIZING CARBOXYMETHYL CELLULOSE NANOPARTICLES: A STUDY ON CHARACTERIZATION, PHYSICAL PROPERTIES, AND IN VIVO TOXICITY

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<https://doi.org/10.55251/jmbfs.10326>

ARTICLE INFO

Received 1. 7. 2023
Revised 27. 2. 2024
Accepted 29. 2. 2024
Published xx.xx.201x

Regular article



ABSTRACT

The research involved the extraction of cellulose (C) from agricultural waste such as rice husk and the creation of nano-cellulose (NC) using Acid hydrolysis and Ultrasound method. Subsequently, carboxymethyl cellulose (CMC) and carboxymethyl Nano Cellulose (NCMC) were prepared using Alkalization and Etherification method. The team analyzed several properties of the compounds, which included the natural polymers' size measured using a Size Analyzer, resulting in 35865.7 nm for CMC. The percentage of nanopolymers yield was also calculated and noted a decline, from 37.36% for CMC to 26.48% for NCMC. FT-IR spectroscopy device was used to identify the functional groups, indicating a higher degree of substitution for NCMC (0.630%) than for natural polymers CMC (0.573%). The structure and size of polymer nanoparticles were examined using AFM, and FESEM, devices, and the diameters ranged from 30.90-49.65 nm for NCMC. The study also analyzed physical properties such as viscosity, which was observed to decrease for nanopolymers compared to natural-sized polymers (80.33 and 52.23 for CMC and NCMC, respectively), and water binding capacity, which decreased for nanopolymers compared to natural-sized polymers (725.42% and 540.76% for CMC and NCMC, respectively). Fat binding capacity was also studied, and it was found to be 511.35% and 230.25% for CMC and NCMC, respectively. The study concluded that NCMC was non-toxic at concentrations ranging from 100-1000 µg/ml.

Keywords: Polymer, Nano, Cellulose, Carboxymethyl cellulose, Carboxymethyl cellulose nanoparticle, Toxicity

INTRODUCTION

Biopolymers are polymers made from natural sources like plants, animals, and microbes or chemically produced from natural components such as polylactic acid. They are capable of being broken down by natural processes into non-toxic materials and do not harm the environment (Bratovčić *et al.*, 2015). Cellulose (C₆H₁₀O₅)_n is a plentiful biopolymer found in nature and is a significant constituent of cell walls. It is made up of uniform polymers that are created by connecting glucose units with β,1-4 linkages. Additionally, each cellulose unit comprises three hydroxyl groups that are capable of forming hydrogen bonds, making cellulose unstable, insoluble in water, and not readily soluble in organic solvents (Khalil *et al.*, 2012). After rice is manufactured and ground, the leftover rice husks can cause environmental problems if burned after the harvest season, as well as during grain cleaning and grinding. Nevertheless, rice husks are a rich source of cellulose and other valuable materials, which make them a valuable resource for extraction. As a result, several studies have investigated different acid and base separation techniques to extract cellulose from this waste material (Kadry, 2019; Rashid and Dutta, 2022; Andalia *et al.*, 2020; Abdulhameed, 2020). Cellulose has limited applications in many food industries due to its high degree of crystallization and numerous hydrogen bonds between polymer chains, which make it insoluble in water. To address this issue, it is necessary to convert cellulose into water-soluble derivatives using various solvents. Chemical reactions are used to produce these derivatives, including Carboxymethyl cellulose (CMC), Methyl cellulose (MC), Hydroxypropyl cellulose (HPC), and Hydroxypropyl methylcellulose (HPMC) (Singh and Singh, 2013). Carboxymethyl cellulose (CMC) is a crucial anionic polysaccharide with a straight chain structure that is produced by replacing the hydroxyl groups of cellulose's glucose molecule with a carboxymethyl group (-CH₂-COOH). The reaction of cellulose with sodium hydroxide (NaOH) and monochloroacetic acid (MCA) results in the formation of CMC. It is characterized by being odorless, tasteless, and non-toxic, and is a significant source for the creation of biofilms (Karataş and Arslan, 2016; Gupta *et al.*, 2020; Karataş and Arslan, 2016). Furthermore, CMC possesses favorable characteristics that allow it to create biodegradable and edible films, and it can also carry various natural active components, nutrients, flavors, antioxidants, and antimicrobial agents. The coatings and films produced from CMC are colorless, tasteless, odorless, and

hypoallergenic (Panahirad *et al.*, 2021). In the production of nanopolymers, acid hydrolysis is a widely used chemical process. This method involves the use of powerful acids to cleave the glycosidic bonds of polysaccharides and disrupt their separation areas. Furthermore, the acid attacks the crystalline sections and then the amorphous regions, resulting in the formation of fibers and nanocrystals. Nanocellulose is produced by acid hydrolysis with sulfuric acid. These nanocrystals have a negative charge because the acid reacts with the hydroxyl groups of cellulose. Sulfur groups with a negative charge on nanocrystal surfaces act as repulsion and inhibit aggregation. These are easy to isolate and purify. Ultrasound increases nanocrystal production and decreases diameter (Lu and Hsieh, 2012; Pandi *et al.*, 2021; Hassan *et al.*, 2022). Kadry (2019) successfully synthesized nanoparticles of carboxymethyl nanocellulose (NaCMNC) using acidic hydrolysis of cellulose particles obtained from rice straw residues. The cellulose particles were converted into nanoparticles and then treated with a solution of 15% NaOH and monochloroacetic acid to produce carboxymethyl cellulose nanoparticles. This approach improved drug delivery and release compared to using regular-sized carboxymethyl cellulose (Kadry, 2019). Kumar *et al.* (2019) investigated the characteristics of nanoparticles made from carboxymethyl cellulose prepared by acid hydrolysis of cellulose particles with 30% sulfuric acid. The resulting cellulose nanoparticles were then treated with a combination of 40% monochloroacetic acid and isopropanol in ratio of 15:85 to generate nano carboxymethyl cellulose. Scanning electron microscopy analysis indicated that the particle size was below 100 nm and the nanoparticles were effective in removing chromium and heavy metals from wastewater (Kumar *et al.*, 2019). Therefore, the main objective of this research was to manufacture nanoparticles of Carboxymethyl cellulose using agricultural food waste, which is one of the most common and environmentally damaging polymers. The study also aimed to investigate the properties of these nanopolymers such as their effective groups using FTIR, their size and shape using SEM and AFM size analyzer.

MATERIAL AND METHODS

Materials

The source of the rice husks used in the study was a nearby mill located in Basra Governorate. Chemicals such as 98% sulfuric acid, NaOH, NaOCl₂, acetone, ethanol, acetic acid, toluene, isopropanol, monochloroacetic acid, as well as laboratory materials from Sikma Company were also employed.

Preparation of carboxymethyl cellulose and Carboxymethyl cellulose nanoparticles

Extraction of cellulose

Cellulose was extracted from rice husks according to the method described by Lu and Hsieh, (2012),

Preparation of nano-cellulose

The method used by Phanthong et al. (2016) was adapted to prepare nanocellulose. Firstly, 10 gm of dried cellulose was weighed and mixed with 90 ml of 47% sulfuric acid in a beaker, stirring at 600 rpm using a magnetic stirrer at 45 °C for 90 min. The reaction was then stopped by adding cold distilled water, followed by centrifugation at 8000 rpm for 15 min at 10°C until the pH reached neutral. The cellulose suspension was transferred to an ultrasound device, set at 400 watts and 20 kHz for 30 min, after placing the sample in an ice bath (Nasri-Nasrabadi et al., 2014). Next, the samples were freeze-dried after placing them in a freezer at -20 °C, which resulted in nanocellulose powder.

Preparation of carboxymethyl cellulose (CMC) and nano-carboxymethyl cellulose (NMC)

Jeddi and Mahkam's (2019) method was used to prepare both CMC and NMC, which involved two steps. Firstly, 2 gm of cellulose or nano-cellulose were weighed, and 150 ml of isopropanol and 20 ml of 20% NaOH were added for alkalization pretreatment. Secondly, 3.5 gm of monochloroacetic acid were added to the mixture, along with 200 ml of ethanol solution, and the pH was adjusted to between 6-8 using acetic acid. The filtrate was discarded, and the product was washed three times with ethanol solution and dried at 40 °C for 24 hours.

Estimation of the degree of substitution for CMC and NCMC

The degree of substitution (DS) was calculated according to the method described by ASTM D-1439-03 (2005).

Characterization of polymers and nanopolymers

Polymer yield

The percentage of the Polymer yield was estimated according to Mohanasrinivasan et al. (2014) by using the following equation (1):

$$\text{Polymer yield (\%)} = \frac{\text{polymer weight}}{\text{sample weight}} \times 100 \quad (1)$$

Measurement of volume of natural cellulose using Size Analyzer

Dimensions of cellulose extracted was measured according to the method used by Chattopadhyay and Patel, (2016) using Size Analyzer type (90 Plus) of the University of Technology/Baghdad.

FESEM examination of polymer nanoparticles

The method used for this measurement was based on Pereira et al. (2014). The polymers' morphology was then examined using a Field-Emission scanning electron microscope (TESCAN Mira3 device, Model: Mira3, made in Czech Republic).

FT-IR spectroscopy for the determination of effective groups of polymers and nanopolymers

The functional groups of both polymers and nanopolymers were identified using FT-IR spectroscopy (FT/IR-4100, Jasco, Japan) at the Polymer Research Center at the University of Basra. The range of wavelengths analyzed was between 400 and 4000 cm⁻¹, following the method of Fatima (2020).

Atomic force microscopy (AFM)

The mean size of both cellulose nanoparticles and carboxymethyl cellulose nanoparticles was determined by utilizing the AA3000 atomic force microscope, as per the procedure described in Sarno and Cirillo, (2019).

Viscosity

The viscosity of both carboxymethyl cellulose and nano-carboxymethyl cellulose was determined by utilizing an Ostwald Viscometer type D, following the procedure outlined by No et al. (2000).

Water binding capacity

The method used by No et al. (2000) was followed to estimate the water binding capacity of carboxymethyl cellulose and carboxymethyl cellulose nanoparticles.

Fat binding capacity

Fat binding capacity (FBC) carboxymethyl cellulose and carboxymethyl cellulose nanoparticles estimated according to the equation (7) by No et al. (2000).

Detection of Toxicity polymers and Nano polymers

The method used by Nair et al. (1989) was followed to determine the toxicity of nano-carboxymethyl cellulose. In this method, fresh human blood (1 mL) was mixed with 20 mL of normal saline. Then, 100 µL of nano-carboxymethyl cellulose (concentration 100-1000 µg.mL⁻¹) was added to 2 mL of human blood suspension. To prepare the control sample, 100 µL of distilled water was added. The samples were then incubated at 37 °C, and the incubation was continued for 10, 30, and 60 min.

Statistical Analysis

The statistical analysis of the data followed a Complete Randomized Design (CRD) and was performed using the SPSS software (2018). The obtained results were subjected to the t-test to compare specific characteristics, and the significance level was set at 0.05. Each experiment was conducted three times.

RESULTS AND DISCUSSION

Production of Carboxymethyl Cellulose and Carboxymethyl Cellulose Nanoparticles

Table (1) displays that the yield percentages of carboxymethyl cellulose nanoparticles were lower compared to those of carboxymethyl cellulose, with values of 37.70% and 26.49%, respectively. These outcomes were similar to the findings of Rashid and Dutta (2022), who observed that the cellulose percentage in rice husks ranged from 38.53% to 54.15%, which varied depending on the rice variety. Additionally, Kargarzadeh et al. (2012) reported that the yield percentage of cellulose nanocrystals, obtained through the acid hydrolysis of cellulose using sulfuric acid, decreased from 59% to 23% as the reaction time was increased from 20 to 120 min.

Table 1 Yield Comparison between, Natural Carboxymethyl Cellulose, and Nano Carboxymethyl Cellulose

Yield%	Natural	Nano
carboxymethyl cellulose	37.36 ± 2.023 ^a	26.48 ± 1.02 ^b

Characterization of the synthesized polymers

Measurement of the size of cellulose using a Size Analyzer

The results depicted in Figure 1 indicate the average size of carboxymethyl cellulose obtained from rice husks. The figure illustrates that the mean size of carboxymethyl cellulose derived naturally from rice husks was 35.865.7 nm.

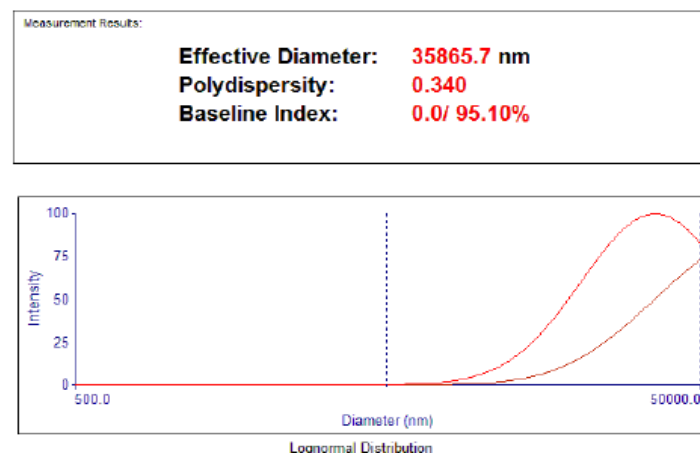


Figure 1 Size ratio using Size Analyzer for cellulose extracted from rice shells.

Characterization of cellulose by FTIR

In Figure (2), the infrared spectra of different samples including cellulose extracted from rice husks, nanocellulose, carboxymethyl cellulose (CMC), and carboxymethyl cellulose nanocrystalline (NCMC) are displayed. The C-H bond is represented by a low-intensity band observed in all samples within the range of (2900.41- 2921.63 cm⁻¹), as reported by **Zhang et al. (2016)**. Additionally, the low intensity observed between (1500-1700 cm⁻¹) in cellulose indicates the effectiveness of the extraction process and the purity of the extracted cellulose, which suggests the absence of amorphous lignin and hemicellulose compounds. These results support the findings of high crystallinity of pure cellulose observed in XRD analysis, as reported by **Ilyas et al. (2018)** and **Rashid and Dutta (2022)**. The infrared spectroscopy analysis of carboxymethyl cellulose and its nanoparticles revealed the presence of two bands at 1599.66 and 1645.95 cm⁻¹, respectively, indicating the C=O substituted carboxymethyl cellulose group. The intensity of these bands was higher in NCMC than CMC, which is consistent with the degree of substitution for these samples, which were 0.573 and 0.663, respectively (**Mondal et al. (2015)**). In contrast, the same figure showed bands between 1419.35-1427.07 cm⁻¹ and 1326.82-1370.18 cm⁻¹, indicating the stretching vibration of CH₂ and C-O-H, respectively. These bands suggest the loss of crystallization during carboxymethylation synthesis, leading to a decrease in the O-H band at 3362.28-3428.8 cm⁻¹ (**Rashid and Dutta, 2022**). The stretching vibration of the C-O-C bond in the pyranose ring in cellulose appeared at 1059.69-1119.48 cm⁻¹ (**Kumar et al., 2019**), while the stretching vibration of the β-1.4 bond was observed at 895.212-898.666 cm⁻¹ (**Bano and Negi, 2017**). These results are consistent with **Kadry (2019)**, who reported that CMC and NCMC have a similar infrared spectrum.

Table (2) displays the degree of substitution of carboxymethyl cellulose and its nanoparticles. The data indicate that the degree of substitution of carboxymethyl cellulose nanoparticles is higher than that of natural carboxymethyl cellulose, reaching 0.573 and 0.630, respectively. This can be explained by the increased surface area of the smaller particles compared to the larger ones. These findings are consistent with the results of **Rahman et al. (2020)**, who found a similar increase in the degree of substitution for smaller-sized particles. Additionally, **Thanakkasane et al. (2021)** observed a similar trend in the degree of substitution of carboxymethyl, where decreasing the size of led to an increase in the degree of substitution due to the increased surface area of smaller molecules.

Table 2 Degree of substitution of cellulose and nano-cellulose from rice husks.

Properties	cellulose	nano-cellulose
Degree of substitution	0.573 ± 0.047 ^b	0.630 ± 0.056 ^a

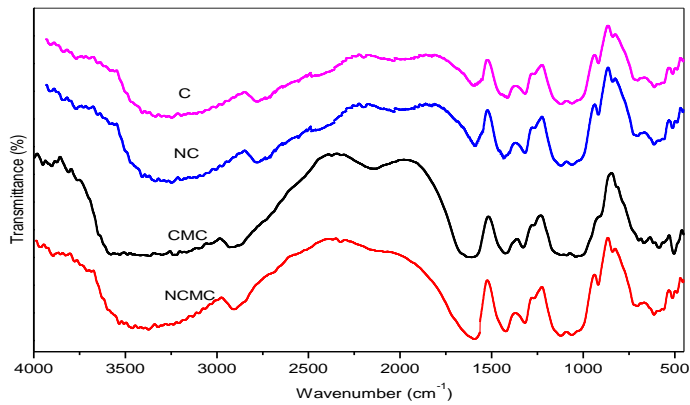


Figure 2 FTIR spectrum of natural cellulose (C), nano-cellulose (NC), natural carboxymethyl cellulose (CMC) and nano carboxymethyl cellulose (NCMC)

Crystallinity index of cellulose, nano-cellulose, carboxymethyl cellulose, and nano-carboxymethyl cellulose from rice husks

Table (3) presents the crystallization values of cellulose, nano-cellulose, carboxymethyl cellulose, and nano-carboxymethyl cellulose extracted from rice husks. The results indicate that the degree of crystallinity of cellulose was 72.27%, while that of nano-cellulose increased to 81.70%. On the other hand, the degree of crystallization of cellulose was lower than the finding reported by **Jiang and Hu (2019)**, which was 53.79%. The discrepancy in the degree of crystallization could be attributed to variations in rice varieties and differences in the extraction methods employed (**Jonoobi et al., 2015**).

The reason for the increase in the degree of crystallization of nano-cellulose is the elimination of non-crystalline regions during its production through acid hydrolysis. The acid attacks and removes the non-crystalline regions of cellulose, thereby preserving the crystalline regions. This phenomenon is dependent on factors such as the source of cellulose, type of acid used, concentration, and temperature of the reaction, as noted by **Rana and Gupta (2020)**. These findings are consistent with those of **Wan Ishak et al. (2018)**, who reported an increase in crystallinity of nano-cellulose to 74% compared to 72% for cellulose extracted

from rice husks. They also agree with **Sarno and Cirillo (2019)**, who found that the degree of crystallinity of cellulose nanocrystals extracted from cellulose residues produced by the acidic method was 83%. Furthermore, the results are in line with those of **Mondal et al. (2015)**, which demonstrated the disappearance of all the characteristic peaks of carboxymethyl cellulose obtained from corn husks after basic treatment, resulting in the disintegration and swelling of the cellulose chains, extending into the double helical regions, and leading to damage to the crystal structure.

Table 3 Crystallinity index of cellulose, nano-cellulose, carboxymethyl cellulose and nano-carboxymethyl cellulose from rice husks.

Properties	C	NC	CMC	NCMC
crystallization index	71.45± 1.00 ^b	80.21± 0.30 ^a	0.00±0.00 ^c	0.00±0.00 ^c

Exploring Surfaces at the Nanoscale with Field Emission Scanning Electron Microscopy (FESEM)

The morphology and diameter of nanoparticles made from rice husks were analyzed using FESEM at different magnifications (200, 100, 50, and 5 kx) in Figures (3) and (4). The cellulose nanoparticles (NC) had diameters ranging from 26.77-37.29 nm, while the carboxymethyl cellulose nanoparticles (NCMC) had diameters ranging from 30.90-49.65 nm and had an irregular spherical shape. These findings were consistent with previous research by **Cao et al. (2020)** and **Wei et al. (2019)**, who reported that carboxymethyl cellulose nanoparticles were spherical in shape and had diameters of 32.6 nm and 35 nm, respectively. Similarly, **Ibrahim et al. (2015)** found that the diameter of cellulose nanoparticles prepared using acid hydrolysis and ultrasound waves ranged from 19-39 nm.

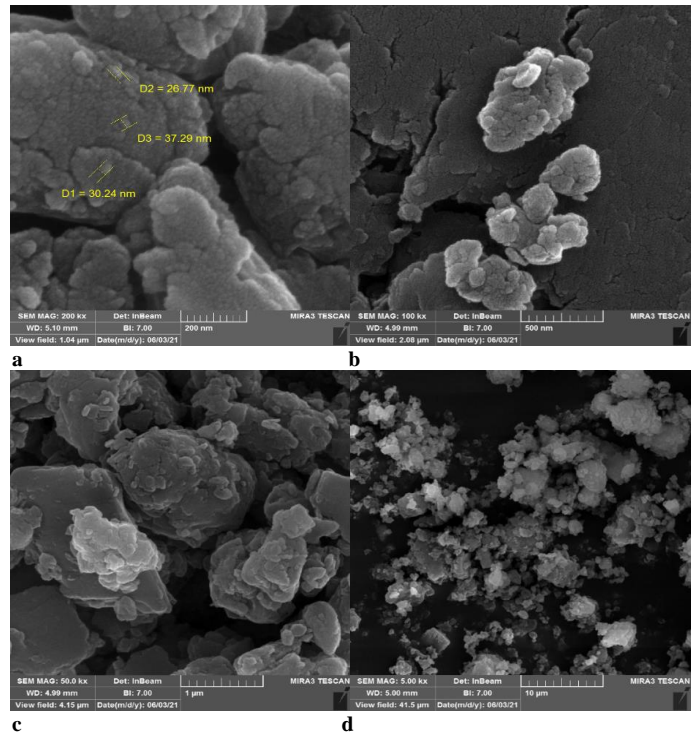
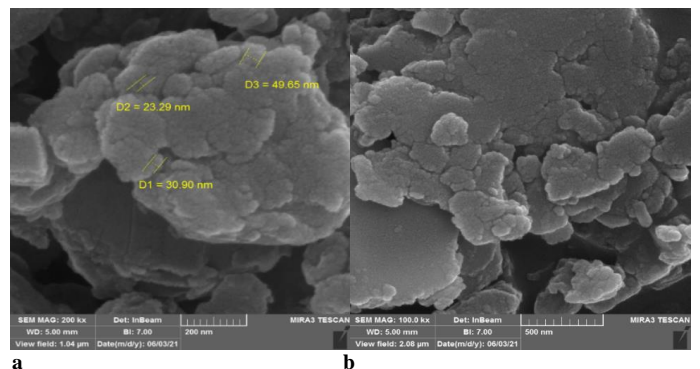


Figure 3 FESEM images of cellulose nanoparticles with different magnification (a): 200kx, (b): 100kx, (c): 50kx, and (d): 5kx.



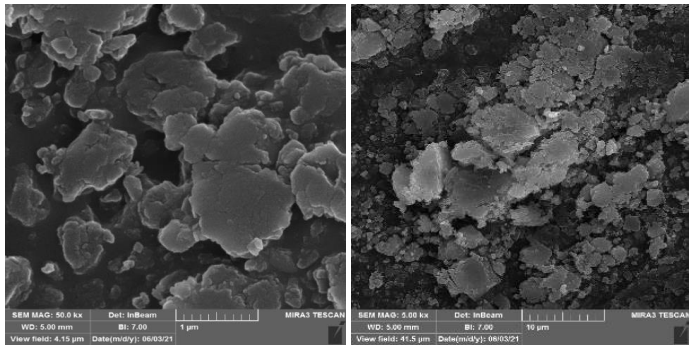


Figure 4 FESEM images of Carboxymethyl cellulose nanoparticles with different magnification (a): 200kx, (b): 100kx, (c): 50kx, and (d): 5kx.

Analysis of Cellulose and Carboxymethyl Cellulose Nanoparticles Size via Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) was used to determine the particle size distribution rate of nanocellulose and carboxymethyl cellulose nanoparticles in Figures (5) and (6). The findings revealed that the minimum size of nanoparticles was approximately 40 nm. The size of carboxymethyl cellulose nanoparticles ranged from 6.53-26.17 nm, and the topography of the surface of CMC nanofilms had an average roughness of 0.906 nm. These results were consistent with previous research by Sarno and Cirillo (2019), who used acid hydrolysis with sulfuric acid to produce cellulose nanocrystals from cellulose residues and found nanocrystal sizes ranging from 23-150 nm. Li et al. (2012) also reported on the dimensions of Cellulose Nanocrystals (CNC) prepared through acid hydrolysis with sulfuric acid, which ranged from 60-112 nm. Our study found smaller diameters for carboxymethyl cellulose nanoparticles compared to Jeddı and Mahkam (2019), who used cellulose nanoparticles with sizes ranging from 50-350 nm to create their nanoparticles, and Kumar et al. (2019), who produced carboxymethyl cellulose (NMC) from cellulose nanoparticles obtained through acid hydrolysis of cellulose fibers with average diameters of less than 100 nm. The reason for the larger diameters observed in other studies may be attributed to the tendency of polymers to aggregate, particularly during the freeze-drying stage. This explanation is supported by Lu and Hsieh's (2012) findings that hydrogen bond formation between molecules of cellulose nanocrystals can result in spontaneous assembly and larger particle sizes.

Physical properties of polymers and nanopolymers

Viscosity

Table (6) displays the viscosity and water-oil binding capacity values for carboxymethyl cellulose and nano-carboxymethyl cellulose. It can be observed that the viscosity of carboxymethyl cellulose nanoparticles was lower at 52.23 cP compared to natural carboxymethyl cellulose at 80.33 cP. The decrease in viscosity for nano-carboxymethyl cellulose compared to natural carboxymethyl cellulose can be attributed to the acid hydrolysis and high heat treatment during the preparation process, which results in depolymerization and lower molecular weight (Moorjani et al. 1975; Huang et al. 2009; Fernandes-Kim 2004). Viscosity is used as a parameter to determine the average molecular weight of a polymer using the Mark-Houwink equation (Chattopadhyay and Inamdar, 2010). These findings are consistent with those of Chattopadhyay and Inamdar (2012), who reported that viscosity decreases as molecular weight decreases.

Water Binding Capacity (WBC)

Table (4) indicates a reduction in the water binding capacity of carboxymethyl cellulose nanoparticles in comparison to their normal-sized counterparts. The water binding capacity of carboxymethyl cellulose and carboxymethyl cellulose nanoparticles was found to be 725.42% and 540.76%, respectively. This decrease in water binding capacity could be attributed to the lower molecular weight of the nanoparticles resulting from acid hydrolysis and high heat treatment during their preparation, as suggested by Moorjani et al. (1975), Huang et al. (2009), and Fernandes-Kim (2004). The results are consistent with the findings of Bidgoli et al. (2014), who reported water binding capacities of carboxymethyl cellulose from different sources ranging from 593-4227%.

Fat Binding Capacity (FBC)

Table 4 displays the fat-binding capacity of carboxymethyl cellulose and carboxymethyl cellulose nanoparticles, with the observation that the fat-binding ability of the nanoparticles is lower than that of regular weight polymers. The fat binding capacity of carboxymethyl cellulose was found to be 511.35%, while that of carboxymethyl cellulose nanoparticles was 230.25%. According to Jin et al. (2017), this is likely because the short chains in the nanoparticles tend to form individual dissolved molecules instead of trapping oil in micelles or matrices.

Table 4 Characterization of the Physicochemical Properties of Carboxymethyl Cellulose and Carboxymethyl Cellulose Nanoparticles

Properties	Carboxymethyl cellulose	Carboxymethyl cellulose nanoparticles
Viscosity	80.33± 2.75 ^a	52.23 ± 1.80 ^b
Water Binding Capacity (WBC)	725.42 ± 14.96 ^a	540.76±1.53 ^b
Fat Binding Capacity (FBC)	511.35 ± 8.70 ^a	230.25± 9.47 ^b

Detection of Toxicity Nano polymer

Figure 7 illustrates the outcomes of the evaluation of toxicity of nano-carboxymethyl cellulose produced from rice husk. The findings indicate that carboxymethyl cellulose nanoparticles, at concentrations ranging from 100-1000 µg.mL⁻¹ and after 10, 30, and 60 min of incubation, did not cause any alterations in human blood cells, such as degeneration or sedimentation. This suggests that carboxymethyl cellulose nanoparticles are not harmful to human blood, and are safe for consumption in all concentrations. Thus, these nanoparticles can be utilized in various food industries. These results are consistent with the research by Moreira et al. (2009), which found that there was no toxic effect of Nano cellulose on male mice.

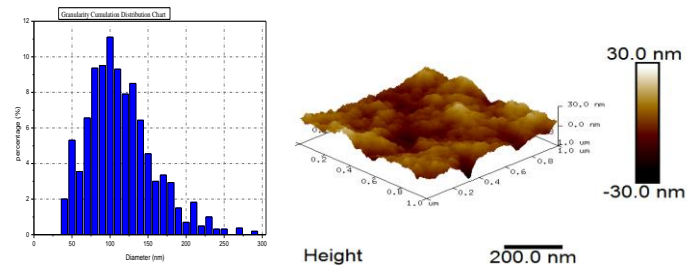


Figure 5 Dimensions of cellulose nanoparticles using AFM.

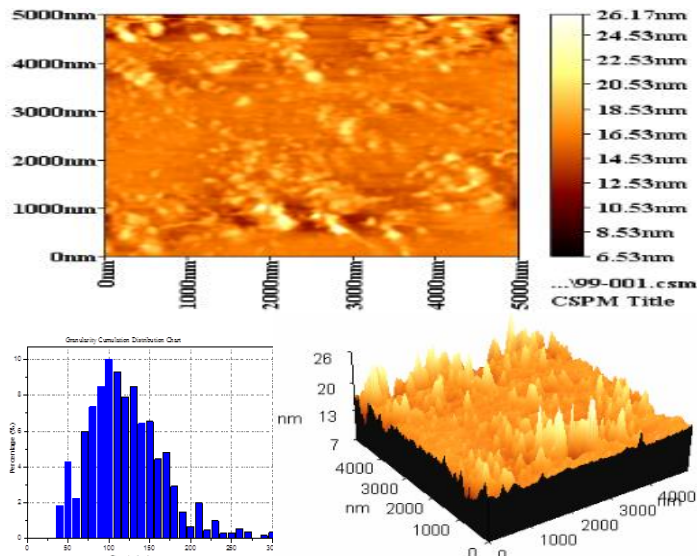


Figure 6 Characterization of Carboxymethyl Cellulose Nanoparticle Size via AFM

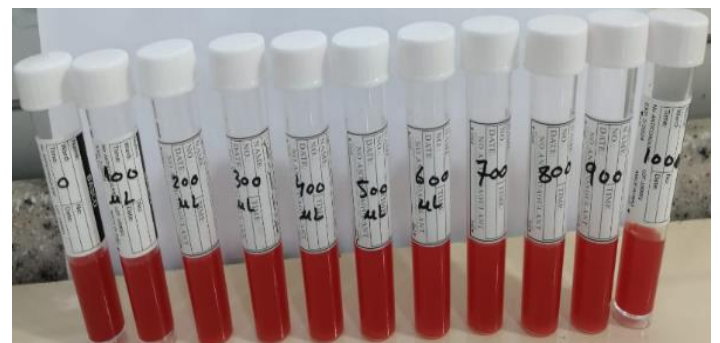


Figure 7 3.4Toxicity detection of carboxymethyl cellulose nanoparticles after 60 min.

CONCLUSION

Cellulose was obtained from rice husk waste and subsequently employed in the production of nano-cellulose, Carboxymethyl cellulose, and Carboxymethyl cellulose nanoparticles. The physicochemical properties of these materials were examined, indicating that the production of Carboxymethyl cellulose nanoparticles declined as a result of variations in their chemical composition. The Carboxymethyl cellulose nanoparticles were observed to possess dimensions inside the nanoscale range, as evidenced by microscopy experiments. The application of Fourier transform infrared spectroscopy for chemical analysis revealed that there were no significant disparities in the chemical makeup of the polymers and nanopolymers. The X-ray diffraction analysis revealed an augmentation in the crystallization index of the cellulose nanoparticles. Conversely, the Carboxymethyl cellulose and Carboxymethyl cellulose nanoparticles exhibited a reduction in crystallization as a result of the replacement of hydroxyl group bonds with Carboxymethyl groups. The results of this study demonstrate a decrease in physical characteristics, such as viscosity, water-binding capacity, and fat-binding capacity, for Carboxymethyl cellulose nanoparticles in comparison to natural polymers. Significantly, it was shown that the synthesized Carboxymethyl cellulose nanoparticles exhibited a lack of toxicity.

Statements and Declarations

Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

Author Contributions: KHAS, AGA, KMZ, ABA, TGA: software, methodology, data curation, validation, formal analysis, Conceptualization, writing-original draft, writing—review and editing.

Consent for publication: All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials: Data is available upon request.

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