PREPARATION AND PROPERTIES OF CINNAMON ESSENTIAL OIL LOADED ANTIBACTERIAL NANOCELLULOSE/CHITOSAN AEROGELS FOR BIOMEDICAL APPLICATIONS

ESAM BASHIR ABDULSALAM YAHYA

UNIVERSITI SAINS MALAYSIA

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by

ESAM BASHIR ABDULSALAM YAHYA

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LIST OF ABBREVIATIONS

A	Aerogel sample.
CFU	Colony Forming Unit.
CNF	Cellulose Nano Fiber.
CNPs	Cellulosic Nano Particles
CS	Chitosan.
DSC	Differential Scanning Calorimetry
DTG	Derivative Thermo-Gravimetry.
E. coli	Escherichia coli.
FTIR	Fourier Transform-Infra-Red.
GC-MS	Gas Chromatography–Mass Spectrometry.
GPa	Giga Pascal.
MBC	Minimum Bactericidal Concentration.
MCC	Micro Crystalline Cellulose.
MFC	Micro Fibrillated Cellulose.
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth.
MIC	Minimum Inhibitory Concentration.
mL	Milliliter
mm	Millimeter.
µL	Microliter
µm	Micrometer.
3D	3 Dimensions.
MPa	Mega Pascal.
NCC	Nano Crystalline Cellulose
Nm	Nano Meter.
S. aureus	Staphylococcus aureus.
$Sc.CO_2$	Supercritical Carbon Dioxide.
SEM	Scanning Electron Microscope.
SPP	Multiple Species
SSA	Specific Surface Area
TEM	Transmission Electron Microscope.

TGA	Thermo Gravimetric Analysis
TMOS	Tetra Methyl Ortho Silicate.
TPA	Texture Profile Analysis.
USM	Universiti Sains Malaysia
UV	Ultra Violet.
XRD	X-ray Powder Diffraction
ZOI	Zone of Inhibition

LIST OF SYMBOLS

%	Percent
+	Plus
-	Minus
±	Plus-minus
°	Degree
°C	Degree Celsius
≥	Greater than or equal to
≤	Less than or equal to
T_m	Melting temperature
T_{onset}	Initial degradation temperature
T_{max}	Maximum degradation temperature
µL	Microliter
µm	Micrometer.
Θ	Theta

PENYEDIAAN DAN SIFAT-SIFAT AEROGEL NANOSELULOSA/KITOSAN ANTIBAKTERIA TERISI MINYAK PATI KAYU MANIS UNTUK APLIKASI BIOPERUBATAN

ABSTRAK

Aerogel adalah bahan berfungsi baru yang terdiri daripada perancah gentian dan udara, tanpa air atau sebarang pelarut lain. Aerogel nanoselulosa tulen mempunyai banyak ciri unik tetapi ia biasanya digabungkan dengan bahan bukan organik untuk mengatasi kelemahan yang tidak diingini seperti penyerapan air yang tinggi dan sifat mekanikal yang lemah, yang membantu penempatan mikrob di permukaannya. Matlamat kajian ini adalah untuk mengasingkan dan mencirikan CNF menggunakan karbon dioksida superkritikal ($Sc.CO_2$) dan menyiasat peranan $Sc.CO_2$ dalam meningkatkan sifat CNF. Kajian ini juga bertujuan untuk mengarang dan mencirikan CNF antibakteria/aerogel kitosan, bersama CNF terpencil dengan kitosan dan memuatkan aerogel dengan minyak pati kayu manis sebagai agen antibakteria utama. Aerogel telah direka menggunakan pendekatan teknologi hijau; terdiri daripada teknik homogenisasi tekanan tinggi dan pengeringan beku. Pengasingan CNF mengesahkan keupayaan $Sc.CO_2$ untuk meningkatkan sifat CNF terpencil berbanding CNF yang diperoleh bukan melalui penglibatan $Sc.CO_2$, dari segi saiz zarah, sifat terma dan nilai potensi zeta. CNF yang diperolehi melalui penglibatan $Sc.CO_2$ mempunyai panjang gentian purata, dan diameter masing-masing 53.72 dan 7.14 nm, manakala CNF yang diperolehi bukan dari $Sc.CO_2$ mempunyai panjang dan diameter gentian yang lebih panjang (302.87 dan 97.93 nm). Kestabilan haba yang lebih tinggi dan nilai negatif yang lebih rendah turut direkodkan untuk CNF yang diperolehi dari $Sc.CO_2$. Pengekstrakan minyak pati kayu manis telah dijalankan menggunakan $Sc.CO_2$, yang

didapati mempunyai hasil yang rendah tetapi ketulenan yang tinggi. Aktiviti antibakteria yang tinggi dari pati minyak yang diekstrak telah dilaporkan walaupun pada kepekatan rendah, perkara ini dijelaskan dari hasil analisis kromatografi Gas yang mendedahkan kandungan Cinnamaldehyde yang tinggi (75.3%). Aerogel yang disediakan mempunyai keliangan dan ketumpatan masing-masing antara 99.1 hingga 90.8 % dan 0.0081 hingga 0.141 g/cm^3. Aerogel CNF tulen mempunyai keliangan tertinggi dan ketumpatan paling rendah tetapi menunjukkan sifat mekanikal yang lemah dan kapasiti penyerapan air yang tinggi. Pengunaan kitosan meningkatkan sifat mekanikal aerogel dengan ketara, mengurangkan interaksinya dengan air dan menjadikan aerogel kekal dalam vitro utuh. Aerogel bermuatan minyak pati kayu manis mempunyai nilai serapan air yang lebih rendah antara 7.84 hingga 14.6 g/g, berbanding dengan sampel yang tidak dimuatkan (20.4 g/g). Kesan antibakteria sinergistik minyak pati kayu manis dan molekul kitosan memberikan aerogel sifat aktiviti antibakteria yang lebih kuat terhadap *S. aureus* dan *E. coli*. Aerogel antibakteria yang disediakan mempunyai potensi besar dalam aplikasi bioperubatan seperti perancah kejuruteraan tisu sebagai tambahan kepada aplikasi pembalut luka.

PREPARATION AND PROPERTIES OF CINNAMON ESSENTIAL OIL LOADED ANTIBACTERIAL NANOCELLULOSE/CHITOSAN AEROGELS FOR BIOMEDICAL APPLICATIONS

ABSTRACT

Aerogel is a novel functional material consists of fibers scaffold and air, without water or any other solvent. Pure nanocellulose aerogel has numerous unique characteristics but it normally combined with inorganic materials to overcome its undesired drawbacks such as the high water absorption and poor mechanical properties, which favour the microbial colonization in its surfaces. The aim of this study was to isolate and characterize cellulose nano fibers (CNFs) from kenaf bast fibers using supercritical carbon dioxide ($Sc.CO_2$) and investigate its role of $Sc.CO_2$ in enhancing the properties of CNFs. The study also aim to fabricate and characterize antibacterial CNFs/chitosan aerogels, cross-linking the isolated CNFs with chitosan and loading the aerogel with cinnamon essential oil as the main antibacterial agent. The aerogel was fabricated using a green approach; consist of high-pressure homogenization and freeze-drying techniques. The results of CNFs isolation confirm the ability of $Sc.CO_2$ to enhance the properties of isolated CNFs compared with non-$Sc.CO_2$ obtained CNFs, in term of particle size, thermal properties and zeta potential value. The $Sc.CO_2$ obtained CNFs had an average fibre length, and diameter of 53.72 and 7.14 nm, respectively, while non-$Sc.CO_2$ obtained CNFs had longer fibre length and diameter (302.87 and 97.93 nm). Higher thermal stability and lower negativity value was also recorded for $Sc.CO_2$ obtained CNFs. Extraction of cinnamon essential oil was conducted using $Sc.CO_2$, which was found to be of low yield but high purity. Great antibacterial activity of the extracted essential oil was reported even at low

concentration, which explained by the results of Gas chromatography analysis that revealed the high content of Cinnamaldehyde (75.3%). The prepared aerogel had porosity and density ranged from 99.1 to 90.8 % and 0.0081 to 0.141 g/cm3 respectively. Pure CNFs aerogel had the highest porosity and lightest density but it showed poor mechanical properties and high water absorption capacity. Introducing chitosan significantly enhanced the mechanical properties of aerogel, reduced its water uptake and made the aerogel remain in vitro intact. Cinnamon essential oil loaded aerogels had lower water absorption values ranged from 7.84 to 14.6 g/g, compared with the unloaded samples (20.4 g/g). The synergistic antibacterial effect of cinnamon essential oil and chitosan molecules give the aerogel stronger antibacterial activity against *S. aureus* and *E. coli*. The prepared antibacterial aerogels have great potential in biomedical applications such as tissue engineering scaffolds in addition to wound dressing application.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Biopolymers defined as naturally occurring materials consist of repetitive monomeric units that covalently bonded to form larger molecules, such as cellulose, collagen, and alginates, etc. In the past few years, biopolymers have been extensively used in production of different materials including films, papers, hydrogels and aerogels for different biomedical applications due to their unique properties such as biocompatibility, non-toxicity and non-immunogenicity, in addition to their availability, biodegradability and eco-friendly (Yahya et al., 2020).

Cellulose is the most abundant biopolymer on earth and has been extracted from a variety of renewable and sustainable sources (Isa et al., 2022). Nanocellulose products have received much attention due to their outstanding properties, which include a high tensile strength, a high Young's modulus, high flexibility, and a low coefficient of thermal expansion (Jaafar et al., 2021). There are a variety of nanocellulose production methods, such as chemical, mechanical, chemo-mechanical, and enzymatic treatments, that have been widely studied and used in research and industry environments. However, most of these methods require the use of cellulose-rich fibers and powders as raw materials. Plants with high lignin contents are unsuitable for nanocellulose production (Jonoobi et al., 2012), which limits the output of nanocellulose and increases production cost. Therefore, a method that can effectively utilize plants with high lignin content to generate cellulose nanofibers is highly desirable. As one of the most traditional lignocellulosic fiber plants, the abundantly available kenaf is a potential source of nanocellulose because of its high cellulose content and high crystallization (Tuerxun et al., 2019).

Chitosan is another biopolymer with cationic nature from the glycosaminoglycan family. It is one of the most abundant biopolymers that has been extracted from multiple sources such as; the shells of crustaceans, cell walls of fungi, and exoskeleton of arthropods etc (Ambarish and Sridhar, 2015). Chitosan has been reported to be insoluble in water and form an aqueous alkaline solution. The convenient mechanism resulting from pH-dependent solubility of this polymer allow its processing under mild conditions and the ability to form various shapes and sizes of hydrogel or aerogel (Rinki et al., 2011).

Aerogel was defined as a solid, ultra-lightweight and lucid open porous network, obtained from gel following the removal of the pore liquid without any significant modification in the network structure (García-González et al., 2019). S. Kistler made the initial aerogel in 1931, as a result of replacing the liquid of the gel with gas without changing the structure intact. He used silica gel as a precursor material in his first preparation (Ziegler et al., 2017). Few years later, aerogel have been steadily developed and it was prepared from organic, inorganic, hybrid and even different composites of materials.

Traditional materials for aerogel preparation like silica, carbon, metals, and synthetic polymers present many drawbacks, which limitate their use in biomedical applications. These drawbacks include immunological rejection by the body and their high cytotoxicity, which may cause undesirable immunogenic response (Rebelo et al., 2017). Thus, most of recent studies have focused on using biopolymers such as cellulose, chitosan and alginates, etc., instead of inorganic or traditional oil-based materials (Zhao et al., 2018). The isolation of such biopolymers, enhancing their properties and the synthesis of biopolymers based materials have witnessed significant

development the past view years, and many scientists have joined the race to achieve novel development in this battle.

Essential oils have been used for centuries as food additives, fragrance and in medical therapies. Their antimicrobial effects have recently been re-evaluated and confirmed. Cinnamon essential oil is one of these essential oils that have been used since ancient times in China, Africa, India and Australia (Zhang, Y. et al., 2016). In recent years, cinnamon essential oil has been applied in numerous application as antimicrobial, antioxidant and anti-carcinogenic agent, as well as food, seasonings and cosmetics applications (Wong et al., 2014). Taking this in consideration, this study was designed to utilize the antibacterial activity of cinnamon essential and incorporate it with biopolymer aerogel, aiming to develop antibacterial aerogel for biomedical applications such as wound healing and tissue engineering scaffolds. The incorporation of cinnamon essential oil as antibacterial agent in biopolymers aerogels can be of great significance in the biomedical field to overcome the toxicity of antibiotic and compete the antibiotic resistance of the pathogens.

1.2 Problem statement

The production of nanocellulose has attracted tremendous attention in the past few years due to its suitability for wide range of applications including medical and nonmedical applications. Numerous techniques have been used to produce CNFs with slightly different properties depending on the extraction conditions as well as used chemical agents. Many of these techniques either using toxic chemicals or expensive approaches, which eventually raise the production costs and/or reduce the quality of CNFs due to the possible chemical contamination. Supercritical carbon dioxide ($Sc.CO_2$) is an environmentally friendly, inexpensive and essentially nontoxic

technique has attracted great attention in the past few years in extraction and isolation applications, which use relatively moderate critical temperature (30 to 80 °C) and pressure (70 to 120 bar) (Baldino et al., 2020; Seghini et al., 2020). The exact role and the mechanism of Sc.CO_2 in CNFs isolation is not yet fully investigated, many researches have reported using Sc.CO_2 approach without comparing the outcomes and highlighting the effect of Sc.CO_2 on the isolated fibers. Thus, this study will initially aim to investigate the role of Sc.CO_2 in CNFs isolation and compare the properties of Sc.CO_2 and non-Sc.CO_2 obtained CNFs to choose the best approach for the further investigations.

The past few years witnessed great advances in fabrication of aerogel scaffolds using different materials able to provide a proper micro-environment for the proliferation and interaction of variety of human cell types, leading to formation of tissue or a whole organ (Nezhad-Mokhtari et al., 2020). The aerogels also have been used in other medical applications such as wound dressing, due to their aeration, which is an important character in wound healing process. In order to develop aerogels for such applications the precursor material should possess superior properties to ensure the proper growth of cells and to avoid any possible toxicity or microbial contamination. The addition of natural antibacterial agent in these scaffolds will significantly enhance their properties, to become superior compared with the current and conventional materials. From different aspect, cellulose molecules contain the preponderance of hydroxyl functional groups, it tend to possesses a strong affinity to water, making the pure cellulose aerogel collapse in liquid environment, which is a major issue in tissue engineering as well as wound dressing application. Cellulose aerogel also attract the moisture on its surfaces, which favour the microbial growth, as it does not possess any antibacterial activity, just like many of conventional wound

dressing materials, which lead to bacterial infection if no disinfection used or the wound was not clean from microorganisms. Moisture from air at high levels is able to diffuse into pure cellulose aerogel in three ways; primarily via the inter-polymer chain micro-gaps (Alhuthali et al., 2012). To a lesser degree, via the fibre and matrix interfaces through capillary transport tracing any gaps and/or flaws caused by incomplete wettability and impregnation, and finally, via micro-cracks in matrix which result from cellulosic fibre swelling (Li et al., 2018).

Taking these issues in consideration, this study was designed to enhance the mechanical properties of pure nanocellulose aerogel by introducing chitosan into the system, which also cross-linked with the cellulose making the aerogel remain intact under water. Furthermore, the antibacterial properties of chitosan wasn't sufficient to inhibit the bacteria, thus cinnamon essential oil was loaded into the aerogel texture to produce antibacterial aerogel able to eliminate the growth of gram positive and gram negative bacteria. Natural antibacterial aerogel has sufficient absorption ability of wound exudates, superior mechanical behaviour and strong antibacterial activity. The use of biopolymers and natural antibacterial agent without any chemicals or toxic approach will produce biocompatible and non-immunogenic material suitable for different biomedical application such as wound dressing or tissue engineering scaffolds. Contrasting many wound dressing materials, aerogels, with its high porosity, allow oxygen permeability, which is prim necessary for the normal healing process and in the same time prevent the possible microbial contamination resulted from exposing the wound to open air (Kur-Piotrowska et al., 2018). The use of natural antibacterial aerogels in biomedical application can be of great significance in the materials and biomedical fields.

1.3 Objectives of the study

The aim of this research was to isolate cellulose nano fibers from Kenaf fibers and fabricate natural antibacterial CNFs based aerogels for biomedical applications. However, in order to achieve this, the research has several objectives including:

1- To isolate and characterize cellulose nanofibers (CNFs) from Kenaf baste fibers using supercritical CO_2 ($Sc.CO_2$) and investigate the role and physical mechanism of $Sc.CO_2$ in enhancing the properties of isolated CNFs.

2- To evaluate the antibacterial activity, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of cinnamon essential oil extracted from raw cinnamon sticks against selected pathogenic bacteria.

3- To prepare and characterize cinnamon essential oil loaded and non-loaded antibacterial nanocellulose/chitosan aerogels for biomedical applications.

1.4 Structure of thesis

This thesis consists of five chapters. The first chapter contains a generalized background of the study, a simplified picture of the aerogel development, preparation and applications, presenting the importance of biopolymer based aerogels in biomedical applications. It also addresses the justification of using supercritical approach in the isolation of CNFs and the defects of using pure CNFs aerogel in biomedical applications in addition to the proposed approaches to overcome these defects. The second chapter is a review of literatures and previous research that have been done in the field of nanocellulose properties and isolation in addition to the development of nanocellulose based aerogels. It also provides a brief picture of the preparation techniques, properties and applications of nanocellulose based aerogel. In the third chapter, materials and methods of the study are presented, explanation of the

preparation and characterization approaches that have been done in this study. The results and their analysis are covered in details in chapter four, which also contains critical discussion of presented results and the suitability of prepared material for biomedical applications. Finally, chapter five addresses the conclusions reached by the research in summary and recommendations that came out.

CHAPTER 2

LITERATURE REVIEW

2.1 Biopolymers

Biopolymers defined as naturally occurring materials consist of repetitive monomeric units that covalently bonded to form larger molecules, such as cellulose, collagen, and alginates, etc. In the past few years, biopolymers have been extensively used in aerogel preparation for different biomedical applications due to their unique properties (Subrahmanyam et al., 2015). Cellulose and chitosan are the most used biopolymers in biomedical applications due to their unique biological characteristics such as hemostatic properties, antibacterial and mucoadhesive properties which are useful for wounds healing, drug delivery and tissue engineering applications.

2.2 Kenaf bast fibers

Kenaf bast fibre (*Hibiscus cannabinus L.*) is an essential natural fibre with antibacterial properties, excellent air permeability, and high mechanical properties. The plant has high economic potential with ecological advantages making it survive a wide range of weather conditions (Li, W. et al., 2016). This has made its cultivation easy, as in three months of plantation it grows to the mature stage of about 3 m tall and with a base diameter of 3–5 cm. In addition to its excellent economic potential, kenaf fibers have gained considerable attention due to their high cellulose content (Rizal et al., 2021a). During the isolation of cellulose, these compounds must be removed or at least eliminated. Lignin is bonded with hemicellulose and cellulose by ester linkages and hydrogen bonds. Lignin present in the cell walls of cellulosic fibres has been essentially responsible for the structural integrity of these fibres together with cellulose. Thus, natural or vegetable fibres in most cases (except for cotton) have been termed as lingo-

cellulosic fibres (George and Sabapathi, 2015). The complex structure of lignin linkages with hemicellulose forms a matrix around cellulose molecules and results in it being very tough and difficult to break the network. The connection between the sugar monomer formed long chains and the interaction between these chains generates strong intermolecular forces between them. The high linearity of the cellulose molecule also results in a tightly packed nature of cellulosic fibres (Hurtado et al., 2016).

The polymeric structure and linkages of the polysaccharide resulted in the fibre's strong intermolecular forces between its chains. The high linearity of the cellulose molecule accounted for the crystalline nature of cellulosic fibres (Hurtado et al., 2016). Many procedures have been developed to produce cellulose fibre, for example, the preparation of spinnable solutions from cellulose using different solvents and conditions (Mahltig et al., 2017). Viscose, modal, or lyocell cellulosic fibres can be generated with different physical properties such as the degree of crystallinity, molecular orientation, and chemical properties (adsorption of water and dyes) (Hokkanen et al., 2016). These fibres are usually produced with a significant number of inorganic additives, which are embedded inside the cellulosic fibres. The embedding of inorganic particles is essential to the creation of new fibres with different functional properties. The embedded inorganic particles act as the carrier of the functional properties. Cellulosic fibres are extensively used in many applications such as the manufacture of different types of apparel due to its comfort and ability to absorb moisture, making it comfortable against the skin (Seabra et al., 2018). In most cases, cellulosic fibres have been extensively blended with other polymeric fibres such as polyester and spandex, especially in the manufacturing of shirting.

2.3 Cellulose

Cellulose has been reported as the most abundant, naturally sourced biopolymer, with 150 billion tons produced yearly (Bajwa et al., 2019). Plants mainly consist of cellulose, which provides them with their structural integrity. The use of naturally occurring polysaccharides for industrial applications has drawn the attention of many researchers.

2.3.1 Cellulose fibre architecture

Cellulose is a natural polysaccharide and consists of repeating units of monomer glucose connected through (1,4) beta D linkages. Plant biomass is composed of cells surrounded by primary and secondary cell walls. Cellulose and hemicelluloses are the main components of plant cell walls and form 34–75% of the primary cell wall and 50–80% of the secondary cell wall (Madsen and Gamstedt, 2013). The aggregation of many cellulose molecules form fibrils. The fibril bundles combine to form cellulose fibres. The fibrils have both crystalline (ordered) and amorphous (disordered) regions that are the main reinforcement segment for plants, trees, algae, some marine creatures, and bacteria (Moon et al., 2011). The purity of bacterial cellulose is higher than that of plant-based cellulose. However, bacteria cellulose and plant cellulose have similar crystallinity, water retention capability, tensile strength, and biocompatibility.

These characteristics of isolated cellulose are dependent on the source and method of preparation (Jozala et al., 2016). Figure 2.1 presents the hierarchical structure of cellulose, cellulose nanocrystals, and micro/nanofibrillated cellulose (the upper part of the figure), while the lower part presents the structure of bacterial cellulose. Both micro- and nanocrystalline cellulose can be produced by the extraction of crystalline regions from native cellulose using acid hydrolysis of disordered/amorphous regions, which link together assemblies of microfibrils (de Campos et al., 2019). Depending on

the conditions of extraction, the crystalline region of the microcellulose can significantly vary in size and aspect ratio. This usually results in different types of fibrils, crystalline, and particle sizes (which may be in micro- or nano-size). However, they are normally anisometric (Sotnikova et al., 2018).

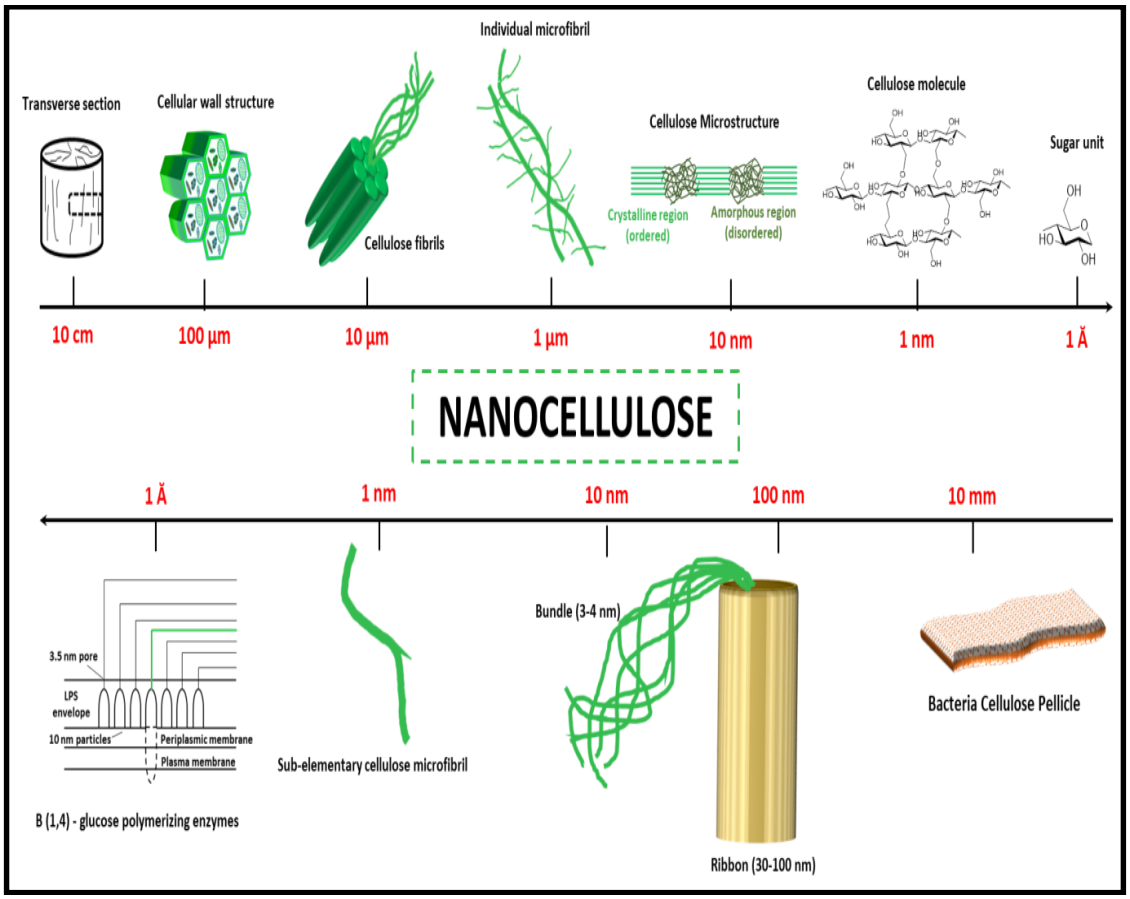

Figure 2.1 The hierarchical structure of cellulose. The upper part: plant cellulose; the lower part: bacterial cellulose. Adapted from (Jozala et al., 2016).

2.3.2 Cellulose Micro and Nano Materials

The micro-sized form of cellulose has two basic forms: micro fibrillated cellulose (MFC) and microcrystalline cellulose (MCC). The definition of these forms is based on the features in the physical and microstructural view of the material. These views differentiate the properties of each form and their effect when used as reinforcement in materials. Cellulose microfibrils are composed of 18 or 24 cellulose

chains with a variety of cross-sectional shapes, which are the original structure present in the natural plant (Wang and Hong, 2016). MFC is produced from lignocellulosic biomass by mechanical treatment. The size distribution of MFC is wide, even if some fibres have diameters in the nanoscale. Microcrystalline cellulose has been described as a purified, partially depolymerized cellulose. It has been sourced and isolated from woody and non-woody lignocellulosic materials and purified cotton linters (Sorieul et al., 2016). It has been reported that cellulose microfibrils vary in size from 0.2 to 374 μm. The micro-fibrillated cellulose chain aggregates together to form macro-fibrillated chains (Sreekumar et al., 2019). However, microcrystalline cellulose has been reported as the non-amorphous region of the chain, which is divided into a well-organized crystal-surface and poorly organised crystal interior (Oehme et al., 2015). However, the amorphous or non-crystalline region has been attributed to mechanical damage or wood pulping treatment rather than the state of native cellulose (Sorieul et al., 2016).

Micro-fibrillated cellulose is a micro-size fibrillated unit obtained through a fibrillation process of cellulose fibres using mechanical shearing with or without enzymatic or chemical pre-treatment (Kataja et al., 2017). The small diameter of 3D fibrils allowed them to form a network of microfibres with an extremely large surface area. Micro-fibrillated cellulose has the typical structure of cellulose with crystalline and amorphous regions. It possesses a high viscosity and yield stress. It is sheer thinning and has a high water-holding capacity. The size distribution of these fibres are wide, their length varies from 50 to more than 900 μm, and the width from nano-size in some cases to micro-size (Saputro et al., 2017). MCC is often prepared by the acid hydrolysis of cellulose at high temperatures. The reactive amorphous regions of the cellulose are selectively hydrolysed, which releases the crystallites out of the chains. The results of the acid hydrolysis of the aqueous suspension have often been referred to as

microcrystalline cellulose suspensions (George and Sabapathi, 2015). The particle size of isolated microcellulose materials is dependent on the cellulose source, pre-treatment operation, and the preparation process.

Nanocellulose materials include materials with one, two, or even three external dimensions in the nanoscale (1–100 nm), and nanostructured cellulose materials with an internal composition of inter-related constituent parts in which one or more of these parts is in the nanoscale. Cellulose nano objects include cellulose nanocrystals, cellulose nanofibrils, and cellulose nanoparticles. At the same time, nanostructured cellulose materials include cellulose microfibrils, micro-fibrillated cellulose, and bacterial cellulose (de Campos et al., 2019). Nanocellulose is often used by researchers to enhance the strength of biomaterials. Nanocellulose possesses high mechanical strength and environmental sustainability (Nuryawan et al., 2020). Many researchers have categorized nanocellulose materials into three main types (Figure 2.2): nanofibrillated cellulose, nanocrystals, and nanoparticles (Kim, J.-H. et al., 2015). These three types have a relatively similar chemical composition, but different degrees of crystallinity, particle size, and morphological properties (Abdul Khalil, H. et al., 2020). Soyekwo et al., describe nanofibrillated cellulose as long flexible nano-size fibres, 1–100 nm in diameter and 500–2000 nm in length, and chemically composed of 100% cellulose with both crystalline and amorphous regions (Soyekwo et al., 2017). The average size of CNF particles varies and depends on the method of preparation. Recently, CNF has been economically produced from various cellulose sources such as pulp and cotton via a series of surface modifications to enhance their performance in the desired application (Lee, 2018).

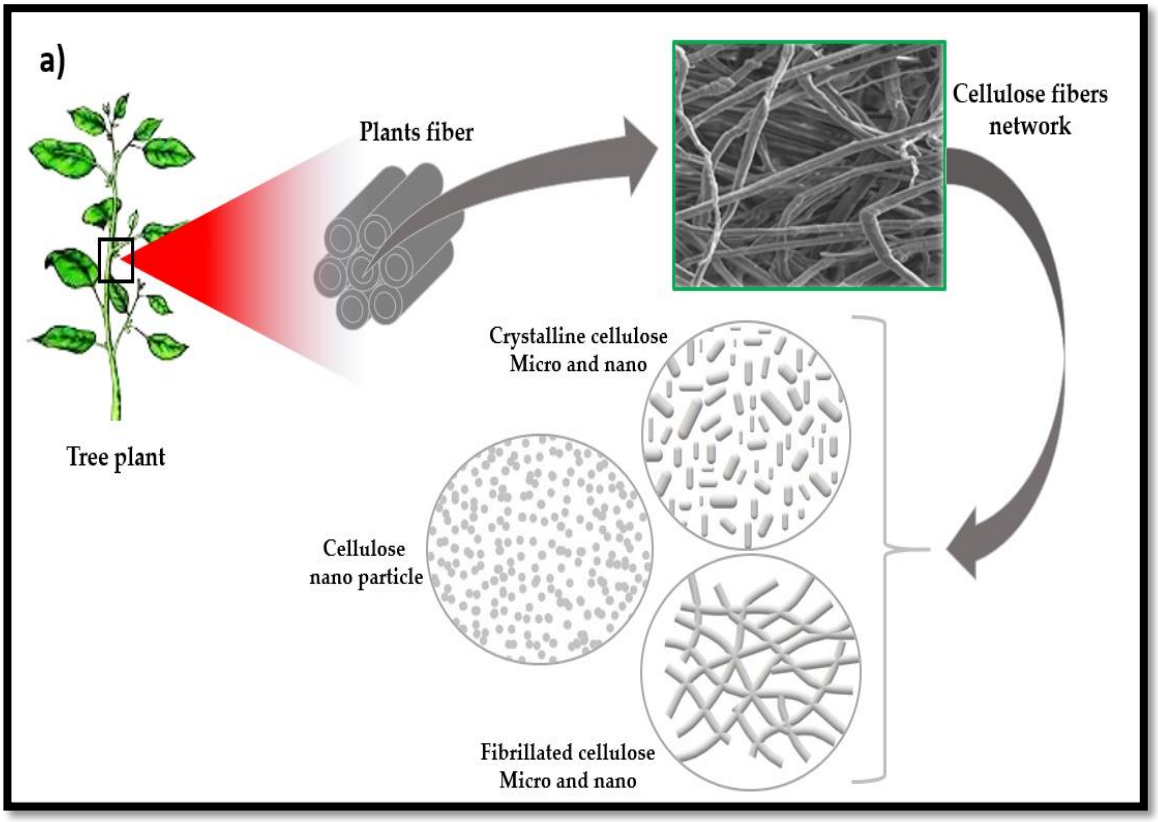

Figure 2.2 Classification of cellulose natural fiber into; micro and nano crystallines, micro and nano fibers and nano particles. Adapted from (Abdul Khalil, H.P.S. et al., 2020b)

Nanocrystalline cellulose (NCC) has been extracted from cellulose fibres by acid hydrolysis and characterised with high strength (Raza et al., 2019). CNF has otherwise been called cellulose nano-whiskers or cellulose nanocrystals (Abraham et al., 2017). Isolated NCC has been described as shorter and has a rod-like shape compared to CNF. The acid hydrolysis method used by (Okahisa et al., 2018) was to reduce the average diameter using an ultra-turrax and micro-fluidizer. A similar diameter has been obtained by (Lu, Y. et al., 2019). The diameter of NFC is diverse based on the preparation process (Jiang and Hsieh, 2013), the authors obtained NFC with a small diameter of 15 nm by using the blending method, which was smaller than the one obtained with the electrospinning process (70–130 nm) by (Ohkawa et al.,

2009). Over the past few years, NCC has been investigated for many medical applications including tissue-engineering scaffolds. Its biocompatibility and other properties that are similar to NFC make it suitable for in vitro and in vivo usage. Cellulosic nanoparticles (CNPs) are another cellulose-based nano-sized short structure material of cellulose. Gu et al. (Gu et al., 2019) characterised CNPs and described them as short needle or ribbon-like particles. Furthermore, their properties are dependent on the source of the cellulose, and it appears as a rod or spherical shape. CNPs have been prepared by various hydrolysis methods, the removal of the amorphous regions of cellulose, and dispersal of the crystalline region (Prud'homme et al., 2020).

2.4 Extraction and characterization of nano cellulose

Cellulose is the most abundant polymer on earth, it presents in all plant cell walls, in algae, some fungi, marine organisms of tunicates family, invertebrates, and some Gram-negative bacteria (Tayeb et al., 2018). Plant fibres are relatively in abundance and renewable. It is widely reported that nanofibres are extracted from plant cellulose fibres. A plant cell wall comprises primary and secondary cell walls. A primary cell wall has a primary wall (thin outer layer), and a secondary wall is composed of three layers.

2.4.1 Methods of nano cellulose extraction

The plant primary cell wall consists of 9–25% cellulose microfibrils, compared to the secondary wall that contains 40-80%. Furthermore, the primary cell wall consists of 25–50% and 10–35% of hemicelluloses and pectin, while the secondary cell is composed of only 10–40% hemicelluloses and 5–25% lignin (Madsen and Gamstedt, 2013). However, the precursor material referred to as lignocellulose biomass should be cleaned and dried. After the isolation of lignocellulose biomass, it contains many

accompanying materials apart from cellulose such as hemicellulose and lignin. The removal or elimination of accompanying materials required as hemicellulose carries carboxylic groups, which gives the final CNFs a slight negative charge causing repulsing between the fibres and prevents their aggregation in the wet state (Fall et al., 2011).

Isolation of CNFs can be conducted in two main steps, which are the pretreatment and production steps. The first step involves the pretreatment of cellulose fibres while the second step is the production of cellulose nanofibers (Lotfiman et al., 2018). In order to isolate NC from lingo-cellulosic biomass, it must undergo delignification, which is a necessary process consisting of pulping to depolymerisation and then solubilises lignin and hemicelluloses, followed by bleaching with chemical agents. Native cellulosic fibres have to be treated in strongly acidic conditions in order to hydrolyse the amorphous fractions of cellulose and form simple highly crystalline rod-like cellulose nano-whiskers. These whiskers have been termed differently in many works of literature such as nano-whiskers, cellulose nano fibres, cellulose nanofibrils, micro fibrillated cellulose or nanofibrillated cellulose. During the pulping step, the fibers are reduced, and lignin is solubilized to obtain cellulose fiber using several chemical methods: (i) Sulfite pulping consists of a process where the wood chips are treated with H_2SO_3 and HSO_2^- ions to disrupt lignin from cellulose fibers (Garcia et al., 2016). (ii) For the Kraft process, the wood chips are submitted to NaOH and NaS_2 treatments using high temperature and pressure to remove lignin from cellulose. (iii) In the soda process, which is the most employed process for agro-industrial residues, and NaOH at high temperatures is used to solubilize lignin from cellulose fibers (Sharma et al., 2020). In addition, chemical-mechanical pulping combines the chemical and mechanical refining treatments, such as wood cooking with NaOH to remove lignin

from cellulose fibers, and then they are submitted to mechanical treatment. After that, the use of enzymes (namely, xylanases and laccases) has been increasingly employed as an environmentally friendly strategy to eliminate the remaining lignin in the pulp and to reduce the chemicals consumption in the process (Chaurasia and Bhardwaj, 2019). Finally, the bleaching process consists in the complete removal of lignin using chemical, gases, and steam, to provide the required color for paper manufacturing. Figure 2.3 shows the steps of cellulose nano fibres isolation from lignocellulose biomass.

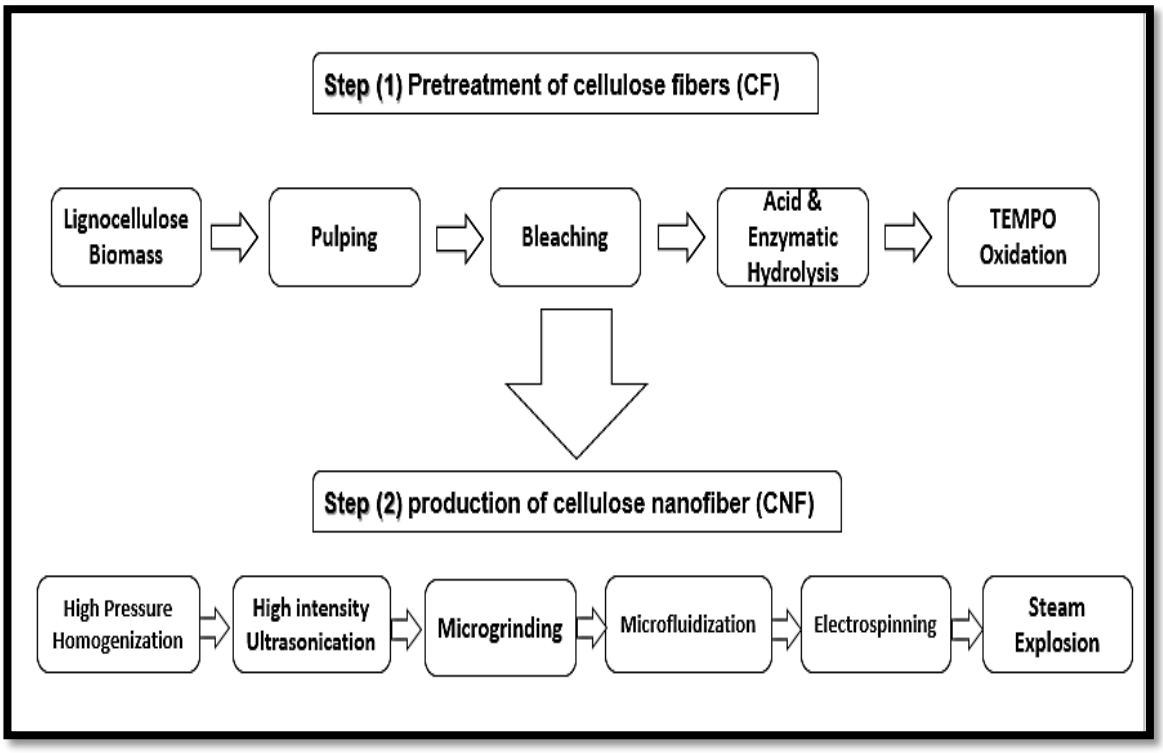

Figure 2.3 Steps of plant cellulose nano fibres isolation from lignocellulose biomass

Cellulose nanofibers can be achieved by isolation from biomass at high shear forces using high-pressure homogenisers. One of the methods of preparing CNF is by the reinforcement of natural and synthetic matrices to form a polymer composite. Bamboo CNF was prepared using high-intensity ultrasonication by the acidification of sodium chlorite at 75 ^{o}C for 1 hour (Keplinger et al., 2019). The result obtained revealed

that the hydrolysis of the enzyme has the potential of enhancing the surface charge and the mechanical activation of the degree of freedom of carboxymethyl. Modification of nanofibrils can be achieved by esterification of the primary and secondary hydroxyl group present in CNF by mechanical disintegration.

2.4.1(a) Extraction of nano cellulose using supercritical carbon dioxide

Supercritical fluid extraction, particularly supercritical carbon dioxide assisted extraction, has been extensively used for the recovery of several types of natural compounds. However, studies reporting the use of supercritical carbon dioxide assisted extraction of recovery cellulose from agro-industrial are limited. Albarelli and colleagues reported the production of nanocellulose from sugarcane bagasse using supercritical carbon dioxide assisted extraction coupled with steam explosion or organosolv as pre-treatments, followed by enzymatic hydrolysis (Albarelli et al., 2016). Cassava pulp waste was treated for 60 min and 120 min at variable temperature (40°C to 80°C) and pressure (8 MPa to 20 MPa). Following supercritical carbon dioxide treatment, surface area of extracted cellulose was enhanced by 16%, due to better dissociation of the cellulose fibrils. Increasing pressure, improved thermal stability. An increase in crystallinity (5%) was observed when extending the treatment time from 60 to 120 min (20 MPa, 60°C). Kenaf fibers, bleached using a chlorine free treatment, were subjected to supercritical carbon dioxide assisted extraction (50 MPa, 60°C, 2 hr), followed by mild acid hydrolysis to produce cellulose nanofibers (Nanta et al., 2019). Recovered cellulose nanofibers had diameter ranging from 10 to 15 nm and a crystallinity index of 92.8% (Atiqah et al., 2019). Cold plasma assisted extraction is considered as an emerging green technology for the recovery of natural compounds. As far as our literature search could ascertain, there is a dearth of scientific studies reporting

the use of cold plasma assisted extraction for the extraction of cellulose from agro-industrial by-products for the production of nanocellulose.

2.4.2 Characteristics of cellulose nano fibers

Cellulose has many advantages over the conventional and the other biopolymers of being the most abundant biopolymer on earth. It can be extracted from different sources, giving material with slightly different characteristics (Manzato et al., 2018; Meng et al., 2019; Yazdanbakhsh et al., 2017). Apart from plants; cellulose have been extracted using static culturing of many types of bacteria such as *Acetobacter xylinum* (Lotfiman et al., 2018) but bacterial cellulose has a the advantage of having higher degree of crystallinity (Long, L.-Y. et al., 2018) and occur in pure form without any impurities such as lignin and hemicellulose. Bacterial cellulose is a type of cellulose produced by certain bacteria such as *Acetobacter xylinum*. *Acetobacter xylinum* is an extracellular product and a unique form of cellulose produced in nature. It is very pure cellulose with a narrow size distribution and high crystallinity (Huang et al., 2017). Table 2.1 presents the main differences between plant-based cellulose and bacterial cellulose.

Table 2.1 The main differences between plant-based cellulose and bacterial cellulose.

Properties	Plants Cellulose	Bacterial Cellulose	References
Purity	Moderate to low	High	(Pacheco et al., 2018)
Crystallinity degree	54–88%	65–79%	(Mishra et al., 2018)
Degree of polymerization	Ranged from 500–15,000	800–10,000	(Klemm et al., 2005)
Availability	Highly available	Limited	(de Amorim et al., 2020)
Industrial-scale production	Limited	Very limited	(de Amorim et al., 2020)

2.4.3 Physical and chemical properties

Cellulose macromolecules are assembled in the semi-crystalline filament. The macromolecules consist of micro fibres and nano fibres. The three-carbon monomeric units in the cellulose chain are bonded to the hydroxyl groups (O'Connell et al., 2008). The hydrogen bonds play an important role in the structure of cellulose and the physicochemical properties of the molecule. The remarkable properties of cellulose nano fibers such as low density (1.6 g/cm^3), high elasticity modulus (110-220 GPa), high purity, attractive aspect ratio (10-100) in addition to high tensile strength (≥ 3 GPa) and surface area, making it one of the most utilized biopolymers in various applications (Long, K. et al., 2018).

The bond between a group of cellulose chains leads to the formation of micro-fibrils, which are jointly grouped to form typical cellulose fibres. Two different ends of the microfibrils in each cellulose polymeric chain possess carbonyl functionality and a supplementary hydroxyl group in carbon number 4. Stiffening of the cellulose chain has been a direct result of the banned free rotation of the cellulose rings by hydrogen bonds along with their linked-glycosidic bonds. However, the interactions of the van der Waals and hydrogen bonds lead to the formation of either crystalline ordered regions or amorphous disordered regions of the unique structure of cellulose (Borges et al., 2015). The crystalline form has been known as type I, or cellulose I, and consists of a mixture of alpha-cellulose, beta-cellulose, triclinical structure, and monoclinical structure. Cellulose II is irreversible thermodynamically and more stable than cellulose I. Cellulose II can be prepared by treating the polymer with a concentrated alkaline solution such as sodium hydroxide. Different treatments of cellulose will lead to the generation of other crystal polymorphs such as cellulose III, IIIII, IVI, and IV (Trache et al., 2016). The term microcellulose describe all forms of cellulose materials with

diameters in the micrometre range, typically from 1 μm to 1000 μm, while nanocellulose describes the forms of cellulose-containing dimensions in the nanoscale (1–100 nm) (Sotnikova et al., 2018).

Nano cellulose particles are rigid and stiff due to the hydrogen bonding, which gives the material high crystallinity, their surface also rich in hydroxyl groups (–OH) leaving it open to endless modification and functionalization possibilities. The high surface area of nano particles and the chemistry of cellulose making it suitable for loading various types of drugs (Panchal et al., 2019). Another advantage of cellulose-based nanocomposites in drug delivery applications is the ability of cellulose molecules to for different types of bonding with drugs and the combining material/s. Galkina et al., developed another cellulose based nanocomposite using titania and cellulose nanofiber for delivering different types of drugs through the dermal route (Galkina et al., 2015). The authors used a radio-labeling analysis to evaluate the drug release kinetics and revealed that cellulose nanofibers/ titania nanocomposites have the potential to deliver antibiotics, anesthetics and analgesics drugs. Another green and ecofriendly cellulose based nanocomposite have been used to deliver anticancer drug, 5-fluorouracil (Sun et al., 2019).

2.4.4 Thermal properties

Thermal stability of materials is an essential parameter that determine its potential use in various industrial application such as packaging as well as the biomedical field (Lin and Dufresne, 2014b). The study of thermal degradation behavior of each material within the composite in addition to the overall degradation of the composite allowed scientists to optimize the desired designing and processing conditions and develop composite with enhanced thermal stability. Various investigations have been conducted to study the thermal behavior of cellulose nano

fibers during its compounding and extrusion. CNFs decomposition temperature found to range from 200 to 300 °C; therefore, its necessary to control the compounding temperature at nearly 200 °C during the fabrication process to avoid any degradation of the CNFs (Dos Santos et al., 2013).

Thermal stability of cellulose nano fibers was found to differ based on the source of the nanocellulose, processing techniques, types of matrices and drying process. The addition of nano cellulose (CNFs or CNCs) can greatly improve the thermal stability of prepared composite, due to the enhancement of the filler–matrix interaction through hydrogen bonding (Mandal and Chakrabarty, 2014). With the addition of cellulose nano fibers (10 %), the thermal decomposition temperature of nanocomposite improved (from 272.5 °C to 339 °C) compared with the control. Generally, the addition of cellulose nano fibers into the nanocomposites significantly improved its thermal properties (Ben Cheikh et al., 2018). Owing to the highly sulfated amorphous regions within the nano cellulose, at higher temperature, it would be degraded even before the decomposition of un-sulfated crystal interior. Sulfation take place at amorphous domains, under extreme hydrolysis conditions in addition to affecting the cellulose chains at the ends of the crystals. The higher the sulfation area in nanocellulose, the more accessible the nanocellulose to thermal degradation (hence the lower the decomposition temperature) (Ben Cheikh et al., 2018). Various studies have investigated the thermal characteristics of cellulose nano fibers obtained from different sources, different preparation techniques and different matrices. The thermal characteristics was found to vary depending on the source of nanocellulose and extraction conditions. Surface modification of cellulose nanofibers have been used to improve the thermal properties, especially in those with hydrophobic polymer matrices.

Introduction of carboxyl groups into nanocellulose was found to enhance the thermal properties of the nanocomposite (Cao et al., 2018).

2.4.5 Biocompatibility and cytotoxicity

The term biocompatibility is described as the ability of a material to function in living tissue without complications. The biocompatibility of a material is often dependent on its cytotoxicity and the immunological response of that material on its exposure to the body fluids or cells (von Recum, 2016). A few pieces of research on the biocompatibility and cytotoxicity of the cellulose-based material have been conducted. Several studies have provided results on biocompatibility and cytotoxicity, but have not been fully explored due to a variety of cellulosic fibre sources, range of different methodologies, and sample preparations. A significant number of studies have confirmed that cellulose could be generally considered to be biocompatible with only moderate or no foreign body responses in vivo (Strätz et al., 2019; Togo et al., 2018). Ul-Islam and co-workers analysed cell–scaffold interaction using a cancer cell line and cellulose/chitosan scaffolds (Ul-Islam et al., 2019). Strong adhesion and negligible aggregation of cancer cell lines were reported in the scaffold matrix and better cell–scaffold interaction than cell–cell interaction (Ul-Islam et al., 2019). Ramphul et al. (Ramphul et al., 2020) reported that due to the presence of several OH groups, cellulose is more hydrophilic and hence will promote cellular interactions. The strong interaction between human cells and cellulose scaffolds have been linked with the downregulation of Notch receptors in the growing cells due to direct interaction between the strange material and the cells. Real-time polymerase chain reaction (RT-PCR) has been used to verify this hypothesis. Primers for notch −1 to 4 receptors have been used and revealed that the cell signalling pathway is responsible for the generation of the expected strong connection of cell lines with the scaffold and a weak connection among the cell lines

themselves (Ul-Islam et al., 2019). According to a study conducted, the interaction of NFC with the cells led to a high level of secreting inflammatory cytokine tumour necrosis factor-alpha, which could be the result of fibre-receptor interactions, which are highly dependent on the surface chemistry of the material (Lopes et al., 2017). The secretion of tumour necrosis factor-alpha could be inhibited by the introduction of surface charges on the surface of cellulose scaffolds, which have been done by the same authors (Lopes et al., 2017). Other studies have suggested that the human body can fully degrade cellulose with cellulolytic enzymes, which would have inevitably caused some incompatibility (Lin and Dufresne, 2014a). The toxicology of cellulose-based materials used in tissue engineering has been conducted on the viability, proliferation of cells, and cytotoxicity. Table 2.3 shows a summary of some recent research into the toxicology of cellulose-based materials. Generally, there has been no evidence for serious impact or damage of cellulose-based materials at both the cellular and genetic level as well as in vivo organ and animal experiments. However, the inhalation of intake of a high percentage of nanocellulose may induce pulmonary inflammation due to the easy self-aggregation and non-degradation of nanocellulose in the body of animals.

2.5 Chitosan

Chitosan is one of the most abundant biopolymers on earth and has been extracted from multiple sources such as; the shells of crustaceans (El Knidri et al., 2018), cell walls of fungi (Zamani, 2010), and exoskeleton of arthropods (Ambarish and Sridhar, 2015) etc. Preparation of aerogels from polymeric nanoparticles such as chitosan are attracting increasing interest among the scientists. The unique properties of resulted aerogels, such as high surface area, mechanical strength, a high degree of polymerization, high purity and high crystallinity make them promising materials for