

Chapter 1

Introduction and Aim

1.1 Background

Cellulose is the most abundant naturally occurring biopolymer [1]. It is present as the most important structural component across various living organisms such as prokaryotes, protists, animals and plants [1]. Cellulose owes its utility to the supra-molecular multi-level hierarchical organisation of β -linked poly-glucan chains into microfibrils [2]. These semi-crystalline microfibrils possess attractive anisotropic mechanical properties. The estimated Young's modulus of cellulose microfibrils by experimental and theoretical approaches is up to 220 GPa [3, 4].

Cellulose has deeply integrated with our society in applications such as paper, cotton, lubricants, fillers, adhesives and also in form of fibres, owing to its abundance, low cost and properties. The current methods of commercial production of cellulose fibres face the following challenges. Till date plants and trees constitute the main resource for cellulose extraction, which leads to a massive deforestation and the chemical treatments required to remove non-cellulosic impurities and produce fibres cause pollution [5]. With the increasing industrial demands and a need for sustainable and environment friendly resources, non-plant based (alternative) sources of cellulose are being explored.

Moreover, the commercially available cellulose fibres do not completely harness the potential of native cellulose. This is because, as an essential part of fibre production process, the extracted cellulose is dissolved and regenerated, which disrupts the native cellulose crystal structure (cellulose I), resulting into a mechanically inferior structure (cellulose II) [6-10]. The lack of orientation, also, prohibits a complete transfer of properties from molecular scale to macroscopic level.

Therefore, in order to utilize the potential of cellulose in applications such as fibres, it is beneficial to preserve the native cellulose crystal structure and produce high degree of alignment. One of the other advantages of maintaining the native cellulose

structure is the preservation of the self-assembled oriented cellulose chains in form of microfibrils, which possess desirable properties. Given this, in order to further transfer the properties to macroscopic level, it is required to devise a strategy to orient the microfibrils. One of the proven ways to produce high performance anisotropic products is to pursue the liquid crystalline processing route. A liquid crystalline phase is state where the material flows like liquid but possesses orientational order like crystals. The involvement of a liquid crystalline phase formation has been found effective in production of high performance man-made fibres such as Kevlar [11] and natural fibres such as silk [12].

1.2 Aim

The primary motivation of this thesis is to develop a novel cellulose processing strategies involving liquid crystalline processing from non-plant based sources of cellulose, which can be utilised for fibre spinning. Cellulose was extracted from bacteria and tunicates (sea-squirts) for this work. Cellulose is present in the protective tunic of the sea squirts [13], and produced by certain species of bacteria as an extracellular membrane [14]. The bacterial cellulose is particular attractive as an alternative cellulose source owing to its higher crystallinity and 100% purity. Tunicate cellulose has also been found to possess high crystallinity, mechanical properties, but requires chemical treatment to remove non-cellulosic inclusions.

During the course of this work, the following areas were explored:

1. Architectural characterisation of cellulose produced by bacteria and tunicates
2. Formation of liquid crystalline phase from bacterial and tunicate cellulose
3. Pilot fibre spinning trials from the liquid crystalline cellulose suspensions
4. Exploratory study into *in situ* modification of bacterial cellulose to tune micro-macro-structure of cellulose

1.3 Thesis outline

The research (in literature and from experiments), carried out towards the objectives stated in the previous section, is presented in the following seven chapters. A summary of all the relevant findings reported in the literature has been presented in chapter 2. The production and extraction of cellulose from the various sources of cellulose has

been discussed. A comprehensive account of the previous work dedicated to formation of liquid crystalline phase from cellulose has been presented. Chapter 2 also summarises the current strategies to produce cellulose fibres. The gap in the cellulose science and technology is evident as in the large amount of work done on cellulose; enough emphasis has still not been laid on utilising bacterial and tunicate cellulose and improving on the current cellulose fibre production practices.

With these objectives on board, the cellulose obtained from three sources: nata-de-coco, bacterial cellulose and tunicate cellulose have been characterised using scanning electron microscopy, atomic force microscopy, wide angle X-ray diffraction and small angle X-ray scattering. The relevant production, extraction procedures and characterisation techniques have been described in chapter 3 and the results are discussed in chapter 4. The three kinds of celluloses have been characterised and compared using the above-mentioned techniques, which are useful for extracting information at different scales.

Following a full characterisation and elucidation of the hierarchical organisation of cellulose, the self-assembly of cellulose nanowhiskers (obtained by acid hydrolysing cellulose microfibrils) into a lyotropic liquid crystalline phase has been studied in chapter 5. A complete phase transition diagrams of cellulose nanowhiskers suspensions have been established using isotropic-liquid crystal phase separation and polarised optical microscopy. The transition behaviours have been compared and the factors those influence the transition have been determined. The formation of a chiral nematic liquid crystalline phase has been reported in the literature but the origin of this interaction has not been understood and thus, the chapter leads into a detailed discussion on this.

Having established formation of liquid crystalline phase from bacterial cellulose and tunicate cellulose nanowhiskers, fibres were spun after standardising spinning conditions. The novel fibres made, have been characterised to obtain information about orientation, microstructure and mechanical properties and the results have been presented in chapter 6.

The bacterial cellulose has often been used as a model system to study cellulose biosynthesis, owing to the fact that bacterial cellulose synthesis allows *in situ*

modification and visualisation [15, 16]. The chapter 7 of this thesis explores the effect of various additives on the bacterial cellulose microstructure. Work has been directed towards developing a rationale to modify cellulose microstructure. *In situ* modification has also been used as a tool to tune the liquid crystalline phase of cellulose.

The final chapter is dedicated to the conclusions made from the work presented in this thesis. The work has laid the basis for several new avenues of research at the interface of technological advancement and efficient utilisation of cellulose.

Chapter 2

Literature Review

2.1 Background

Amongst the various classes of naturally occurring polymers such as polysaccharides, polyamides, polyamines, and polyolefins, polysaccharides constitute the structural materials [17]. The polysaccharide, cellulose, is the most abundant biopolymer which forms the major part of plants and trees [18]. *'In terms of the massive quantity of natural polymer biosynthesis, Nature is alive and well with respect to cellulose'* – R. M. Brown [1].

Natural cellulose based materials, such as wood, hemp, cotton, and linen, have been used by our society as fuel, garments and engineering materials for thousands of years and their use continues today, as verified by the enormity of the world-wide industries in forest products, paper and textiles [19, 20]. The cellulose derivatives such as methyl cellulose, hydroxyl propyl methyl cellulose, ethyl cellulose, nitrocellulose and cellulose nitrate, are used in lubricant, binder, explosives, lacquer, controlled release drug tablet, and detergents [21]. Cellulose is also extensively used for technical applications such as tire cords, dialysis membranes, and reinforcements, in form of fibres [5].

The development of scientific and technical understanding of cellulose started relatively late. It was only in the period 1837-1942, French agricultural chemist Anselme Payen showed that plant tissue, seeds, cotton linters, root tips and leaves, when purified led to a chemical substance with a uniform composition ($C_6H_{10}O_5$): a carbohydrate composed of glucose residues and isomeric with starch (44.4% C and 6.2% H), which was called cellulose [22-24].

Cellulose has gained importance in current global scenario as it is the most abundant natural renewable biodegradable polymer and has special technical importance. While cellulose applications found technological space, the establishment of its chemical and physical structures underwent multitudinous periods of struggle, which reflects its complexity and uniqueness. Some of the important questions about its synthesis and assembly still remain unanswered.

This review first describes the production and extraction of cellulose from various sources and its chemical structure. The most interesting aspects of cellulose is its hierarchical organisation into semi-crystalline microfibrils. The following few sections are dedicated to the hierarchical organisation, crystal structure and biosynthesis of cellulose. The rest of the chapter is dedicated to the extraction, properties and applications of cellulose nanoparticles. The final section summarises some of the gaps in the current understanding and identifies the areas with scope of improvement, which has largely served to outline the goals of this Ph.D work.

2.2 Sources, extraction and production of cellulose

Cellulose is present as structural component in a wide variety of living organisms. It is produced by primitive prokaryotes, protists and also by higher forms of living organisms such as plants trees and sea squirts (Table 2.1) [1].

Currently, plants and trees constitute the main commercial source of cellulose [25, 26]. The cellulose content varies significantly with source as it is present along with other impurities such as hemicelluloses and lignin, for example, cotton balls contains 90-99% cellulose, while wood contains 40-45% [27]. The extraction of cellulose from plants and trees needs elaborate chemical and mechanical treatments including removal of lignin, hemicellulose, bleaching, degradation and removal of low molecular weight cellulose. The most common chemical pulping process is the Kraft pulping process, which is an alkaline process utilising sodium hydroxide and sodium sulphide as active delignification chemicals. Another family of delignification methods comprises various sulphite pulping processes based on sulphur dioxide with varying cation, liquor pH and temperature or bleaching agents [5]. Considering the deforestation and pollution caused by extensive chemical treatment, while using plants and trees as the cellulose source, the alternative greener sources are needed.

Table 2.1: Representative genera of living organisms which have been found to produce cellulose [17]

Prokaryotic	Gram positive anaerobic bacteria- <i>Sarcina</i> Gram negative purple bacteria- <i>Acetobacter</i> , <i>Rhizobium</i> , <i>Alcaligenes</i> <i>Agrobacterium</i>
Eukaryotic	A. Photoynthetic organisms a. Chlorophyta (green algae) b. Charophyta (stoneworts) c. Phaeophyta (brown algae) d. Chrysphyta (yellow-green, diatoms) e. Rhodophyta (red algae) f. Vascular plants (mosses, ferns, angiosperms etc) B. Non photosynthetic organisms a. Protists – <i>Dictyostelium discoideum</i> Fungi- <i>Saprolegnia</i> , <i>Achlya</i> b. Animals AscidianTunicates- <i>Metandrocarpa</i> , <i>Hyalocynthia</i>

Out of cellulose obtained from various sources, bacterial cellulose holds a special significance. Bacterial cellulose is obtained as a 100% pure, highly crystalline pellicle consisting of long endless cellulose microfibrils and almost 95-99% water (Figure 2.1(a)). Various species of bacteria, such as *Acetobacter* and *Rhizobium*, have been identified to produce cellulose [28]. In early 1950s, Hestrin and Schramm made essential contribution to the development of process of production of cellulose from *Acetobacter Xylinum* [29-33]. The bacteria were grown in medium consisting of glucose, peptone, citric acid, yeast and distilled water. Bacterial cellulose is produced as a floating gelatinous pellicle by layered deposition (Figure 2.1 (b)) [14]. The thickness of cellulose pellicle increases with time and reaches a maximum thickness [14]. Since the past few decades, a lot of biochemistry and microbiology research has been directed towards optimising the carbon source [34-36], and culture conditions to maximise cellulose yield and properties [37, 38] depending on the bacterial strain. Owing to these efforts, the production of bacterial cellulose has also been successfully scaled up in laboratories and is commercially available [39]. Bacterial cellulose has been used as a Phillipinese dessert food ingredient for a long time and is commercially available as

nata-de-coco [14]. It is produced by the fermentation of coconut milk in food industries. The nata-de-cocco was first identified as bacterial cellulose in 1960s [40, 41].

Tunicate, also known as urochordates or sea squirt, is the only animal known to produce cellulose [42]. The name “Tunicata” is derived from the unique integumentary tissue, the tunic, which entirely covers the epidermis. Cellulose formed as crystalline microfibrils, is a major component of the tunic, and the cellulose microfibrils are deposited in a multi-layered texture with a bundled structure parallel to the epidermis as shown in Figure 2.1 (c, d) [43]. Some of them secrete a balloon-like gelatinous structure that acts as a feeding apparatus, and is constituted of cellulose microfibrils. The tunic contains approximately 60% cellulose, 27% nitrogen-containing components and, in the fresh condition, approximately 90% water [44]. Non-cellulosic material has to be removed by chemical and mechanical treatment [45]. Tunicate cellulose has shown to have distinguishing properties such as high crystallinity and better mechanical properties [4, 46, 47].

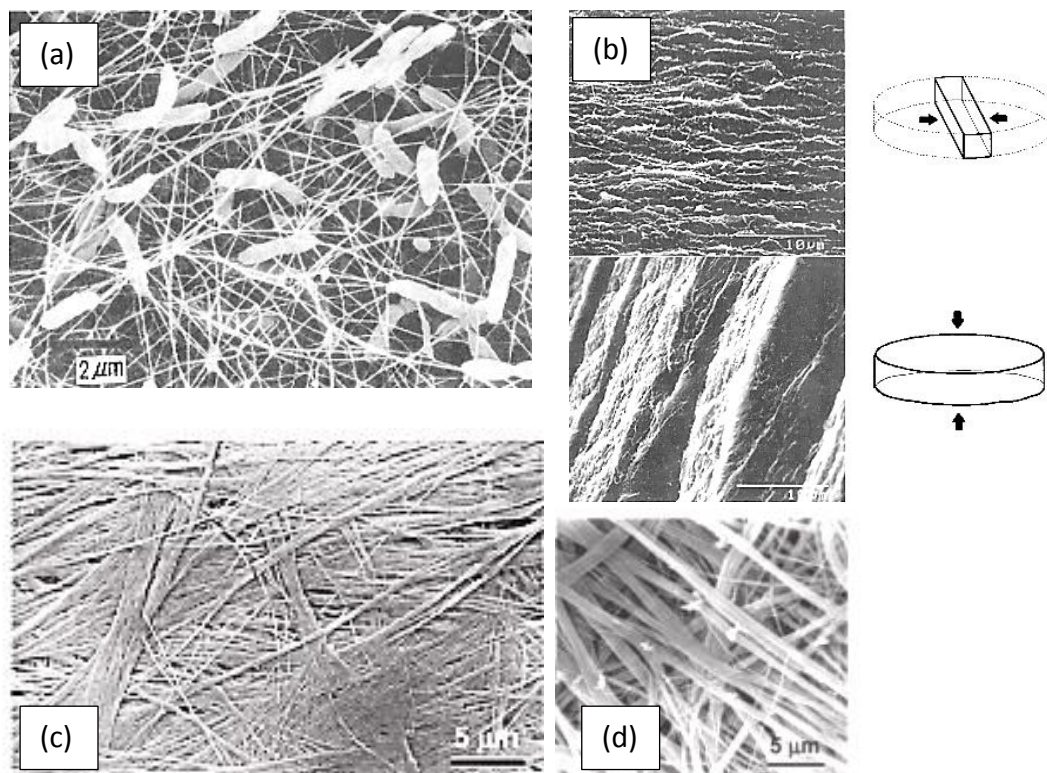


Figure 2.1: Scanning electron micrograph of a) freeze dried surface of bacterial cellulose gel showing network of microfibrils along with bacteria [14] b) fracture edge of bacterial cellulose film along and perpendicular to the direction of growth, showing layered structure [48] c) innermost layer of tunic in *Metandrocarpa uedai* [49] d) innermost layer of tunic in *Polyandrocarpa misakiensis* [13], comprising of cellulose microfibrils and ribbons

2.3 Chemical structure

Cellulose is a homo-polymer consisting of β -1,4 linked ringed D-glucose monomers with a syndiotactic configuration [50]. The repeat unit is comprised of two anhydroglucose rings ($(C_6H_{10}O_5)_n$; $n=10,000$ to $15,000$ depending on its source [50]), as shown in Figure 2.2. The β -D-glucopyranose rings adopt 4C_1 chair conformation and as a consequence, the hydroxyl groups are positioned in the equatorial plane while the H atoms are in the axial position. Each monomeric residue is oriented 180° to the next. The intra-chain H-bonding in cellulose leads to a linear configuration.

The hemi-acetal formation between the hydroxyl at C-4 and C-1 can result in two different stereo-centres at C-1 as the hydroxyl group at C-4 can attack the C-1 carbonyl from both sides of glucose, resulting into α and β configurations. The glucose monomers are linked by a β -linkage in cellulose and by α -linkage to form starch as shown in Figure 2.3.

Although the utilization of cellulose has a long history, the understanding of chemistry and structure is relatively new. Anselm Payen determined the chemical formula in 1838, but Haworth *et al.* proved that cellulose was the building block of cellulose polymer only in 1930 [51, 52]. The polymeric structure of cellulose was determined in Staudinger which finally made evident the macromolecular structure of cellulose [53].

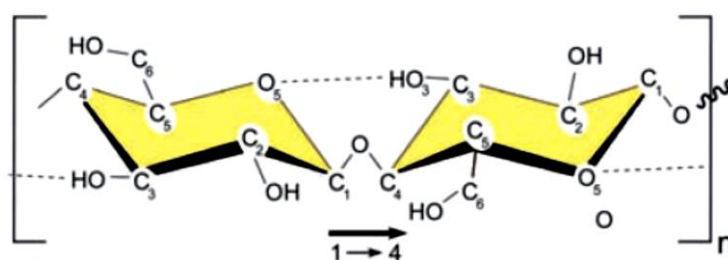


Figure 2.2: Chemical formula of cellulose showing β -1,4 linkage, where cellulose is the basic repeating unit [4]

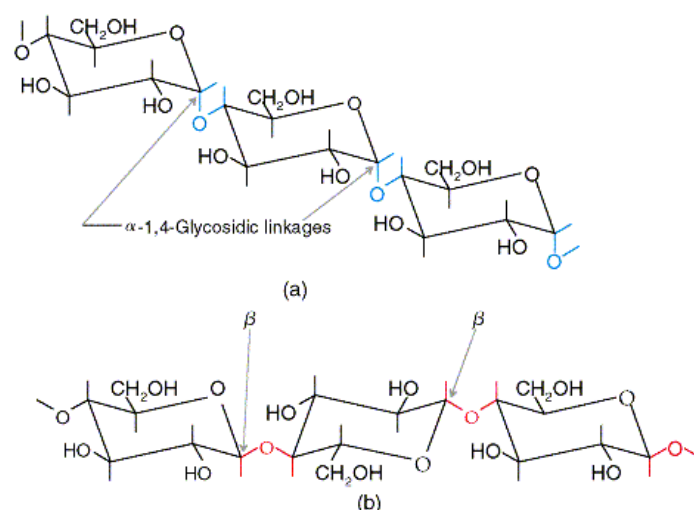


Figure 2.3: Chemical structures of cellulose and starch showing the difference in the glycosidic linkages: α linkage in starch and β in cellulose [W1]

2.4 Crystal Structure

Six different crystalline forms of cellulose are known- I, II, III, IV, VI. However, with the emergence of X-ray diffraction, it was observed that most of the native cellulose belonged crystallographically to Cellulose I. Cellulose I has been identified to exist as two types Cellulose I α and I β [54]. They are similar in chain conformation, with the principal difference being the manner in which the chains are staggered longitudinally. This gives triclinic crystallographic symmetry to the I α form and monoclinic symmetry to cellulose I β . The X-ray diffraction pattern of cellulose I is shown in Figure 2.4, three major reflections are seen from the planes with d-spacing 3.9, 5.3, and 6.2 Å which correspond to planes (110), (010), (100) in I α and (200), (110) and (1-10) in I β . The most accurate structures for the two polymorphs were reported by Sugiyama *et al.* using X-ray and neutron diffraction [54] as shown in Figure 2.5 (a, b). The I α unit cell belongs to P1 space group and contains one cellulose chain and the unit cell parameters are $a=0.672$ nm $b=0.596$ nm, $c=1.040$ nm $\alpha =118.08^\circ$ $\beta=114.80^\circ$ $\gamma=80.375^\circ$. The I β unit cell is space group P2₁, contains two cellulose chains, and unit cell parameters are $a=0.778$ nm $b=0.820$ nm $c=1.038$ nm $\gamma=96.5^\circ$. The two polymorphs in spite of the structure parameter differences, when looked down the cellulose chains, do not appear too much shifted. Their relative orientation is shown in Figure 2.5(c).

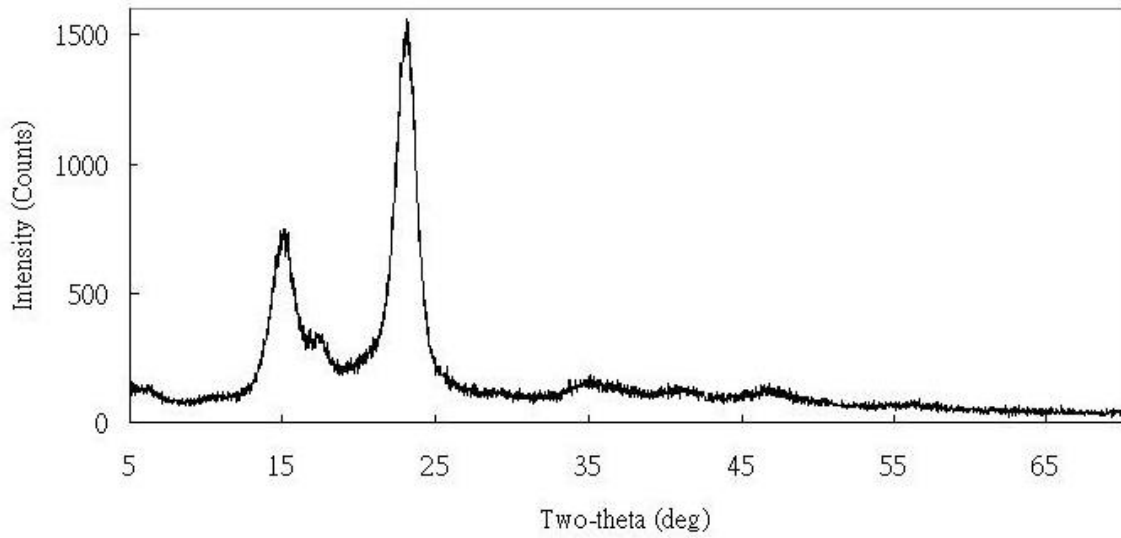


Figure 2.4: X-ray diffraction pattern for bacterial cellulose which represents native cellulose, produced in a bioreactor, exhibiting 3 main reflections from planes (100), (010) and (110) from $I\alpha$, and (1-10), (110) and (200) from $I\beta$ corresponding to the d-spacing about 6.2, 5.3 and 3.9 Å, respectively [55]

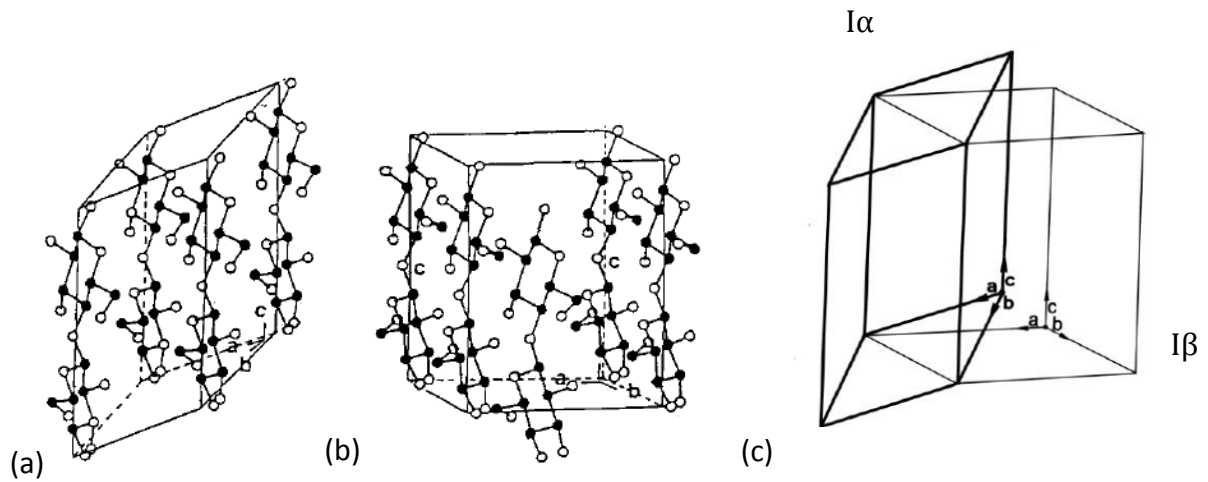


Figure 2.5: Crystal structure of the cellulose I polymorphs a) $I\alpha$ (triclinic), b) $I\beta$ (monoclinic) [56] c) relative orientation of the two polymorphs [54]

Cellulose II is formed from regeneration after cellulose I dissolution and is a thermodynamically more stable form of cellulose. Hence, once cellulose I is converted to cellulose II structure, it is irreversible. Figures 2.6 and 2.7 show the arrangement of molecules in cellulose I and cellulose II [6-10]. Cellulose II has lower mechanical

properties than cellulose I. The tensile Young's moduli for cellulose I and cellulose II were measured to be 140 -220 GPa and 90 GPa, respectively. On the other hand the shear moduli were found to be 1.5 GPa and 2.5 GPa for cellulose I and cellulose II respectively. The tensile properties are better for cellulose I while shear properties are better for cellulose II. This is because of different H-bonding pattern, two intra-molecular H-bond between anhydroglucose units in cellulose I and better inter molecule H-bonding in cellulose II as compared to cellulose I as shown in Figures 2.6 (b) and 2.7 (b) [10].

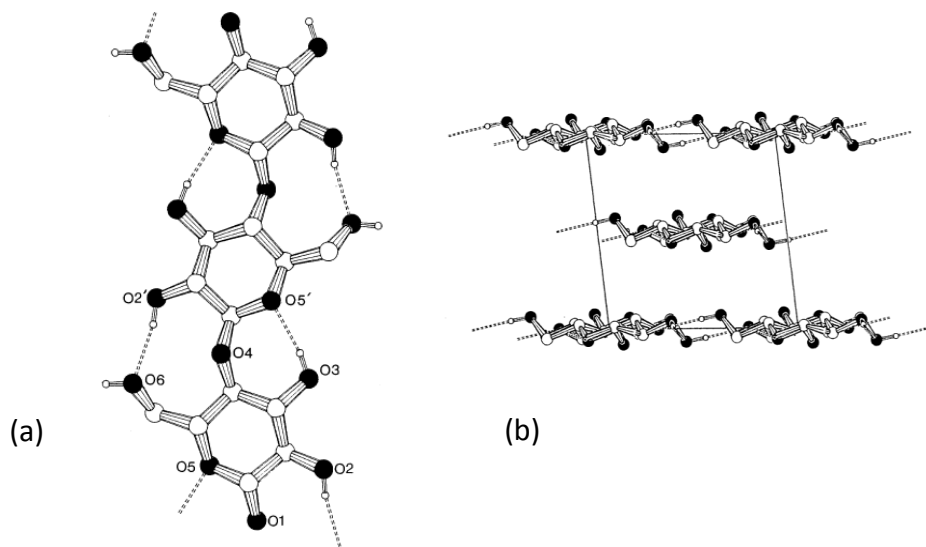


Figure 2.6: a) The chain conformation of cellulose I b) The crystal structure of cellulose I viewed along the chain axis. Dashed lines indicate H-bonding [6-10]

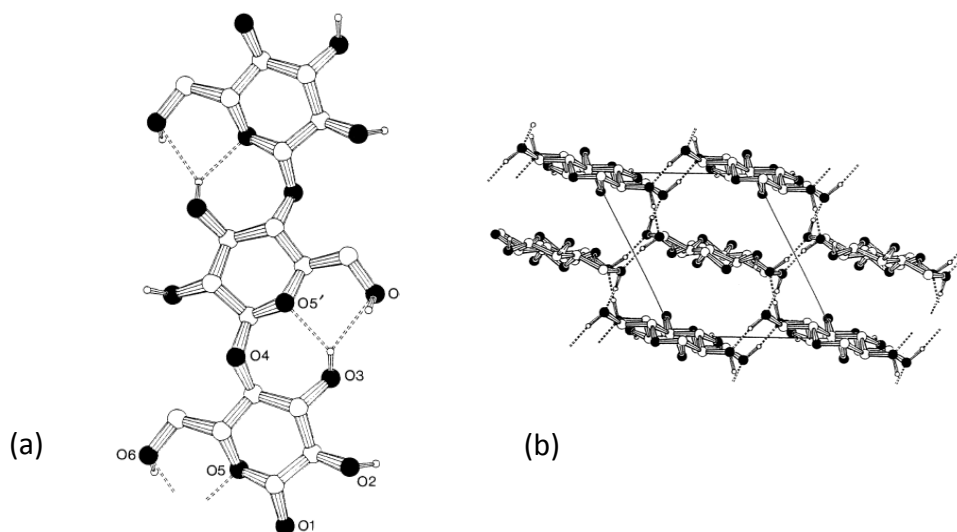


Figure 2.7: a) The chain conformation of cellulose II b) The crystal structure of cellulose II viewed along the chain axis. Dashed lines indicate H-bonding [6-10]

2.5 Organisation of cellulose

a) Chain configuration

With the study of crystallisation of polymers made progress, it was found that the folded chain crystallisation leads to a thermodynamically more favourable structure. Same was first believed to be true for cellulose. Manley observed string of beads on cellulose microfibrils, which he thought was because of the folded chain structure of cellulose crystals [2, 57]. On the other hand, Muhlethaler stated that these microfibrils were uniform [58] and proposed the extended chains model, which was further supported by Frey-Wyssling [59]. Marx-Figini and Schulz proposed spinning mechanism for cellulose from microtubules present on the cellulose producing cell, where the polymerisation of cellulose also occurred [60]. Under normal spinning circumstances, a flexible polymer would crystallize in a folded chain configuration when spun, but later, it was found that the orientation of microtubule and fibrils did not coincide [61]. Also, a chain folded crystallisation is characterised by a sharp meridional reflection at low angle when exposed to X-ray owing to the fold period, which has not been observed for native cellulose [62, 63]. While there is no direct evidence for extended chain configuration, the lack of evidence to support a folded chains configuration has led to the acceptance of an extended chain configuration [64].

b) Hierarchical organisation and crystalline structure

Cellulose occurs as a supra-molecular arrangement of poly-glucan chains in the form of thin long semi-crystalline microfibrils in all native cellulose forms, irrespective of its source. Various techniques such as electron microscopy, X-ray diffraction, and small angle scattering, have been used to investigate the hierarchical organisation and the elementary unit of cellulose. However, the results have not been congruent and the supra-molecular organisation of cellulose remained an active area of polymer research.

One of the earliest models of cellulose was by Von Nageli [65], where the cellulose fibre-like elements visible under microscope were subdivided into sub-microscopic anisotropic crystalline particles called micelles (Figure 2.8 (a)). This idea was extended by Seifriz [66] and Meyer [67] where the cellulose fibrils were arranged in a bricklike pattern (Figure 2.8 (b)). However any of these ideas did not gain much attention. Nishikawa and Ono found cellulose crystallites were rod-like and Nishikawa

was also amongst first to postulate discontinuity of crystallinity in cellulose [68]. Herzog and Jancke analysed X-ray data from different sources of cellulose and led to common terminology of native cellulose [69] and established orientation of crystallite parallel to the fibre direction.

Mukherjee and Woods suggested four crystalline continuous primary filaments, each of which are 700 nm and 300 nm thick were cemented together by paracrystalline region to form a cellulose microfibril [70]. Manley further showed microscopic evidence for the existence of the elementary fibril with thickness 35 Å in cellulose from many sources [2]. Figure 2.8 (c) shows the model of elementary fibril proposed by Muhlethaler [58, 71] which was modified by Rowland [72] into Figure 2.8 (d). Preston discarded the concept of similar elementary fibril across cellulose from various sources [73]. He showed crystallites of different sizes were present in cellulose and in many cases was found to exceed the value suggested by Manley.

Cellulose microfibrils are composed of crystalline and non-crystalline regions. Experimental work has been done to understand the arrangement of crystalline region inside cellulose microfibril and accordingly models of the arrangement of cellulose chains inside microfibril have been suggested [20, 74-76]. Various techniques such as accessibility methods (swelling, water sorption), acid dissolution, decrease in chains length, were used to get an understanding of the amorphous region. Sharples presented work based on acid hydrolysis and concluded the non-terminal linkages are resistant to acid attack and the hydrolysis occurs at terminal linkages [77]. Frey Wyssling [59] and Kratky suggested crystalline and amorphous regions alternate with less well ordered amorphous regions on the lines of fringed micelle theory [78], in which the poly-glucan chains gradually transit from crystalline to amorphous regions as shown in Figure 2.9 (a). Fringed micelle theory fitted quite well with the X-ray diffraction and acid hydrolysis observations. The presence of ordered and disordered regions has been recognised using small angle neutron scattering in deuterium labelled cellulose II fibres but no evidence has so far been obtained for cellulose I fibres [79].

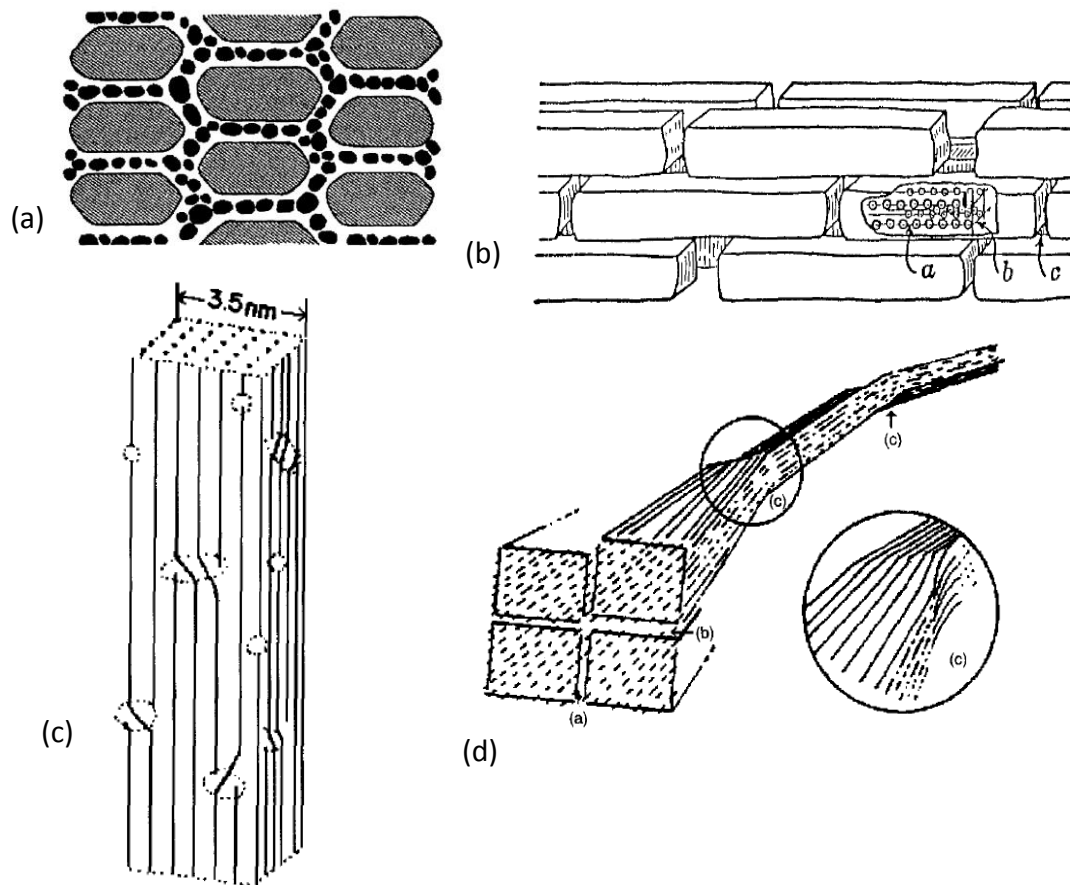


Figure 2.8: a) Schematic of cellulose fibril cross section showing Von Nageli's concept of the micelles [65] b) Arrangement of micelles proposed by Seifriz and Meyer (a,b and c showing different valence forces) [66] c) Muhlethaler's structural model of elementary fibril consisting of chains ends [58, 71] d) Schematic representation of Rowland's elementary fibril (showing a) coalesced surface of high order b) readily accessible slightly disordered surface and c) readily accessible surface of strain-distorted tilt and twist regions) [72]

Another school of thought about the structure of cellulose microfibrils is based on the observations of correlation between the crystallinity and the surface area of the crystallite. The surface of microfibrils is prone to defects and thus leads to a reduction in crystallinity. Ranby [80] suggested that the crystalline micelles are linked by paracrystalline cellulose chains containing residue glucose and other sugars as shown in Figure 2.9 (b). Preston [75] extended his work and suggested one central crystalline core embedded in a paracrystalline core [20]. Later Rowland accounted for irregularities such as surface imperfection (distorted surface and twisted or strained region of the crystalline elementary fibril (Figure 2.8 (c, d)). Using small angle X-ray scattering, X-ray diffraction and scanning electron microscopy, Astley *et al.* proposed

another model for the arrangement of crystalline and non-crystalline regions where more than one microfibrils were believed to be embedded in a paracrystalline matrix (Figure 2.9 (c)) [81]. More recently the non-crystalline or amorphous regions have been further investigated [82-84]. It has been found that there is some range of order in amorphous regions. These have been more appropriately called nematic ordered cellulose [84]. With so much done and established, the field remains a great area of interest.

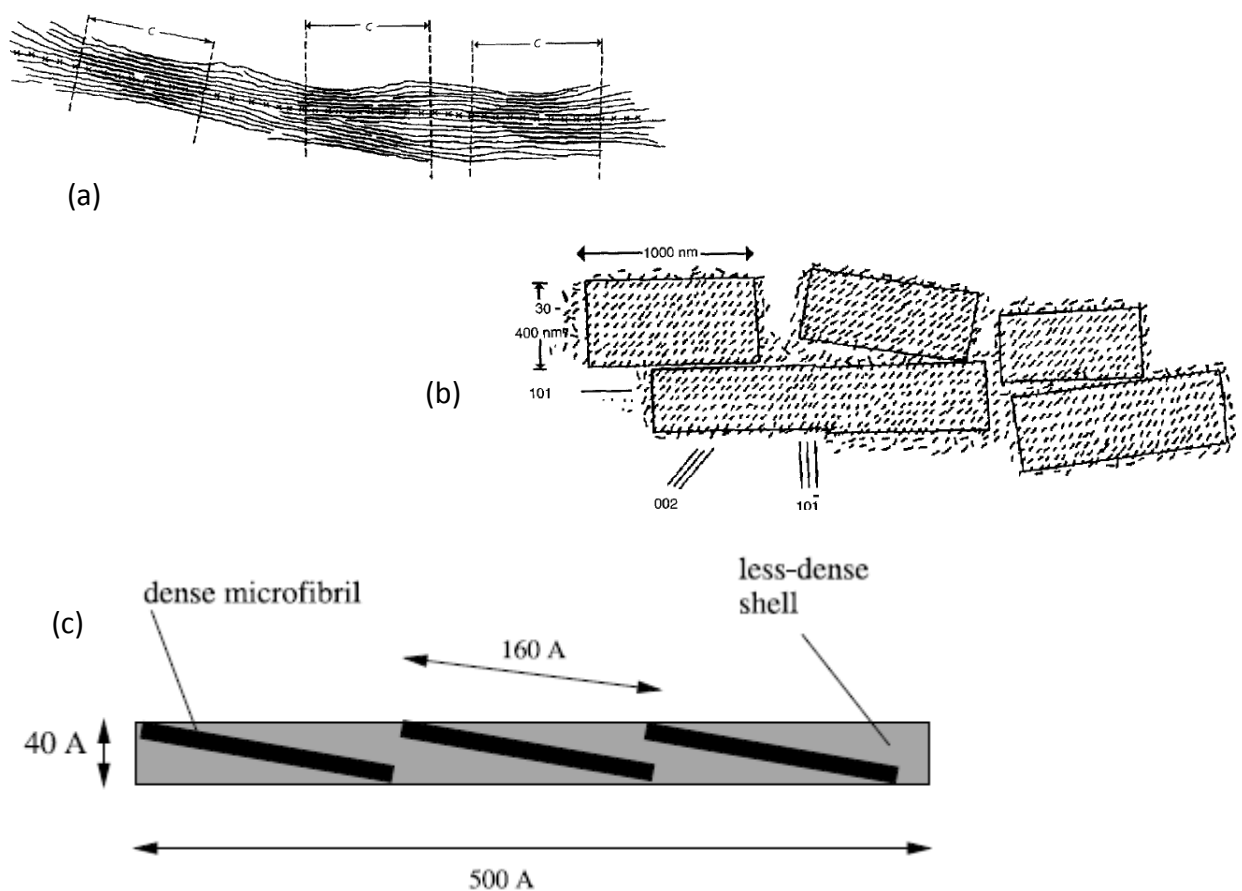


Figure 2.9: a) Main valence chain going through more than one micelle, C is the length of the crystallised region, according to the fringed micelle theory [59, 78, 85] b) Schematic representation of Ranby model with lamellae of cellulose [80, 85] c) A model for the cross-section of the ribbon produced by *Acetobacter* in the wet state. Dense (and not completely crystalline) microfibrils are surrounded by a less-dense shell of polymers [81]

2.6 Cellulose Biosynthesis

The mechanism of aggregation of poly-glucan chains into crystalline cellulose is of great importance in order to understand diverse physical properties of cellulose. Roelofsen proposed [86] that the assembly of cellulose microfibril occurs under the precise control of an enzyme complex located on the growing tip of the microfibril. Preston added that this structure must have a high degree of organisation, which he called ordered granule hypothesis [87, 88]. This system dictates the assembly and orientation of cellulose microfibril. This ordered structure was first observed by Brown and Montezinos using freeze fracture studies of the growing tip of the microfibril in the alga *Oocystis apiculata* and was nominated as terminal complex (TC) [89].

The investigation has shown that sites of glucose polymerisation are situated in clusters according to two basic degrees of organisation [87]. On the enzymatic level the catalytic sites (forming synthase) of glycosidic bond formation operate within sufficient proximity and with the appropriate orientation to ensure rapid laterally aligned aggregation. Each synthase comprises of multi-polymerizing unit, and at cyto-structural level, synthase complexes are tightly grouped to ensure poly-glucan chain aggregation into microfibrils. Although many self-assembly events take place extra-cellularly, the process is tightly bound to morphology of the cellulose producing cell [15].

The TCs determine the shape and size of cellulose microfibril. A large amount of research has resulted into a wealth of information about TC geometry and microfibril architecture [1, 49, 90-94]. The known geometries of TCs are shown in Figure 2.10. The correlation between the geometry of the TC in relation to the size and shape of microfibril synthesized has been illustrated in case of alga *Erythrocladia* in Figure 2.11 [1]. A large variation in biosynthetic environment and machinery amongst the various cellulose producing organism has made it difficult to gain a complete understanding of these.

An extensive amount of work to understand cellulose biosynthesis and organisation has been done on bacteria as a model system. In bacteria, various pieces of evidence have proven that TCs are linearly arranged on surface of the bacterium [15]. The TCs form a massive basket shaped subunit, which traverses the cell membranes periplasmic space and outer membrane. Each TC consists of 3 subunits parallel to the

longitudinal axis of the bacterium, which ensures not only mini-crystal assembly into a microfibril, but also close association and extensive intermolecular H-bonding between microfibril which propels the cells through the medium (Figure 2.12 (a)).

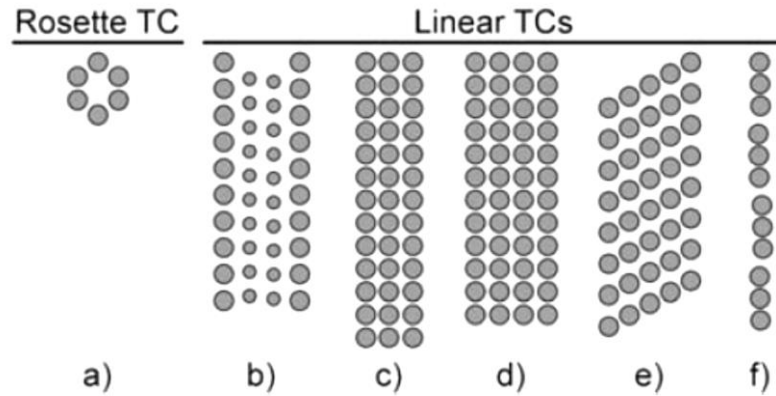


Figure 2.10: Schematic of rosette and linear TCs (Adapted from Brown *et al.* [1]) for a) wood, plant, green algae b) tunicate c) green algae d) red algae e) yellow green algae f) bacterial (each dark circle represents a single TC subunit)

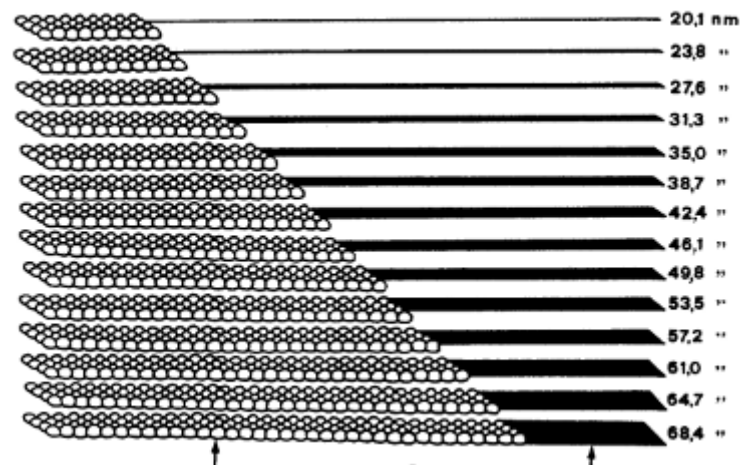


Figure 2.11: *Erythrocladia subintegra* relationship between TC length and cellulose microfibril width. The number of transverse particles gradually increases, 2 rows per increase, each transverse particle row synthesise 12 glucan chains which constitute a mini crystal component [94]

In ascidians, TCs are present in the plasma membrane of the epidermal cells facing the tunic (Figure 2.12 (b)). The microfibrils are formed in a multilayer texture parallel to the epidermal cells. The linear shaped TCs consist of two types of membrane particles, investigated in four species. Across these species of ascidians, the lengths of

the TCs have been found to vary, leading to different microfibrils dimensions. The grouping of TCs leads to the formation of microfibrils bundles [13, 93].

In alga *Vaucheria*, TC consists of rows of subunits arranged diagonally [95]. The primary aggregation of glucan chains occurs via diagonal rows of TC subunits. Each unit is postulated to possess only one catalytic site, three of these units produces a mini-sheet and four of these mini sheets produced from a single diagonal row makes a mini-crystals (Figure 2.13 (a)). In alga *Erythrocladia* [94], TCs are precisely arranged in 4 rows of subunits which leads to a constant thickness of about 1.5-2.0 nm but variable widths are correlated with the TC length (Figure 2.11). Both of these algae have TC with one catalytic site each. On the other hand, another alga *Valonia*, has as many as 10-12 catalytic sites in each TC subunit [96]. In *Valonia*, linear TC has three subunits, and the product of a transverse row of three subunits is a massive mini-sheet of more than 30 glucan chains and all transverse rows together contribute to form a highly crystalline microfibril with 1000 glucan chains.

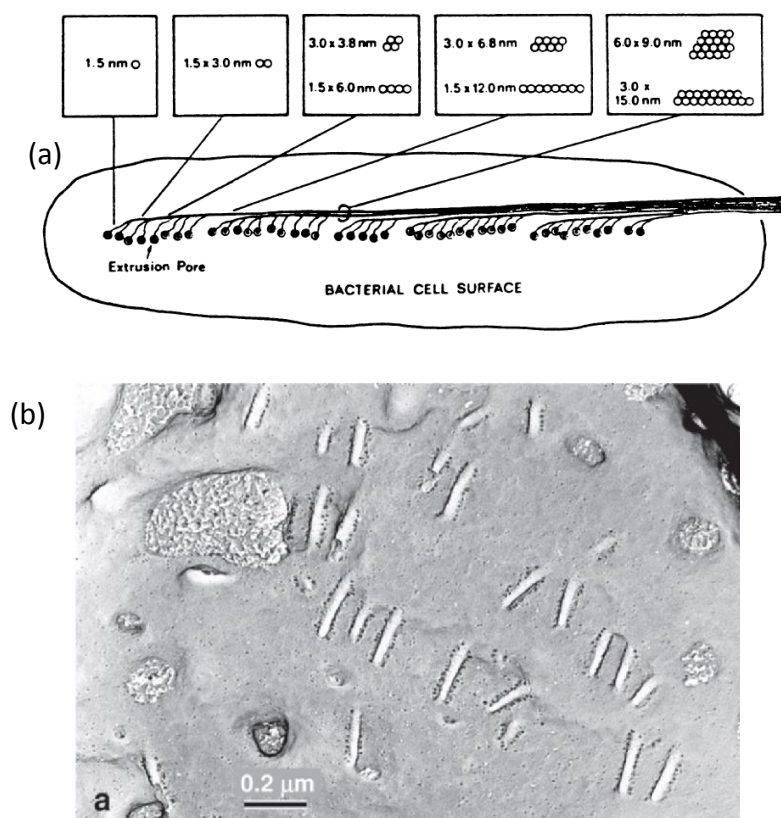


Figure 2.12 a) Generalised model of ribbon assembly in *Acetobacter Xylinum* showing a possible mechanism of origin of microfibrils and separate fibrillar subunits within the ribbon [97] b) Freeze-fracture images showing cellulose-synthesizing linear TCs consisting of two kinds of membrane particles in *Metandrocarpa uedai*, TCs are grouped in almost the same direction on epidermal cell membrane [93]

In higher living organisms such as plants and trees, a different geometry of TC has been observed, termed as the rosette arrangement [98]. The subunits are arranged with a 6 fold symmetry with each subunit with 6 catalytic sites producing 36 chains fibril from each TC, as shown in Figure 2.13 (b). This arrangement is found in all land plants including angiosperms, gymnosperms, ferns and mosses.

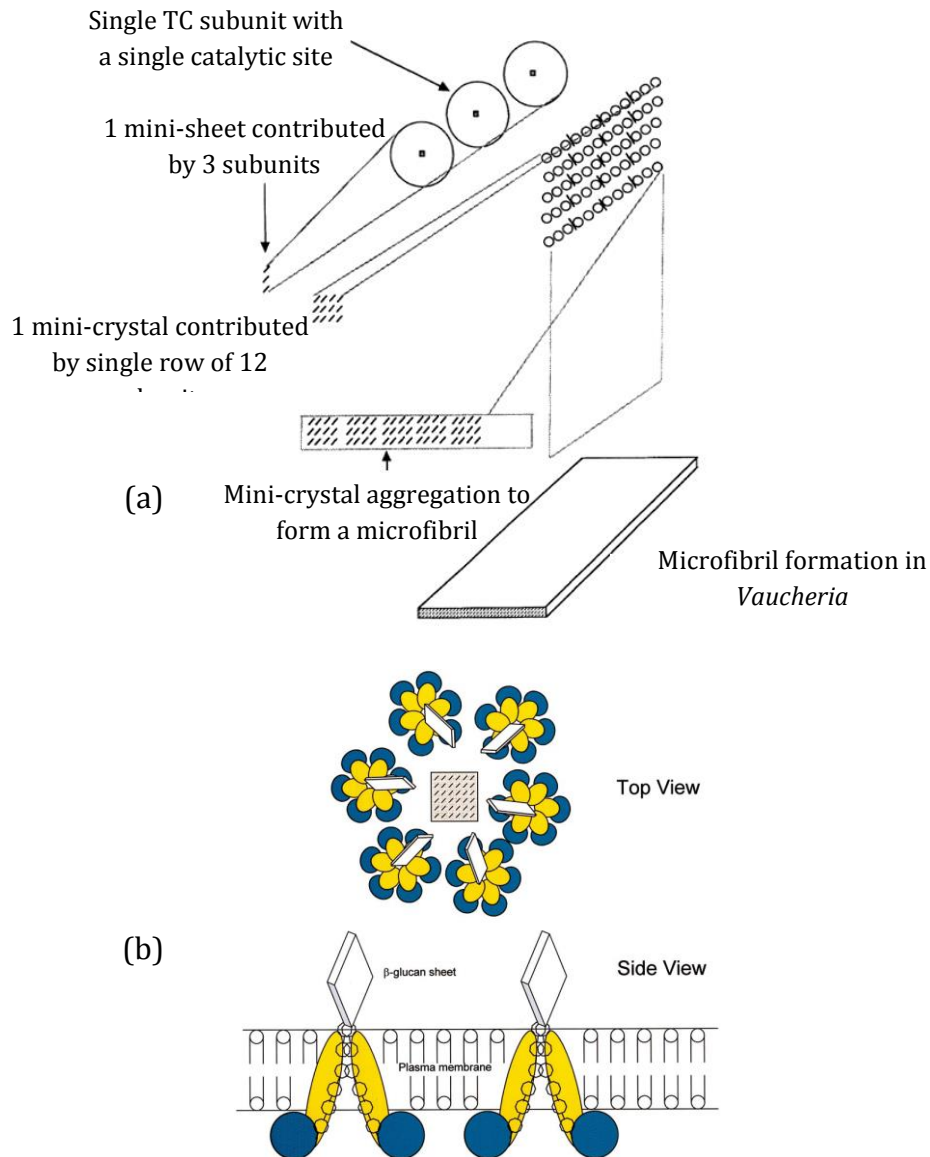


Figure 2.13: Schematic representation of the geometry of the TC in relation to the size and shape of the microfibril synthesized a) *Vaucheria* [1] b) plants (each of the six subunits of the rosette must consist of six glucan synthase molecules, each particle is believed to synthesize glucan chain sheets, these glucan chain sheets assemble by H-bonding to form the metastable cellulose I microfibril) [99]

The cellulose microfibrils are produced in two steps: first formation of mini-sheets by cellulose chains within each TC subunit and second, these mini-sheet are assembled to form a mini-crystal [1]. This is supported by some of the computational calculations done by Cousin *et al.* [100]. The assembly of mini-sheets to form mini-crystals is also supported by more recent experiments involving sonication assisted delamination of mini sheets [101]. The direct evidence for mini-sheets has not been seen but have been hypothesized on basis of dye altered studies, delamination observed on sonication and energy analysis. However, another piece of work involving ultrasonic treatment has suggested nano-structural reorganisation of cellulose fibrils to form larger crystallites and result into higher crystallinity. This has been attributed to the fusion of neighbouring ribbon due to cavitation effects [102].

The terms used in the cellulose literature for various levels of hierarchical organisation could be confusing. A terms used throughout the thesis are defined below:

1. Poly-glucan chain : polymer of beta 1-4 linked cellulose units produced by each catalytic site on cellulose synthase enzyme complex
2. Mini-sheets: Association of few to large number of glucan chains by hydrogen bonds and/or van der Waals forces as a single monolayer after extrusion from catalytic site within a TC subunit.
3. Mini-crystals: Association of glucan chains from a single TC subunit formed by association of mini-sheets, also referred to as protofibril, elementary fibril
1. Microfibril: Single crystalline entity comprised of 1 or more mini-crystals depending on the TC subunit arrangement. The term microfibril refers to the thinnest cellulose fibrillar structure visible in the field of electron microscope.
2. Ribbons: bundles of microfibrils; could be the end result of TC arrangement or more than one cellulose synthesizing cell.

2.7 Bacterial cellulose – model to study cellulose biosynthesis

Cellulose producing bacteria have often been used as a model to study cellulose biosynthesis [15, 16, 90, 103-105]. In the last five decades, a plethora of information on cellulose synthesis, organisation has been derived from studying bacterial cellulose. In the process, effect of various *in situ* modifications on bacterial cellulose morphology and microstructure has been studied. Some of these experiments are summarised here.

Yamanaka *et al.* have presented their work on modification of bacterial cellulose using antibiotics such as nalidixic acid and protein synthesis inhibiting agents such as chloramphenicol and reducing agents such as dithiothreitol and 2-mercaptoethanol [106]. The effect of these agents was seen on the bacterial cells and on the width of microfibrils. The width of microfibril increased with the increase bacterial cell length as shown in Figure 2.14.

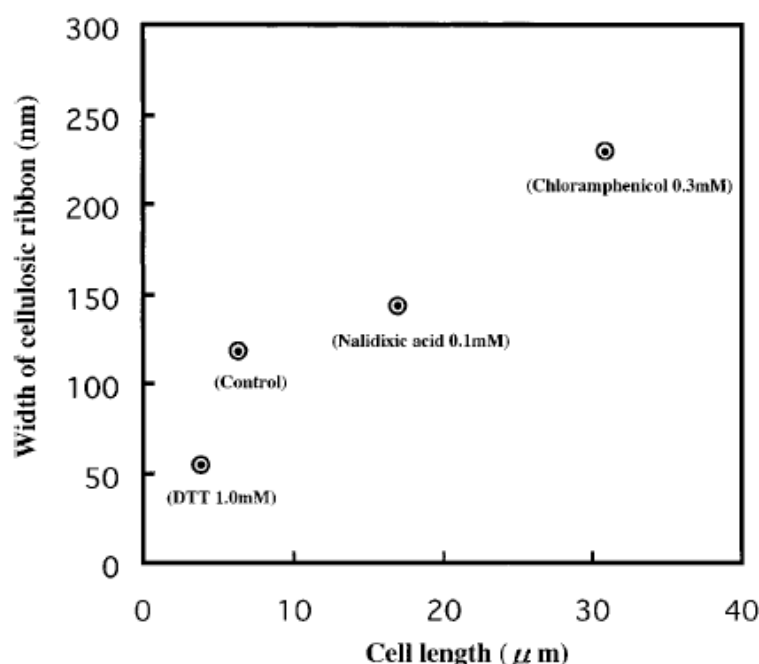


Figure 2.14: Relationship between average cell length and average width of cellulose ribbons or aggregates of ribbons affected by chemical reagents [106]

Calcofluor white ST (CF) is a fluorescent brightener used commercially to whiten cellulose textiles and paper. CF was used to separate the processes of polymerisation and crystallisation. It was found that CF interrupted crystallization while the glucan polymerisation continued and rather increased (Figure 2.15 (a)). This suggests that these are two separate processes [16]. The effect of CF decreases as the dye is consumed and normal biosynthesis is resumed. A previous study reported that 69 Å crystallites are obtained if 0.025% of CF is used and 28 Å crystallite are obtained for larger concentrations. CF has also been reported to affect assembly in other cellulose-producing organisms [107]. Introduction of CMC also leads to disruption in cellulose assembly [103, 108, 109] (Figure 2.15 (b)). The effect of carboxy methyl cellulose (CMC) was found to vary with the degree of substitution in CMC. Haigler has explained the

effect of CF and CMC by emphasising on the hierarchical assembly process and that these agents interfere at different sites to cause effect on the different hierarchical levels [16, 103]. CF interferes at the glucan aggregation level while CMC interferes in later assembly in fasciation of bundles of fibrils.

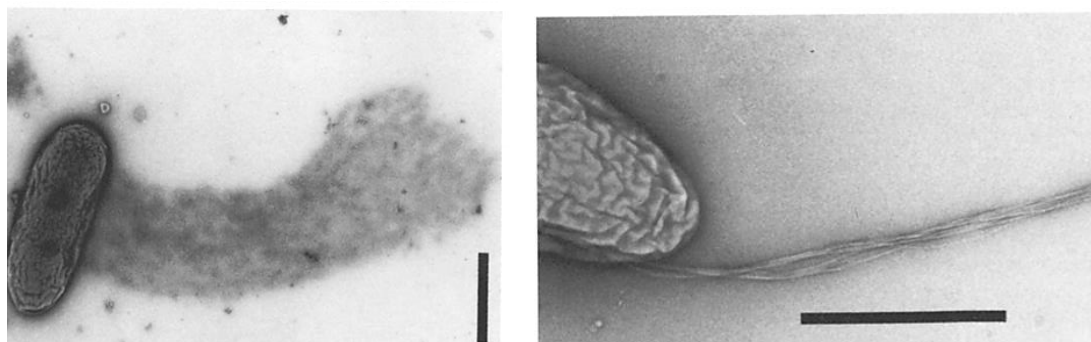


Figure 2.15: Transmission electron micrograph image of cellulose microfibril synthesized in the presence of a) fluorescent brighteners where microfibril crystallization is disrupted (scale bar 1 μm) b) CMC that lacks the tight organization (scale bar 1 μm) [103]

These experiments do not only emphasize the importance of bacterial cellulose as a model system but also showed that it could be used to produce modified bacterial cellulose. Some of the work reported have included effect of poly-ethylene glycol and starch to modify the microstructure of bacterial cellulose [110]. In addition production of composites has also been attempted, for example with carbon nanotubes [111].

Several groups have worked on obtaining bundles of oriented cellulose microfibrils. Effect of kinematic viscosity has been studied by varying the thickness of silicone oil layer in order to introduce orientation of bacterial cellulose microfibrils [112]. In an another attempt by Putra *et al.* the orientation of cellulose microfibrils was introduced by nanopatterning on a polydimethyl siloxane (PDMS) substrate, as shown in Figure 2.16 (a) [113]. The motion of cellulose producing bacteria can be controlled by electric fields, which was also found to be an effective methods to produce oriented microfibrils, as evident from Figure 2.16 (b) [114].

As a result of these experiments, control over the supra-molecular structure and properties of bacterial cellulose by choice of strain, additives, carbon source, culture conditions has been attempted.

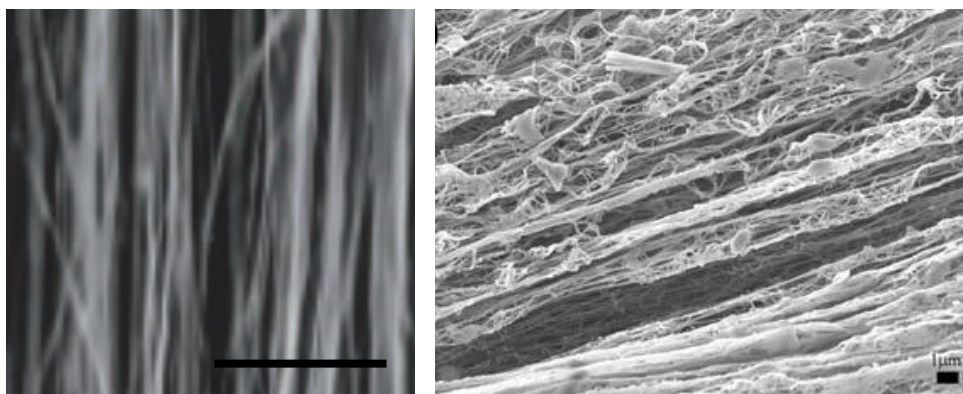


Figure 2.16: a) Bright field images of bacterial cellulose gel produced at ridged PDMS/liquid interfaces (scale bar 50 μm) [113] b) SEM images of the cellulose networks produced under voltage gradient 0.45 V/cm (scale bar 1 μm)[114]

2.8 Cellulose Nanoparticles

The properties, functionality, durability and uniformity that are required for the next generation of cellulose based products and their engineering applications cannot be achieved with traditional cellulosic materials. Cellulose nanoparticles are ideal materials on which these expectations can be based.

Cellulose nanoparticles of various kinds can be extracted, owing to various available cellulose sources and processes for nanoparticle extraction (Figure 2.17). Nine particle types are considered to describe the main cellulose-based particles, which typically differ from each other based on cellulose source materials and the particle extraction method. There are wood fibre (WF) and plant fibre (PF), microcrystalline cellulose (MCC), microfibrillated cellulose (MFC), nanofibrillated cellulose (NFC), cellulose nanocrystals (CNC), tunicate cellulose nanocrystals (t-CNC), alga cellulose nanocrystals (AC) and bacterial cellulose particles (BC) [4]. The main important and widely used methods for cellulose nanofibres isolation include mechanical (such as crushing, cryocrushing), chemical (such as acid hydrolysis, alkaline hydrolysis, organic solvent treatment and ionic liquid treatment), physical (such as ultra-sonication, microwave, gamma rays irradiation) and biological (cellulose degrading enzyme extracted from various organisms) treatments or a hybrid of the above [115]. SEM images of some of the cellulose nanoparticles are shown in Figure 2.18, and their details are summarised in Table 2.2.

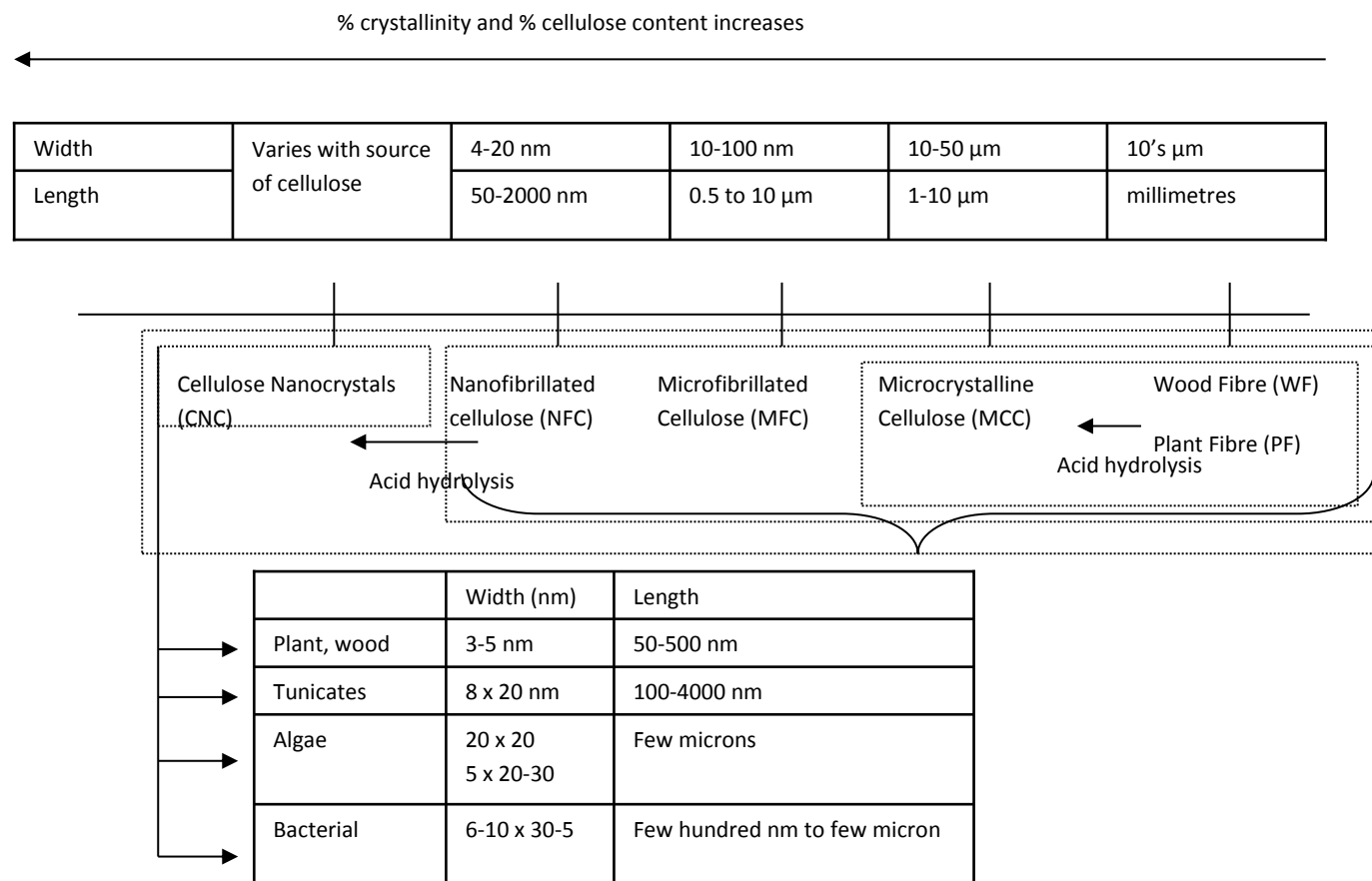


Figure 2.17: Summary of the dimensions and production of different kinds of cellulose nanoparticles (constructed on the basis of [4])

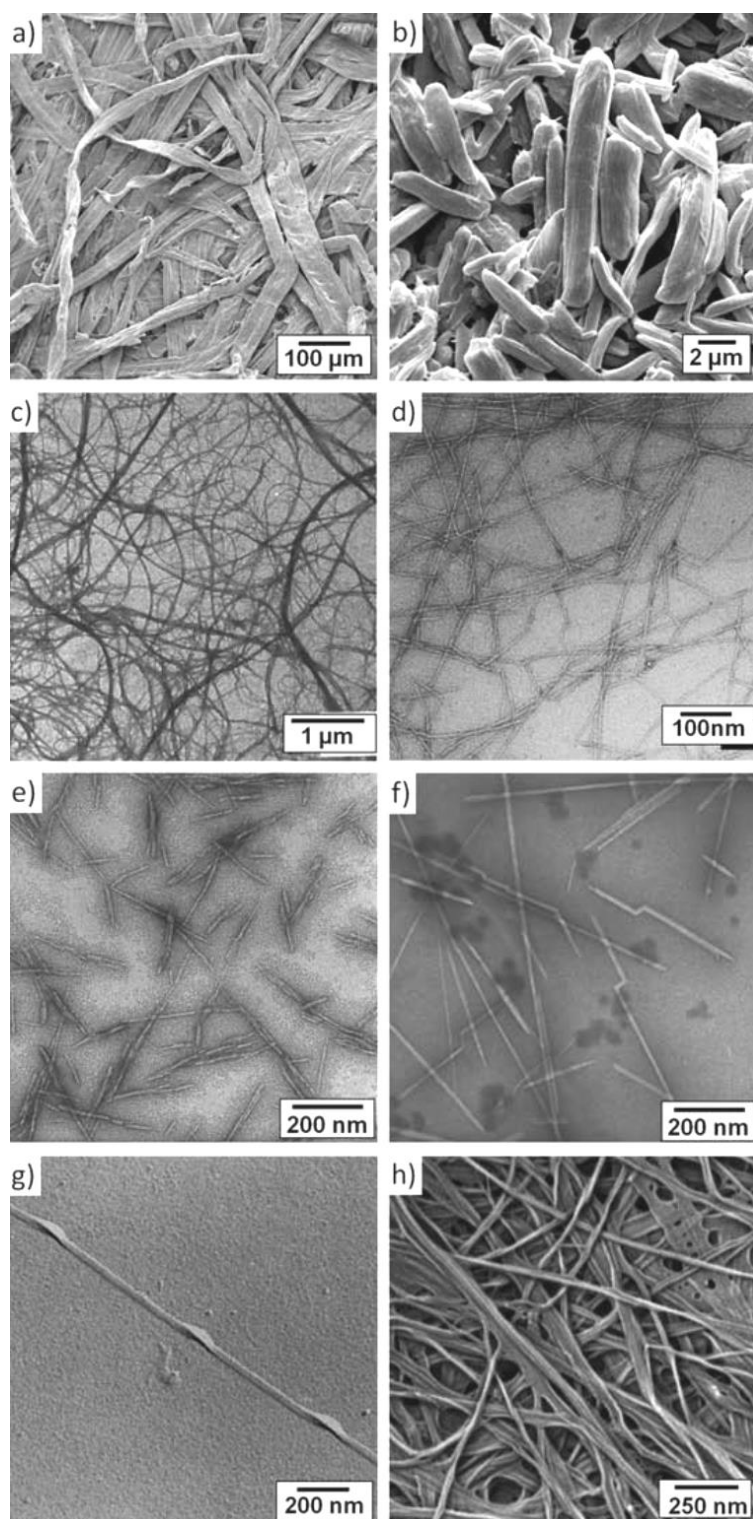


Figure 2.18: Cellulose nanoparticles a) SEM image of WF [116] b) SEM image of MCC that has been de-agglomerated [117] c) TEM image of MFC [118] d) TEM image of Tempo mediated oxidised NFC [119] e) TEM image of wood CNCs f) TEM of t-CNC g) TEM of AC [120] h) SEM image of BC (adapted from [4])

Table 2.2: Summary of different nanoparticles obtained from cellulose (adapted from [4])

Particle Type	Particle size			Cross section	Crystallinity (%)
	Length (μm)	Width (nm)	Height (nm)		
WF and PF[116]	>2000	20-50	20-50		43-65
MCC [117]	10-50	10-50	10-50		80-85
MFC[118]	0.5-10's	10-100	10-100		51-69
CNC[117, 121-123]	0.05-0.5	3-5	3-5	Square	54-88
t-CNC [46, 121, 124]	0.1-4	20	8	Parallelogram	85-100
AC [125]					>80
(valonia)	>1	20	20	Square	
(Micrasterias)	>1	20-30	5	Rectangular	
BC[126]					65-79
(Acetobacter)*	>1	30-50	6-10	Rectangular	63
(Acetobacter)	>1	6-10	6-10	Square	

*modified bacterial cellulose

Out of the various nanoparticles possible, the cellulose nanocrystals (CN), also called cellulose nanowhiskers, hold special importance because of certain exceptional qualities discussed below. CNs have high aspect ratio, low density (1.6 g/cm^3), and a reactive surface of -OH side groups that facilitates grafting chemical species to achieve self-assembly, controlled dispersion within a wide range of matrix polymers, and control of both the particle-particle and particle-matrix bond strength. Some variety of CN composites produced to date can be, transparent, have tensile strengths greater than cast iron, and have very low coefficient of thermal expansion (CTE). Potential applications include barrier films, antimicrobial films, transparent films, flexible displays, reinforcing fillers for polymers, biomedical implants, pharmaceuticals, drug delivery, fibres and textiles, templates for electronic components, separation membranes, batteries, super-capacitors, electro-active polymers, and many others. Loads of reviews are available on cellulose nanowhisker production, properties and applications [4, 127, 128]. The production of cellulose nanowhiskers and properties with special emphasis on mechanical properties and liquid crystallinity, are discussed in the following section.

2.9 Cellulose nanowhiskers

2.9.1 Production of nanowhiskers

The formation of a colloidal suspension of cellulose nanocrystals, also called nanowhiskers, produced by sulphuric acid hydrolysis was first reported by Ranby and

Ribi [76, 129]. Cellulose nanowhisker production is a two-step procedure, chemical hydrolysis and mechanical dispersion. The acid hydrolysis of cellulose microfibrils is a heterogeneous acid diffusion process where the less ordered regions are attacked by acid as shown in Figure 2.19. The overall efficiency of acid hydrolysis is determined by the kinetics of the process which in turn is dictated by time temperature and acid concentration. The reaction continues until all the amorphous regions are hydrolysed to glucose and then slows down significantly as the levelling off degree of polymerisation (LODP) is reached and the remaining acid attacks the surface as well reducing end groups of cellulose. After LODP is reached, there are two further mechanisms which support further hydrolysis of cellulose nanowhiskers. First was suggested by Nickerson and Harble that the material dissolves by the surface chain oxidation which in turn increases the aspect ratio of cellulose nanowhiskers [130]. The second mechanism was developed by Sharples [77] which involved acid attack on the ends of the crystallites. This leads to decrease in the aspect ratio. Dong *et al.* have conducted a detailed investigation on the effect of hydrolysis condition on the nanowhiskers properties and supported the later theory [131]. They also studied the effect of temperature on the nanowhiskers production and showed that at low hydrolysis temperature (26°C), the reaction took 18h and produced whiskers with a poor yield while at 65°C the hydrolysis could not be controlled due to dehydration. At 45°C the reaction proceeded smoothly and produced good yield within 1h [131].

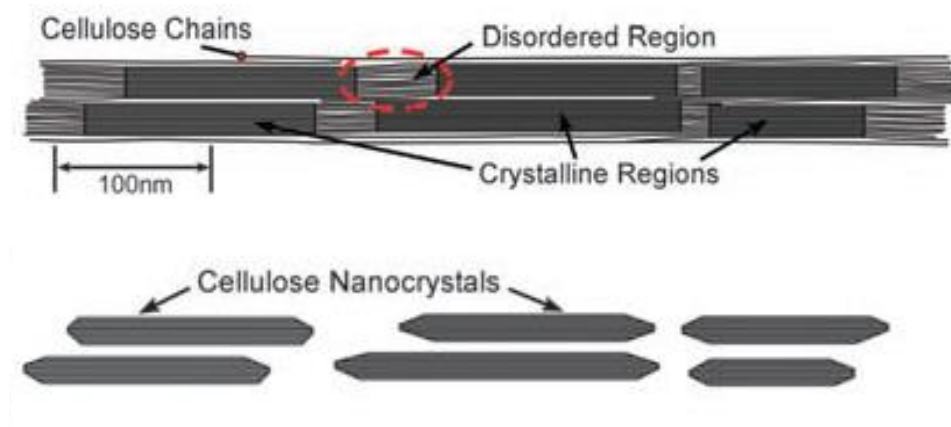


Figure 2.19: Schematic representation of the effect of acid hydrolysis on microfibril [4]

Conventionally sulphuric acid (65 wt%) has been used for the acid hydrolysis treatment of cellulose to produce nanowhiskey [117, 121, 132-137]. In addition, hydrochloric acid and phosphoric acid have also been used. Sulphuric acid induces sulphate group on the surface of cellulose while HCl does not. This is the reason why sulphuric acid derived cellulose nanowhiskers form electrostatically stabilised suspension in water but the HCl derived cellulose nanowhiskers do not [138]. The crystallinity of the starting cellulose material also determines the effect of acid hydrolysis [139]. The effect of acid concentration, temperature, solid to acid ratio, time of reaction has been studied on cellulose morphology and some common observations have been made [117]. The conditions also affect other properties of CNCs such as thermal stability, mechanical strength.

In the last five decades, the native cellulose nanowhiskers have been produced from a variety of cellulose sources such as microcrystalline cellulose (MCC), cotton, tunicates, bacterial cellulose. Wood cellulose nanowhiskers are 3-5 nm in width and 100-200 nm in length [122, 140, 141], while tunicate cellulose nanowhiskers are about 10-20 nm in width and 500-2000 nm in length [142, 143]. Similarly, cotton derived nanowhiskers are 5-10 nm wide and 100-300 nm long [144, 145] and nanowhiskers derived from a sea plant, *Valonia*, are 20 nm wide with 1000-2000 nm length [146]. The images of nanowhiskers from various sources obtained by electron microscopy are shown in Figure 2.20 and dimensions of these obtained in various studies are listed in Table 2.3. From the data illustrated in the table, it can be seen that there is a large scatter in the length and width of the nanocrystals.

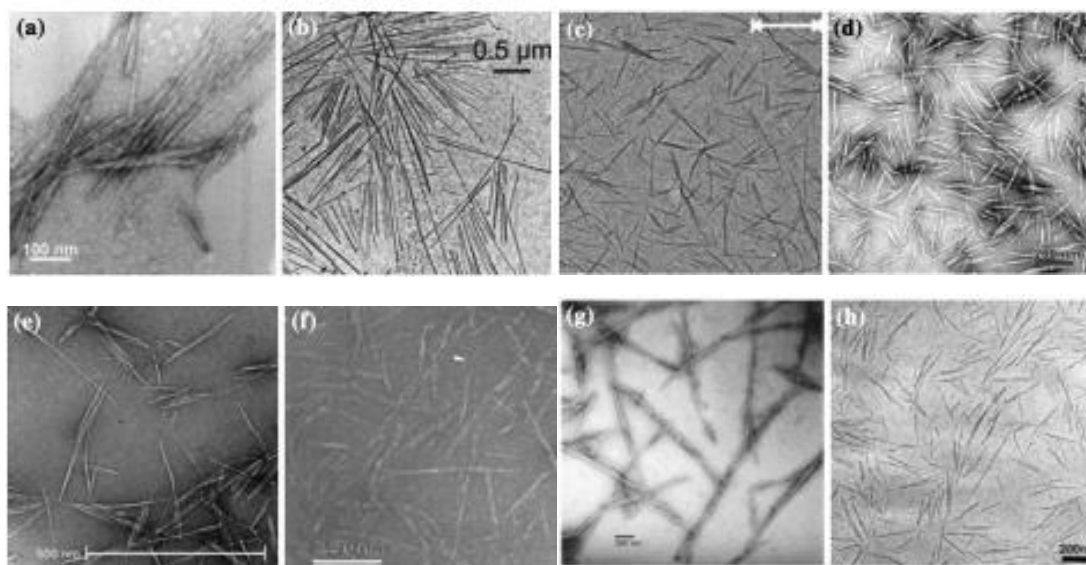


Figure 2.20: TEM images of cellulose whiskers obtained from acid hydrolysis of a) microcrystalline cellulose [147] b) tunicate [142] c) cotton [148] d) ramie [149] e) sisal [150] f) straw g) bacterial cellulose [151] h) sugar beet [152] (Reproduced from [153])

Table 2.3: Length and width of cellulose nanocrystals obtained from various sources [137]

Source	Length (nm)	Width (nm)	Technique	Reference
Bacterial	100-1000	10-50	TEM	[144]
	100-1000	5-10×30-50	TEM	[154]
Cotton	100-150	5-10	TEM	[155]
	70-170	7	TEM	[156]
	200-300	8	TEM	[145]
	255	15	DDL	[157]
	150-210	5-11	AFM	[158]
Cotton linter	100-200	10-20	SEM-FEG	[159]
	25-320	6-70	TEM	[121]
	300-500	15-30	AFM	[160]
MCC	35-265	3-48	TEM	[121]
	250-270	23	TEM	[161]
Ramie	150-250	6-8	TEM	[162]
	50-150	5-10	TEM	[163]
Tunicate		8.8×18.2	SANS	[164]
	1160	16	DDL	[157]
	500-1000	10	TEM	[142]
	1000-3000	15-30	TEM	[143]
	100-1000	15	TEM	[145]
	1073	28	TEM	[121]
Valonia	>1000	10-20	TEM	[146]
Soft wood	100-200	3-4	TEM	[140]
	100-150	4-5	AFM	[122]
Hard wood	140-150	4-5	AFM	[122]

2.9.2 Properties of cellulose nano-crystals

Cellulose nanoparticles are attractive from various perspectives as compared to the native cellulose material. The mechanical, thermal, self-assembly, rheology and optical properties of cellulose nanoparticles have them suitable for modern applications. Cellulose nanocrystals have garnered in the materials community a tremendous level of attention that does not appear to be relenting. However, from the view point of this thesis, only mechanical and self-assembly behaviour have been discussed in details.

a) Mechanical properties

The techniques such as Raman, AFM indentation, AFM 3 point bend and inelastic X-ray scattering have been used for measurement of mechanical properties of cellulose nanoparticles. Table 2.4 lists the mechanical properties of various cellulose nanoparticles both obtained experimentally and calculated. A wide distribution of properties have been reported, owing to the variation in the measurement techniques, and variation within the material such as crystallinity, relative distribution of the two polymorphs, anisotropy, and defects.

The elastic properties of cellulose I particle have been investigated since 1930s by theoretical and experimental techniques. Cellulose nanocrystals have higher modulus than the other cellulose nanoparticles (Table 2.4). This is because nanocrystals are made by the removal of amorphous regions, leaving behind crystallite. One of the common techniques has been *in situ* tensile test combined with XRD to measure strain. The values for elastic modulus in axial direction (E_A) ranging from 120 to 138 GPa have been reported [3, 165]

However, in this technique, a perfect load transfer and orientation is assumed and thus might underestimate the true values. Inelastic X-ray scattering (IXS) avoids the perfect load transfer issue and the reported values for elastic modulus in axial direction (E_A) = 220 GPa and elastic modulus in transverse direction (E_T) = 15 GPa [3].

Bacterial cellulose (BC) and tunicate cellulose (TC) nanocrystals have been used for direct measurements by AFM due to their higher crystallinity, uniform and large particle cross section. A study by Iwamoto [46], where AFM three point bending was performed, the E_A for TC nanocrystal was found to be 151 GPa. Another technique,

involving Raman spectroscopy in combination with tensile testing, resulted into similar values [47]. Other values obtained from these techniques in different pieces of work are listed in Table 2.4. The reported values of E_A for BC nanocrystals were 78 GPa [166] and 114 GPa [167] measured by AFM- three point bend and Raman, respectively.

It is known that TC is rich in $I\beta$ polymorph while BC in $I\alpha$ polymorph. Some researchers [168] have predicted modulus of $I\alpha$ more than $I\beta$ and others [169] have reported vice versa, by modelling. However, in experimental studied using Raman, the modulus was found to be larger for t-CNC than that for BC [47, 169]. AFM measurements were also found to be in line with this result [46, 166].

Table 2.4: Summary of properties of cellulose nanoparticles (Adapted from [4])

Material	E_A (GPa)	E_T (GPa)	σ_f (GPa)	ε_f (%)	Technique	Reference
WF	14-27		0.3-1.4	4-23	Tensile	[170]
PF	5-45		0.3-0.8	1.3-8	Tensile, Raman	[153]
MCC	25±4				Raman	[171]
CNC						
Plant	57,105				Raman	[172]
Wood		18-50			AFM indentation	[173]
t-CNC	143				Raman	[47]
	151 ±29				AFM-3pt bend	[46]
BC	78 ±17				AFM-3pt bend	[166]
	114				Raman	[167]
Cellulose $I\beta$						
Experimental	120-138				XRD	[3, 165]
	220 ±50	15±1			IXS	[3]
Modelling	137-168	10-50	7.5-7.7			[169]
Cellulose $I\alpha$						
Modelling	128-155	5-8				[169]

where E_A = elastic modulus in axial direction, E_T = elastic modulus in transverse direction, σ_f = tensile strength ε_f = strain to failure

b) Liquid crystallinity of Cellulose Nanocrystals

Cellulose and cellulose derivatives have been reported to form chiral nematic phases both in solution (lyotropic liquid crystal)[174] and in absence of solvent (thermotropic liquid crystal) [175]. The preservation of helicoidal organisation of liquid crystalline phase of cellulose and its derivatives has been seen in films [176] and cross-linked gels [177].

The analogy between the molecular orientation in liquid crystalline phase and that present in microfibrils in biological systems has been indicated [178]. A natural microfibril liquid crystalline suspensions was observed in form of glucuronoxylan stabilized suspension of cellulose microfibrils in quince seed mucilage [179]. The first in-vitro self-assembly of cellulose nanowhiskers, obtained by acid hydrolysis on microfibril, into a chiral-nematic (cholesteric) liquid crystalline phase was reported in 1992 [180]. The formation of rigid rod-like entities was a result of acid hydrolysis which can overcome entanglements and bundling.

The transition from isotropic to liquid crystalline suspension occurs across a biphasic concentration range, similar to that observed for other liquid crystal entities such as virus [181], deoxyribose nucleic acid (DNA) [182], and carbon nanotubes [183]. The transition concentrations depend on the aspect ratio of the nanowhiskers obtained after hydrolysis, which in turn depends on the cellulose source as discussed in the previous section (Table 2.3) [137]. The individual cellulose nanocrystallites obtained from acid hydrolysis of filter paper were 7 nm wide by 70-170 nm long which showed transition between 4 and 13 wt% as evident from the phase diagram shown in Figure 2.21 (a) [156, 184]. The formation of a liquid crystalline phase for nata-de-coco (food grade bacterial cellulose) nanowhiskers was found to initiate at 0.3 wt% and reach completion at 1.2 wt%. The dimensions of cellulose nanowhiskers were 10 nm by 50 nm by 1-2 μm , i.e. an aspect ratio of 50-100, which was larger than that of filter paper nanowhiskers in the previous study [135]. The cellulose nanowhiskers obtained from tunicate cellulose are known to have an even higher aspect ratio and the formation of a liquid crystalline phase was found to start at 1 mg/ml and completes at 5-6 mg/ml (POM photographs included in Figure 2.21(b)) [138]. Thus in the literature, for bacterial and tunicate cellulose nanowhiskers, the transition is observed at much lower concentration owing to higher aspect ratio compared to nanowhiskers from other cellulose sources. Cotton nanowhiskers of different lengths were prepared by varying the hydrolysis temperature and dispersed in model apolar solvent using ethoxylated phosphoric ester of nonylphenol (BNA) [134]. It was thus demonstrated that the transition to liquid crystalline phase shifts to a higher concentration with a decrease in aspect ratio and a broader biphasic region due to polydispersity and increased attractive interaction between nanowhiskers as compared to water as a solvent.

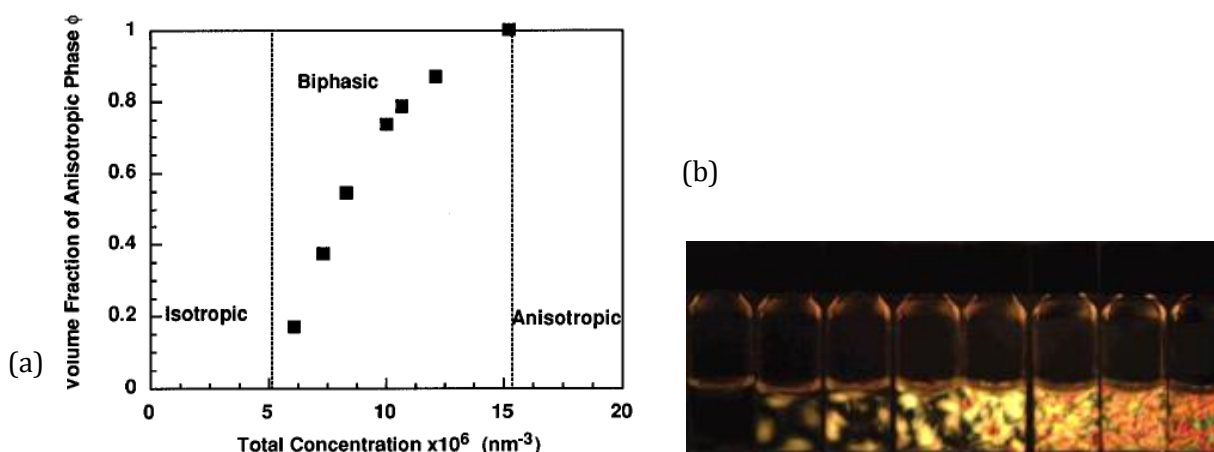


Figure 2.21: a) Phase behaviour of BC nanocrystals dispersed in deionized water as a function of the total concentration [156] b) Photographs of dispersions of Tunicate nanowhiskers in water [138]

Almost all the work done on liquid crystalline phase from cellulose nanowhiskers has shown formation of a finger print texture, illustrated in Figure 2.22 (a), when observed in polarised optical microscope which is a signature of chiral nematic phase [134, 143, 145, 156, 180]. This implies the cellulose nanowhiskers are arranged in several nematic planes (planes with orientational order) but each nematic plane is stacked on top of the other with their orientation director forming an angle with each other, as shown in Figure 2.22 (b). Araki *et al.* showed the first instance of the formation of a nematic liquid crystalline phase by nata-de-coco nanowhiskers [144, 185] where the addition of electrolyte leads to phase change from nematic to chiral nematic phase as demonstrated in Figure 2.23.

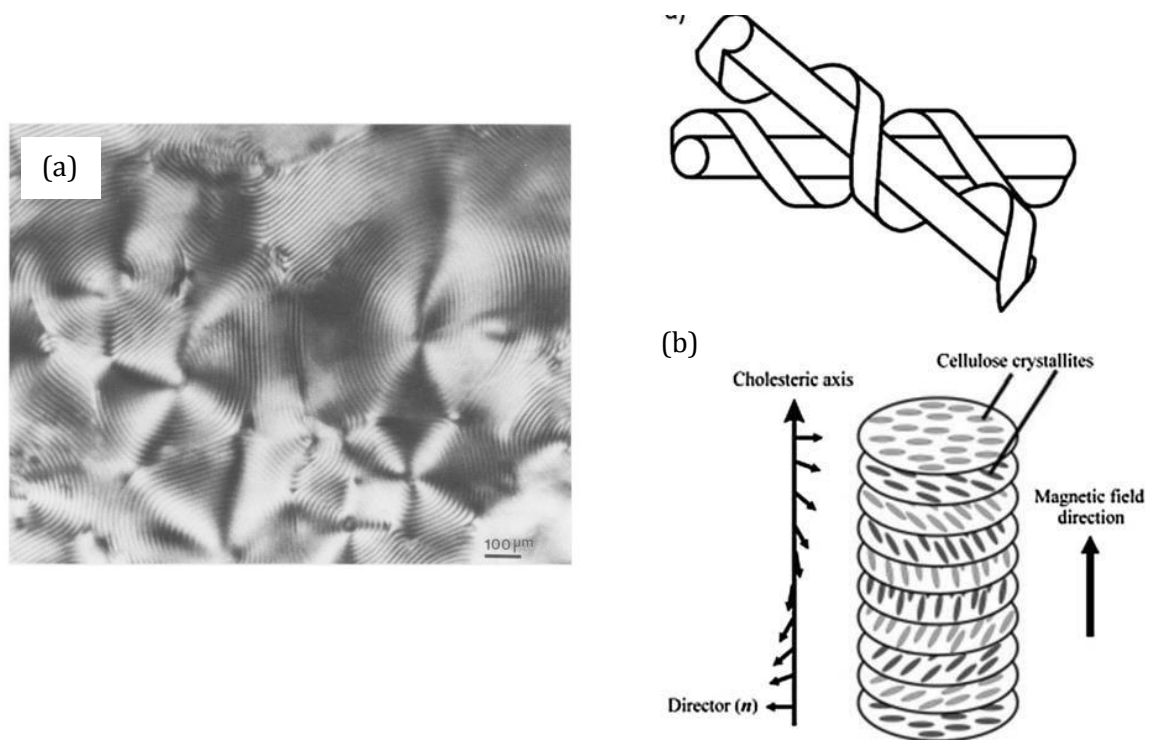


Figure 2.22: a) CNC suspension viewed via optical microscopy showing the fingerprint texture of chiral nematic structure as viewed through crossed polarizers [180] b) Schematic of a chiral nematic phase (magnetic field is indicated to just show the resulting orientation of nanowhiskers with respect to field) and probable twisted morphology of nanowhiskers [186]

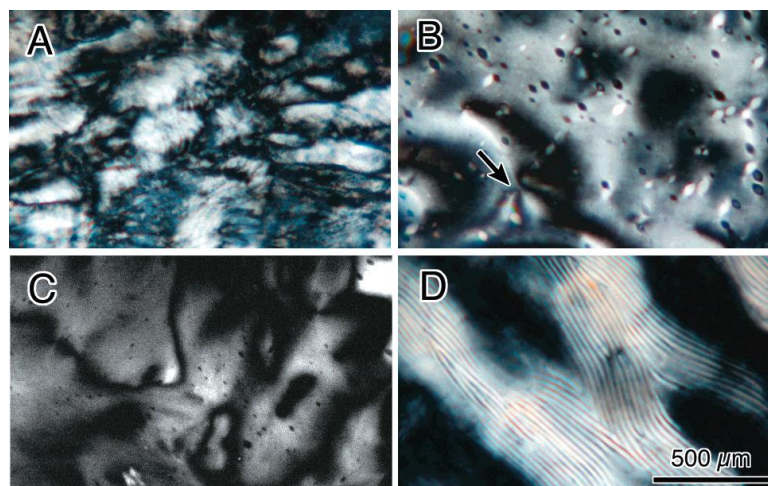


Figure 2.23: Polarised optical micrographs of BC nanowhiskers anisotropic phase. (A-C) no salt, cellulose concentration 1.23%; (D) 0.1 mM NaCl, cellulose concentration 1.58%. [135]

Apart from cellulose nanowhiskers, various other rod-like particles such as DNA and viruses, form chiral nematic phase formation. In regularly built structures like those obtained from DNA or viruses the origin of chiral interaction can be attributed to the natural regular helical conformation [187-189]. In the case of irregular nanorods like cellulose nanocrystals, the origin of chiral interaction is complicated to understand. The work reported in the literature is reviewed here.

The most accepted theory for the origin of chiral interaction between cellulose nanowhiskers is that the cellulose nanowhiskers are also thought to possess natural repeating twists. This presence of twist in cellulose microfibrils has been supported by various experimental observations [120, 135, 190-192]. The twist has been observed under electron microscopes in the case of bacterial cellulose microfibrils [144, 191], tunicate nanowhiskers [192] and green alga microfibrils (Figure 2.25 (a)) [193]. The twist was found to repeat every 700nm in case of the green alga. The helical nature of cellulose nanowhiskers has been interpreted by small angle neutron scattering [190]. In case of cotton nanowhiskers and also in bacterial cellulose nanowhiskers, the pitch of the chiral nematic phase was found to decrease with increases in salt concentration [135]. These observations suggest that the chiral interactions become stronger on addition of salt which screens the surface charges and increases the interaction (Figure 2.24). So, upon addition of salt the nanowhiskers interact and form chiral nematic phase, which can be explained only by the twisted nature of cellulose nanowhiskers.

On the other hand, some researchers criticize the natural twist hypothesis because of the following reasons. First of all, the twists in the microfibrils have not observed by microscopy of all cellulose sources. Second, it should also be noted that the distance between nanowhiskers is more than five times the diameter of the nanowhiskers, which suggest that the geometric twist alone would not be sufficient to mediate chiral interactions. PEG grafted cellulose nanowhiskers lead to a decrease in pitch which means stronger chiral interaction in spite of screening of the shape by PEG [155]. This observation is non-intuitive, if the chirality arises from geometric twist alone. Elazzouzi Hafraoui *et al.* investigated the formation of chiral nematic phase by replacing strongly repulse electrostatic interactions for a smoother steric repulsion [134]. Cellulose nanowhiskers were grafted with surfactants and dispersed in cyclohexane. Given the low ionic dissociation constant and insignificant role of the

remaining charges, any direct transfer of chirality of cellulose at a molecular level would be prohibited, owing to large inter-particle distance and covering layer of surfactant. In this situation, according to Straley's theory, the pitch should show dependence on inverse of the square of the concentration, which was not observed experimentally. Hafraoui and co-workers argued that if the chiral interactions arose from the geometrical constraints, then the interaction should depend on the distance between the nanocrystals (25-40 nm). For the same value of inter particle distance in water, the pitch was found to be much larger than that in cyclohexane even when the electrostatic interaction was screened by salt in aqueous suspensions. This work emphasises the importance of solvent dielectric properties for the chiral nematic interactions and disregards the natural twist theory.

However, despite all these arguments and observations, the aforementioned natural twist hypothesis is more accepted [135]. Various investigations have shown that bacteria produce twisted and bundled cellulose microfibrils (Figure 2.25 (b))[191]. The twists seen for bacterial cellulose are consistent with those seen for cellulose microfibrils from other sources. The occurrence of twists in bacterial cellulose microfibrils has been attributed to either the bacterial cell rotation [56] or intrinsic chirality of cellulose chains [125, 194]. The former does not explain the presence of twist in cellulose microfibrils from other sources, moreover the bacterium possess no flagella or other mechanism of locomotion and thus the twist should be attributed to the cellulose molecules or cell surface interaction [194]. The latter is in line with the observation that the chirality of cellulose is manifested as a helical structure of the cellulose chains which has been supported by induced circular dichroism, nuclear magnetic resonance and the formation of the chiral nematic phase [195, 196]. Simulation of cellulose nanofibrils in various carbohydrate environments has also shown the development of a helical twist in the fibril [158,159]. In systems comprising of fibrils with 36 and 59 chains simulated in a GROMACS environment with optimized potential for liquid simulations, twisting was caused by reorientation of cellulose chains as a result of H-bonding for both the fibrils (Figure 2.25 (c)) [197]. A similar study of the 36 chain model done using a different simulation package employing CHARMM force field also yielded a structure with a helical twist [198]. In another work, the degradation of bacterial cellulose by a fungi derived cellulase enzyme revealed rotation of

microfibrils during the treatment. The degradation altered the crystal structure, allowing the linear cellulose polymer to relax to a lower energy state thus relieving the strain induced by crystallization of nascent glucan chains during the biogenesis [199].

The transfer of twisted nature to macroscopic products has also been observed. For example, the fibres made from electro-spinning of cellulose derivative suspensions [161] exhibited same phenomenon as do the plant tendrils which are three or four orders of magnitude larger, curling into spiral or coiling into helices, the latter being able to reverse their handedness [200]. So far, the relationships between expressions of chirality at different morphological levels remain unclear [120, 199, 201].

In all the parts of evidence seen so far, both right as well as left handed twist have been observed, for example, a left handed occurs twist in bacterial cellulose and primary wall cellulose microfibrils [202] and a right handed twist is observed in the green alga microfibrils [120]. However, only left handed chiral nematic phase formation has been observed from cellulose nanowhiskers [180] .

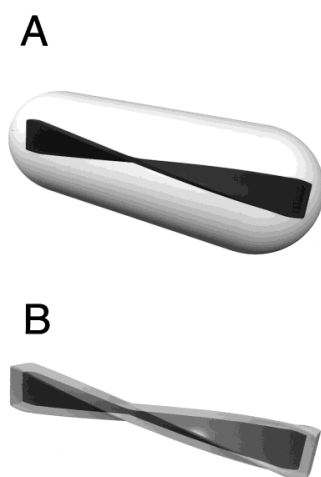


Figure 2.24: Schematic illustration of BC microcrystals with surface charge (A) in water, repulsion by surface charge extends to long range, resulting in an apparently non-chiral rod; (B) addition of NaCl decreases repulsion range and effective particle becomes twisted rod [135]

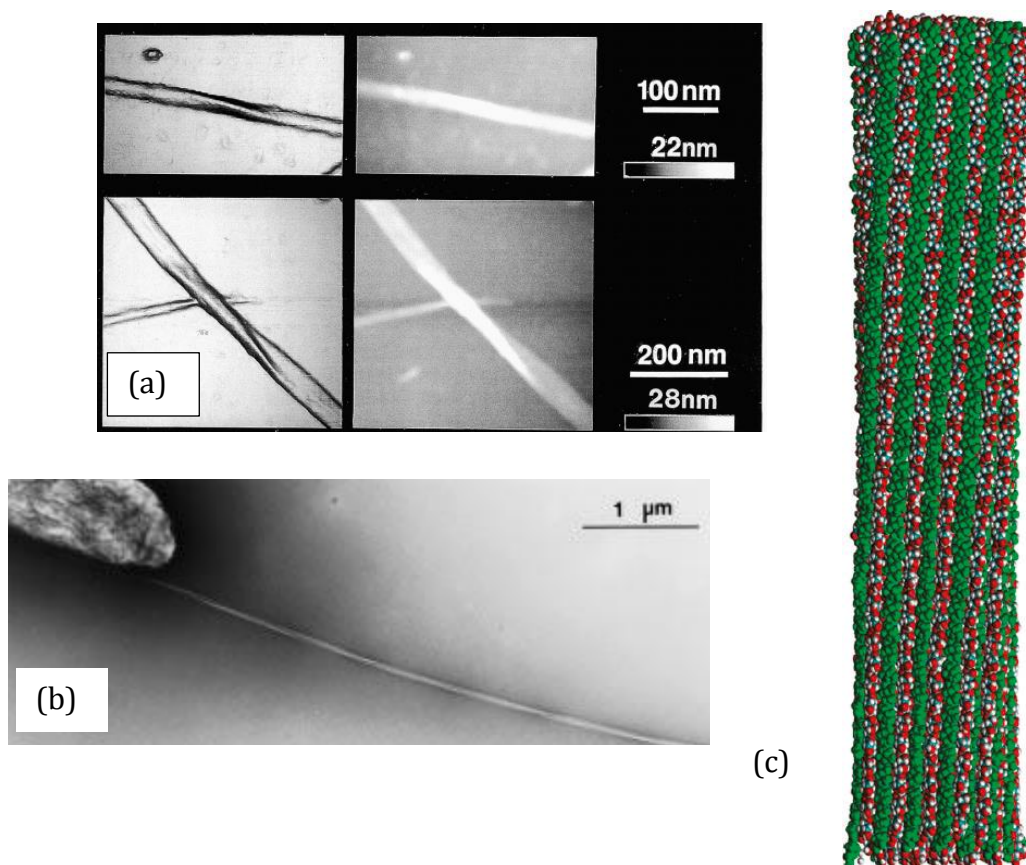


Figure 2.25: a) TEM image of alga microfibrils showing regular right handed twists [120] b) TEM image of bacterial cellulose producing twisted microfibrils [109] c) A simulated 59 chains fibril with twist [197]

2.9.3 Applications of cellulose nanocrystals

Cellulose nanocrystals are being considered in applications such as transparent films, barrier films, reinforcements, protein immobilisation and drug delivery [4, 128]. Because of the ability of nanowhiskers to form aligned domains, they are being used in various applications such as forming iridescent films and as a medium to align proteins.

Chiral nematic films can be cast from cholesteric suspension of cellulose nanocrystals as the chiral nematic phase is conserved [203]. Iridescent films have been prepared from nanocrystalline cellulose derived from wood and effect of ionic strength, temperature, concentration, exposure to magnetic field on the chiral nematic properties has been used to tune its properties [204]. In addition cellulose nanowhiskers been used to make security papers due to its liquid crystalline behaviour [205, 206].

Owing to high mechanical properties of cellulose nanowhiskers, they are suitable as fillers. The use of nanocrystalline cellulose as a filler was first reported by Favier *et al.* in poly styrene co-butyl acrylate [207]. The properties of composites would depend on the morphology of the cellulose nanowhiskers and the matrix used. The aspect ratio of nanowhiskers determines the percolation threshold and higher aspect ratio leads to better mechanical properties. For example, tunicate cellulose nanowhiskey with aspect ratio 67 served as better filler than bacterial cellulose nanowhiskers with an aspect ratio of 60 and cotton nanowhiskers with an aspect ratio of 10 [50].

Various applications such as drug carrier of cellulose nanowhiskers in pharmaceutical research have been conceived and are being investigated. Nanocrystalline cellulose, being non-toxic, has been shown to be a suitable carrier for therapeutics [208]. Dong and Roman have reported a method to label nanocrystalline cellulose with fluorescein-5'-isothiocyanate (FITC) for fluorescence bioassay and bio-imaging techniques [209].

The interaction of nanowhiskey has been used to mimic biological systems like that in a sea cucumber which allows it to reversibly change stiffness. In the designed nanocomposite of nanowhiskers in rubbery ethylene oxide, a small amount of water uptake caused dramatic modulus reduction from 800 to 20 MPa [210]

Flexible transparent nanocomposites were obtained by reinforcing resins with high nanofibre content with enhanced mechanical properties and low CTE [211].

2.10 Summary and the aims of the thesis

The abundance and properties of cellulose has prompted the wide variety of usage. The vegetal sources of cellulose constitute the main source of cellulose. This leads to extensive deforestation and pollution due to the heavy chemical treatment required. Bacterial cellulose, an alternative cellulose source, is better in terms of purity, crystallinity and mechanical properties. However, the utilization of alternative cellulose sources such as bacterial and tunicate cellulose remains limited.

The important aspect of cellulose is the hierarchical organisation of cellulose chains into microfibrils. The supra-molecular organisation allows efficient transfer of properties of cellulose chains to microfibrils resulting into microfibrils with attractive

mechanical properties. Cellulose nanoparticles can be obtained by various treatments such as fibrillation, chemical treatments, out of which nanowhiskers obtained by acid hydrolysis are particularly useful as they have shown to self-assemble into a liquid crystalline phase. However a very limited amount of work has been reported on the liquid crystalline phase formation from non-vegetal celluloses.

One of the most important applications of cellulose is in form of fibres. So far wood has been the major source of the raw material. And also the commercial cellulose fibres are made by cellulose regeneration which leads to cellulose II structure which is inferior in properties. So far no fibres have been made where the natural cellulose crystal structure is conserved. Limited literature is available on liquid crystal phase formation of bacterial cellulose and fibre from cellulose with cellulose I structure.

Chapter 3

Materials and Methods

In the following sections, the protocols used for producing and extracting cellulose and cellulose nanowhiskers and the procedure used for fibre spinning have been described. Various characterisation techniques used for assessing the composition, internal structure, and properties of the as-produced cellulose, cellulose nanowhisiker and fibres have been also discussed.

3.1 Cellulose production

Three kinds of cellulose were obtained for this work: nata-de-coco (NdC), laboratory cultured bacterial cellulose (BC) and tunicate cellulose (TC). The production, extraction and procurement of all them is described in the following sections.

3.1.1 Nata-de-Coco

Nata-de-coco (NdC), the commercially available bacterial cellulose, was obtained from Yeguofood Pvt. Ltd, China in form of small cubes of dimension 1 cm (Figure 3.1). NdC is produced in food industries by the fermentation of coconut milk as follows [W2]:

The coconuts are grated in water and coconut milk is extracted. Sugar and acetic acid are fermented with the bacteria *Acetobacter Xylinum*. The slurry is mixed well to dissolve sugar. Then, it is poured into shallow containers and tightly covered with paper and maintained at 28-32°C. A thick gel-like layer of cellulose is obtained at the surface of the container in 8-10 days. The culture may be kept static or agitated. The material obtained for this study was prepared in a static environment. The bacteria contained in the nascent gel are removed by immersing it in dilute alkaline solution and washing with water. The gel obtained was more than 95% water.



Figure 3.1: Photograph of nata-de-coco cubes [W3]

3.1.2 Bacterial cellulose

The bacterial cellulose (BC) was produced in laboratory using three different strains of bacteria *Acetobacter Xylinum* or *Gluconobacter Xylinum*, namely AX, AY and ATCC. All the strains for bacterial cellulose production were procured and cultured at Dr. Dana Kralisch and Dr. Nadine Heßler's Laboratory, Friedrich Schiller University, Jena, Germany to produce cellulose and details about the bacterial strain are included in the Table 3.1. The Hestrin-Schramm culture medium (Figure 3.2 (a)) was used for all the strains, which consisted of distilled water with glucose (20 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate (Na_2PO_4) (3.4 g/L), citric acid ($\text{C}_6\text{H}_8\text{O}_7$) (1.15 g/L) (Table 3.2) [30-33]. The bacterial cell cultures were set up in 5 petridishes (25 mL media) for each strain of bacteria as shown in the Figure 3.2 (b). The cellulose was collected at the end of 14 days for all the strains. Figure 3.3 shows the cellulose produced in the petridishes for each of the strains. The as-produced cellulose pellicles were boiled in 1M NaOH solution and washed with distilled water (Figure 3.4) to obtain cellulose free of the culture media and bacterial cells. The cleaned pellicles, shown in Figure 3.5, were sterilized in an autoclave to preserve till further use. The cellulose produced by the bacteria AX, AY and ATCC are referred as BC_AX, BC_AY, and BC_ATCC, respectively. The three strains produced cellulose in different quantities and in different morphologies. The strain AX form a thick tough pellicle, while ATCC makes a much thinner pellicle of less than half the thickness of those produced by AX. AY on the other hand failed to form a pellicle.

Table 3.1: Details of the strain of the bacteria *Acetobacter Xylinum* used to produce BC

Strain	Designation	Source
AX5	AX-DSM 14666	Institute for organic chemistry and macromolecular chemistry, Jena
AY	ATCC 23769	Courtesy Dr. R.M. Brown Jr (USA)
ATCC	ATCC 10245	Courtesy Dr. R.M. Brown Jr (USA)

Table 3.2: Details of the Hestrin-Schramm bacterial cell culture medium

Chemical	Details	Source
Glucose	D-(+)-Glucose (anhydrous)	Fluka purity $\geq 99.5\%$
Peptone	Bactopeptone (enzymatic degraded protein)	Difco Laboratories, Detroit, USA
Yeast extract	Yeast extract (autolyzed yeast cells)	Difco Laboratories, Detroit, USA
Sodium phosphate	$\text{Na}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Disodium hydrogen phosphate dehydrate)	Fluka, purity $\geq 99.5\%$
Citric acid	$\text{C}_6\text{H}_8\text{O}_7$ (Citric acid monohydrate)	Fluka, purity $\geq 99.5\%$

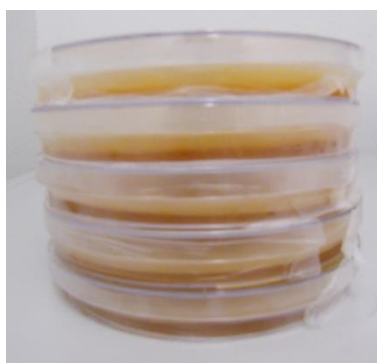


Figure 3.2: a) Photograph of a glass bottle containing Hestrin-Schramm culture medium b) 5 petridishes containing 25 mL of the culture media, inoculated with bacteria and covered with parafilm

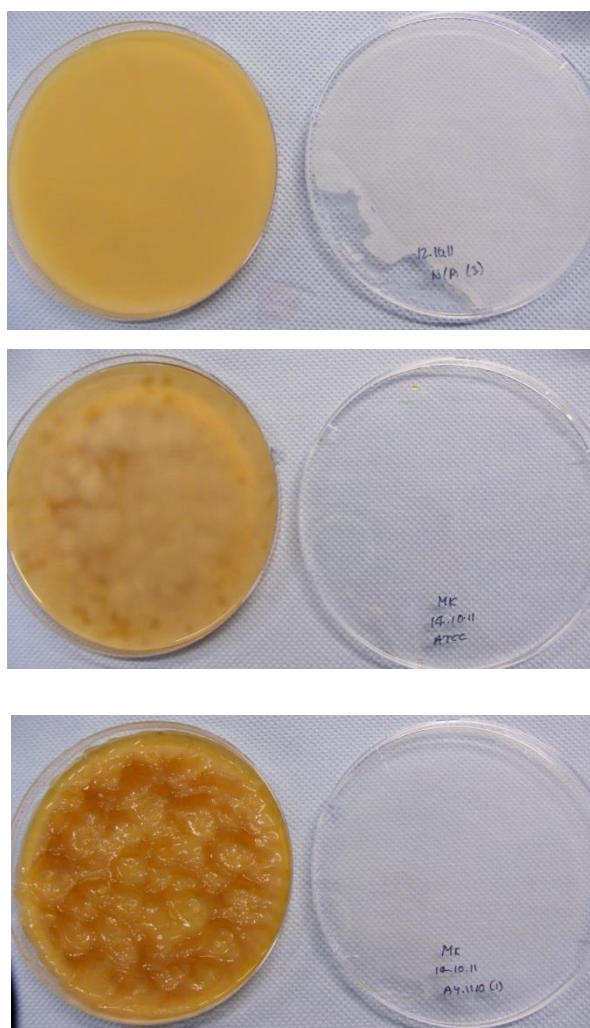


Figure 3.3: Pellicles obtained after 14 day cultivation from the strains a) AX b) ATCC c) AY



Figure 3.4: Treatment of cellulose pellicles with 1M NaOH with constant stirring to remove media and bacterial cell debris



Figure 3.5: Pellicles after removal of bacteria and the media a) BC_AX b) BC_ATCC c) BC_AY

3.1.3 Tunicate Cellulose

The tunicates or sea squirts were obtained from Lochfyne seafarm Ltd., UK, in order to extract cellulose. The following procedure was used to extract cellulose from tunicates (This protocol was obtained from Dr. James Dugan, student of Prof. Stephen Eichhorn). The gutted tunics were cleaned in running water and incrustations/other organisms such as mussels and dirt were removed. The cleaned tunics were chopped using an ordinary kitchen knife and chopping board into pieces measuring about 1 or 2 cm across. About 50g chopped tunic were treated in 300ml of 5% (w/w) KOH (aq.) and stirred at 80°C overnight. The cooled mixture was filtered through a course filter and washed thoroughly in running tap water and then several times in deionised water. The pieces of tunic were then added to 300ml deionised water and set stirring on a hot plate at 60°C. Glacial acetic acid (0.5 mL) and sodium hypochlorite solution (4% Cl) (1mL) were added and continuously stirred for a further 5 hours. The mixture was allowed to cool and then rinsed thoroughly and ground into a pulp for later use.

3.1.4 Modification of Bacterial Cellulose

Polyethylene glycol (PEG), carboxy methyl cellulose (CMC), calcofluor (CF) and nalidixic acid (NA) were used as additives to modify the bacterial cellulose microstructure. The additives used are listed in Table 3.3 along with their role and amounts used in the experiments. The additives were added before the bacterial inoculation. The required amount (indicated in Table 3.3) were added in the culture medium and autoclaved. CF, NA and PEG easily dissolved while CMC dissolved only after autoclaving.

The modified medium (25 mL) were poured in 5 petridishes and incubated at 25°C to allow cellulose production. The cellulose pellicles were collected at the end of 14

days and the procedure described above in section 3.1.2 was used for removing the media and the bacterial cell debris. This work was done at Dr. Dana Kralisch and Dr. Nadine Heßler's Laboratory, Friedrich Schiller University, Jena, Germany

Table 3.3: Details of the additives used to modify bacterial cellulose

Modifier	Source	Amount (g/100mL)	Role
Calcofluor (CF)	Calcofluor white strain, (Sigma Aldrich)	0.1	Calcofluor is a dye used to locate polysaccharides [16]
Nalidixic Acid (NA)	≥98% (Sigma-Aldrich)	0.1mM	Nalidixic acid is an antibiotic which is bacteriostatic at low concentration and bactericidal at high concentration [106].
Polyethylene Glycol (PEG)	Poly ethylene glycol 4000 (Sigma Aldrich)	4	PEG is a water soluble polymer which also increases the viscosity of the system [110].
Carboxy methyl Cellulose (CMC)	Carboxymethyl cellulose sodium salt (Sigma Aldrich)	2	CMC is cellulose with one hydroxyl group substituted with the carboxymethyl group [103].

3.2 Methods

3.2.1 Production of cellulose nanowhiskers – Acid hydrolysis

The dried cellulose from the three sources was treated with dilute sulphuric acid to obtain dispersion of cellulose nanowhiskers [76, 180]. About 0.2 g of dried cellulose was treated with 20ml of 40% (vol/vol) sulphuric acid (H_2SO_4 , Sigma Aldrich >98% purity). The mixture was stirred with a magnetic stirrer and the temperature was maintained to be 45-50°C (Figure 3.6 (a)). This was continued for about 5 hours, till an off white to yellow coloured dispersion was obtained. However, for TC, it took longer to about 8-10 hours. The suspension was neutralised by exchanging the acid with deionised water using a 0.2 µm PTFE filter paper placed in a Buchner funnel (Figure 3.6 (b)). This was repeated till all acid was squeezed out and the pH of the suspension passing through the filter was close to 5. Finally, the suspensions (Figure 3.7) were collected from the funnel. Centrifuge (4000 rpm, 60 min) was also used to concentrate the suspensions to 15 wt%.

The surface charge was determined by titrating 50 mL of 1 wt% cellulose nanowhisker suspension in water with 0.01 M NaOH. The change in pH was observed. The volume of NaOH solution used at the point of inflection of pH change was used to calculate the milimoles required to titrate the initial amount of nanowhiskers in the suspension. The average dimensions of cellulose nanowhiskers were used to calculate the surface area of each nanowhiskers and number of nanowhiskers present in the suspension. The total surface area was used to calculate the surface charge density.

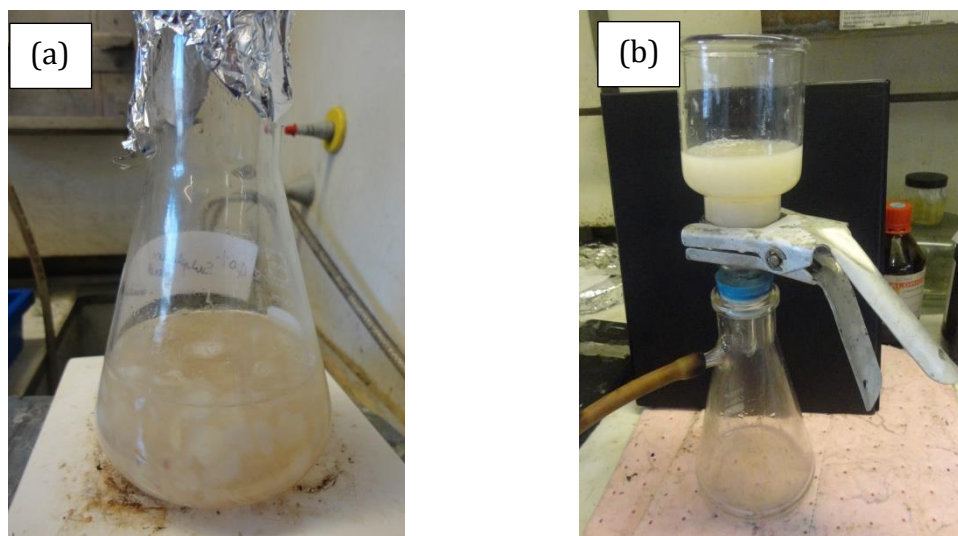


Figure 3.6: a) Flask containing 40% sulphuric acid (200 mL) with cellulose (2 g) at 45-50°C and constant stirring b) Filtration setup: funnel containing nanowhisker suspension with acid passing through a PTFE filter into the flask connected to a vacuum pump

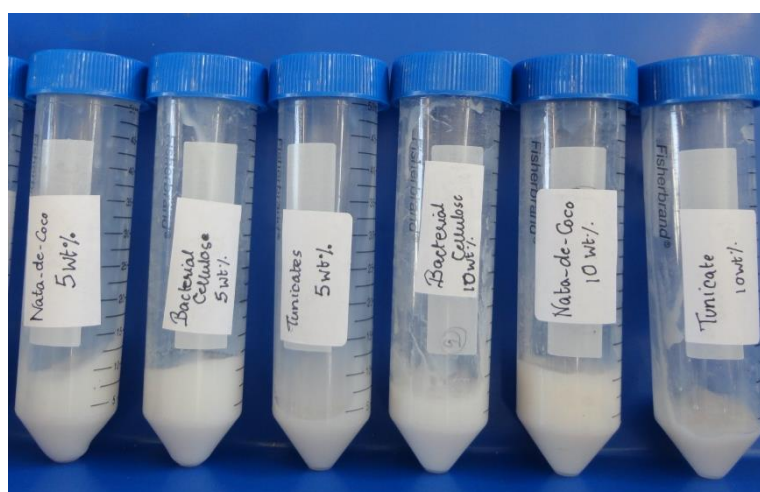


Figure 3.7: Photographs of tubes containing NdC, BC and TC nanowhisker suspension (5 wt%, 10 wt%)

3.2.2 Establishment of phase transition

The nanowhiskers suspensions, collected from the treatment described in section 3.2.1, were diluted multi-fold to prepare different concentrations (ranging from 0.01 wt% to 15 wt%) of cellulose nanowhiskers in order to develop an isotropic to a liquid crystalline phase transition diagram. The suspensions (10 mL) were allowed to undergo phase separation under the influence of gravity for 4 weeks and the volume fractions of phases obtained after phase separation were noted. The phase separation was also observed under the influence of enhanced gravitational pull using 1 mL of suspension in a MISTRAL centrifuge at 3500 rpm. The time of centrifuge speed was standardised by varying the time and observing the volume of phase collected at the bottom and choosing the time beyond which no significant change in volume was seen. After phase separation, the volumes of the top and the bottom phases were measured to plot a phase transition diagram, indicating the onset and completion of phase separation. The phase transition was also observed by polarised optical microscopy, which is discussed in section 3.3.4.

3.3.3 Fibre spinning

The fibre spinning setup for cellulose fibre spinning comprised of a plastic B.D syringe (5 mL, 12 mm diameter) with a 22G needle (0.4 mm diameter) (Figure 3.8(a)). The tip of the needle was grounded using emery papers (WS Flex 2500) . The extrusion speed of the syringe pump was controlled using a syringe pump (kd scientific, Linton instrumentation) (Figure 3.8 (b)). The extruded fibre was extruded through an air gap (Figure 3.8(c)) of 5 cm between the needle tip and the conveyer belt (Figure 3.8(d)), which was used to collect the fibre. The speed of the conveyer belt was controlled by varying the voltage using a power supply (Farnell instruments Ltd.) (Figure 3.8(f)) to the motor attached to the conveyer belt (Figure 3.8(e)). The belt was covered with a white Teflon tape in order to avoid the sticking of the fibre to the belt surface after drying. The optimisation of the process is included in section 6.3. The temperature and humidity were monitored using a humidity sensor (Figure 3.8 (g)) (Oregon scientific). The fibres were air dried on the conveyer belt and collected later.

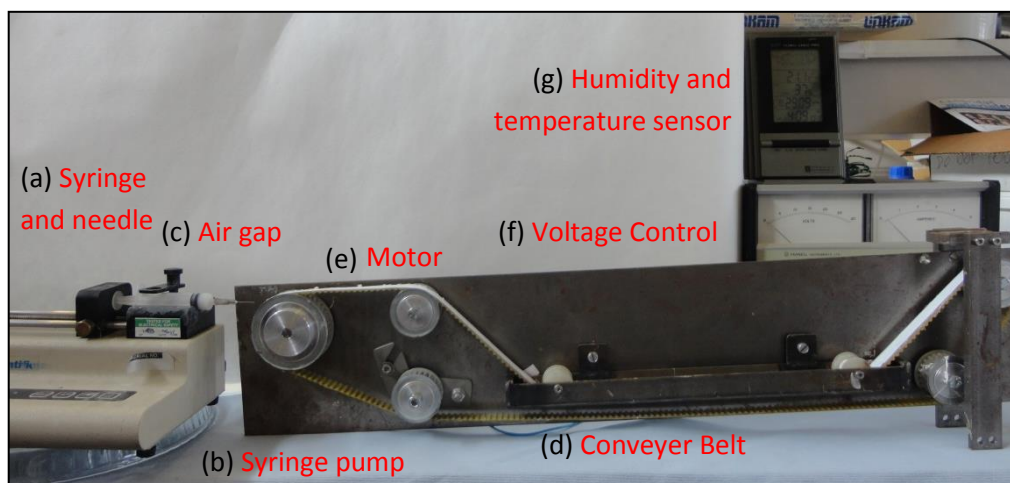


Figure 3.8: Spinning setup indicating the (a) syringe and needle, (b) syringe pump, (c) air gap, (d) conveyer belt and (e) motor driving it using the (f) voltage source and (g) humidity and temperature sensor

3.3 Characterisation Techniques

3.3.1 Powder X-ray Diffraction (PXRD)

X ray diffraction is generally used to determine the composition of the samples. In the current work this techniques has been used for determination of the structure of cellulose, crystallinity and crystallite size. The diffraction maxima are obtained when X-rays (with wavelengths comparable to atomic spacing) scattered from lattice planes in a specimen undergoes constructive interference in accordance with Bragg's law. The inter-planar distance d_{hkl} is related to the scattering angle θ and the X-ray wavelength λ by the equation 3.1:

$$2d_{hkl}\sin\theta = n\lambda \quad \text{where } n \text{ is an integer.} \quad (3.1)$$

All the XRD patterns were analysed using a consistent protocol involving determination of the background and amorphous contribution to the diffraction pattern. Using either high score plus or origin, the baseline or background was determined. Then the peaks were identified at 2 theta around 14°, 17°, 21° and 22°. In most of the samples the amorphous halo could be easily spotted between 18°- 20°. The peaks were fitted using the algorithm in 2 separate softwares (highscore plus and origin), till a satisfactory fitting was obtained. The result of fitting obtained in case of dried BC is shown in Figure 3.9.

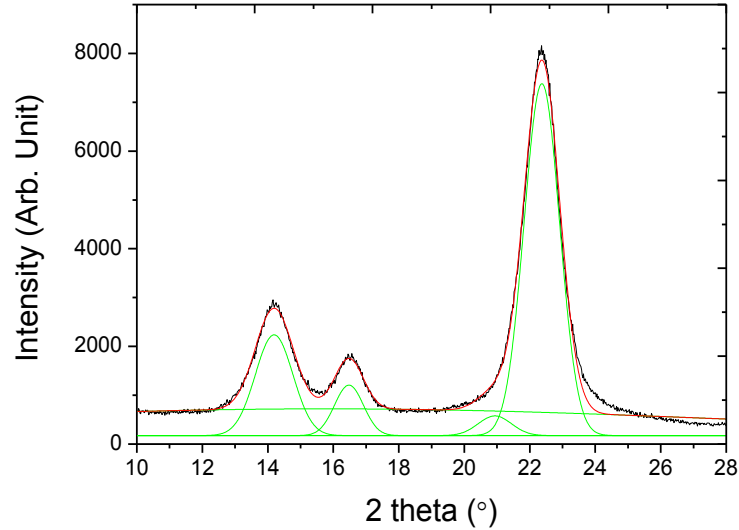


Figure 3.9: X-ray diffraction pattern for BC showing the original data (black line), fitted data (red line) and individual peaks and background (green line)

a) Determination of crystallinity index

The determination of cellulose crystallinity has always been difficult. Various methods have been devised for the calculation of the crystallinity index (CrI) [212, 213], however the following method to determine the crystallinity index has been widely used because of its simplicity [212].

$$CrI = \frac{I_{200} - I_{am}}{I_{200}} \times 100 \quad (3.2)$$

Where I_{200} and I_{am} are the peaks for (200) and amorphous region.

The intensity of the fitted peak, obtained as after removing background and determination of peaks from crystalline and non-crystalline regions, corresponding to the 200 plane and the amorphous region were used to calculate the crystallinity index.

b) Determination of the crystallite size

The estimation of crystallite size can be done from the line broadening. The width of the peak is generally estimated by either measuring the full width half maxima (FWHM) of the peak or the area under the peak divided by the height of the peak. The former was chosen for the calculations in this work. In order to accurately determine the crystallite size, various contributions to the peak broadening have to be taken into account. For a perfectly infinitely long crystal, the peak width will be very tiny. However, the

broadening of the peak happens due to the limit in the crystallite size, and in addition due to the instrumental broadening strain and paracrystalline distortions in the material.

The contribution by the instrumental broadening used in the current work is about 0.1° , which is negligible in comparison to the widths of the peak obtained [personal communication with Dr. Mary Vickers]. In order to estimate the contribution to the peak broadening from paracrystalline distortion, it is required to identify the peaks from higher order. Once the widths of the peaks from higher orders of a set of planes are measured, the plot of peak width with s^2 , ($s_{hkl} = h/d_{hkl}$ where h is the order of reflection which is originated by a family of planes with spacing d_{hkl}) follows a linear relationship and the extrapolation to $s = 0$ yield the real crystallite size [252].

However, in the current work it was difficult to de-convolute the peaks of higher order due to overlapping contributions from various sets of planes from the two polymorphs of cellulose I structure present. Thus, the analysis could not be performed and the Sherrer's equation [214] (equation 3.3) was directly used for crystallite size (D) determination.

$$D = \frac{k\lambda}{\beta \cos(\theta)} \quad (3.3)$$

, where $k=0.94$ [215], β is the FWHM and θ is the peak location

However, it must be remembered that the contribution of paracrystallinity to peak broadening may be as large as 50% as has been observed in some studies on polyethylene [252].

All work has been performed on Phillips PW1830 vertical diffractometer using a Cu_α line. The data was recorded from 10° to 30° with a step size of 0.05° and dwell time 1s. Dry cellulose samples were placed on silicon wafer and exposed to X-rays. The pattern obtained was compared against the JCPDS database and literature. Phase identification and XRD peak fitting were carried out with Highscore Plus software and Origin 8.

3.3.2 Wide angle X-ray scattering (WAXS)

WAXS analyses Bragg reflections scattered to wider angles yielding information on sub-nanometer interplanar separations and is used, in the view of fibres, for measuring the degree of orientation of the cellulose nanowhiskers along the fibre axis. The azimuthal scan was performed and the full width half maxima the intensity distribution was determined. The degree of orientation (f_c) calculated by

$$f_c = \frac{180 - f_{whm}}{180} \quad (3.4)$$

A Phillips generator with accelerating voltage of 40 V and current of 40 mA. The exposure time used was 3 hours. These patterns were obtained on X-ray photographic films, which were developed and scanned to obtain soft copy. The scattering pattern was collected on a photographic film which was developed with a standard protocol of 2min 30 s in developer, 30 s in stopper and 8 min in fixer, followed by washing in water for 4 hours and dried in the oven.

3.3.3 Small Angle X-ray Scattering (SAXS)

SAXS was used to obtain the dimensions of the elements larger than those accessible by the PXRD. The data was obtained as 2 D distribution of intensity which was converted to Intensity (I) vs scattering vector (q) distribution by azimuthal integration of intensity where $q=4\pi\sin(\theta)/\lambda$. The SAXS data was used to determine the cross section dimensions 2a and 2b of the scattering element as follows:

In the low q region ($1/L \ll q \ll 1/r_c$, where r_c is the radius of gyration of the cross section of the scattering element and L is the length of the scattering length), the scattering from long rods should follow Guinier approximation given by

$$qI(q) = G \exp\left(\frac{-q^2 r_c^2}{2}\right) \quad (3.5)$$

where, G is the scaling constant.

The critical radius can be obtained from the slope of the $\ln(qI(q))$ vs. q^2 plot. The critical radius obtained from the Guinier plot can be expressed in terms of the sides of the cross section of the diffracting element.

$$a^2 + b^2 = 3r_c^2 \quad (3.6)$$

Another relation between a and b can be obtained by calculating the cross-sectional area (S) as follows

$$S = 4ab = \lim_{q \rightarrow 0} \frac{2\pi q I(q)}{Q} \quad (3.7)$$

where Q is the invariant calculated as

$$Q = \int_0^\infty q^2 I(q) dq \quad (3.8)$$

The Guinier plot was used to extrapolate the data to obtain the intensity from $q = 0$ to $0.01 \text{ \AA}^{-1}$. The invariant Q was calculated as the area under the curves from the plots of $I(q)$ vs. q^2 . The value of G was also estimated by extrapolating the Guinier plot.

Bruker Nanostar diffractometer was used to record the patterns using Cu K α ($1.54 \text{ \AA}$) X-radiation, with exposure times of 10,000 s. For these X-ray scattering experiments, Silver Behenate (AgBh) was used for calibration. The transmission coefficient of the sample was determined using an exposure time of 1000s, using the following equation:

$$t_s = \frac{I_{GC+s} - t_{GC} I_s}{I_{GC} - t_{GC} I_0} \quad (3.9)$$

, where t_s is the transmission coefficient of the sample, I_{GC+s} is the scattering intensity with glassy carbon and sample, t_{GC} is the transmission coefficient of glassy carbon (which is 0.16), I_{GC} is the background intensity with glassy carbon and I_0 is the background intensity without glassy carbon.

After the determination of the transmission coefficient, the background was subtracted as follows

$$I_s = I_{s+bg} - t_s I_{bg} \quad (3.10)$$

, where I_s is the scattering intensity from sample as a distribution of scattering vector, I_{s+bg} is the intensity collected comprising of scattering from sample and background, t_s is the transmission coefficient of sample calculated from equation 3.9 and I_{bg} is the background intensity.

For the nanowhisker suspensions in water, the background was collected for glass capillary filled with water. Data analysis was carried out with Bruker software applications along with suitable spreadsheet software (Origin Pro).

3.3.4 Polarised Optical microscopy

POM is a powerful technique to observe anisotropy in the material as anisotropy leads to birefringence. The manner in which the molecules are arranged within the phase can be detected by careful analysis of the microscopic defect texture. Since the polarizer in the microscope are crossed at 90° to each other, then with no sample in place light is extinguished and so blackness is seen. Similarly, if an isotropic liquid is analysed the polarised light remains unaffected by the sample (isotropic) and so no light passes through the analyser (second, upper polarizer). However, when an anisotropic, birefringent medium is present, light is not extinguished and an optical texture appears that gives information relating to the arrangement of the molecules within the medium. The texture not only depends on the phase structure but also on the alignment. There are two basic forms of alignment. When the axis of the constituting phase is at right angle to the supporting surface and in case sample placed between slide and coverslip, the axis is in direction of observation, the phase is homeotropic. In this case, the polarised light cannot pass through the material and complete darkness is observed, even on the rotation of sample between crossed polars. In homogeneous alignment, the constituting molecules are aligned parallel to the supporting surface.

The cellulose chains are positively birefringent with refractive indices 1.53 and 1.58 perpendicular and parallel to the chain axis, respectively. In native cellulose microfibrils and derived nanocrystals, the cellulose chains are present parallel to the longitudinal axis of the cellulose microfibrils/nanocrystals, making them strongly positively birefringent. Therefore, when the nanocrystals or microfibrils are present in parallel, a birefringent domain is formed. When this domain is observed between crossed polars, the relative orientation of the director axis of the domains and the axes of the polarisers determine its visibility, as indicated in Figure 3.10. If the axis of the domain is parallel to either the polariser or analyser, it appears dark and if present at 45° , it appears with maximum brightness. When a retardation plate is inserted between the crossed polars, depending on the relative orientation of the fast and slow axes of the retardation plate and the birefringent unit, increase or decrease in retardation occurs,

resulting into blue or yellow colours. In case of cellulose crystals, which possess a slow axis along the chain axes, yellow coloration is observed when the crystals are parallel to the long axis of the retardation plate, which is the fast axis for the plate. However, blue coloration is observed, if the crystals are at right angles to the long axis of the plate, as illustrated in Figure 3.10. The regions without orientation or director in the direction of observation appear magenta in colour [216]. Thus, the microscopic domains can be easily distinguished with the aid of crossed polars and a retardation plate.

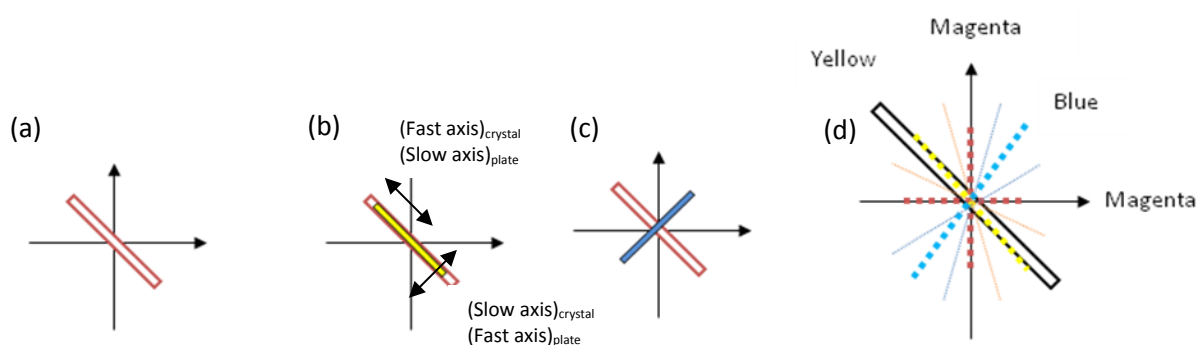


Figure 3.10: a) Position of retardation plate between crossed polars b) Crystal appears yellow when the slow axis of retardation plate is parallel to the fast axis of the crystal c) Crystals appear blue when the fast axis of crystal and the retardation plate are parallel d) Variation in visible colour on varying the relative orientation of crystal with respect to retardation plate

a) Nematic phase

On rotating the sample between crossed polars, the bright regions become dark and dark regions become bright, depending on the orientation of the ordered domain. However, the isotropic regions would always appear dark. The Schlieren brushes appear black because of optical extinction caused by the crossed polarisers. Optical extinction occurs when the molecules are either aligned parallel to axes of any of the polarisers. The alignment of molecules converges in a point which can occur in many ways. The textures resulting into Schlieren brushes are shown in Figure 3.11(a).

b) Chiral nematic phase:

A chiral nematic phase and nematic phase are close in the energy but a chiral molecule would form a chiral nematic phase rather a nematic phase. In a chiral nematic phase, molecules form nematic phase but the director of each nematic plane is rotated by an angle with respect to the previous phase and thus phase is characterised by a pitch

when the director makes a 360° rotation (Figure 3.11 (b)). Again depending on the homeotropic and heterotropic orientation, two kinds of texture may be seen: Grandjean texture and Finger print texture. The most commonly seen is the finger print texture (Figure 3.11 (c)).

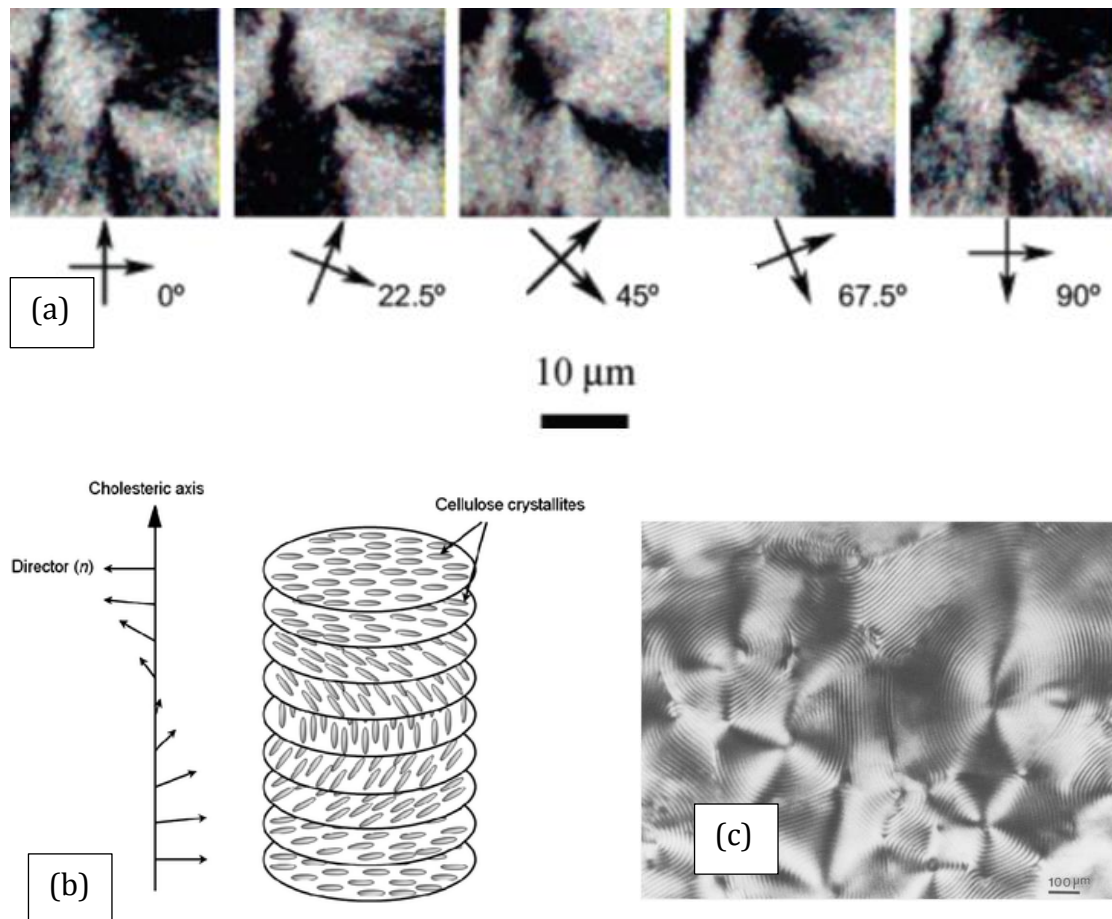


Figure 3.11: (a) Schlieren texture exhibited by multiwall carbon nanotubes (nematic liquid crystalline phase) [183] b) Schematic representation of chiral nematic phase [217] c) Finger print texture exhibited by cellulose nanocrystals forming a chiral nematic phase [180, 217]

For the polarised optical microscopy investigations, two kinds of samples were prepared: 1) The suspension ($\sim 2\ \text{mL}$) was placed on a glass slide and covered with a coverslip and analysed (Figure 3.12 (a)) 2) the suspension was filled in a capillary $0.4\ \text{mm} \times 4\ \text{mm}$ cross section (Figure 3.12 (b)). All samples were analysed on an optical

microscope (Olympus) with and without a sensitive colour plate with a retardation of 530 nm inserted between the crossed polars at 45° with respect to each of the polariser axes.

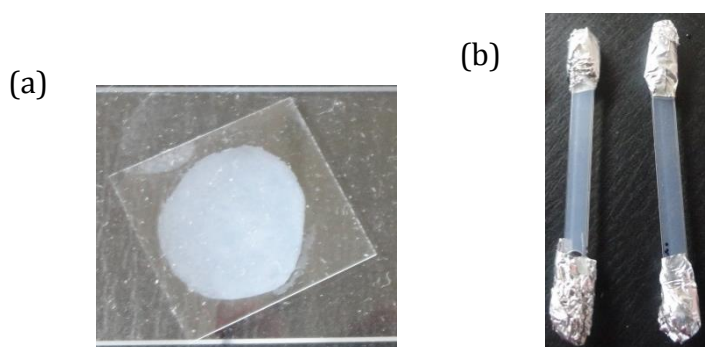


Figure 3.12 Sample preparations for polarised optical microscopy a) Droplet between cover slip and glass slide and b) Suspension filled in a capillary sealed by parafilm and aluminium foil

3.3.5 Scanning electron microscopy

SEM uses a beam of electron to image the specimen. A high resolution can be achieved by electron beam as compared to an optical microscope due to the short wavelength of the incident electrons. The electron beams interact with the sample, loose energy and lead to the emission of secondary and backscattered electrons. These emissions when detected and measured can be used to obtained topological and compositional information about the sample. For these purposes, the secondary electron imaging (SEI) mode was routinely used. The secondary electrons originate from within a few nanometres of the sample surface and the information obtained by the SEI mode is representative of the sample-surface rather than the bulk.

A JEOL 6340 FEGSEM was used at voltages of 5kV and a working distance of about 4 mm. The cellulose samples were coated with gold for 60s with a deposition current of 40 mA. All cellulose samples were sensitive to the electron beam and thus it was required to quickly observe and take images. High resolution work was done on SEM-FIB Helios with an accelerating voltage of 1kV.

3.3.6 Atomic Force Microscopy

AFM was carried out to investigate structural parameters (dimensions) of the cellulose microfibrils and nanowhiskers. Tapping mode was used on a Nanoscope SPM microscope (Veeco Instruments) with a Bruker RTESP tip. Samples were prepared by drying a droplet of a very dilute dispersion of nanowhiskers on a silicon wafer.

3.3.7 Rheological measurements

Rheological measurements were performed on ARES rheometer, TA instruments in parallel-plate geometry. The 50 mm diameter plates were used for measurement at room temperature, maintained by a water jacket. The gap between the parallel plates was kept to be 1mm. Variation in viscosity, elastic modulus and loss modulus was measured with increase in strain. The measurements were also taken with variation in frequency at a constant strain.

3.3.8 Mechanical testing

Tensile tests were carried out using a dedicated fibre-testing equipment (Textechno Favimat125a) on single fibres (held at a small initial pretension) at a standard gauge length varying from 5 mm to 20 mm. Typical test-speed of 1 mm min⁻¹ was used to record the elongation of the fibre with increasing force until fibre fracture. The force and displacement measurement ranges of the load cell were 0-2 N (resolution=0.0001 cN), respectively. Using the diameter values acquired by optical microscopy, the strength, and stiffness values were calculated and expressed in MPa.

Chapter 4

Architecture and Morphology of Cellulose

4.1 Introduction

Cellulose is synthesized by diverse organisms including prokaryotes, protists, plants and animals [1]. Irrespective of the source, cellulose is present in form of semi-crystalline fibrils, conventionally called microfibrils (with nano-scale lateral dimension) resulting from the supra-molecular organisation of poly-glucan chains [1]. Cellulose is known to be produced by a cell directed self-assembly process, and thus, the biosynthesis machinery of each organism determines the characteristics [16] such as dimensions, shape and number of levels in the hierarchical organisation, as discussed in section 2.5-2.7. In addition to the physical machinery involved in the synthesis, any variations in the biosynthesis environment also affect the morphology and properties of the cellulosic structures [218].

In the current work, cellulose was obtained from two different sources, namely, bacterial cellulose and tunicate (sea squirt). Various species of bacteria produce cellulose in the form of exo-polysaccharide membranes, and these membranes are believed to serve a variety of functions including protection to the bacterial cells against heavy metals, ultra-violet radiation and foreign organisms, and maintaining optimum oxygen and nutrient supply. The other source of cellulose used in this work is tunicates where cellulose is present as a protective tunic, and is the only animal known to produce cellulose [1].

The terminal complexes (TCs), which are enzymes present on the cell surfaces, are responsible for cellulose production in all cellulose producing organisms [1]. In the case of bacterial cellulose, linear arrays of TCs with subunits take part in the biosynthesis. Each subunit is believed to assist in the making of a bundle of cellulose chains many of which aggregate to form a protofibril, which further forms crystalline fibrils and subsequently microfibrils, which then finally assemble to form ribbons [103]. In tunicates, various arrangements of the TCs have been identified, and a majority of the TC-assemblies have been observed to belong to the linear type [13, 219]. The

correlation between TC and microfibril crystallisation has been studied [94], however a complete understanding is still underway.

This chapter attempts to characterize the supramolecular architecture of cellulose obtained from bacteria and tunicates. Two varieties of bacterial cellulose, including commercially available food-grade bacterial cellulose (known as nata-de-coco) and laboratory cultured bacterial cellulose have been investigated. The tunicates were obtained from the Lochfyne Seafarms Ltd. for cellulose extraction in this work. The production and extraction of celluloses is described in section 3.1.4. The characterisation techniques were chosen to extract information at different length scales in order to elucidate the hierarchy of the cellulose chain organisation in bacterial cellulose and tunicate cellulose. The techniques included scanning electron microscopy (SEM) and atomic force microscopy (AFM) to obtain the dimensions of the microfibrils. Powder X-ray diffraction (PXRD) to determine the crystallinity and crystallite size. Small angle X-ray scattering (SAXS) was used to obtain information about other organisational levels in the range that cannot be accessed by the above techniques.

These techniques have been frequently used to characterize cellulose, however little literature reports utilization of multiple techniques together to build hierarchical model of cellulose. One such work has been reported by Astley *et al.* where SAXS and SEM were used together and a model for bacterial cellulose microfibril was constructed comprising of semi crystalline rectangular cross section microfibrils forming ribbons with width of 500Å [81]. In another investigation, SAXS was used complementary to WAXS and TEM for *Picea abies* cellulose to identify the elementary cellulose fibril of 25 ± 2 Å [220]. From these techniques a wide range of dimensions have been reported. This variation may be due to various reasons such as varying sources of cellulose, usage of different cellulose nanoparticles and methods used to obtain and analyse the data.

In this chapter, the information obtained from various characterisation techniques, as mentioned before, has been used to build models to describe the native hierarchical organisation of cellulose obtained from the two sources. In order to fully understand the hierarchical bottom-up organisation of cellulose chains, it is required to relate the models developed by the top down characterisation tools to the biosynthetic

machinery of the cellulose producing organism. This has been addressed for bacterial cellulose in the later part of this chapter.

4.2 Characterisation by SEM

The freeze-dried samples of the three kinds of cellulose, nata-de-coco (NdC), tunicate (TC) and bacterial cellulose (BC) were investigated by SEM. Figure 4.1, 4.2 and 4.3 represent the SEM micrographs obtained from NdC, TC and BC, respectively. All the samples were found to comprise of thin, long and uniformly flat tape-like cellulose fibrils, consistent with the observations on all naturally occurring cellulose from other sources [2].

The fibrils with the smallest width observed in the SEM images of the sample are called microfibrils and the fibrils with larger widths are referred to as ribbons. The microfibrils are known to be composed of sub-fibrils which cannot be directly seen by the SEM [2]. The SEM images revealed that the microfibrils and ribbons were densely entangled and at many points, bundling of microfibrils and de-bundling of ribbons was seen, which resulted in a branched morphology. A quantification of the association of microfibrils in terms of diameter distribution and branched-unbranched microfibril segment has been done in the next section.

Before discussing further, it is worth mentioning about the layered structure of bacterial cellulose pellicle. Figure 4.4 represents the SEM images of the cross section of a bacterial cellulose pellicle, along and perpendicular to the direction of pellicle growth, as indicated in the image. In the image representing the cross section perpendicular to the direction of growth (Figure 4.4 (d)), the separate layers of cellulose can be easily identified. This observation is consistent with the previously reported observations on bacterial cellulose [221]. The bacterial cellulose is produced in layers, with the newest formed layer on the top of the last formed layer.

4.2.1 Width distribution

The width-distributions of cellulose microfibrils and ribbons were obtained from the SEM images for all cellulose samples, and are illustrated in Figure 4.5. The measured width varied from 20 nm to over 120 nm for all the cellulose samples. The wide distribution is attributed to the variable degrees of aggregation of the microfibrils to

form the ribbons. However, from the width distributions, it can be seen that the diameters of the majority of the fibrils are in the range of 40-50, 20-30 and 40-50 nm for NdC, TC and BC, respectively. The smallest fibril width, which is same as the microfibril width was found to be in the range of 10-20 nm for TC and 20-30 nm for NdC and BC. These values are consistent with the values reported in the literature for microfibril width as well as for some derived nanoparticles [46, 124, 126, 132]. The average widths were 58 ± 33 nm, 106 ± 80 nm and 65 ± 36 nm for NdC, TC and BC, where larger average width indicates a higher degree of aggregation.

It was found that sonication aids in separating ribbons into constituent microfibrils. Figure 4.6 (a, b) shows the SEM images of NdC, before and after sonication. The sonication treatment causes the ribbons to break into individual microfibrils and thus, the width distribution shifts to the lower values, as evident from Figure 4.6 (c). Intense ultra-sonication has shown to cause delamination and nanostructural reorganisation of cellulose microfibrils [102]. However, a very minimal amount of sonication was applied to avoid disruption of microfibrils in this work.

The formation of cellulose ribbons by the association of microfibrils is advantageous both for bacteria and for tunicates. In the case of bacterial cellulose, ribbons aid in the formation of pellicles of low density, which assist the bacteria to float. In the case of tunicates, the ribbons provide structural integrity to the tunic, leading to better protection. Various cellulose producing species of tunicates have been observed to exhibit a wide degree of aggregation of cellulose microfibrils, which has been related to the location of terminal complexes and their proximity [222].

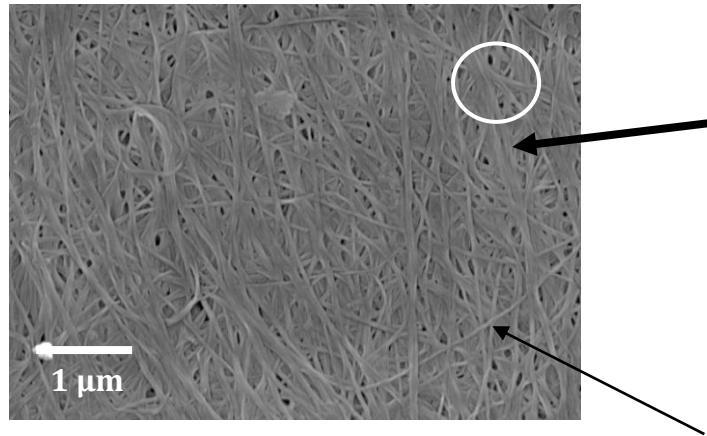


Figure 4.1: SEM image of dried NdC (microfibril indicated by a thin arrow, ribbon by a thick arrow and branching point is encircled) (scale bar 1μm)

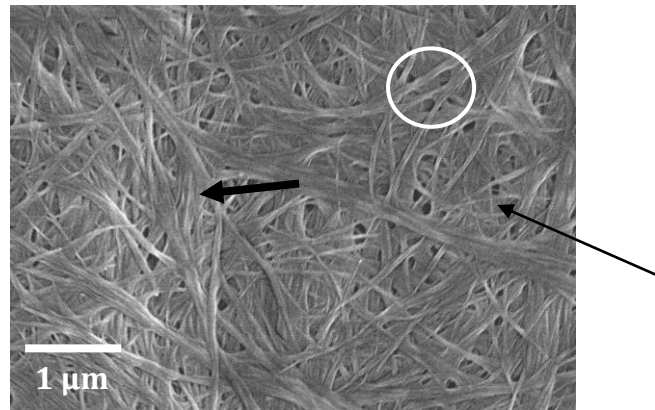


Figure 4.2: SEM image of TC (microfibril indicated by a thin arrow, ribbon by a thick arrow and branching point is encircled) (scale bar 1μm)

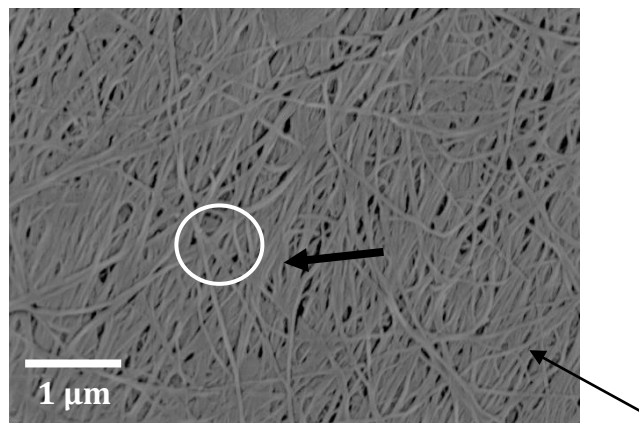


Figure 4.3: SEM image of BC (microfibril indicated by a thin arrow, ribbon by a thick arrow and branching point is encircled) (scale bar 1μm)

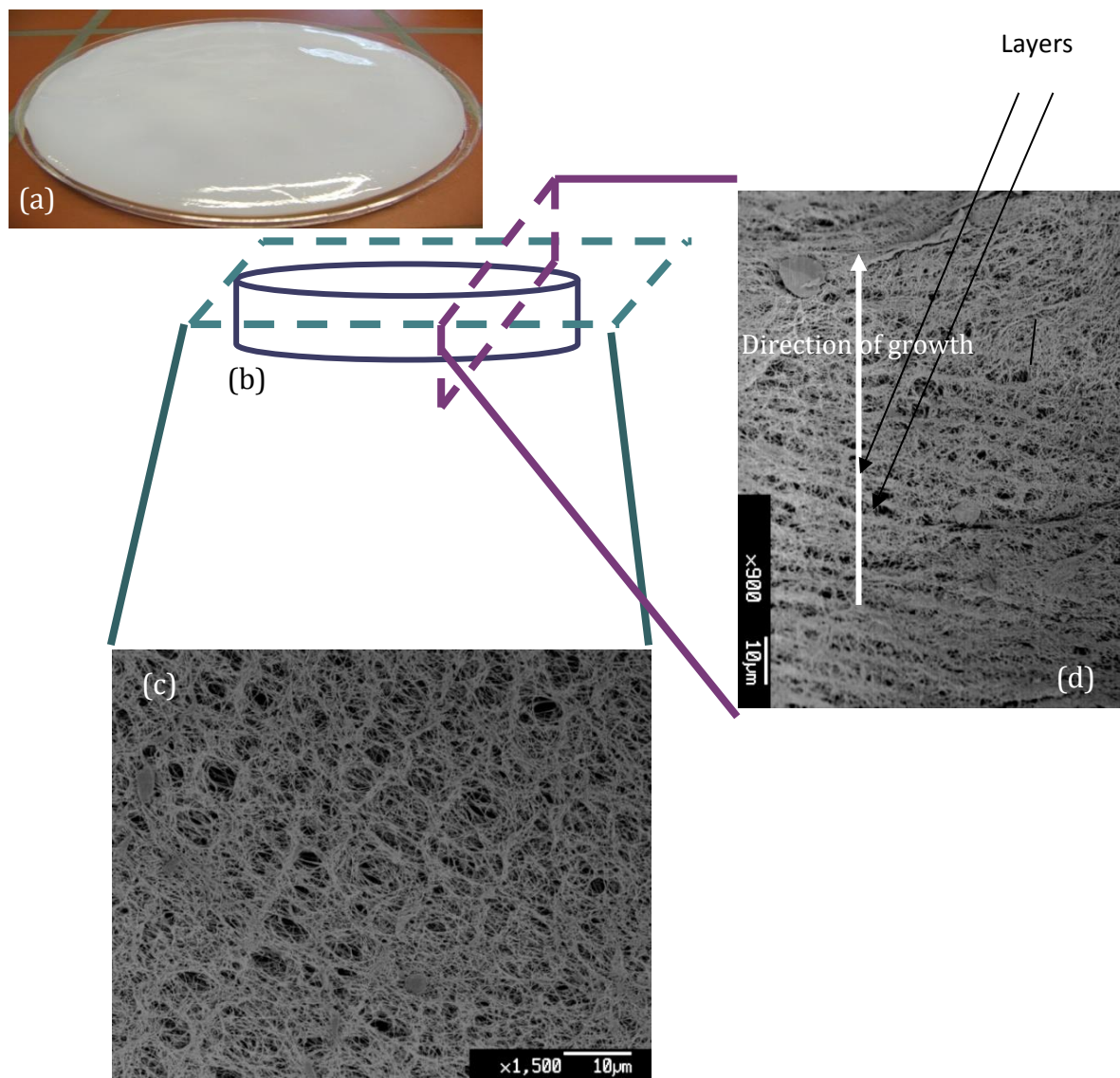


Figure 4.4: a) BC pellicle b) schematic showing sections of cellulose pellicle. SEM images of the different sections of the BC pellicle c) parallel to the pellicle surface (scale bar 10 μm) and d) perpendicular to the plane of the pellicle (scale bar 10 μm) (direction of growth is indicated in (d))

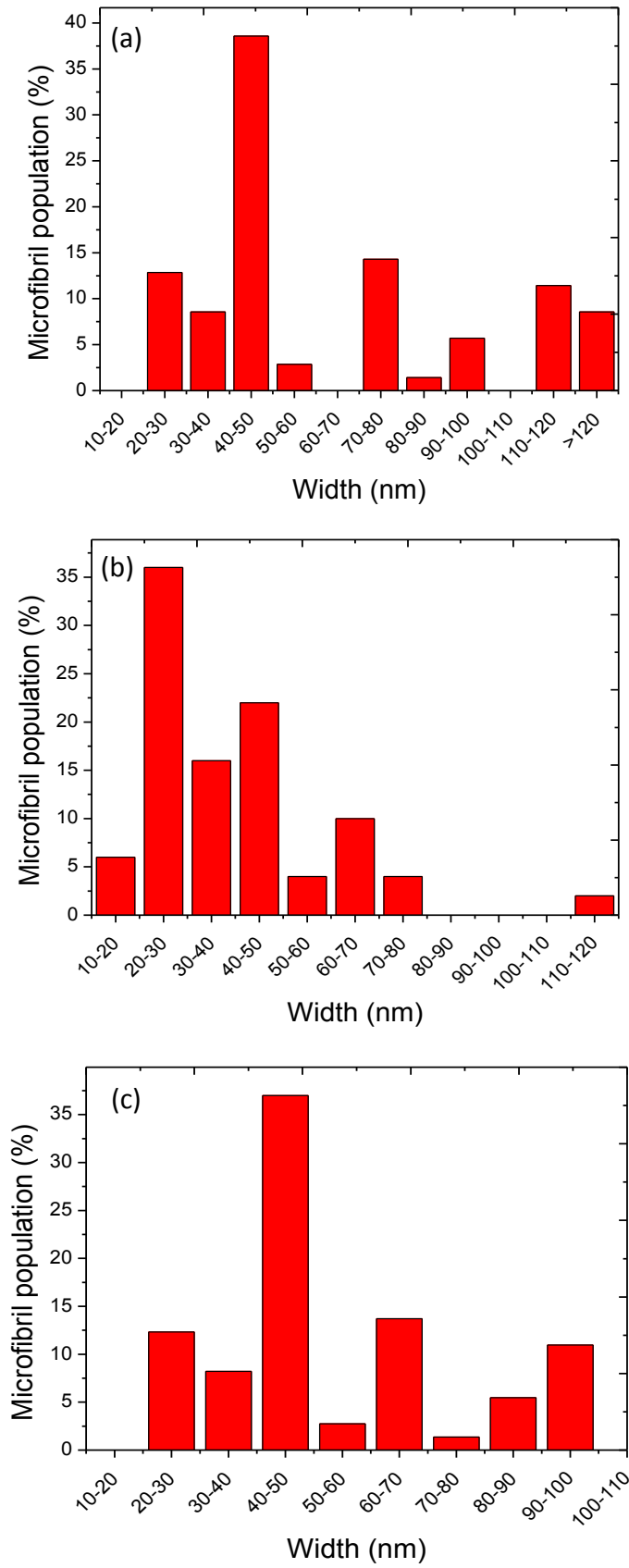


Figure 4.5: Diameter distributions of a) NdC (58 ± 33 nm) b) TC (106 ± 80 nm) c) BC (65 ± 36 nm) microfibrils obtained from SEM images from 200 measurements

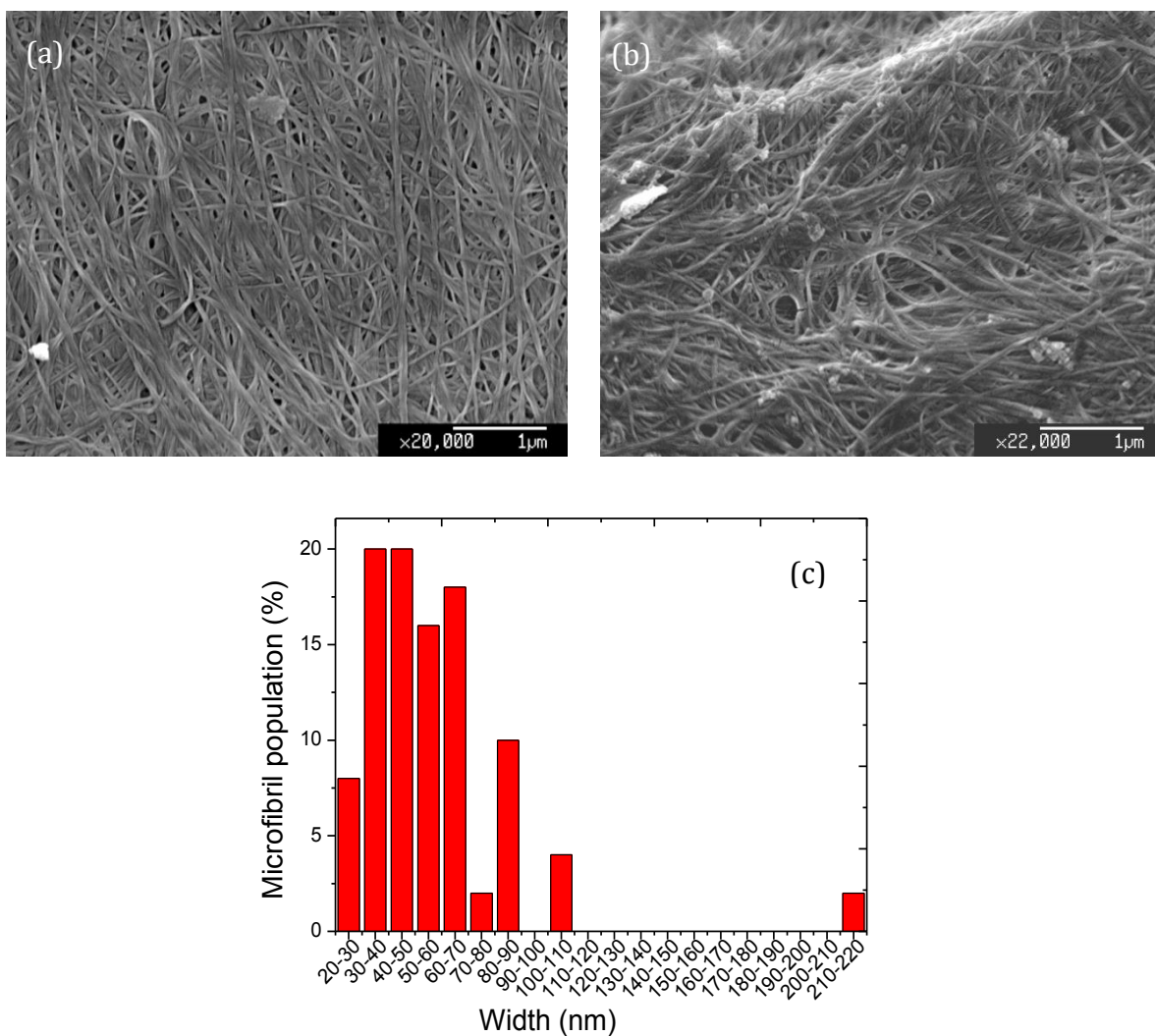


Figure 4.6: SEM image of NdC samples (scale bar 1 μm) a) before and b) after sonication. c) Microfibril width distribution after sonication

4.2.2 Branching

The association of microfibrils into ribbons and de-bundling of ribbons into microfibrils results into a branched morphology. Some researchers have stated that, in case of bacterial cellulose, the formation of a three-way branching may result from continued secretion of cellulose microfibrils during the natural bacterial cell division [14, 48]. This would result into narrower fibrils at the branching point. Moreover, the length between branching points would correspond to the length of fibrils produced in a life time of bacteria, which can be estimated from doubling time of the bacteria (3-4 hours) and rate of production of microfibrils (2 μm/min) [48], to be 360-480 μm. The lengths of the microfibrils/ribbons between branching points were measured for over 200 segments

from many SEM images, for all the cellulose samples obtained from the different sources, and the segment-length distributions are furnished in Figure 4.7. The average separation or length of microfibril segment between branching points is 900 ± 800 nm, 1080 ± 845 nm and 1265 ± 776 nm for NdC, TC and BC, respectively. Thus, the experimentally observed spacing between branching points in the samples here was always less than that estimated for segment, corresponding to branching resulting from bacterial cell division.

If the branching was due to bacterial cell division, an addition of matter would be seen after branching as the daughter cell restores its number of extrusion pores with growth. Now, if we follow a ribbon in bacterial cellulose, the numerical addition of widths of microfibrils after branching is similar to the width of the ribbon observed prior to branching. This contradicts the theory suggesting the branching of microfibrils originating from the bacterial cell division. The branching observed in the samples may be due to the intersection or aggregation of microfibrils produced by different bacteria.

Moreover, the individual microfibrils are sometimes distinctly visible constituting the ribbons. Hence, the branching is observed when a ribbon splays into its constituent microfibrils or microfibrils produced by different bacterial cells aggregated to form ribbons. This hypothesis is supported by higher resolution SEM images presented in the next section.

As mentioned before, nata-de-coco is also a kind of bacterial cellulose. However, it varies from the lab-cultured bacterial cellulose in terms of microstructure, because the two bacterial cellulose forms are produced in different conditions and from different bacterial strains. NdC, produced on a commercial scale, is obtained by fermentation of coconut milk. However, the lab cultured bacterial cellulose is prepared using an in-house protocol, employing a static cultivation technique, using glucose as the carbon source. The bacterial strains and the cultivation conditions, have a profound effect on the morphological features such as microfibril dimensions, degree of entanglement, bundling of the cellulose microfibrils [218], which is reflected in the aforementioned observations of spacing between microfibril bundling- ribbon splaying point. The average separation between branching or bundling points is twice for BC as compared to NdC.

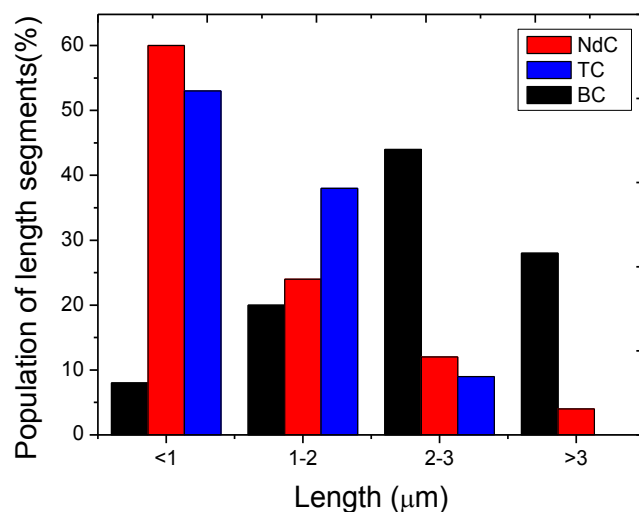


Figure 4.7: Distribution of length of microfibril segments between branching points observed for NdC (900 ± 800 nm), TC (1080 ± 845 nm) and BC (1265 ± 776 nm)

4.2.3 Additional features observed from high-resolution SEM

Before discussing the results obtained from other techniques, some additional features obtained by high resolution SEM are highlighted in this section. The discussion so far revealed that the cellulose samples from all the sources show similar features like bundling, microfibril width distributions and branching. Here, the results of the in-depth study of BC using high resolution SEM are furnished.

Figure 4.8 (a) shows a high magnification image of gold coated BC sample with a bead-like texture on the cellulose microfibrils. These beads were about 15-20 nm in width and were uniformly present on the fibril surface. In polymer science, gold deposition has been found to be helpful in distinctly identifying the low density amorphous regions as it helps to project amorphous regions as bulged areas. However, in the present work, when a stub (without any sample) was coated with the same amount of gold, beads with identical dimensions (Figure 4.8 (b)) were observed. This implied the beaded pattern seen on the microfibrils, were merely gold deposits, or gold islands. No evidence for bands of amorphous material with crystalline regions could be seen along the microfibrils in BC.

As discussed in the previous section, bundling-splaying of cellulose microfibrils and ribbons was commonly observed. In the images shown in Figure 4.9, ribbons and constituting 20-30 nm wide microfibrils can be easily identified. In addition, a 30 nm wide twisted microfibril can also be seen. The twist was generally observed only in thinner fibrils, that is, when microfibril existed individually. Once the microfibrils bundle into large ribbons, twists were not observed. This might suggest a critical fibril width which can retain a twist (possibly originating from the intrinsic chirality of cellulose chains).

The SEM image in Figure 4.8 (a) shows a ribbon composed of three twisted microfibrils where a closer observation of the end of the ribbons enables us to visualise its composition. The splayed end of the ribbon revealed three microfibrils, each 20-30 nm wide, which matches well with the inferences made from the low-resolution SEM images. Often, the ends of polymer chains can reveal some information on the crystalline and amorphous regions. The ends of the microfibrils in the analysed samples were clean with absence of any tapering (as seen in Figure 4.8 (a) and Figure 4.9 (b)), similar to the observation by Colvin *et al.* on the growing tip of bacterial cellulose microfibrils [223]. This implies that the sub-fibrils present within the microfibrils, end at the same point, which might be construed as evidence that the crystalline and the amorphous regions occur serially along the microfibril length. However, the amorphous regions could not be spotted in the high resolution SEM implying a sparse distribution, if at all present. This inference will be further discussed later in this chapter.

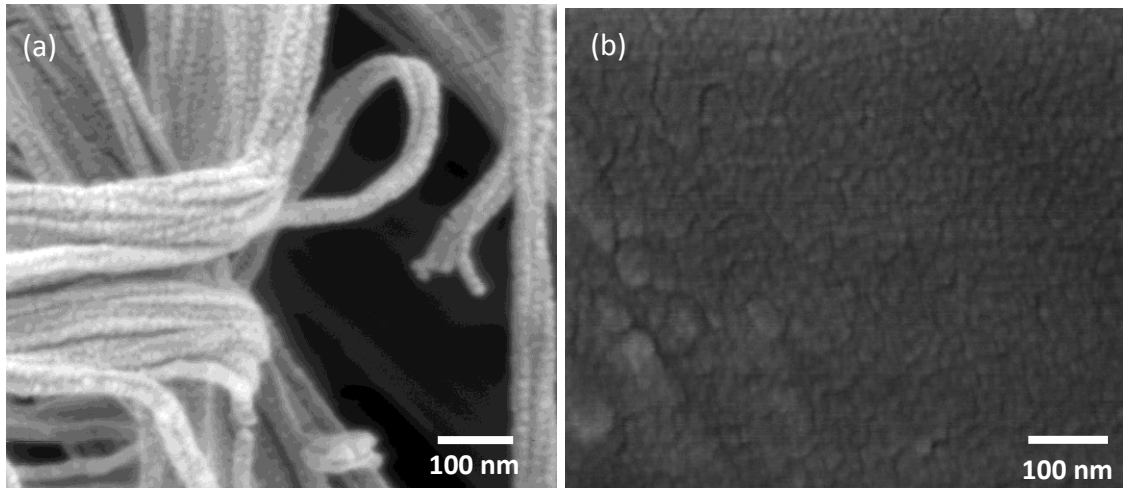


Figure 4.8: SEM images of a) BC b) stub sputtered with gold for 60s (scale bar 100 nm). Both show bead of characteristic size of 15-20 nm

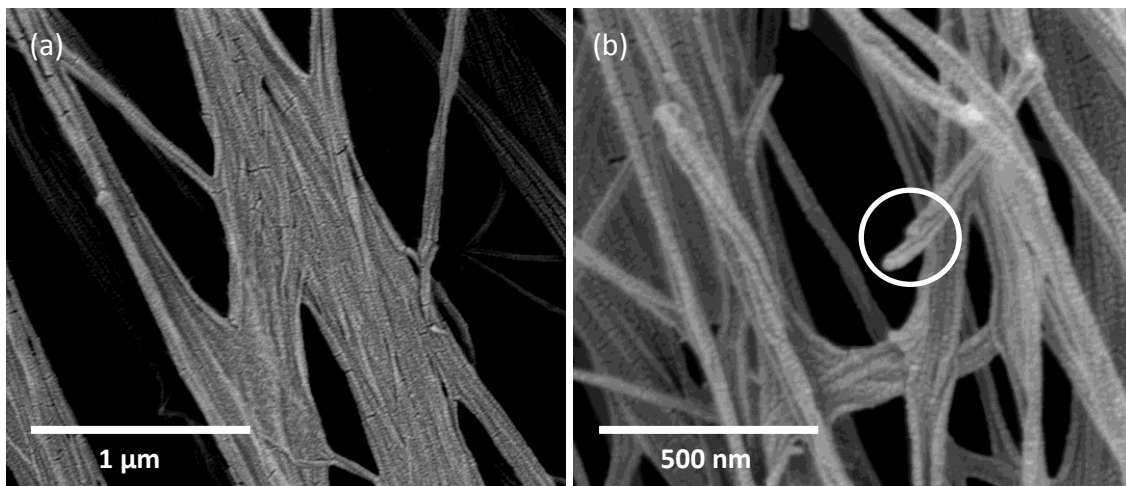


Figure 4.9: High resolution SEM images of bacterial cellulose showing a) bundling and (scale bar 1 μm) b) splaying of ribbons and ends of a few microfibrils (encircled) (scale bar 500 nm)

4.3 Characterisation by AFM

AFM was used to obtain the dimension of the microfibril perpendicular to the substrate plane which can not be determined by SEM, as AFM can acquire height information as it scans along a line. For AFM analysis, dilute suspensions of microfibrils were prepared by sonication. However, the drying of the droplets on a silicon substrate for microscopy resulted into aggregation and thus the measurements were carefully done to avoid error due to overlap. Figure 4.10 shows the image of the BC microfibril suspension

obtained by AFM. Like SEM, AFM confirmed the presence of long, uniform and even endless ribbon-like morphologies of all the cellulose samples. From the AFM images (Figure 4.10), the heights of the ribbons or the individual microfibrils was obtained. The height of the ribbons was found to vary from 6 to 20 nm for TC microfibrils, 7 to 25 nm for BC and 8 to 25 nm for NdC microfibrils, and the average height for all the cellulose samples were in the range of 8-10 nm. These results are consistent with the data presented in section 5.2 obtained for cellulose nanowhiskers derived from these cellulose sources where the measurements are more accurate due to very little aggregation. Overall the dimensions are also consistent with the values reported in the literature [46, 126, 154, 164]. The AFM was not used in measuring the width of the microfibrils due to the tip broadening effect. The ratio of the two lateral dimensions of the microfibrils width and thickness obtained from SEM and AFM, respectively, is in the range of 2-6, which confirms that the microfibrils are non-circular in cross section, in line with the previous observations [4, 46, 126].

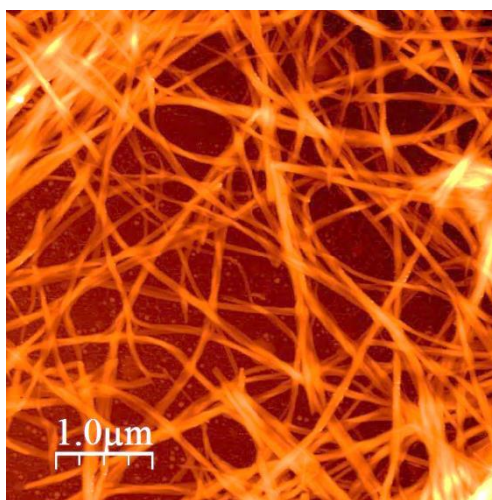


Figure 4.10: AFM image of BC microfibril dispersions in water (scale bar 2 μ m)

4.4 Characterisation by PXRD

The characterisations by SEM and AFM revealed that the dimensions of the microfibrils are of the order of a few tens of nm. However, it is known that the cellulose microfibrils are semi-crystalline and related attributes such as crystallinity, crystallite size vary with

the source of cellulose [73, 186], Thus the cellulose samples were further characterised by PXRD to obtain the crystallite sizes and the percentage of crystallinity.

The PXRD patterns acquired from all the samples (shown in Figure 4.11) are typical of the patterns shown by native cellulose, irrespective of its source. The data was analysed by determining the background and reflections from crystalline and non-crystalline content as discussed in section 3.3.1. The three most intense peaks are present at 2-theta value of about 14.4°, 16.7° and 22.5° corresponding to the d-spacing of approximately 6.19, 5.31 and 3.96 Å for NdC, 14.45°, 16.35° and 22.48° corresponding to the d-spacing of approximately 6.12, 5.42 and 3.95 Å for TC, and 14.3°, 16.6° and 22.5° corresponding to the d-spacing of approximately 6.15, 5.29 and 3.98 Å for BC, calculated using the Bragg's equation. These values are consistent with the experimentally observed values for cellulose I native cellulose structure [54].

It is well known that cellulose I exists as two polymorphs, I α and I β , which are triclinic and monoclinic structures respectively [11]. Figure 4.12 shows the arrangement of the cellulose chains in the two crystal structures, in which the various planes and corresponding d-spacing have also been indicated. The I α unit cell belongs to P1 space group and contains one cellulose chain and the unit cell parameters are a=0.672 nm b=0.596 nm, c=1.040 nm α =118.08° β =114.80° γ =80.375°. The I β unit cell is space group P2₁, contains two cellulose chains, and unit cell parameters are a=0.778 nm b=0.820 nm c=1.038 nm γ =96.5° [54]. The two polymorphs in spite of the structure parameter differences, when looked down the cellulose chains, do not appear too much shifted. Both the polymorphs contribute to the three main reflections observed in the XRD patterns, because the various sets of planes in both polymorphs have similar d-spacings. The planes which contribute to the most intense peaks are (100), (010) and (110) from triclinic cellulose, and (1-10), (110) and (200) from monoclinic cellulose [11]. In the XRD patterns, the contribution of each of these phases to the peaks can be obtained, although the relative amounts of the polymorphs is determined by the cellulose source. These will not be discussed further here, although, it may be mentioned from chapter 3 that tunicate cellulose is rich in I β cellulose, while cellulose of bacterial origin is richer in I α cellulose [11].

The PXRD data was used to estimate the crystallite sizes normal to the planes and the corresponding d-spacing. Further, depending on the d-spacing of the planes, the crystallite cross sections was determined.

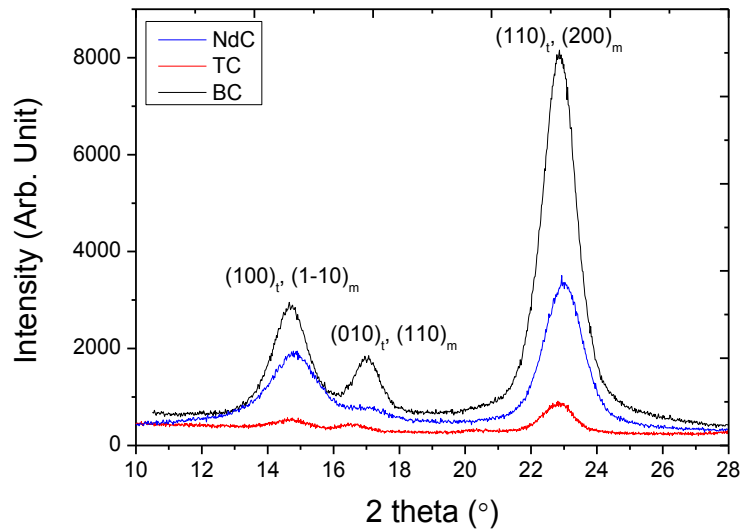


Figure 4.11: XRD patterns for BC, TC and NdC, showing three main reflections corresponding to $(100)_t$, $(010)_t$ and $(110)_t$ from $I\alpha$ (t = triclinic) , and $(1-10)_m$, $(110)_m$ and $(200)_m$ from $I\beta$ cellulose (m =monoclinic)

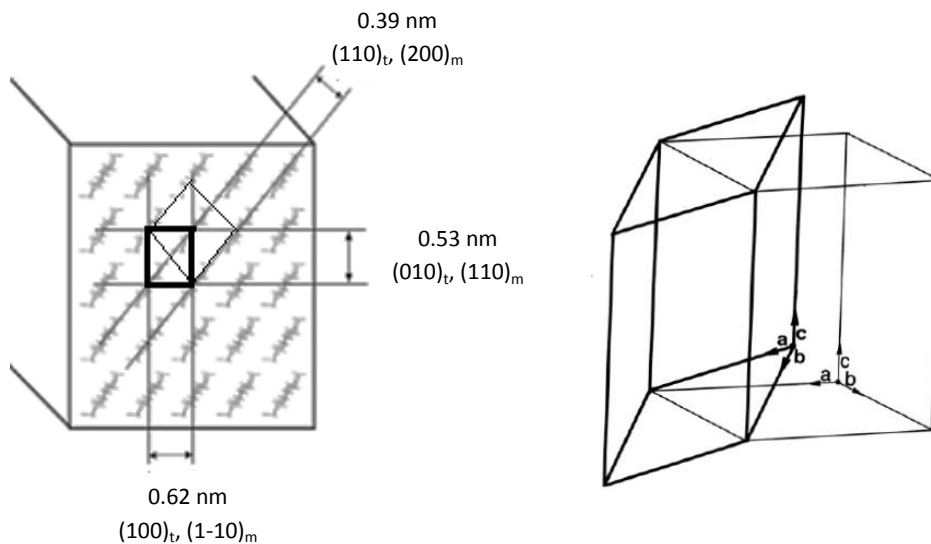


Figure 4.12: a) Arrangement of the cellulose chains in the cellulose I crystal indicating the spacing between the various planes for the two polymorphs $I\alpha$ and $I\beta$ (t = triclinic, m = monoclinic) b) relative orientation of $I\alpha$ and $I\beta$ [54]

4.4.1 Crystallinity

The crystallinity index (%) was calculated using the following equation [224] :

$$crystallinity (\%) = \frac{I_{200} - I_{am}}{I_{am}} \times 100 \quad (4.1)$$

where I_{200} and I_{am} correspond to the intensity of the peak correspond to amorphous region and (200) plane, respectively.

The cellulose ribbons or microfibrils are composed of amorphous, paracrystalline and crystalline regions. The calculated crystallinities for all the samples are listed in Table 4.1 and was found to be the maximum for BC (~85%), followed by NdC and TC exhibiting crystallinities >75% and >65%, respectively. The variation of crystallinities amongst cellulose from various sources is due to the different amounts of amorphous or non-crystalline regions, which are dictated by the specific biosynthetic machinery and environment. In general, the crystallinity reported for tunicate cellulose is higher than that determined in this work. This can be attributed to the variation of the crystallinity with the source-species and the handling and processing techniques used.

Table 4.1: Crystallinity of celluloses obtained from the various sources

Material	Crystallinty (%)
Bacterial cellulose	84.1 ± 7.1
Nata-de-coco	78.3 ± 8.3
Tunicate Cellulose	68.5 ± 2.1

4.4.2 Crystallite size

The Scherrer's equation was used to estimate the crystallite size[214] :

$$D = \frac{K}{\beta \cos(\theta)} \quad (4.2)$$

Tables 4.2-4.4 lists the calculated crystallite sizes and d-spacing obtained from the PXRD data for NdC, TC and BC, respectively. The crystallite sizes were determined to

be 5.1, 8.6, and 5.2 nm for NdC, 6.4, 6.6 and 7.8 nm for TC and 6.4, 8.5, and 6.5 nm for BC in the crystallographic directions [1-10], [110] and [200]. The crystallite sizes calculated here represent the lower limit of the crystallite size as the contributions of the instrument and the paracrystallinity in the material to the peak broadening have not been accounted for in the calculations. The instrumental broadening in the setup used for the current study is of the order of 0.1° (section 3.3.1), which is insignificant compared to the peak widths of $2-3^\circ$ observed here. On the other hand, determination of the contribution of the paracrystallinity of the samples was difficult. In order to estimate the broadening originating from the paracrystallinity of the samples, peak widths of reflections from the higher orders of the plane (200) (such as (400), (800)) are required. In the patterns obtained in the present study, these higher order peaks could not be easily distinguished due to low intensities and contribution from multiples planes. The intensity decreases with the higher order planes rapidly due to the presence of numerous atoms in the proximity, which spreads the electron density more widely resulting in the narrowing of the fourier transform, which in turn is reflected in the peak intensity. In addition, it was difficult to separate the contributions from the reflections from the multiple hkl planes originating from the two polymorphs. Therefore, all the crystallite sizes obtained using the Scherrer equation (furnished in Tables 4.2-4.4) correspond to the average lower limit of the actual sizes. However, in literature, the contribution from paracrystallinity to the peak broadening is often neglected in the crystallite size determination of cellulose [192]. However, it may be emphasized here that the contribution of paracrystallinity may be as large as 50% [252].

Table 4.2: Crystallite sizes and d-spacings obtained from the XRD profiles of NdC

Pos. [2θ .]	d-spacing [$\text{\AA}$]	Crystallite Size (nm)
14.4 ± 0.34	6.19 ± 0.15	5.1 ± 0.60
16.7 ± 0.43	5.31 ± 0.11	8.6 ± 0.95
22.5 ± 0.39	3.96 ± 0.06	5.2 ± 0.41

Table 4.3: Crystallite sizes and d-spacings obtained from the XRD patterns of TC

Pos. [$^{\circ}$ 2Th]	d-spacing [$\text{\AA}$]	Crystallite size (nm)
14.45 $\pm$ 0.15	6.11 $\pm$ 0.06	6.4 $\pm$ 0.2
16.38 $\pm$ 0.19	5.41 $\pm$ 0.06	6.6 $\pm$ 0.1
22.5 $\pm$ 0.14	3.95 $\pm$ 0.03	7.8 $\pm$ 0.3

Table 4.4: Crystallite sizes and d-spacings obtained from the XRD patterns of BC

Pos. [$^{\circ}$ 2Th.]	d-spacing [$\text{\AA}$]	Crystallite Size (nm)
14.3 $\pm$ 0.14	6.15 $\pm$ 0.06	6.4 $\pm$ 0.25
16.6 $\pm$ 0.15	5.29 $\pm$ 0.05	8.5 $\pm$ 0.78
22.5 $\pm$ 0.16	3.98 $\pm$ 0.02	6.5 $\pm$ 0.33

From the crystallite sizes and d-spacing derived from the XRD patterns, the number of planes in the cross section of individual crystallites can be determined. For example, a BC crystallite with a crystallite size of 6.4 nm normal to planes (1-10) (with a d-spacing 6.19 $\text{\AA}$) would consist of about 10 (1-10) planes (calculated by crystallite size/d-spacing) along the direction [1-10] in its cross section. Similarly, the number of planes in the cross sections of the BC, NdC and TC crystallite, were calculated and was used to construct a representative of the crystallite cross sections, which is illustrated in Figure 4.13. The non-rectangular shape of the crystallite cross section is because the combination of the crystallite dimensions obtained cannot be fitted to a shape with adjacent sides normal to each other. Similar approach has been used before by Elazzouzi-Hafraoui *et al.* in order to determine the cross-section of avicel, tunicate crystallites [192]. Moon *et al.* have reviewed the observations from various techniques and illustrated that cellulose from various sources is present as crystallites with rectangular or parallelogram cross section [4].

It can be seen that the cross sections of NdC and BC crystallites resemble truncated rectangles while, those of TC crystallites resemble truncated square. The dimensions of the crystallites are almost half the dimensions of the microfibrils obtained from SEM and AFM measurements (section 4.1, 4.2). This implies more than one crystallite may be present in the microfibril cross section. However, it may be remembered that paracrystalline distortion has not been corrected in the crystallite size. It can be concluded on the basis of the XRD analysis that the cellulose samples are not completely crystalline. The contribution to the non crystallinity is by the paracrystalline and amorphous regions in the cellulose chain arrangement. The presence of boundaries between the crystallites constituting an individual microfibril may also lead to decrease in crystallinity.

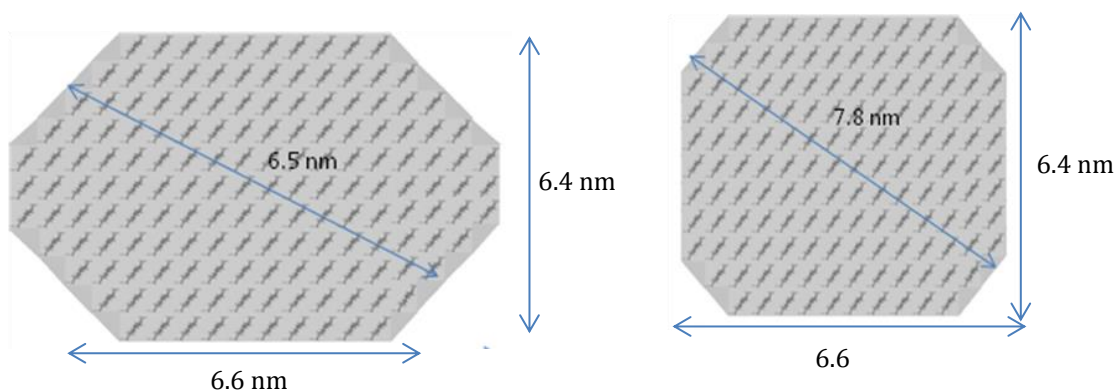


Figure 4.13: Cross sections of BC and TC crystallites constructed from results of PXRD analysis

4.5 Characterisation by SAXS

From SEM, AFM and XRD analyses, it may be inferred that each microfibril comprises of multiple crystalline fibrils. In order to obtain information about the hierarchical level, which is comprised of elements with size larger than crystallites and smaller than the microfibrils, scattering data from the small angle region was obtained and analysed. Figure 4.14 shows the original data collected by SAXS from very thin sheets of NdC, TC and BC. The point where the intensity is maximum (shown in Figure 4.14) represents the position of the beam stop which is located at $q = 0.01 \text{ \AA}^{-1}$ where q is the scattering vector defined as $4\pi\sin(\theta)/\lambda$. Thus, the reliable data lies at q values greater than $q = 0.01 \text{ \AA}^{-1}$.

In order to extract the dimensions of the small angle scattering element, the procedure similar to that demonstrated by Astley *et al.* [81] was followed. It may be assumed the lengths of the entities are too long to scatter in the angle range covered by our setup and therefore, the analysis from small angle region would be useful to extract information about the cross section of the scattering element. The theory predicts that in the low q region ($1/L \ll q \ll 1/r_c$, where r_c is the radius of gyration of the cross section of the scattering element and L is the length of the scattering length), the scattering from long rods should follow Guinier approximation given by

$$qI(q) = G \exp\left(\frac{-q^2 r_c^2}{2}\right) \quad (4.3)$$

where, G is the scaling constant. The approximation is valid in the range $qr_c < 1$, although marginally higher maximum values of qr_c do not necessarily give rise to significant error [81].

More importantly, the Guinier's approximation is valid for only very dilute systems. The system studied here cannot be considered as a dilute system. Therefore the microfibrils were treated with acid to form smaller fragments (called nanowhiskers), which possesses the same cross section dimensions as the starting cellulose microfibrils. The very diluted suspension of cellulose nanowhiskers dispersed in water was also analysed by SAXS. It was found that the results obtained from the analysis of SAXS data analysis of nanowhiskers suspensions were very similar to those for the as-received hydrated and un-hydrolysed sheet-like samples. For the sake of consistency with the literature findings, the SAXS data analysis is discussed for NdC, TC and BC sheets.

The Guinier equation can be rewritten as

$$\ln qI(q) = \ln G - \exp\left(\frac{q^2 r_c^2}{2}\right) \quad (4.3)$$

Therefore, in order to obtain the critical radius (r_c) of the scattering element, $\ln(qI(q))$ was plotted against q^2 as shown in Figure 4.15. The critical radius can be obtained from the slope of the $\ln(qI(q))$ vs. q^2 plot (in accordance with equation (4.3)). It can be observed that the slopes of the plots change at about $q=0.02 \text{ \AA}^{-1}$ for all the samples. This

change in slope may be due to the interference of scattering from the fibrils. However, as a very thin sample was used similar to the one reported [81], the contribution from the interference may be ignored for the purpose of comparative study to the data available in the literature. The change in the slope at low q value was treated as a contribution from entities of two different dimensions. Hence, the entire analysis was carried out considering two slopes for all materials and is presented elaborately only for bacterial cellulose in this section. The SAXS data from the other cellulose samples was similarly analysed and included in the Appendix A.

The critical radius obtained from the Guinier plot can be expressed in terms of the sides of the cross section of the diffracting element. It has been shown in the previous investigations that the cross section of the small angle x-ray scattering element is non-circular and nearly rectangular [4]. Therefore, if the sides of the 'rectangular' cross-section are $2a$ and $2b$, then r_c can be expressed as

$$a^2 + b^2 = 3r_c^2 \quad (4.4)$$

This gives the first relation between a and b . Another relation between a and b can be obtained by calculating the cross-sectional area (S) as follows

$$S = 4ab = \lim_{q \rightarrow 0} \frac{2\pi q I(q)}{Q} \quad (4.5)$$

where Q is the invariant calculated as

$$Q = \int_0^\infty q^2 I(q) dq \quad (4.6)$$

The value of $qI(q)$ when $q \rightarrow 0$ is same as the scaling constant in the Guinier equation and thus

$$S = \frac{2\pi G}{Q} \quad (4.7)$$

The Guinier plot was used to extrapolate the data to obtain the intensity from $q = 0$ to $0.01 \text{ \AA}^{-1}$. This extrapolation was carried out for the two slope values separately as discussed above. The extrapolated and experimentally obtained $\ln(qI(q))$ vs. q^2 plots

are shown in Figure 4.16. The invariant Q was calculated as the area under the curves from the plots of $I(q)$ vs. q^2 , shown in Figure 4.17, for both the slopes. The value of G was also estimated by extrapolating the Guinier plot. The values of the invariant Q , scaling constant G and scattering cross section area S are listed in Table 4.5. Thus, second relation between a and b was obtained (equation 4.5). The two equations (4.4 and 4.5) can be solved graphically, as illustrated in Figure 4.18, to obtain the possible values of a and b . The dimensions of the rectangular cross sections of the small angle X-ray scattering BC entity were found to be 32 nm by 16 nm and as 21 nm by 10 nm for slope 1 and slope 2 respectively.

The dimensions obtained using slope 1 are larger than the dimensions of crystallites as well as microfibrils, which is inconsistent with the idea that small angle scattering was used to extract information about the hierarchical level with elements smaller than microfibrils and larger than crystallites (Table 4.6). The scattering in the region corresponding to slope 1 may be a result of interference or air gaps of the calculated size. In either case, this analysis using slope 1 can be eliminated. On the other hand, dimensions obtained using slope 2 are larger than the crystallite sizes and in similar range with the microfibril dimensions. Therefore, the small angle x-ray scattering data implies that the microfibrils are the elements scattering in the small angle region. This suggests that crystallites are directly organised into microfibrils and the width and the thickness of microfibrils obtained from SEM and AFM respectively are consistent with the two dimensions obtained from SAXS.

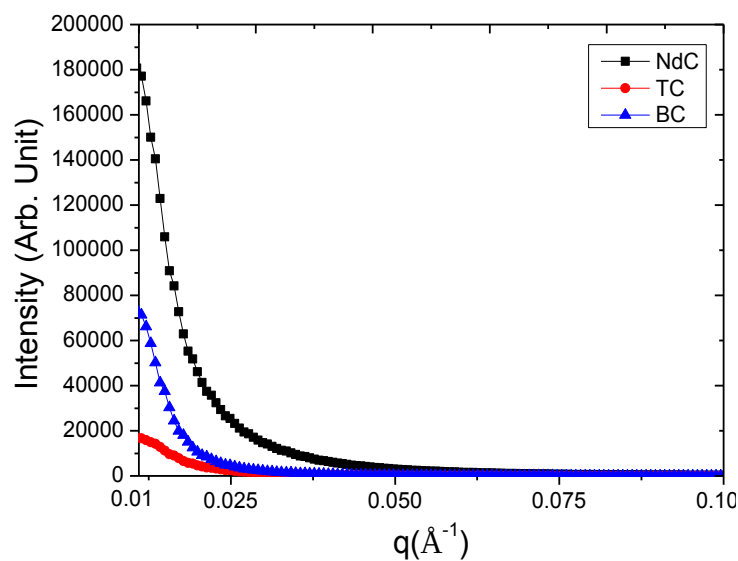


Figure 4.14: Variation in scattering intensity $I(q)$ vs. scattering vector q (from SAXS) for NdC, TC and BC with beam stop labelled

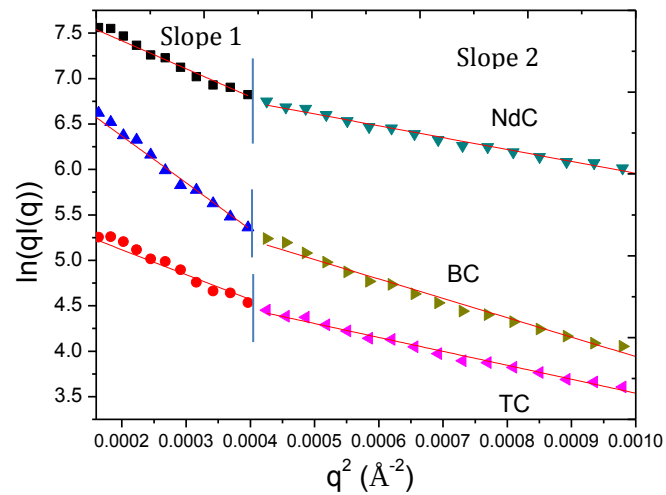


Figure 4.15: Guinier plot, $\ln(ql(q))$ vs. q^2 for NdC, TC and BC showing two distinct regions having different slopes

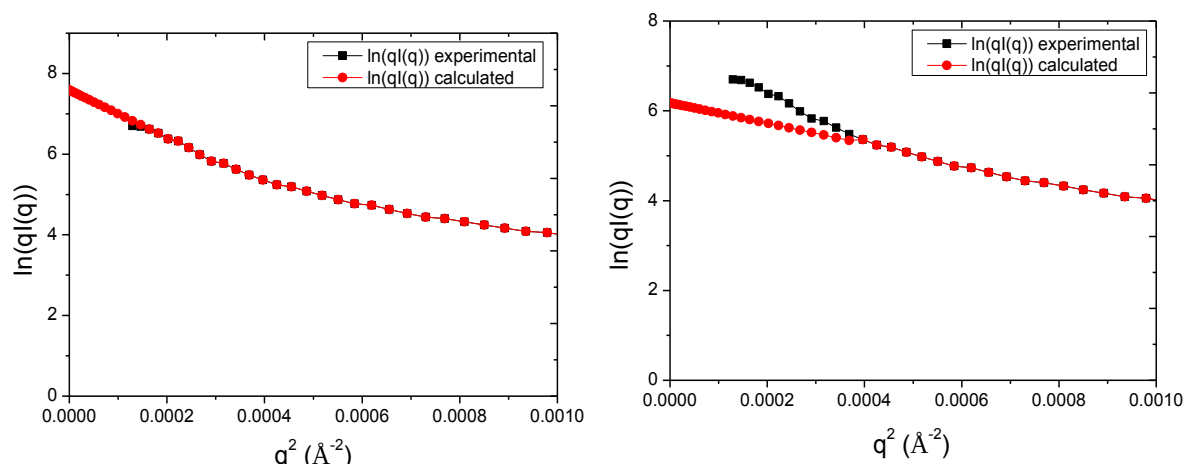


Figure 4.16: $\ln(ql(q))$ vs. q^2 showing both experimental and extrapolated data for BC for a) slope 1 and b) slope 2 in Figure 4.15

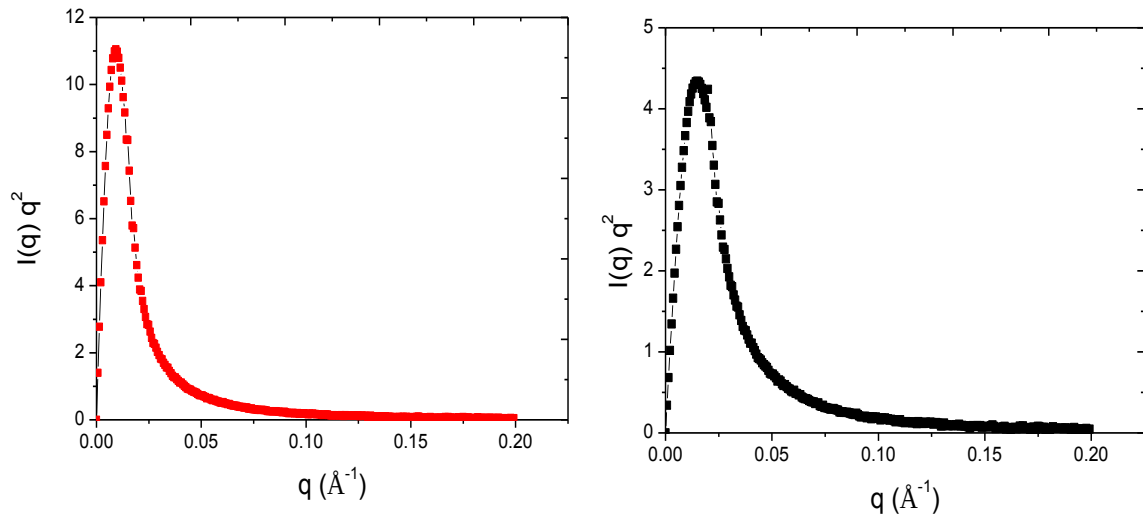


Figure 4.17: $I(q)$ vs. q^2 for the calculation of the invariant Q (according to equation (4.6)) for BC

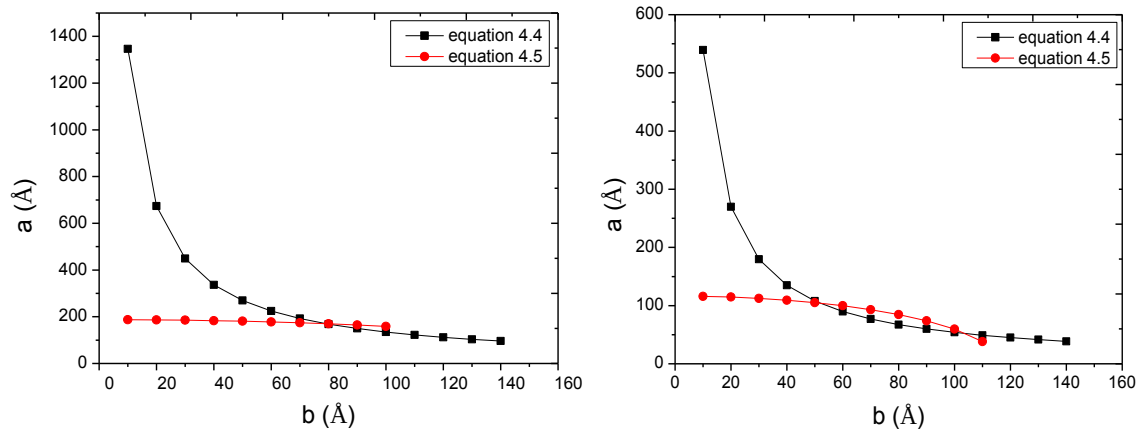


Figure 4.18: Graphical solution of the two equations (4.4) and (4.5) for BC

Table 4.5: Summary of the data extracted from SAXS analysis for BC

	Slope 1	Slope 2
Q (Invariance)	0.23	0.23
G (qI(q) at $q \rightarrow 0$)	197	481
S ($\text{\AA}^2$)	53871	21579
r_c ($\text{\AA}$)	108.35	67
a ($\text{\AA}$)	80	50
b ($\text{\AA}$)	169	105
Dimensions (nm^2)	16×34	10×21

, where slope 1 refers the initial slope region and slope 2 refers to the later slope region in Figure 4.15

Similar analysis was performed on SAXS data for TC and NdC to obtain the cross section dimensions. The dimensions obtained were $25 \text{ nm} \times 8 \text{ nm}$ and $14 \text{ nm} \times 6 \text{ nm}$ for NdC and $25 \text{ nm} \times 10 \text{ nm}$ and $15 \text{ nm} \times 8 \text{ nm}$ for TC using slope 1 and slope 2 (Figure 4.15) respectively. The values obtained using both the slopes for NdC are in the range of the microfibrils dimensions obtained from SEM and AFM, unlike in the case of TC where the dimensions obtained from slope 1 are larger than the microfibril dimensions similar to the case BC discussed above.

So far, while SAXS analysis of cellulose has been widely reported in literature, there has been little consensus in the findings. The range of values reported varies from few $\text{\AA}$ to tens of nm. In case of bacterial cellulose, Astley *et al.* estimated the cross section to be 10 by 160 $\text{\AA}$ for bacterial cellulose microfibrils [81], and Terech *et al.* determined the cross section dimensions of tunicates nanowhiskers to be 88 by 182 $\text{\AA}$ [164]. Therefore, it is essential to use SAXS in conjunction with other supporting and complementary techniques.

Table 4.6: Summary of the dimensions obtained by SEM, AFM, XRD and SAXS

	NdC		BC		TC	
	D1(nm)	D2 (nm)	D1 (nm)	D2(nm)	D1(nm)	D2(nm)
SEM	20-30	N/A	20-30	N/A	10-20	N/A
AFM	N/A	8	N/A	7	N/A	6
SAXS (Initial slope)	25	8	34	16	25	10
SAXS (Later slope)	14	6	21	10	15	8
PXRD	8.6	5.1	8.5	6.4	6.6	6.4

, Where D1 and D2 are dimension in two perpendicular dimensions

4.6 Model: Fibrous cellulose material

In this section, the information obtained from different techniques has been merged together to construct a model for cellulose. The NdC and BC microfibrils were 20-30 nm $\times$ 7-10 nm and TC microfibrils were 10-20 nm $\times$ 6-10 nm on the basis of observations by AFM and SEM. The dimensions obtained from SAXS experiments also resoundingly agreed with that obtained by SEM/AFM analysis.

A variation in the width of the cellulose microfibrils and ribbons was seen in all types of cellulose by SEM, which was attributed to the association between microfibrils produced by different bacteria, resulting into a branched morphology. High resolution SEM revealed twists in some of the microfibrils. This is suggestive of a critical microfibrils width which can retain a twist (arising from the intrinsic chirality of cellulose chains) and beyond this size the microfibrils either untwists or inter-twins to form fibrils with larger dimension. This concept is further discussed in section 5.5.

The cross-sectional sizes of the crystallites obtained by PXRD analysis were 6-7 nm $\times$ 8-9 nm for the BC and NdC and 6-7 $\times$ 6-7 nm for TC. As discussed in section 4.4.2, the contribution from the paracrystallinity to the peak broadening in the PXRD patterns has not been accounted for and these values presented only represent the lower limits of the crystallite sizes. However, the size of the microfibrils obtained by SEM and AFM analyses are twice the values of the crystallite cross-section dimensions obtained from PXRD studies. It may be assumed that introducing line broadening corrections for paracrystallinity to the PXRD peak widths is less likely to result in the shifting of the crystallite sizes obtained such that the dimensions obtained by XRD and SEM/AFM

analyses are at par. Some possible arrangements of the crystallites in the microfibrils are shown in Figure 4.19.

The crystallinities of NdC, BC and TC were determined to be about 75, 85 and 65%. This suggests presence of non-crystalline regions in the cellulose microfibrils. While the presence of distinct amorphous regions in microfibrils is still debated, no evidence of amorphous regions has been seen in the high resolution microscopy work presented in this thesis. However, the loss of crystallinity may be attributed to the paracrystalline distortion and crystallite interface.

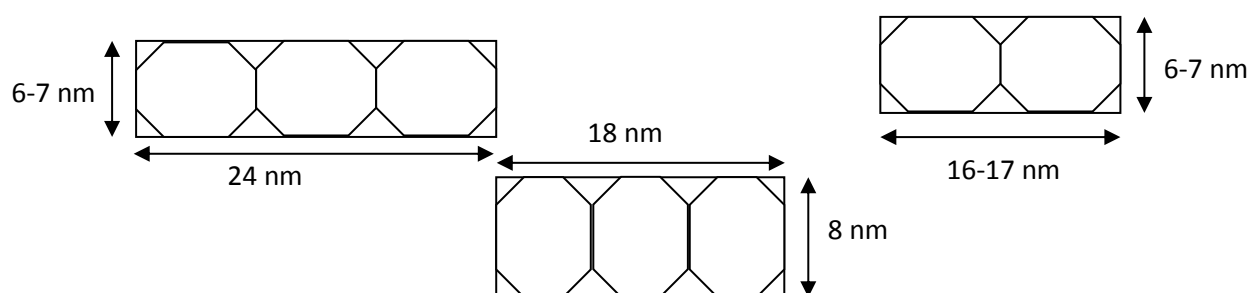


Figure 4.19: Possible models of cross sections of the crystallites that form a microfibril

Biosynthetic assembly in bacterial cellulose

The biosynthetic assembly process of BC on the basis of the work reported in this chapter and literature is illustrated in detail in Figure 4.20. In bacterial cellulose biosynthesis, each pore on the bacterial cell surface is known to produce a bundle with dimensions of about 1.5×1.5 nm [90], containing about 10-15 cellulose chains. These bundles from the pores in proximity assemble to form larger units, generally referred to as protofibrils, which are about 3-4 nm in diameter [5]. The observed crystallite sizes from PXRD analysis are about 8 nm $\times$ 6 nm, which implies the contribution from about $(8 \times 6) / (1.5 \times 1.5) = 20$ -30 pores. These crystalline structures assemble to form individual crystalline microfibrils with dimension 20 nm $\times$ 10 nm. The number of pores that contribute to the formation of a microfibril are $(20 \times 10) / (1.5 \times 1.5) = 88$ pores. The number of pores or biosynthetic sites have been observed to vary from just 12 in a dividing cell to 70 in a elongating non dividing cell in a particular strain of bacteria

[104]. The required number of biosynthetic pores required to produce a microfibrils is just about the maximum number of pores a standard 1 μ m bacterium might possess. The microfibrils produced by different bacterial cells interact leading to formation of ribbons and overall a branched morphology.

4.7 Summary

The structural and hierarchical organisation in cellulose from different origins (nata-de-coco, lab cultured bacterial cellulose and tunicate cellulose) has been studied by a range of characterisation techniques including SEM, SAXS, PXRD and AFM, to extract information at different length-scales in this chapter.

Irrespective of the source, the cellulose chains were organised into microfibrils and ribbons by supramolecular organisation aided by H-bonding and van der Waals interactions between the cellulose chains. The cellulose microfibril was found to be rectangular in cross sections with cross section dimensions of BC and NdC microfibrils and TC to be 20-30 nm $\times$ 7-10 nm and 10-20 nm $\times$ 6-10 nm, determined using SEM and AFM. The branching/splaying of cellulose ribbons and bundling of microfibrils was commonly observed. The length of segment between the branching points was found to be maximum in BC and minimum for NdC. On the basis of the average length between branching points and width of microfibril before and after branching, the branching was attributed due to aggregation of microfibrils from different bacteria and not to bacterial cell division suggested by Yamanaka *et al.* [48]. It has been seen in this chapter that intertwining and twisting in microfibrils with dimension 20-30 nm was observed but not in all cases, which leads to the concept of critical size of twisted microfibril, which will be invoked later in section 5.5.

The crystallite-sizes, representing the dimensions of the cross section of the crystalline fibril, obtained from the PXRD patterns, were 5-6 nm $\times$ 8-9 nm for NdC and BC, and 6-7 nm $\times$ 6-7 nm for TC, without taking into account the paracrystalline distortion. Considering the dimensions of crystallites and microfibrils, there is a possibility of more than one crystallite constituting a microfibril. The crystallinities of BC, NdC and TC were found to be 85, 75 and 65%. However, no evidence of distinct amorphous regions was observed by high resolution SEM.

SAXS was used to obtain additional information about the hierarchical level present between those that are accessible by SEM and XRD. The dimensions of the cross section of the scattering element were found to be in range of the results obtained from the SEM/AFM analysis and therefore did not reveal any additional information. It is suggested that a few crystallites aggregate to form microfibrils and the interfaces between the crystallites contribute to the non-crystalline regions, in addition to the defect prone surfaces.

A biosynthetic assembly model of bacterial cellulose has been proposed. It is suggested that if each pore produced bundles of cellulose chains of 1.5 nm in diameter, then the cellulose chains from about 20-30 pores form a crystalline fibril. Subsequently, in the proximity of the synthesis site, crystalline fibrils associate to form a microfibril. Further microfibrils from different bacteria aggregate to form ribbons which results into a branched morphology.

Having dealt with the natural hierarchical supra-molecular organisation in cellulose microfibrils, the next chapter discusses the next level of organisation where the microfibrils self-assemble to form a liquid crystalline phase characterised by long range positional order.

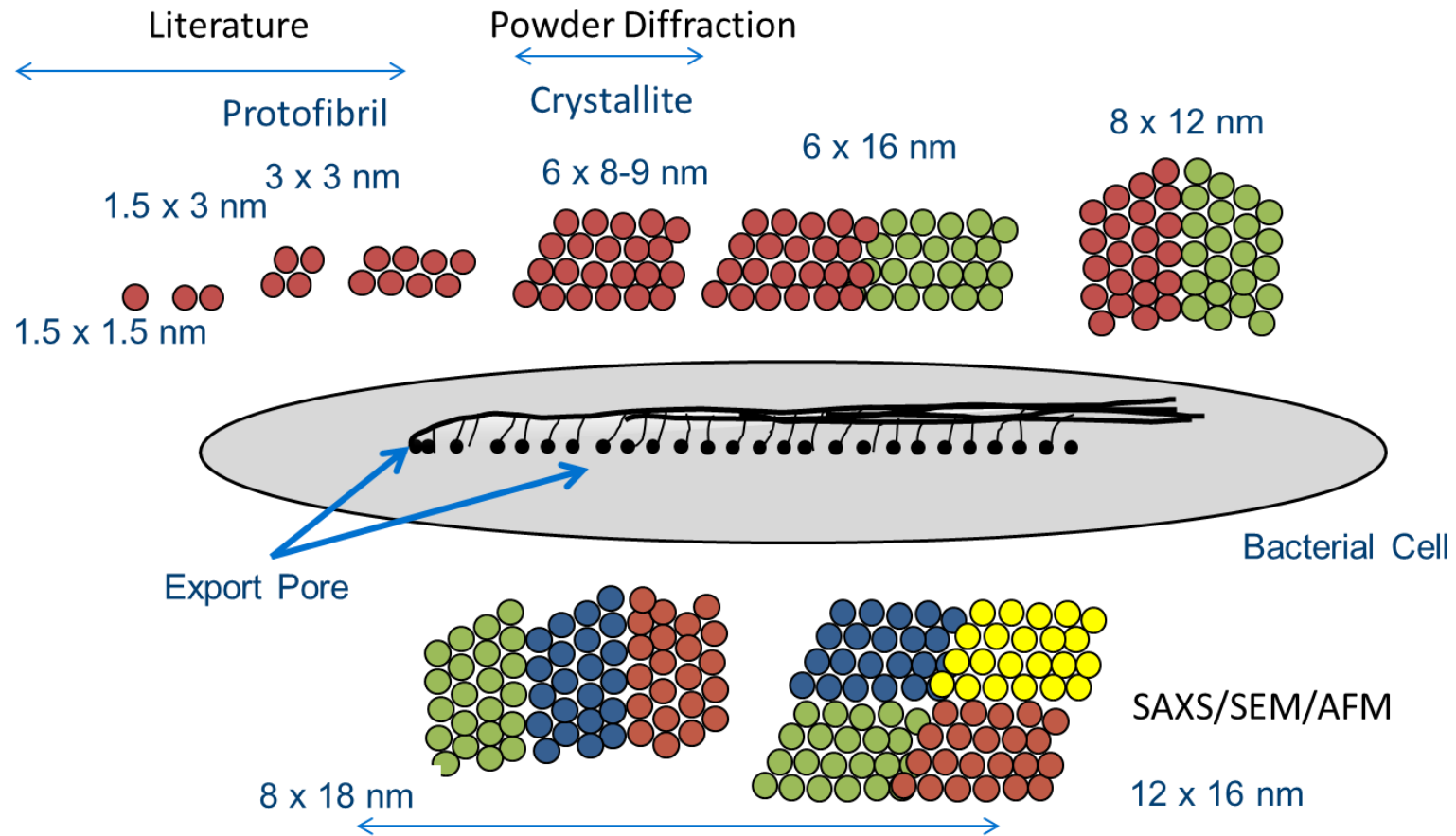


Figure 4.20: Correlation between biosynthesis and the assembly of basic cellulose units into microfibrils, each export pore contributes 15-20 polyglucan chains (literature), contribution from a few pores (approximately 20-30 pores) forms a crystallite, and a few crystallites form microfibrils. The elementary unit protofibril mentioned in the literature also included in the assembly

Chapter 5

Liquid Crystalline Processing of Cellulose

5.1 Introduction

In the previous chapter, it has been shown that the bacterial and tunicate cellulose, like celluloses from other common sources, occur in a multi-level organisation of cellulose chains assembling into microfibrils and ribbons. This hierarchical organisation of cellulose chains has been elucidated using techniques (SEM, AFM, SAXS and PXRD), which probe the sample at different length scales.

The mechanical properties and overall rigidity of cellulose microfibrils is determined by their crystallinity. The cellulose microfibrils comprise of amorphous, crystalline and also less ordered paracrystalline regions, the relative fractions of which varies with the cellulose source [20]. Apart from the naturally present defects and amorphous regions, the defects in the crystalline arrangement can also be created during various mechanical treatments such as fibrillation and so on. The fibril surfaces are prone to the defects and could play an important role in reducing the crystallinity of cellulose [85, 225]. The crystallinity also determines the chemical accessibility in a cellulose microfibril, as lesser crystallinity implies larger amount of non-crystalline (amorphous and paracrystalline) regions that are less dense and hence more prone to chemical attack.

As discussed above, the cellulose microfibrils owe their significant mechanical properties to the crystalline regions and since the crystallinity varies with the cellulose source, the properties of cellulose also depend on its source. The cellulose crystallites exhibit exceptional mechanical properties due to extensive H-bonding and van der Waal's interaction. This ensures efficient transfer of properties from molecular level to microfibril during the supramolecular organisation of cellulose chains. There are numerous reports and reviews that describe the mechanical properties of cellulose fibrils obtained from various sources, based on both experimental results and theoretical calculations [4, 137]. Several methods (including XRD, inelastic X-ray scattering, AFM and Raman spectroscopy) have been used to estimate the stiffness and

the elastic modulus of cellulose crystallites and a wide spectrum of values have been reported [10, 46, 47, 166, 167, 169, 171, 172, 226-228]. The variation in elastic modulus measured or calculated for cellulose microfibrils and crystallites varies from about 120 to 220 GPa [4, 10, 46, 47, 137, 166, 167, 169, 171, 172, 226-228] (section 2.9.2, Table 2.4). The mechanical properties of nanocrystals (obtained via the removal of amorphous regions) were better than that of other cellulose particles such as microfibrils and micro-fibrillated cellulose. This holds true specifically for the nanocrystals obtained from bacterial and tunicate cellulose rather than plant-based cellulose, ascribed to the higher crystallinity of the former than the latter (section 2.8, Table 2.2).

In order to transfer the properties to macroscopic products such as fibres, it would be advantageous to align cellulose microfibrils. One of the well proven strategies to do this, is to involve a liquid crystalline phase formation. Various high performance products such as silk and Kevlar fibres are obtained from rigid rod-like polymers processed by a route involving formation of a liquid crystalline phase [11, 12].

The feasibility of the formation of a liquid crystalline phase in cellulose or cellulose derivatives was first reported by Chanzy *et al.* in a solution of cellulose in N-Methylmorpholine N-oxide [229, 230], although cellulose was believed to be a polymer of fairly low rigidity. The first report on the formation of a liquid crystalline phase from cellulose microfibrils was presented by Ranby and Ribi [76, 129] where the cellulose microfibrils were hydrolysed to obtain nanowhiskers which possessed the capability to self-assemble into a chiral-nematic liquid crystalline phase. The literature review on the liquid crystallinity of cellulose has been presented in section 2.9. It is noteworthy that most of the reports in the literature focus on plant-based cellulose [122, 134, 137, 144, 156, 184]. The evidence of formation of a nematic phase remains sparse and the origin and nature of chiral interaction between nanowhiskers is still not completely understood (section 2.9). In the current work the formation of liquid crystalline phases from cellulose obtained from non-plant based (tunicate and bacterial) sources is reported and investigated.

The tunicate and bacterial celluloses are both produced as a very dilute hydrogels, in which the microfibrils are densely entangled. On drying, both of these are

reduced to about 2-5 wt% of the initial weight. In order to obtain individual microfibrils capable of forming an ordered phase, it is essential to disentangle these microfibrils. The most common procedure followed in polymer science, is to dissolve the polymer and then, either increase the concentration of the polymer or evaporate the solvent in order to facilitate self-assembly. Various solvents have been used for dissolving cellulose hydrogels. However, common solvents (such as acetone, toluene, N-methyl-2-pyrrolidone, and so on) failed to dissolve cellulose. The solvents only induced swelling of the hydrogels resulting in increase in volume of about 300-400% without leading to dispersion or dissolution. An account of these experiments and results are discussed in Appendix B. Other treatments, such as oxidation, grinding, crushing, and sonication, aided dispersion, albeit only to a limited extent. In most of these forms, the cellulose fibrils are still entangled and obtaining uniformly oriented fibrils in the bulk is difficult.

It has been shown in the literature that during acid hydrolysis, the length of microfibrils is shortened, which facilitates formation of an ordered phase. In this chapter, the nanowhiskers obtained from the acid hydrolysis of bacterial cellulose (BC), nata-de-coco (NdC) and tunicate cellulose (TC), are characterised using XRD, SEM and AFM to quantify crystallinity, and geometrical parameters. The phase transition diagrams from isotropic to liquid crystalline phase for these nanowhiskers are established using polarised optical microscopy and phase separation. The formation of various liquid crystal phases, viz. nematic and chiral nematic (cholesteric), has been investigated. Last part of this chapter is dedicated to the nature and origin of chiral interaction between nanowhiskers which leads to the formation of chiral nematic phase.

5.2 Acid Hydrolysis

The dried cellulose from NdC, BC and TC were treated with dilute sulphuric acid (65%) to produce nanowhiskers, as described in section 3.2. The nanowhiskers obtained from acid hydrolysis of cellulose from all the sources in this work have been characterised using XRD, SEM, AFM and the results are discussed in the next section. The complete characterisation is essential in order to understand the self-assembly into a liquid crystalline phase, which is discussed subsequently and constitutes the major part of this chapter.

Characterisation of nanowhiskers

5.2.1 XRD

The XRD patterns for the dried NdC nanowhisiker suspension and un-hydrolysed NdC on a silicon substrate are shown in Figure 5.1. It can be observed from the peak pattern that the cellulose I crystal structure is preserved after hydrolysis. Thus, the acid hydrolysis does not cause any disruption of the native cellulose crystal structure in contrast to the dissolution processes, which lead to the formation of the thermodynamically more stable cellulose II structure. This was found to be true, irrespective of the kind of cellulose.

The crystallinities of the three cellulose samples before and after hydrolysis were calculated from the XRD data (by the method described in section 3.3.1) and are listed in Table 5.1. The crystallinity was found to increase from 75 to 85%, 85 to 92% and 65 to 80 % for NdC, BC and TC, respectively, after hydrolysis with respect to the parent material. This is because acid preferably hydrolyses the less crystalline regions resulting into an overall increase in crystallinity. However, it may be mentioned here that all non-crystalline regions are not equally accessible and paracrystalline distortion also contributes to reduction in the overall crystallinity.

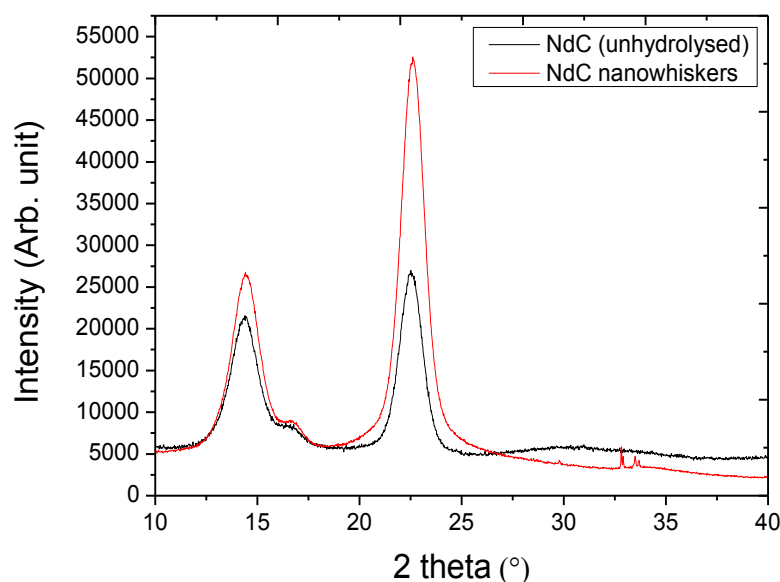


Figure 5.1: XRD pattern of dried un-hydrolysed and hydrolysed NdC (nanowhiskers) showing 3 main reflections corresponding to the planes

5.2.2 SEM

Figure 5.2 (a-d) shows the SEM images of BC, before acid hydrolysis and the nanowhiskers obtained after the hydrolysis, at low and high magnifications. The images show the drastic shortening of the microfibrils after hydrolysis and the width of the microfibrils remained unchanged. A similar effect was observed in the case of the other celluloses also.

High Resolution SEM was used to observe the ends of the microfibrils and the nano-whiskers. The ends of microfibrils as well as nanowhisaker are flat with no tapering, similar to that in case of a brittle fracture. This is consistent with the observation on the growing tip of bacterial cellulose microfibrils and nanowhiskers observed before and reported in the literature [191]. This suggests that all the cellulose chains forming the microfibrils are hydrolysed at same point along their length leaving behind a clear end. This supports the hypothesis that the cellulose microfibrils are comprised of amorphous and crystalline regions in series (section 2.3). On the contrary, the SEM investigations in the previous chapter did not provide evidence for any distinct amorphous regions. In this light, there are two other possibilities: the amorphous regions are too small to be seen by the microscopes or the amorphous regions do not exist distinctly and are present as surface defects which act as the primary site for acid attack. In the latter case, a defect can be expected to act as a nucleating point for cleavage across the fibril, leaving behind a flat end. The majority of the recent literature supports the serial arrangement of amorphous and crystalline regions hypothesis [74].

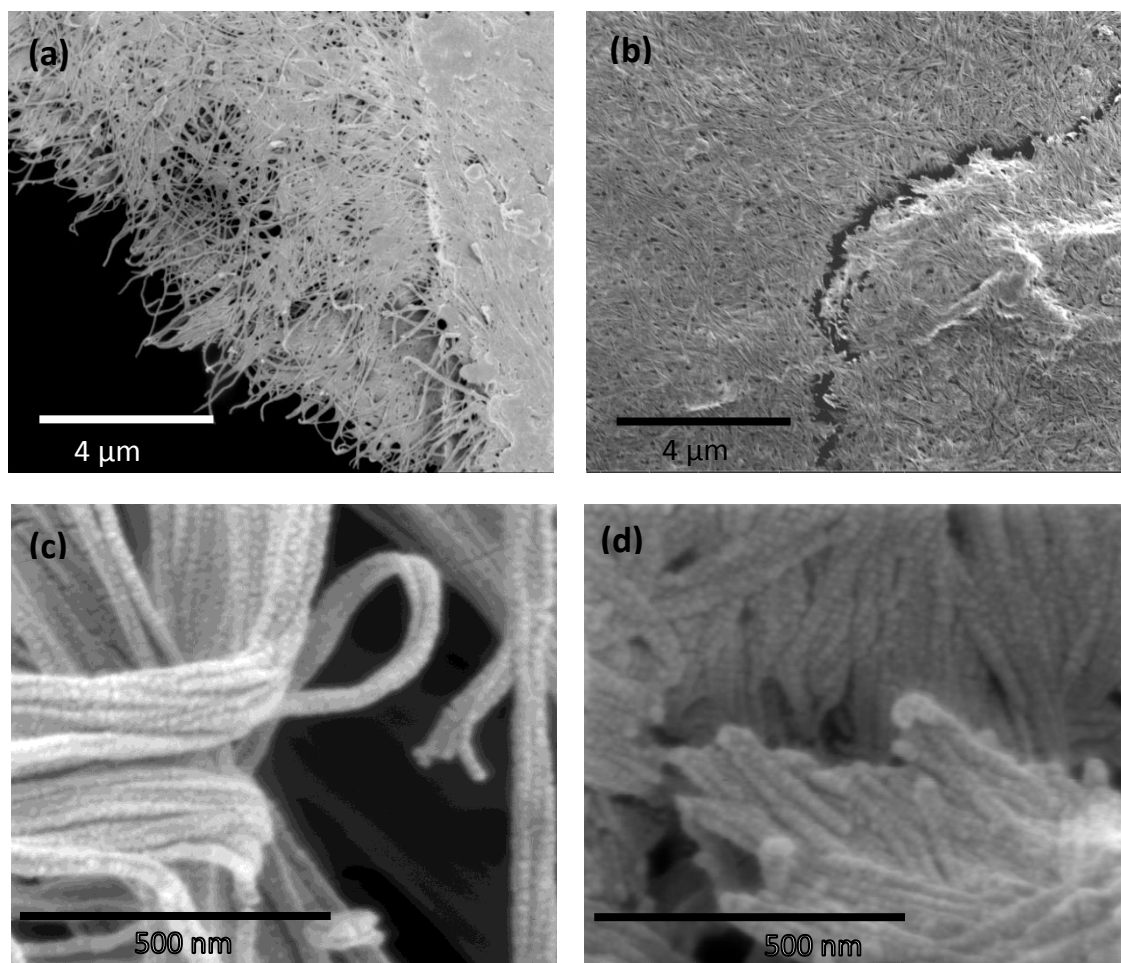


Figure 5.2: SEM image of bacterial cellulose at low magnification (scale bar 4 μm) (a) before and (b) after acid hydrolysis and at high magnifications (scale bar 500 nm) (c) before and (d) after acid hydrolysis

5.2.3 AFM

Figure 5.3 (a-c) shows the AFM images of the nanowhiskers obtained from NdC, TC and BC. It can be seen that these nanowhiskers are finite in length while the un-hydrolysed material comprised of endless microfibrils (section 4.2 and 4.3). This is consistent with the observations obtained from the SEM, as mentioned above. It is known that cellulose microfibrils possess a non-circular cross section [4, 46, 126], as shown in Figure 5.3(d). Thus, in addition to SEM, AFM is required in order to measure the height of the nanowhiskers.

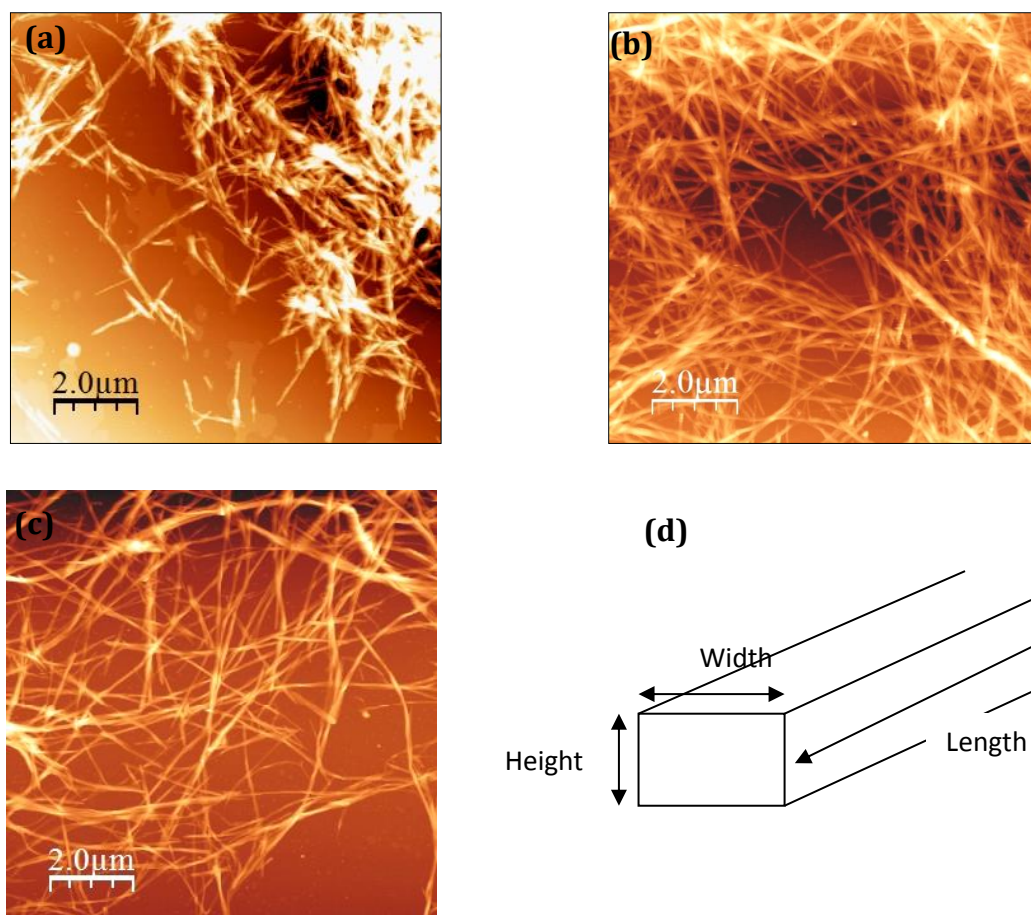


Figure 5.3: (a-c) AFM images of the nanowhiskers obtained from NdC, TC and BC and (d) schematic of cellulose nanowhisker

Figure 5.4 (a, b, c) shows the distribution of the lengths, heights and aspect ratios of the whiskers obtained from the different sources obtained by AFM analysis. From the length distribution of nanowhiskers illustrated in Figure 5.4 (a), it can be observed that the NdC nanowhiskers exhibited the smallest average length of about 1 μm while BC whiskers exhibited the largest average length of over 2 μm. Interestingly, a similar trend was observed for the length segments between branching points in SEM images as was mentioned in the section 4.2.2. Amongst the three materials, BC has the largest average length between branching points and NdC has the smallest. This corresponds with the average length of nanowhiskers obtained after acid hydrolysis as shown in Figure 5.5 (a-c). The correlation between the length distribution of nanowhiskers and the branching pattern has been further investigated in chapter 7.

The height distribution shown in Figure 5.4 (b) indicates that the maximum population of NdC nanowhiskers are 8-10 nm high while the heights of the TC

nanowhiskers and BC nanowhiskers are in the range of 6-10 nm. The minimum height observed in each of the samples are consistent with the height measured for individual microfibrils before hydrolysis (section 4.3). The average length of nanowhiskers reported in this work is larger than that reported so far in the literature for nanowhiskers derived from cotton [145, 156], wood [122, 140] and comparable to those for valonia [146], nata-de-coco [135, 144, 154] and tunicates [142, 143, 192]. The measured heights are almost similar to those reported in the literature.

The width of nanowhiskers could not be accurately measured by AFM because of tip broadening effects. However, the width was approximated as the full width half maximum of the peak obtained by scanning across the nanowhisger and these values have been used for the calculation of aspect ratios. The definition of aspect ratio varies in the literature [135, 156]. Most researchers use the ratio of length to either height or width depending on the technique used. Hirai *et al.* [135] have defined aspect ratio (AR) in their work on the liquid crystallinity of NdC nanowhiskers as

$$AR = \frac{Length}{\sqrt{width \times height}} \quad (5.1)$$

This definition has been used in the present work for the sake of comparison. From Figure 5.4 (c), it can be seen that while the aspect ratios of 65% of NdC nanowhiskers are below 50, the aspect ratio of 50 % of TC nanowhiskers are between 50 and 100 with a substantial population (of about 30%) exhibiting aspect ratios less than 50. A significant proportion (~20%) of the TC whiskers also exhibit higher values (over 100). In the case of BC, the maximum population (55%) of nanowhiskers show aspect ratio values between 50-100 with a significant proportion exhibiting values higher than 100 (30%) and aspect ratios of over 150 have been observed for a smaller portion (5%) of nanowhiskers. Thus, all the systems exhibited a high polydispersity. It is useful to bear in mind that the population distribution of aspect ratios of the whiskers is essential to predict and understand the phase transition behaviour of each sample. Aspect ratios obtained in this work were larger than those reported in the literature for cotton nanowhisger [145, 156], softwood nanowhisger [122, 140], and comparable to those reported in previous work on from tunicate cellulose [142, 143, 192] and nata-de-coco [135, 144, 154].

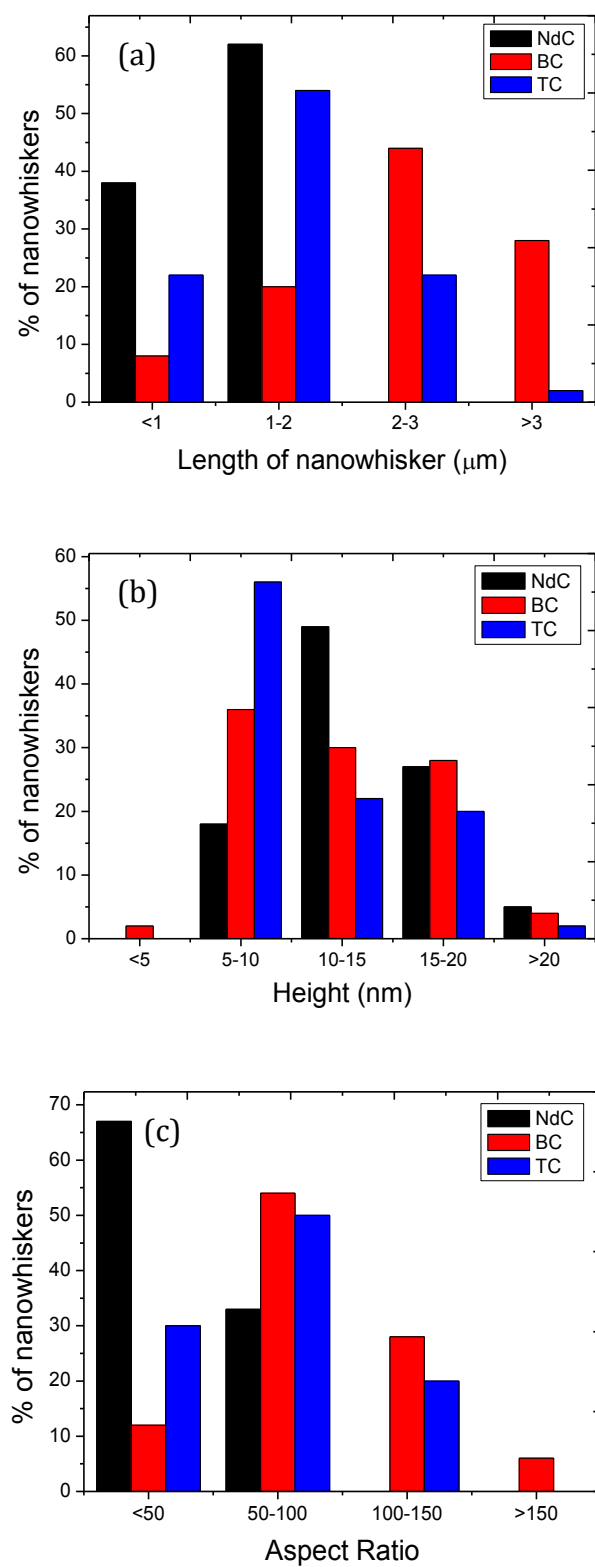


Figure 5.4: Distribution of a) length, b) height and c) aspect ratio obtained from AFM for the NdC, BC and TC nanowiskers, generated from 200 measurements

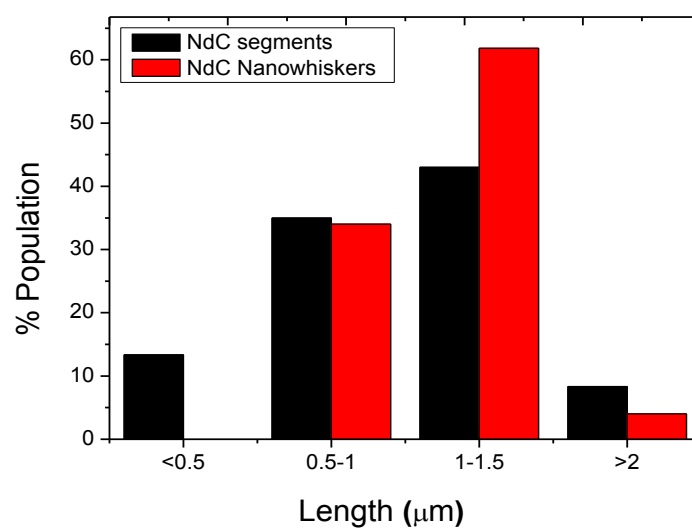
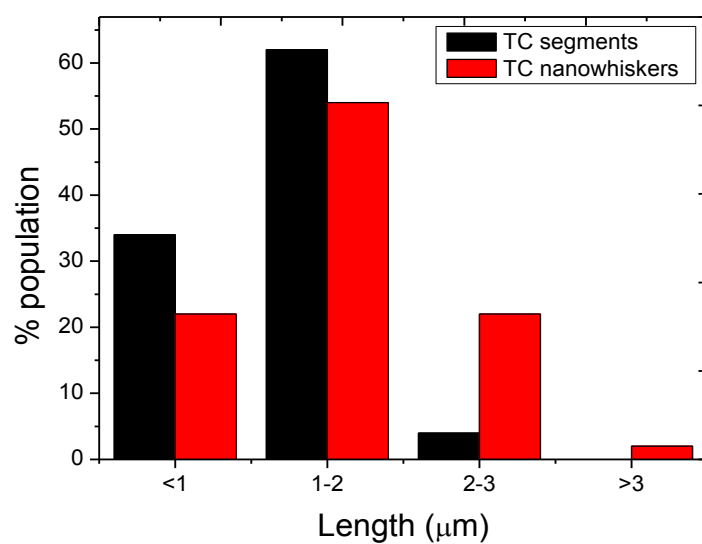
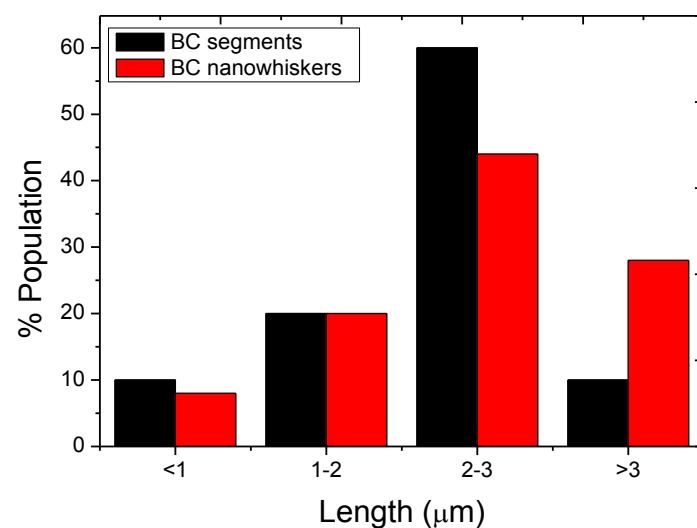


Figure 5.5: Distribution of nanowhiskers length and the microfibril segment length between branching points for a) BC, b) NdC and c) TC, generated from 200 measurements

A few additional features about nanowhiskers have been observed. Figure 5.6 shows AFM image of dispersed BC nanowhiskers where bundling and intertwining is evident to some extent. The intertwining can originate from the twisted conformation of nanowhiskers, which has been observed in celluloses of various origins [191]. Cellulose microfibrils have been reported to form intertwined aggregates in the parenchyma cell wall [253] and bacterial cellulose [191]. Microfibrils, like noodles, become intertwined by the capillarity action of the solvent during drying. There are other interesting implications of twisting of microfibrils which will be discussed later in section 5.5.

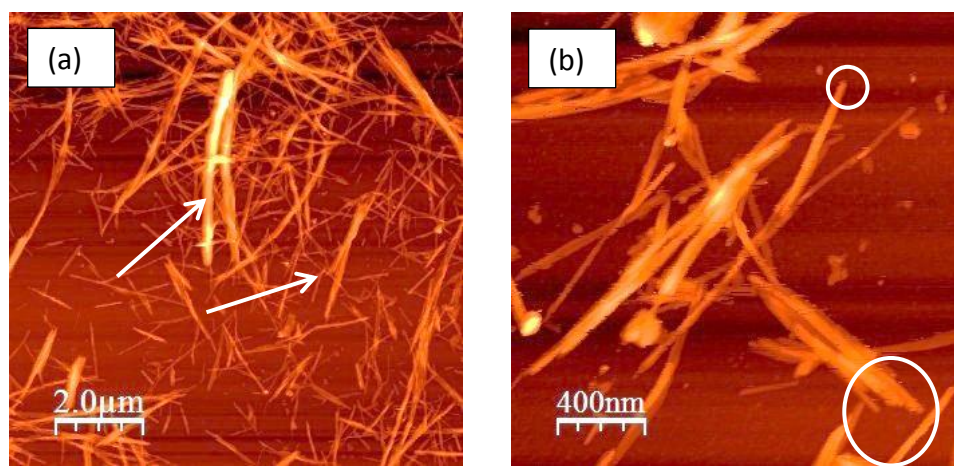


Figure 5.6: AFM images of BC nanowhiskers showing a) twisting-intertwining nanowhiskers indicated by arrow (scale bar 2μm) b) nanowhiskers with flat ends- enclosed in circle (scale bar 400 nm)

A closer look at the area where the nanowhiskers are in close proximity to each other reveals information about their interaction and structure. The nanowhiskers were rarely found to be present end to end or in a side by side position along the longitudinal axis. When the nanowhiskers were present on top of each other, their orientation was rotated by a small angle with respect to each other, as shown in Figure 5.6. This is an indication of the presence of some kind of chiral interaction between the nanowhiskers, which could be ascribed to the twisted configuration discussed above.

AFM images are also helpful in observed the ends of nanowhiskers. The nanowhiskers seemed to contain clean ends as shown in Figure 5.6, which is similar to observation made with SEM (section 5.4(c)).

5.2.4 Surface charge

As the hydrolysis progresses, apart from hydrolytic cleavage of the glycosidic bonds, the $-OH$ groups present on the surface of the nanowhiskers, convert to $-SO_3H$ groups. This imparts negative surface charge, which in turn stabilises the nanowhisiker suspensions in water owing to electrostatic repulsion. The amount of stabilisation and consequently the stability of the dispersion are dependent on the surface area of the nanowhiskers, which is in turn determined by the source of cellulose. Cotton nanowhiskers have been shown to acquire higher surface charge of about 0.155 e/nm^2 than BC of about 0.05 e/nm^2 [135, 156]. The surface charge calculated for the nanowhiskers obtained in this work is of the order reported in previously reported investigations on BC. The surface charge density for all samples ranged between 0.05 to 0.07 e/nm^2 , which is similar to that reported for bacterial cellulose [135, 156]. The ratio of the chains on the surface to the total number of chains in BC and TC nanowhiskers is about 0.1 which is much lesser than that for nanowhiskers obtained from other sources of cellulose and might result into low surface charge [4]. After completely characterising the systems involved, the self-assembly of cellulose nanowhiskers has been studied and results are provided in the next section.

5.3 Phase Diagram

The cellulose nanowhiskers obtained from all the cellulose materials studied here are rigid rod-like entities and thus possess the capability to form a liquid crystalline phase [231]. The phase transition from isotropic to anisotropic liquid crystalline phase is characterised by two concentrations: onset of phase transition and the completion and the narrow concentration range in between is biphasic and exhibits phase separation into isotropic and liquid crystalline phase. Similar trends were observed in the suspensions of nanowhiskers from all the cellulose sources studied here.

In order to develop a phase transition diagram for the nanowhiskers suspensions at various concentrations from the three sources, were allowed to stand still to facilitate phase separation. The volume of the bottom anisotropic liquid crystalline phase was measured. Another method is to measure the area fraction of the liquid crystalline domains observed by polarised optical microscopy. Both of the methods were used and the results are described below.

5.3.1 Phase separation

The phase separation does not occur instantaneously. Typically, it takes about 2 weeks to stabilise. A photograph of falcon tubes, containing 10 mL of suspensions at various concentrations, after 2 weeks of phase separation is shown in Figure 5.7. The equilibrium is determined by the composition of the suspension in terms of aspect ratio of the cellulose nanowhiskers and the solvent environment. The speed, at which this equilibrium is achieved, depends on the ease of the nanowhiskers to move in the suspension to take their positions in the phases.

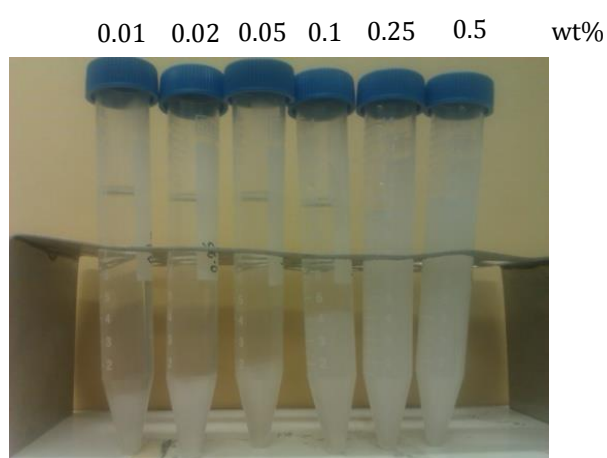


Figure 5.7: Falcon tubes containing suspensions (10 mL) with different BC nanowhisker concentration (increasing left to right) showing the phase separation into top clear phase and opaque bottom phase

The volume fraction of the anisotropic phase was found to increase with total concentration of nanowhiskers and the plot of volume fraction of anisotropic phase with total concentration of cellulose nanowhiskers forms the phase transition diagram, which is shown in Figure 5.8. For all the cases, the onset concentration for anisotropic phase formation was around 0.05 wt%, which was much lower than the values reported in the literature for nanowhiskers from other cellulose sources such as cotton linters [134], filter paper [156, 184] and comparable to that for NdC [135, 144] and TC [138]. The phase separation completed at 0.7, 0.4, and 0.5 wt% for NdC, TC and BC respectively. The transition region was observed to be broadest for TC nanowhiskers and narrowest for BC nanowhiskers. Apart from visual observation of phase separation, optical microscopy is a more reliable tool to observe phase transition and liquid crystal phase identification.

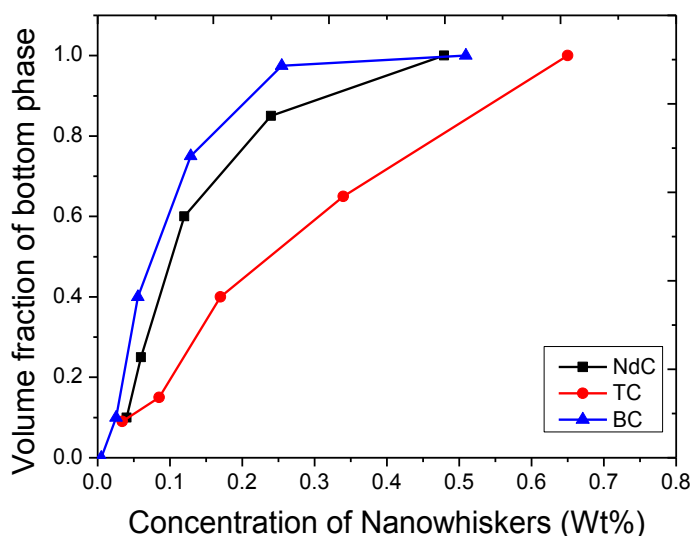


Figure 5.8: Variation in volume fraction of liquid crystalline/ anisotropic phase (bottom phase in Figure 5.7) with total concentration (wt%) of NdC, TC and BC nanowhisker showing the onset and completion of phase separation

5.3.2 Optical microscopy

In the native cellulose microfibrils and derived nanocrystals, the cellulose chains are present parallel to the long axis of the cellulose microfibrils/nanocrystals, making them strongly positively birefringent. Therefore, when the nanocrystals or microfibrils are present in parallel, a birefringent domain is formed. When this domain is observed between crossed polars, the relative orientation of the director axis of the domains and the axes of the polarisers determine its visibility. The details on this phenomenon are included in section 3.3.4. When a retardation plate is inserted between crossed polars, depending on the relative orientation of the fast and slow axes of the retardation plate and the birefringent unit, increase or decrease in retardation occurs, resulting into blue or yellow colours (Figure 3.13). The regions with no orientation or director in the direction of observation appear magenta in colour. Thus, the microscopic domains can be easily distinguished with the aid of crossed polars and retardation plate.

Figure 5.9-5.11 shows the the optical images of samples between glass slide and coverslip between crossed polars with (bottom images) and without (top images) a sensitive tint plate. The birefringent area observed in the crossed polar optical

microscope increases with the concentration of cellulose nanowhiskers, as the number and size of ordered domains increases with total concentration. Conventionally, if more than 50% area of the sample shows birefringence, then the suspension is believed to be liquid crystalline. For the concentrations believed to be the transition completion concentrations in the previous section, showed very little birefringent region. When the concentrations of the nanowhiskers was further increased, birefringent area increased and finally became completely liquid crystalline at about 4, 8, and 10 wt % for BC, TC and NdC suspensions respectively.

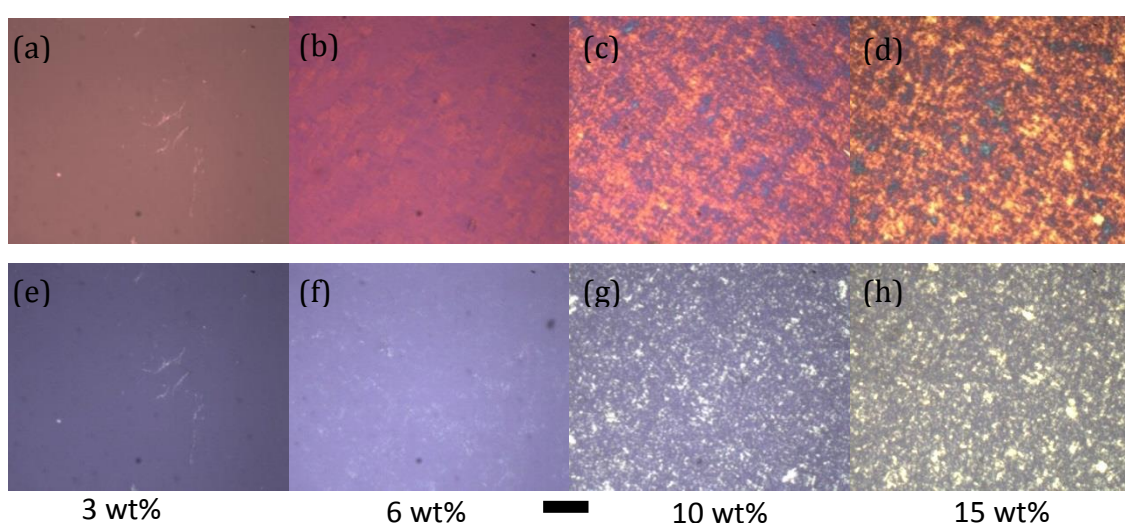


Figure 5.9: POM images of NdC nanowhisiker suspensions at indicated concentrations (a-d) with and (e-h) without a 530 nm retardation plate placed at 45° between crossed polars (scale bar 10 μ m)

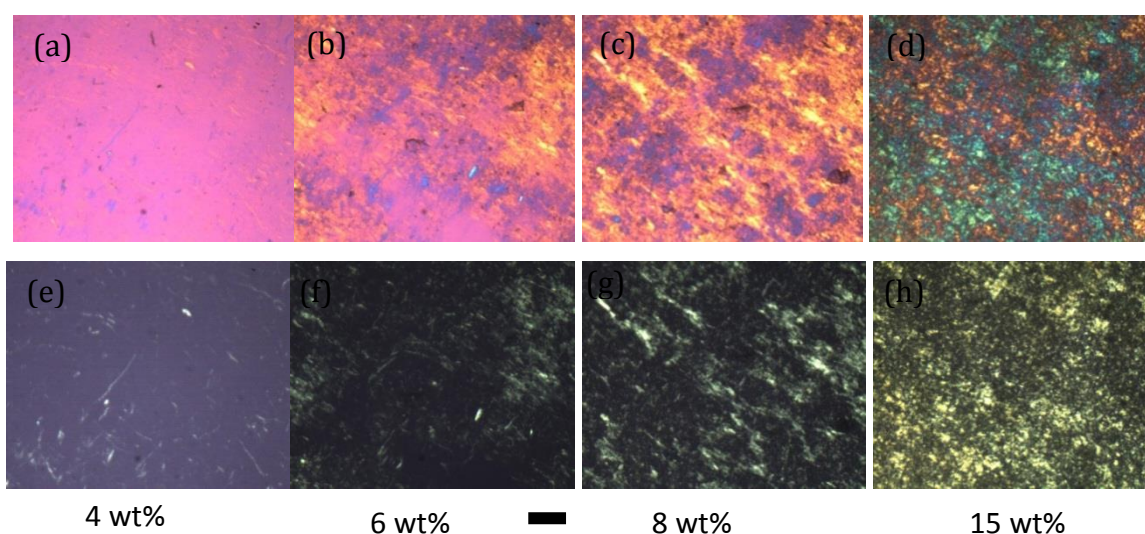


Figure 5.10: POM images of TC nanowhisiker suspensions at indicated concentrations (a-d) with and (e-h) without a 530 nm retardation plate placed at 45° between crossed polars (scale bar 10 μ m)

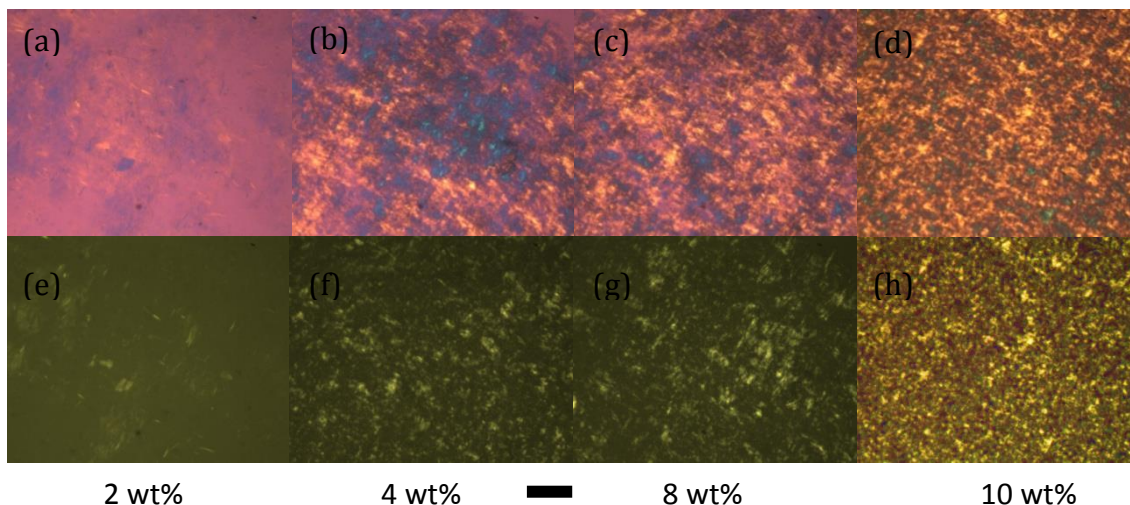


Figure 5.11: POM images of BC nanowhisiker suspensions at indicated concentrations (a-d) with and (e-h) without a 530 nm retardation plate placed at 45° between crossed polars (scale bar 10 μm)

Both of the methods suggest that the transition initiated and completed earliest for BC nanowhisikers. However, the actual values of the transition-concentrations predicted are not the same and were determined to be much higher by the POM. The transition-concentrations predicted by phase separation and POM are off by a factor of 10 with respect to each other. This offset is unusually high.

To explain this, the process of liquid crystal domain formation and phase separation can be considered at two levels: microscopy and macroscopic phase separation. It can be hypothesised that thermodynamically, depending on the concentration of the suspension, microscopic micelles of the liquid crystalline phase are first formed. Macroscopic phase separation occurs because the micelles which are denser than the surrounding isotropic material settle to the bottom. In a system with poly-disperse particles, the liquid crystalline micelles are preferentially formed by the longer particles and thus the isotropic-liquid crystalline phase separation also leads to fractionation. As the overall concentration of the system increases, the micelles grow in size and number. Owing to the high density of micelles, steric hindrance comes in to play in the micellar interaction and gel-like suspensions are formed, which might prevent the macroscopic phase separation [232]. The isotropic phase is trapped as the phase separation is kinetically hindered.

Therefore, in order to obtain a complete phase separation, the suspensions were centrifuged to enhance gravitation pull aiding the phase separation and the results are reported in the next section.

5.3.3 Phase separation under accelerated conditions

First of all, the kinetics of phase separation for a 0.5 wt% BC nanowhiskey suspension (which did not undergo any phase separation under 1g) was studied under conditions of centrifugation at 3500 rpm. The suspension (1mL) was centrifuged for different durations and the volume of the dense phase was measured (Figure 5.12 (a)) and is plotted in Figure 5.12 (b). No phase separation was seen before 2 min of centrifuging. The phase separation initiated after 2 min of centrifuging and a clear top layer was observed along with a diffused boundary between the two phases. The volume of the phase at the bottom decreased with increase in centrifuging time and then became constant as plotted in Figure 5.12 (b). An aliquot of the bottom phase, when observed between crossed polars, revealed liquid crystallinity, while the top layer did not show much birefringence and thus was isotropic (refer to section 5.3.4). Thus, the volume fraction observed after centrifuging is the amount of anisotropic phase in the suspensions. As the volume fraction collected at the bottom almost stabilised after 64 min, 90 min was taken as a standard time for centrifuging to allow complete phase separation to occur.

Suspensions (1mL) of various concentrations of nanowhiskers from the three sources were centrifuged at 3500 rpm for 90 min to obtain the volume of anisotropic phase (Figure 5.13). The volume fractions of the anisotropic phase collected at bottom was plotted against the total concentration (wt%) of cellulose, as shown in Figure 5.14 for NdC, BC and TC. The transition to liquid crystalline phase completed at 4.5 wt%, 11 wt% and 10 wt% for BC, NdC and TC nanowhiskers, respectively. These values are in close agreement with the values predicted by POM in the previous section. According to the results obtained by POM, the transition completes at around 4 wt%, 10 wt% and 8 wt% for BC, NdC and TC, respectively, which is similar to the results obtained from the phase separation studies after centrifugation. Figure 5.15 shows the phase diagram of BC nanowhiskers along with the POM images at 3 different concentrations indicating the transition.

Thus, the trends of phase transition concentrations predicted by POM are consistent with those by phase separation experiments. The transition completes earliest for BC nanowhiskers and last for NdC nanowhiskers. Under gravitational acceleration, the network formation by liquid crystalline micelles and the Brownian motion inhibited the phase separation, which was overcome by centrifuging. All the results discussed above with regard to the phase transition are summarised in Table 5.2. The transition into a liquid crystalline phase was obtained at a much lower concentration than that reported for plant based cellulose nanowhiskers (table 5.1), for example, cotton nanowhiskers undergo transition between 5 wt% and 13 wt%, owing to smaller aspect ratio [156].

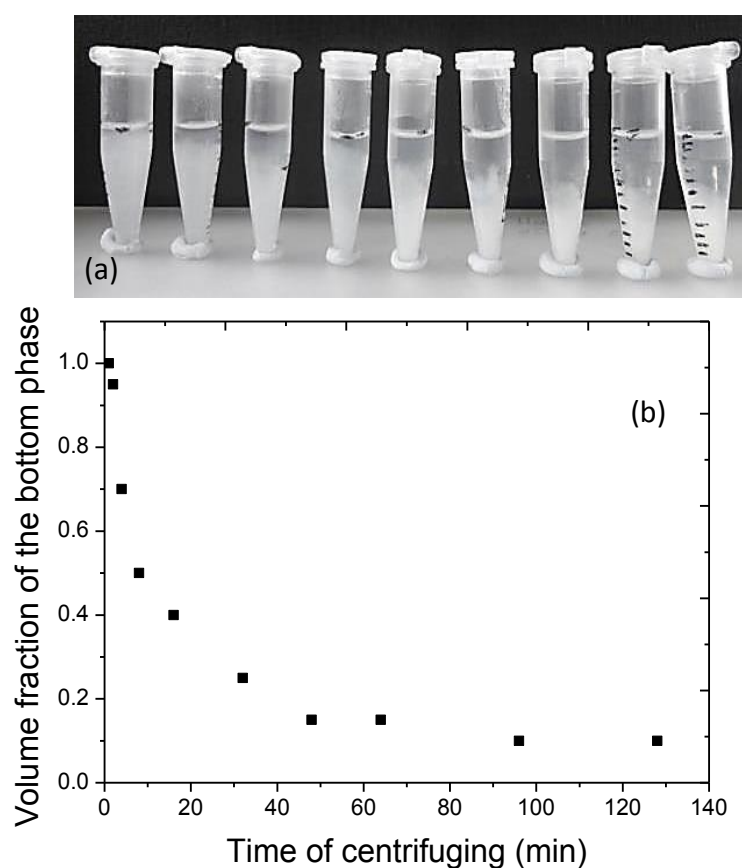


Figure 5.12: a) Photograph of eppendorf tubes containing 1 mL of 0.5 wt% BC nanowhisker suspension showing phase separation after indicated centrifugation time at 3500 rpm b) Variation in volume fraction of the anisotropic phase measured from Figure 5.12 (a) with time of centrifugation

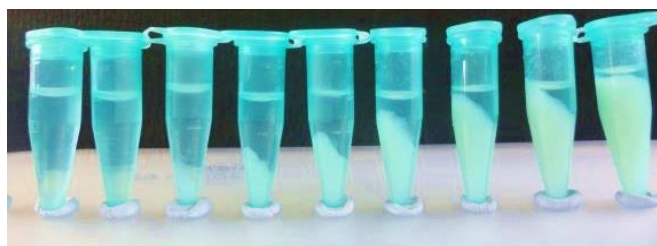


Figure 5.13: Photograph of eppendorf tubes containing 1 mL of various concentration of BC nanowhisker showing phase separation after 90 min of centrifugation at 3500 rpm

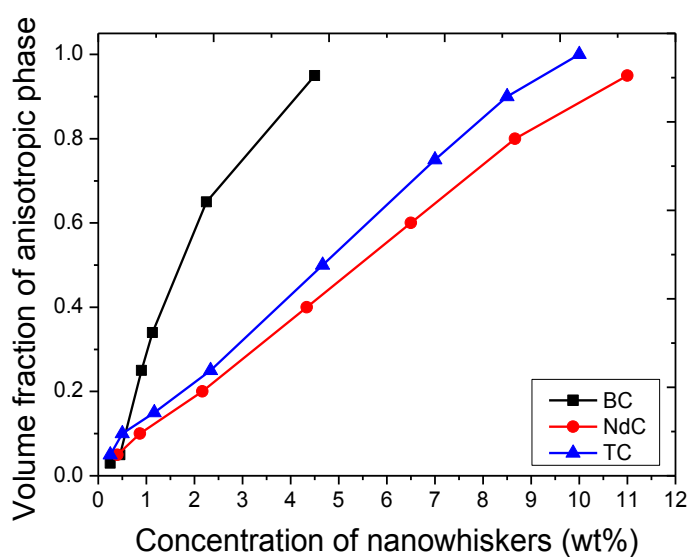


Figure 5.14: Variation in volume fraction of bottom phase after centrifugation at 3500 rpm for 90 min (Figure 5.13) with total concentration (wt%) of NdC, TC and BC nanowhisker showing the onset and completion of isotropic to liquid crystalline phase transition

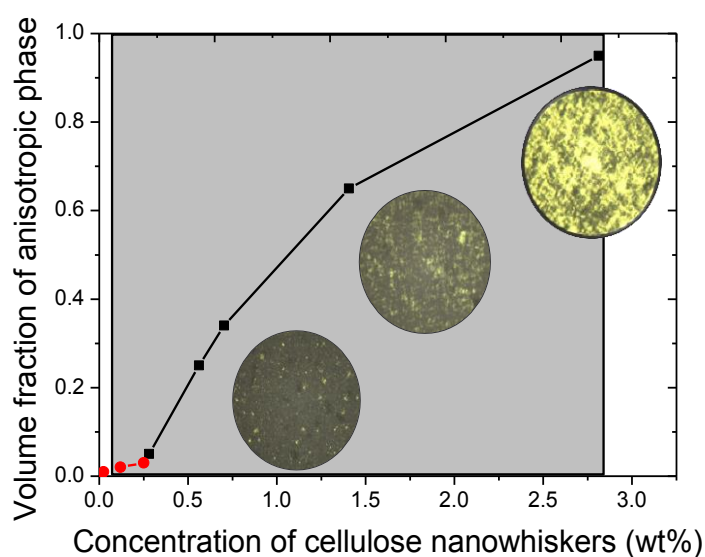


Figure 5.15: Phase transition diagram for BC nanowhisker suspension showing isotropic, biphasic and anisotropic phases as indicated by the birefringent bright regions in POM images (with 90 min centrifuging at 3500 rpm) (Red data points correspond to the concentrations which were also studied by phase separation under gravity alone)

Table 5.1: Summary of aspect ratio and phase transition concentration range (onset to completion of isotropic to liquid crystalline phase transition) for cellulose nanowhiskers from this work and literature

Source	Transition region (onset-completion concentration) (wt%)	Range of aspect ratios	Reference
NdC	0.05-12	20-80	This work
TC	0.05-10	15-140	This work
BC	0.05-4	25-190	This work
NdC	0.42-5	Below 44 –above 73	[135]
TC	1-7 mg/mL	30-110	[138]
Filter paper	4-13	7-10	[156]
Wood	4-12	30	[122]

The phase transition concentration can be theoretically predicted on the basis of the Flory [231] and Onsager theory [233] which are the two most widely accepted theories that deals with isotropic to liquid crystalline transition [234, 235]. According to

Onsager, the transition is dependent on the aspect ratio of the rigid rod-like entities according to the following equations:

$$C_i = 3.3d/l \quad (5.2)$$

$$C_a = 4.5d/l \quad (5.3)$$

where C_a and C_i are the concentrations at which transition from isotropic to biphasic and biphasic to liquid crystalline occur, respectively and d and l are the diameter and length of the involved liquid crystal rigid rod. Since in this work, a poly-disperse system is considered, the smallest aspect ratio was used to calculate the C_a and largest value of aspect ratio was used to calculate C_i as the rods with largest aspect ratio enter the liquid crystal phase first and the ones with the smallest aspect ratio forms the liquid crystalline micelle the last. The onset and completion concentrations for transition, calculated using largest and the smallest aspect ratio, are 0.065 wt% and 0.33 wt% for NdC, 0.038 wt% and 0.48 wt% for TC and 0.028 wt% and 0.28 wt% for BC nanowhiskers, respectively.

However, the system in this work is different from the ideal system due to high polydispersity, charge on the surface, interaction between nanowhiskers and nanowhisiker-water and twisting effect due to chirality of the molecule. This results in variation between the predicted and the experimentally observed concentrations. Surface charge has two main consequences: the effective aspect ratio decreases as the effective diameter increases due to the presence of charged double layer and there is a twisting effect due to surface charge that disfavours alignment. Overall, the first effect stabilises liquid crystalline phase, while the later opposes the liquid crystalline phase formation. In this work, the surface charges were found to be similar for all the samples and hence its effect can be eliminated. All the experimental and calculated transition concentrations are listed in Table 5.2.

Table 5.2: Transition concentrations (calculated and experimental), maximum and minimum aspect ratios, polydispersity index for the nanowhiskers obtained the three sources

Sample	C_{ie}	C_{ae} (setup under gravity)	C_{ae} (setup centrifugation)	C_a/C_i	C_{ic}	C_{ac}	AR	
							AR_S	AR_L
NdC	0.05	0.4	12	240	0.065	0.33	22	81
TC	0.02	0.65	10	500	0.038	0.48	15	139
BC	0.015	0.3	4	260	0.028	0.28	26	189

C_{ie} = experimental transition concentration from isotropic to biphasic (determined by extrapolation to volume fraction to 0 using linear fit to first three experimental data point in Figure 5.14 for each curve), C_{ae} = experimental transition concentration from biphasic to liquid crystalline, C_{ic} = calculated transition concentration from isotropic to biphasic, C_{ac} = calculated transition concentration from biphasic to liquid crystalline AR = Aspect ratio, AR_L = largest aspect ratio, AR_S = smallest aspect ratio

5.3.4 Isotropic and anisotropic phase

Aliquots from the two phases obtained after phase separation were taken and characterised by AFM and POM. Figure 5.16 shows the AFM image and polarised optical micrograph of the two phases. It is seen that the nanowhiskers constituting the upper phase are smaller in length as compared to those present in the bottom phase. The POM images (Figure 5.16 (a, b)) shows very little birefringence in the suspension collected from the upper phase in comparison to the birefringence shown by the bottom phase.

The nanowhiskers with higher aspect ratio enter the anisotropic phase and the smaller ones preferentially go to isotropic phase. This is entropy driven effect where the system tries to maximise the excluded volume. The excluded volume is given by $2L^2d\sin(\gamma)$, where L and d are length and the diameter of the rod and γ is the angle between rods. As the length or the diameter increases the excluded volume increases. As the excluded volume increases, the tendency to minimise excluded volume and hence to align, increases. In the systems of interest here, the diameter is almost same but the length variation is large. Thus, for larger length nanowhiskers, the tendency to align is larger and forms the liquid crystalline micelles the earliest. The extent of fractionation depends on the sluggish diffusion between the two phases with a consequent deviation from the equilibrium compositions remaining at finite times. The upper phase being less dense and composed of smaller aspect ratio nanowhiskers, is isotropic and the bottom more dense phase being composed of larger aspect ratio nanowhiskers is liquid

crystalline which explains the observations of the POM in Figure 5.16 (c, d). Similar mesogenicity driven fractionation by aspect ratio has been reported for similar rigid rod like entities such as MWNTs [236]. The AFM images of fractionated phases of TC and NdC nanowhisiker suspensions are also included in Figures 5.17 and 5.18, respectively. Similar observation of fractionation of nanowhisikers by length can be made.

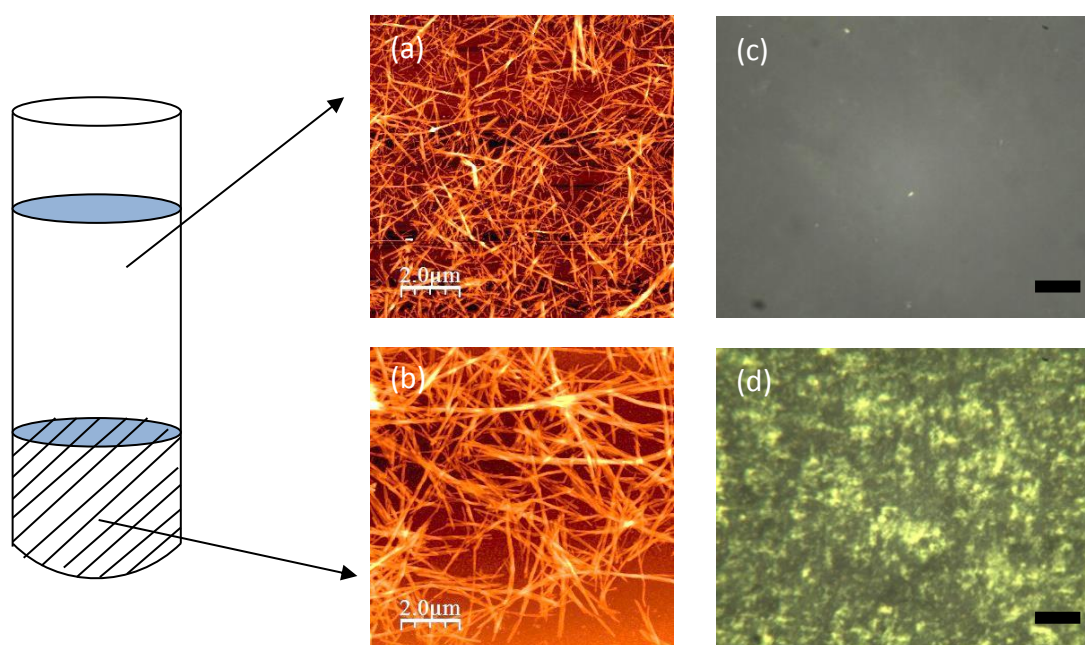


Figure 5.16: Schematic of phase separated nanowhisiker suspension. AFM image (scale bar 2 μm) of the a) top and b) bottom phase of a 1 wt% BC nanowhisiker suspension. POM images (scale bar 10 μm) of c) top and d) bottom phase of a 1 wt% BC nanowhisiker suspension, The images show the top phase is isotropic with smaller aspect ratio nanowhisikers and bottom phase is liquid crystalline and comprise of nanowhisiker with larger aspect ratio

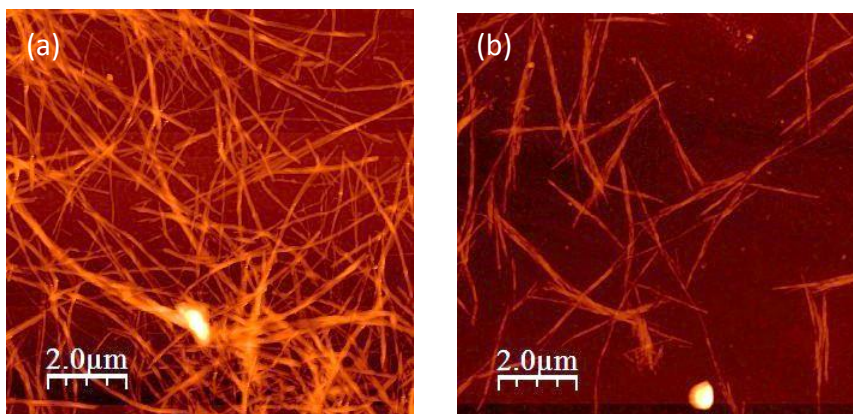


Figure 5.17: AFM image of (a) top and (b) bottom phase of TC nanowhisker suspension (scale bar 2 μm)

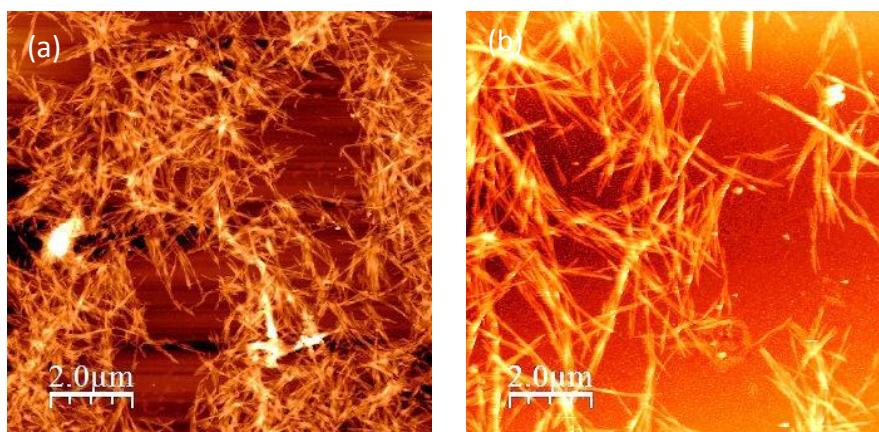


Figure 5.18: AFM image of (a) top and (b) bottom phase of NdC nanowhisker suspension (scale bar 2 μm)

5.4 Liquid crystal phases

POM is useful in identifying the liquid crystalline phase as all kinds of liquid crystalline phases have their typical characteristic texture. As mentioned in the section 3.3.4, two kinds of samples preparation have been used for POM analysis. In one kind of sample, a drop of the suspension (of various concentrations) was placed between the glass slide and the coverslip, which was used in identifying the phase transition in section. In the other kind of sample, a thin capillary filled with the suspension was observed.

5.4.1 Nematic phase

Figure 5.19-5.21 are the optical images of samples (10 wt%) between glass slide and coverslip between crossed polars with (bottom images) and without (top images) a sensitive tint plate. When the sample was rotated between crossed polars, the bright

regions became dark and the dark regions became bright when observed without the tint plate and yellow regions became blue and vice versa with a tint plate. The texture, which exhibits this phenomenon is referred to as a scheliren texture and is a typical characteristic of a nematic phase. A schematic of nematic phase is shown in Figure 5.22 (a).

One characteristic difference shown by the suspensions from three sources, apart from different transition concentrations, is the size of the domains at the same concentrations. A domain is defined as a region of closely associated liquid crystal entities, nanowhiskers in this work, where they have a common director and thus the domains can be easily identified between crossed polars and a retardation plate. The average domain size of NdC suspensions at 10 wt% was about $404\ \mu\text{m}^2$ while that of TC and BC nanowhisiker suspensions was about 865 and $703\ \mu\text{m}^2$ respectively. The NdC nanowhiskers are much smaller in aspect ratio than the others. The longer nanowhiskers have a higher probability of finding other nanowhiskers for a given concentration and thus a higher possibility of expanding the oriented domains. This explains the larger domain size exhibited by BC nanowhiskers in comparison to other materials. The domain size is an important parameter because it is an indicative of the long range order in the liquid crystalline phase.

5.4.2 Cholesteric phase

When the same cellulose nanowhisiker suspensions were filled in a capillary, formation of a finger print texture, comprising of dark and bright bands, was seen as shown in Figure 5.23 (a-c). This indicates the formation of a chiral nematic or a cholesteric phase and which implies the presence of a chiral interaction between nanowhiskers. The cholesteric phase is characterised by the pitch of the fingerprint texture as shown in Figure 5.22 (b). The pitch, which is the distance between bright bands forming the fingerprint texture, was observed to be between 5 and $10\ \mu\text{m}$ in this work.

The difference in texture in the two preparation methods may be explained on the basis of confinement effects. In case of confining a suspension between a glass slide and coverslip, cellulose nanowhiskers lay along the glass slide due to the influence of the surface (the gap between the surfaces is about $50\text{-}100\ \mu\text{m}$). This either prohibits the

formation of the chiral nematic phase or the axis of the phase lies along the direction of observation. In either case, the finger print texture will not be seen.

The origin of chiral nematic interaction in cellulose has been widely investigated. Various reasons such as morphological twist in cellulose nanowhiskers [135, 180, 185], and cellulose nanowhisiker-solvent interactions [134], have been explored and both theoretical and experimental evidences have been reported. In the next section, a detailed discussion on chiral interaction between nanowhiskers is presented.

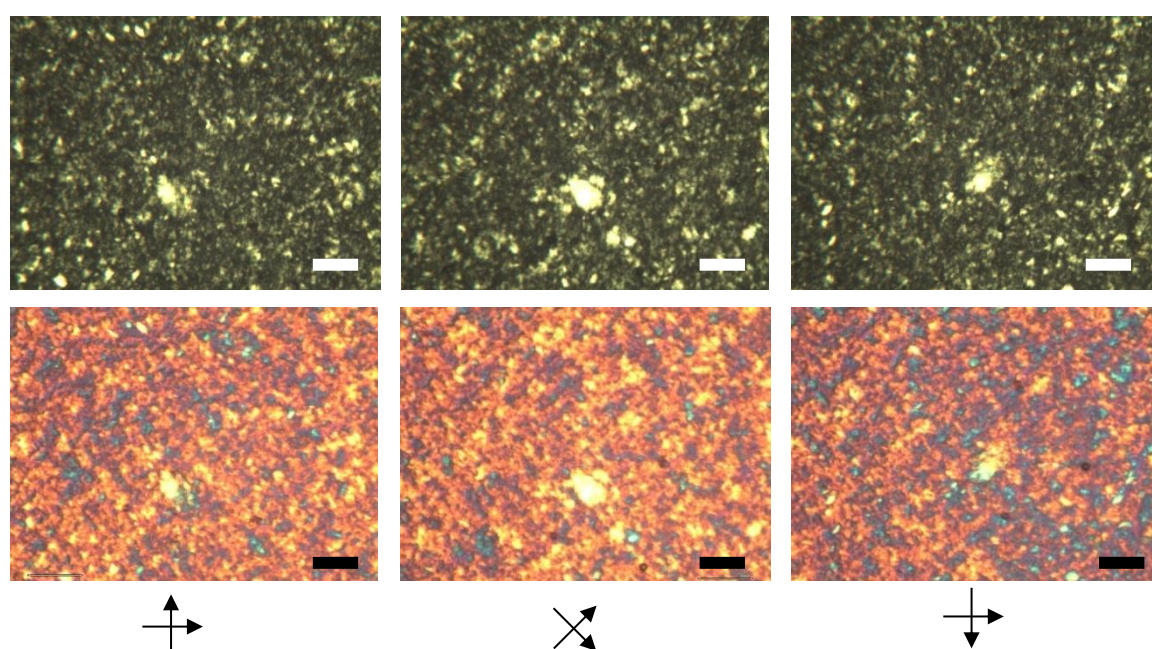


Figure 5.19: POM images of NdC nanowhisiker suspensions placed between a glass slide and a cover slip viewed between cross polars without (top images) and with (bottom images) a 530 nm retardation plate (bar 100 μm) with sample rotated between crossed polars by 45° twice

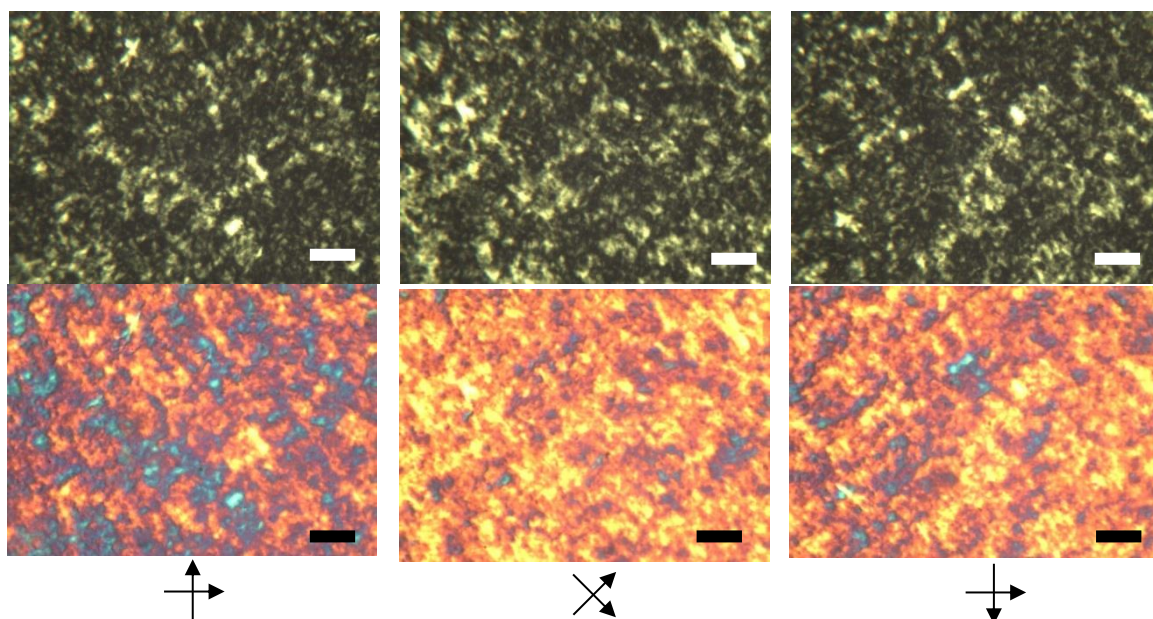


Figure 5.20: POM images of TC nanowhisker suspensions placed between a glass slide and a cover slip viewed between cross polars without (top images) and with (bottom images) a 530 nm retardation plate (bar 100 μm) with sample rotated between crossed polars by 45° twice

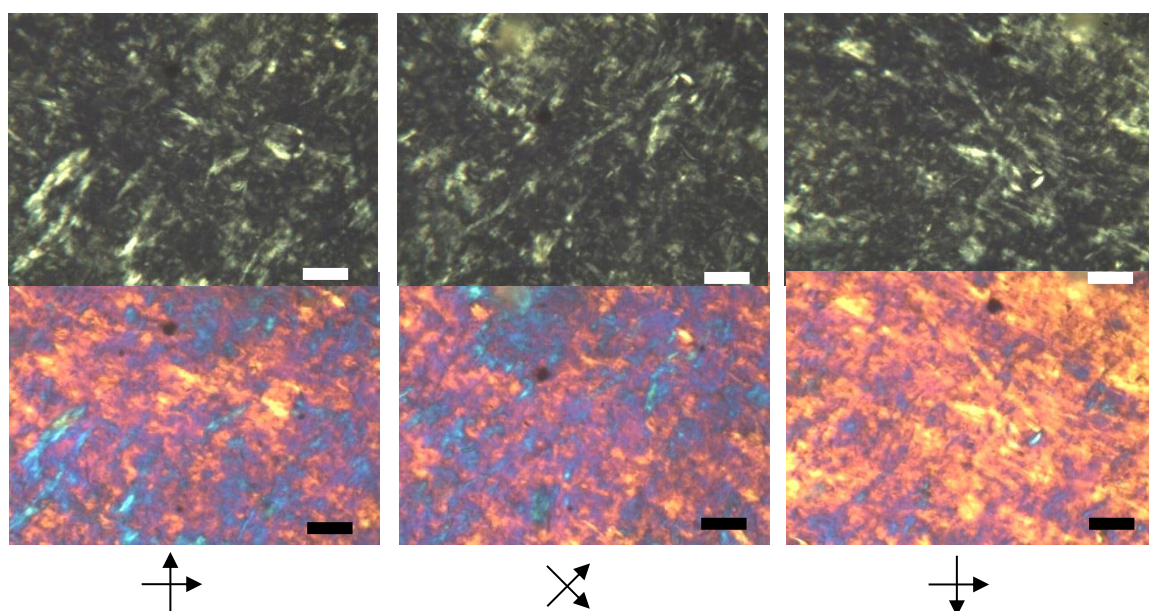


Figure 5.21: POM images of BC nanowhisker suspension between placed between a glass slide and a cover slip viewed cross polars without (top images) and with a 530 nm retardation plate (bottom images) (bar 100 μm) with sample rotated between crossed polars by 45° twice

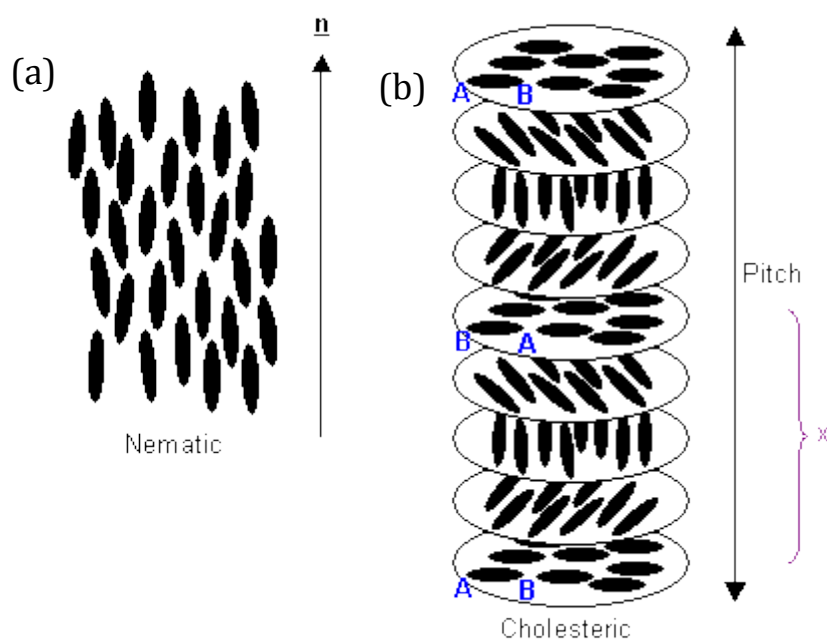


Figure 5.22: Schematic representation of a) nematic and b) chiral nematic or cholesteric phase, A and B are two ends of rod-like entity and x is half the pitch [W4]

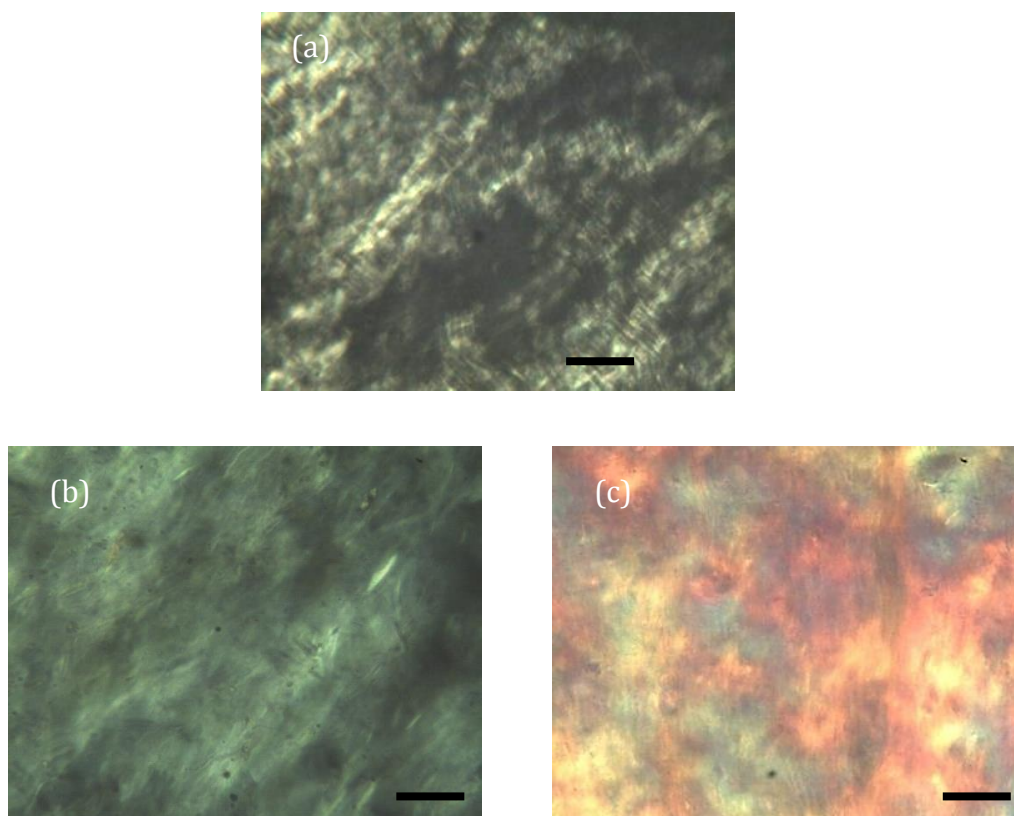


Figure 5.23: POM images of a) NdC, b) TC and c) BC nanowhisker suspension filled in glass capillary (bar represents 50 μm) (image C is taken with tint plate inserted for better contrast)

5.5 Chiral Interaction

The formation of the chiral nematic phase indicates the presence of a chiral interaction between cellulose nanowhiskers. Almost all the work in the literature on the formation of liquid crystalline phase from cellulose and cellulose derivatives has shown formation of a chiral nematic phase. Considering the chiral character of cellulose molecules which has five chiral carbons, chiral interactions between chains of cellulose and cellulose derivatives are intuitive. The chiral carbon at the glycosidic linkage leads to the helical conformation of cellulose chains which has been proven by nuclear magnetic resonance (NMR) and induced circular dichroism (ICD) [195, 196] and ultimately also results into a chiral nematic phase.

The cellulose nanowhiskers also form a chiral nematic or cholesteric phase, as shown in the previous section, and is consistent with the literature on the formation of a chiral nematic liquid crystalline phase from cellulose nanowhiskers obtained from various sources of cellulose [134, 143, 145, 180, 203]. Energetically, there is no difference between a chiral nematic and a nematic phase, although a chiral nematic configuration is preferred when the liquid crystal entity is chiral or is under the influence of a chiral entity. In such a case, the liquid crystals behave like two screws with the same handedness, which do not align, when stacked, but are rotated with respect to each other.

The origin of chiral interactions is not well understood in cellulose nanowhiskers but various reasons such as actual morphological twist [144], twist induced due to anisotropic dispersion energy, and dielectric properties of the medium [134] have been proposed to contribute to the chirality of cellulose macromolecules. Therefore, the two relevant questions with respect to the formation of chiral nematic phase concern

1. Origin of chirality
2. Chiral interaction

In this section, the above-mentioned questions are addressed on basis of some experimental evidences and inferences obtained from the current work. Following pieces of evidence of the twisted morphology of cellulose microfibrils and nanowhiskers has been found in the present work:

a) Microfibrils

In the un-hydrolysed BC, at some instances microfibrils exhibited twist every 250-300 nm, as shown in Figure 5.24 (a-b). This twist repeat distance is less than the average length of nanowhiskers. This implies that the nanowhiskers, which are smaller in length than this repeat unit, may not possess a twist and thus might not efficiently participate in the chiral interaction. Hence, shorter the nanowhiskers, lesser would be the possibility of their participation in chiral interaction and rarer would be the formation of a chiral nematic phase. This was in-fact observed in the current study, although further investigation is warranted to establish this concept. The BC nanowhiskers, which are longer than other, demonstrated a chiral nematic phase more frequently than the NdC nanowhiskers. The twisted morphology results into intertwining and coiling of microfibrils, as shown in Figure 5.24 (c).

b) Nanowhiskers

The curling of cellulose nanowhiskers is evident from the HRSEM image shown in Figure 5.24 (d) and also indicated in AFM images shown in Figure 5.25. It can be observed that where two nanowhiskers are in contact (encircled in the image) the nanowhiskers do not lay edge on but are twisted with each other, leading to a small area of contact. The AFM image of nanowhiskers, obtained from hydrolysis of BC modified by a dye Calcofluor, which is known to interfere with the assembly of poly-glucan chain, is shown in Figure 5.26 (a). The image shows the twisted nature of nanowhiskers. When a nanowhisiker was scanned along the length, a regular pattern in the variation in the height profile of a nanowhisiker was observed implying a regular twist at about 300 nm (Figure 5.26 (b)). Coiling of longer nanowhiskers and absence of edge-edge contact between nanowhiskers are further implications of twisting of nanowhiskers.

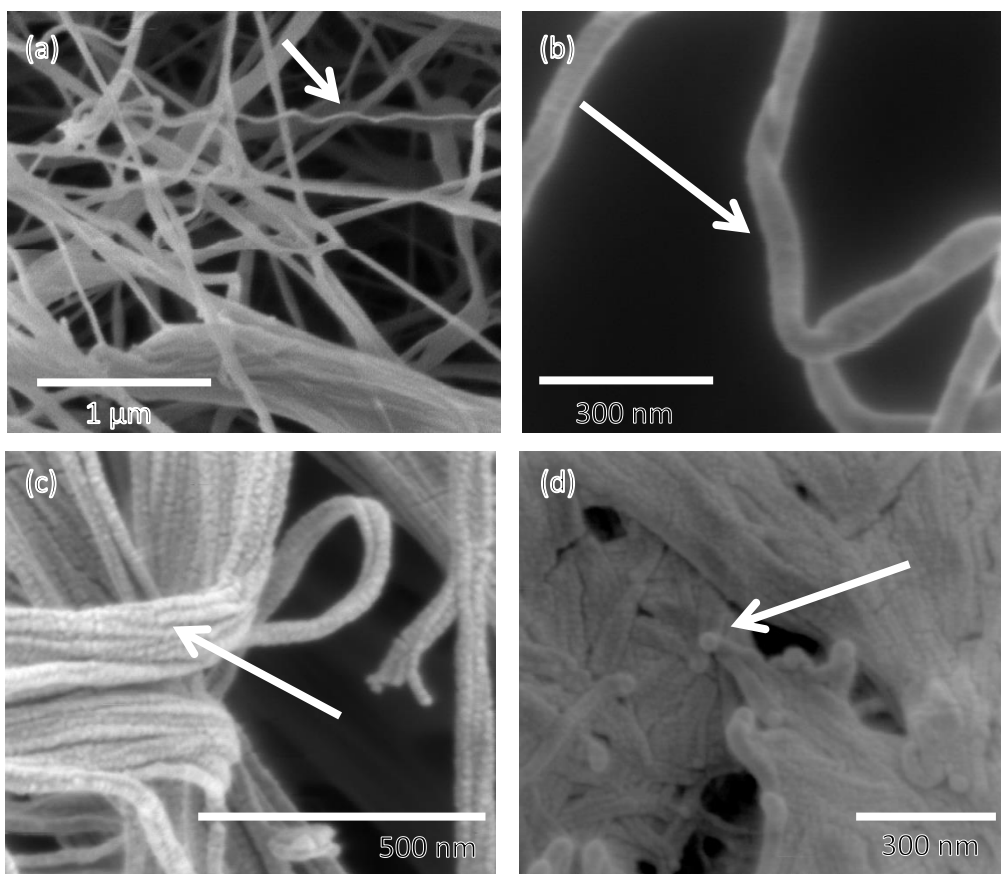


Figure 5.24: SEM images showing twists in (a-c) microfibrils and d) nanowhiskers (Arrows indicate the twisted nature of microfibrils and nanowhiskers)

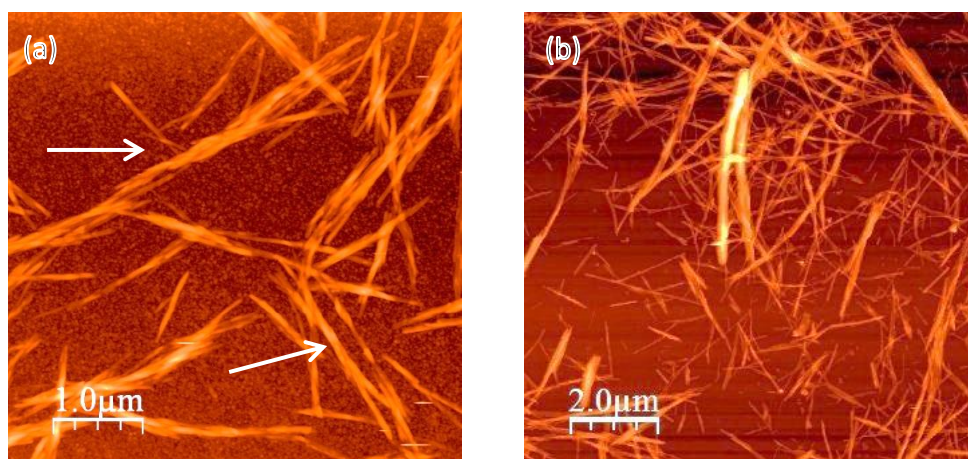


Figure 5.25: AFM image of nanowhiskers showing twists and bundling with arrows

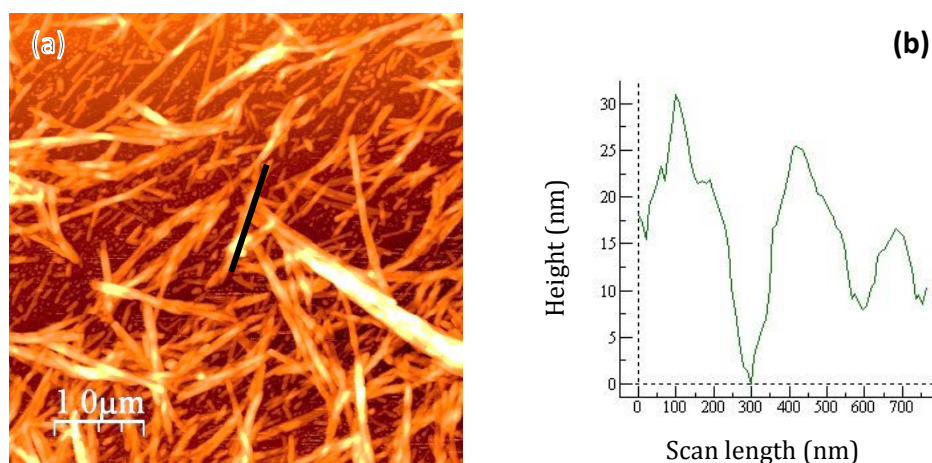


Figure 5.26: a) AFM image of BC nanowhiskers showing helical intertwining b) Height of nanowhisiker along the scan indicated in (a)

If the nanowhiskers are twisted, considering cellulose nanowhiskers may be large crystals and a twisted conformation might not be beneficial to the overall energetics of the system. Because of this the concept of critical size of the twisted microfibrils was introduced in section 4.2.3. However, it is important to understand the underlying mechanism for formation of a twist. The twisted morphology of cellulose nanowhiskers or microfibrils is believed to be a manifestation of the chirality of cellulose chains [125, 194, 197, 201]. The transfer of chirality at various length scales have been studied in the literature, some of which are summarised here and illustrated in Figure 5.27. The Figure 5.27 (a) indicates the helical nature of cellulose chains has been supported by NMR, ICD and by the formation of chiral nematic phase by cellulose derivatives [195, 196]. The basic cellulose protofibrils, shown in Figure 5.27 (b, c), which is believed to comprise of 36 chains, has been computationally modelled in various force field and the development of twist has been observed [197, 198]. The direct observation of bacterial cellulose [191], and algal cellulose [120] (Figure 5.27 (d)) microfibrils and ribbon formation has also revealed twisted morphology. The twists observed in the microfibrils and ribbons are consistent with our observations in SEM and AFM. However, in the present work, the observed twisting was found to be irregular and also the formation of the non-uniform chiral nematic phase was observed.

It is interesting to observe that the transfer of the chiral nature of cellulose chains is also revealed when thin wet strips of newspaper are hung with a paper clip on

one end to dry, the strip develops a twist, right handed or left ended depending on the direction of the newspaper cutting (Figure 5.27(f)). Further, it can be seen that most of the trees, which are further up in hierarchical organisation of cellulose chains, are also twisted (Figure 5.27 (g)). These examples do not prove but support the argument that the twisted nature of cellulose fibrils and nanowhiskers lead to the formation of chiral nematic phase.

An alternative concept that might lead to a chiral interaction in cellulose nanowhiskers is that of a chiral surface. The cellulose crystallites are monoclinic or triclinic [54]. In either case, an arrangement of cellulose chains in such a lattice would imply dissimilar surfaces. Thus, the structures are non-superimposable. The concept of chiral surfaces has never been applied to explain the chiral interactions in cellulose crystallites. Figure 5.28 shows the various possible faces of crystallites along the length of the microfibrils. When two nanowhiskers with different faces come in contact they might reorient in order to minimise energy by rotating or twisting with respect to each other, similar to that seen in Figure 5.24 (d). As a result even the surface charges would be distributed in a way that the mirror image of the charges cellulose surface would be chiral. However, validation of this concept needs further work.

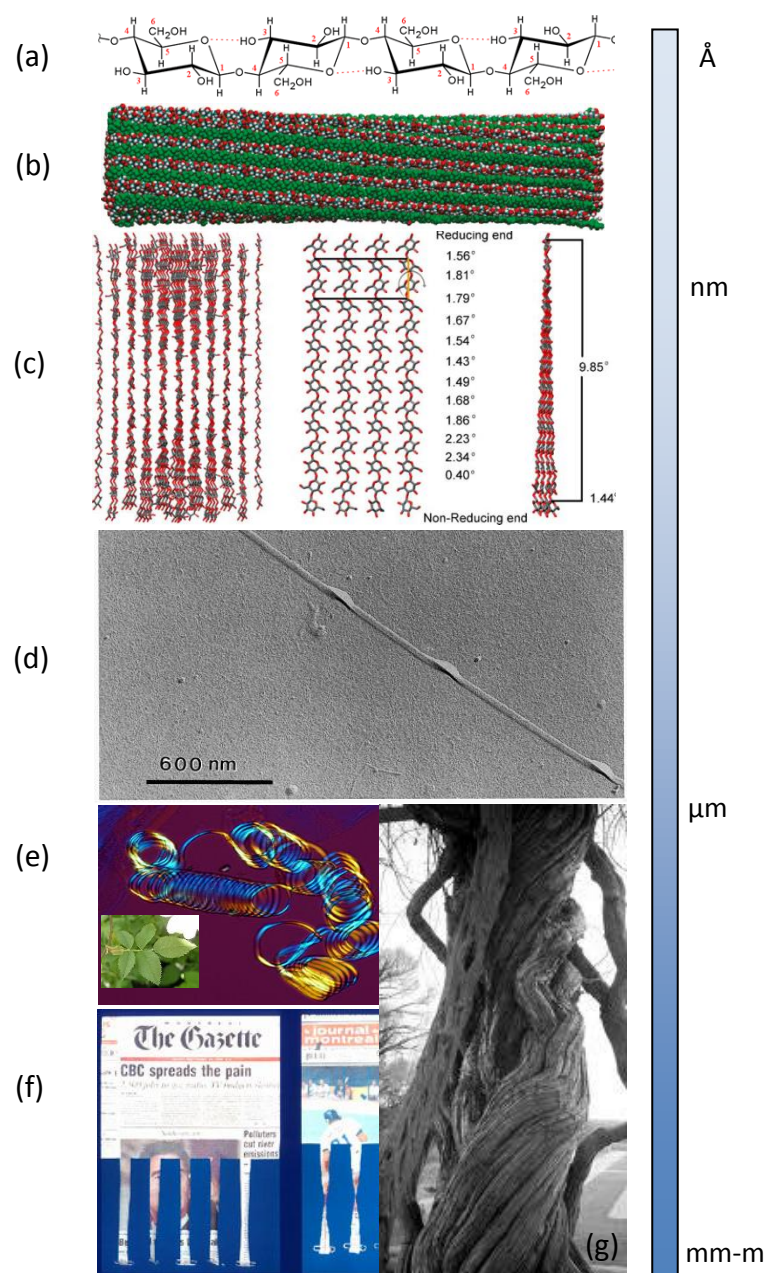


Figure 5.27: Transfer of chirality from cellulose chains to trees a) Twisted configuration of cellulose chains b) A 59 chain cellulose nanofibril simulated for 140 ns at 310 K demonstrating the stable twisted morphology, carried out by the GROMACS software and OPLS force field [197] c) Trajectory average of a 36 chain cellulose nanowhisker, with a section of the central plane of the crystal seen from the above and the side, illustrating the twist, simulated using CHARMM molecular mechanics program [198] d) TEM image of *Micrasteras denticulate* microfibril showing 4 right handed 180° twist [120] e) Helical twisting in cellulose fibril extracted from petiole of a leaf [W5] f) Strips of newspaper wetted with water develops helical twist of drying [W5] g) Photograph of a tree showing a twist [W6]

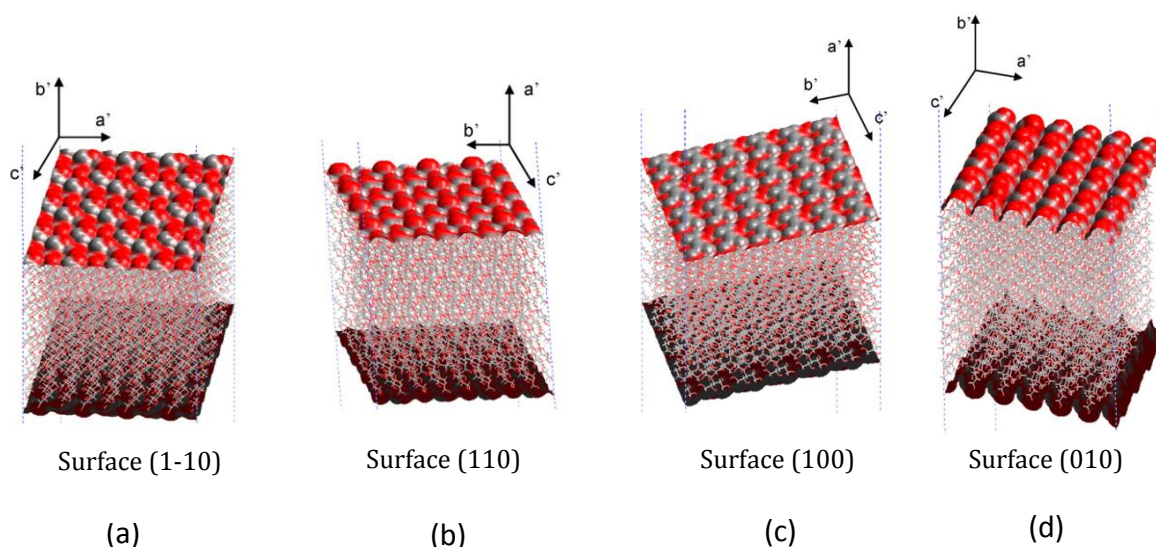


Figure 5.28: Molecular models of four surfaces of the I β allomorph. In each case, c' is the chain axis [237]

We have reported the formation of a chiral nematic phase at a very low concentration as compared to those reported in the literature (Table 5.1) [156]. However, the amount of chiral nematic phase was non-uniformly formed. If we consider the dilute concentration, the average separation between two nanowhiskers would be large producing a challenging distance barrier to generate chiral interaction. One of the ways the two chiral nanowhiskers can communicate is by randomly formed points of contact. Given the dependence of possibility of contact on concentration and length of nanowhiskers, the interaction should increase with concentration and nanowhisker length. As the concentration increases, the chiral nematic phase becomes more widespread. However, there is no direct evidence for existence of such physical contact and its role in manifestation of chiral interactions. The chiral nematic phase is more readily formed by bacterial cellulose nanowhiskers, which are longer than the other sources, which supports the point of contact hypothesis. However, further work is warranted in order to support this hypothesis.

5.6 Summary

All the celluloses (NdC, TC and BC) were hydrolysed with sulphuric acid to produce rigid rod-like cellulose nanowhisker. The overall crystallinity was found to be higher for cellulose nanowhisker as compared to the un-hydrolysed cellulose. This is attributed to the removal of non-crystalline parts of microfibrils by acid. Nanowhiskers with a wide

distribution of aspect ratio were obtained, which varied with the source. The BC nanowhiskers were found to be largest while the nanowhiskers obtained from the hydrolysis of NdC were the smallest.

The cellulose nanowhiskers formed a lyotropic liquid crystalline phase in water. The transition from an isotropic to a liquid crystalline phase was obtained via a biphasic concentration range. The transition was studied by phase separation into denser anisotropic phase and upper clear isotropic phase, as well as, by polarised optical microscopy by which anisotropic birefringent region was seen. Both the techniques revealed different transition concentrations. This was because the formation microscopic liquid crystalline micelles inhibited phase separation into isotropic and liquid crystalline phase. Thus, centrifugation was required to aid phase separation. The onset of transition started at around 0.05 wt% and completion of transition was observed to be earliest for BC nanowhiskers at 4 wt%, followed by TC nanowhiskers and NdC nanowhiskers at 8-10 wt% and 11-12 wt%, respectively. The transition from an isotropic to a liquid crystalline phase was found to depend on the distribution of the aspect ratio. The BC nanowhiskers which possessed the nanowhiskers with the largest aspect ratio underwent transition at the lowest concentration.

The formation of nematic as well as chiral nematic phase was observed, depending on the sample preparation. The origin of the chiral interactions between the cellulose nanowhiskers has been attributed to the twisted morphology of nanowhiskers and microfibrils. The various pieces of evidence have been presented including high resolution SEM images and AFM images showing twisted morphology of cellulose nanowhisiker as well as microfibrils. The concept of twist originating from chiral surface of the crystalline surface of the nanowhiskers has also been introduced. It has also been proposed that the chiral interactions between the nanowhiskers originate from their points of contact. However, further work is required in order to validate these hypotheses.

Chapter 6

Fibres from liquid crystalline cellulose suspension

6.1 Introduction

Cellulose fibres are used in a wide variety of application such as textiles, tire cords, ropes and dialysis membrane. The various common cellulose fibres production strategies include viscose, cuprammonium, and fortisan [5]. All of these processes involve cellulose dissolution and regeneration using heavy chemical treatment, leading to problems such as uncontrolled thermal instability of the involved complexes and irreversible environment degradations. In these processes, the native cellulose I crystal structure changes to cellulose II crystal structure and cellulose II is inferior in mechanical properties to cellulose I [6] (discussed in section 2.4). Moreover, the current cellulose requirements are fulfilled by plants and trees, which lead to deforestation.

Thus, the objective of this chapter is to present a novel method of making cellulose fibres, which can address the above-mentioned issues. During this work, bacterial cellulose and tunicate cellulose was used as the cellulose source to make fibres. The production scalability of bacterial cellulose is testified by the commercial availability of nata-de-coco (bacterial cellulose made from fermentation of coconut milk) as a food ingredient and bacterial cellulose produced by various laboratories [39].

In chapter 5, it has been shown that on acid hydrolysis, bacterial cellulose, nata-de-coco and tunicate cellulose produced nanowhiskers are capable of forming a liquid crystalline phase. The transition was found to occur at a lower concentration than those reported in literature for plant based cellulose nanowhiskey, owing to higher aspect ratio (section 5.3). The liquid crystalline solutions have been found to be particularly useful in producing high performance fibres. The spinning from a liquid crystalline phase allows transfer of the attractive properties possessed by the liquid crystal entities at nano-scale to anisotropic macroscopic products. This advantage is evident in both natural fibres such as silk as well as man-made fibres such as Kevlar. However, there are only a few reports on cellulose fibres production involving a liquid crystalline phase. One of these involved dissolving cellulose in ortho-phosphoric acid to form a liquid

crystalline solution and followed by cellulose regeneration [6]. Enhanced orientation was observed and improvement in properties was seen. Since this process involved regeneration of cellulose, the process resulted into fibres composed of cellulose II crystal fibre.

In this chapter, it is described how the cellulose fibres were made using the liquid crystalline suspension of nanowhiskers obtained from bacterial cellulose (BC), nata-de-coco (NdC) and tunicate cellulose (TC) nanowhiskers as the spinning dope. First of all, the rheological measurements have been performed to measure viscosity, storage and loss modulus at various concentrations of cellulose nanowhiskers, which in turn affects the spinnability. A brief account of optimisation of the spinning process has been discussed. A preliminary characterisation of mechanical properties, orientation and internal and external structure of cellulose fibre produced by the novel route has been reported in the later part of this chapter.

6.2 Characterisation of the spinning dope

All the celluloses studied in this work, NdC, BC and TC, owing to the entanglement of microfibrils in their native state, failed to dissolve or disperse in general solvents (Appendix B). Thus, the celluloses were hydrolysed to produce nanowhiskers, which formed stable aqueous suspensions. In the following sections, the rheological characteristics of as-received hydrated cellulose (before hydrolysis) and nanowhisiker suspensions have been discussed. The isotropic-liquid crystalline phase behaviour of these suspensions, studied in detail in chapter 5, has also been briefly summarised

6.2.1 Rheological behaviour of suspensions

(a) Un-hydrolysed cellulose and cellulose nanowhisiker suspensions

The rheological measurements were performed on the as-received hydrated NdC sheets and the NdC nanowhisiker suspensions (10 wt%) to measure viscosity (η), storage or elastic (G') and loss or viscous modulus (G''). The rheological characterisation comprised of strain sweep and frequency sweep, which involved measurement of viscosity (η), G' and G'' with increase in strain (Figure 6.1) and frequency (Figure 6.2) in the linear viscoelastic region at constant strain (determined from the strain sweep), respectively.

It is seen from the strain sweep that the values of G' , G'' (Figure 6.1 (a)) and viscosity (Figure 6.1 (b)) decreased with increase in strain. The measured values of G' were found to be higher than the G'' values for both, the un-hydrolysed NdC and the nanowhisiker suspension. However, the G' exceeds G'' by much larger extent in case of as-received hydrated (un-hydrolysed) NdC in comparison to the NdC nanowhisiker suspensions and converges at a higher strain than in the case of nanowhisiker suspension as can be seen in the Figure 6.1 (a). The dominance of G' over G'' in both the cases, indicates a gel-like behaviour. The gel-like behaviour of suspensions was also evident by the observations presented in section 5.3, where its gel-like behaviour prevented phase separation (section 5.3). The decrease in viscosity with increase in strain is related to the breakage of the network, leading to the free movement of microfibrils or nanowhisikers. At 10% strain the viscosity of the gel was about 1000 Pa.s and that of suspension was close to 100 Pa.s. This is despite of the fact that the solid fraction in suspension is 10 wt% and that in NdC sheets is less than 2 wt%.

Similar observations can be made from the frequency sweep measurement shown in Figure 6.2. The viscosity of the suspension (10 wt%) is much lower than that of the hydrogel. Initially the viscosity of NdC sheet was above 100000 Pa.s which decreased to about 1000 Pa.s, while in case of suspensions, the viscosity decreased from about 300 Pa.s to below 10 Pa.s, as shown in Figure 6.2 (a). The values of both the modulus (G' and G'') are higher for sheets than that for suspension (Figure 6.2 (b)).

All the above mentioned observations imply that the sheets have a higher resistance to shear deformation and a stronger gel-like behaviour. The NdC sheets are composed of long, almost endless cellulose microfibrils which are densely entangled and aggregated (SEM images in section 4.2), as compared to nanowhisiker suspension where the nanowhisikers are much shorter in length, and thus minimising the entanglement (section 5.2). This explains the insolubility and in-spinnability of the un-hydrolysed NdC sheets. Such a behaviour is common for polymers, owing to entanglement resulting into the overall high resilience of the structure.

The results have been presented only for NdC but invariably holds true for TC and BC. It is worth mentioning that the fibre suspensions generally have remarkably higher viscosity and elasticity than spherical particle suspensions of equal volume

concentration [238]. The rheological parameters vary with concentration and the trends obtained in case of cellulose nanowhiskers are presented in the next section.

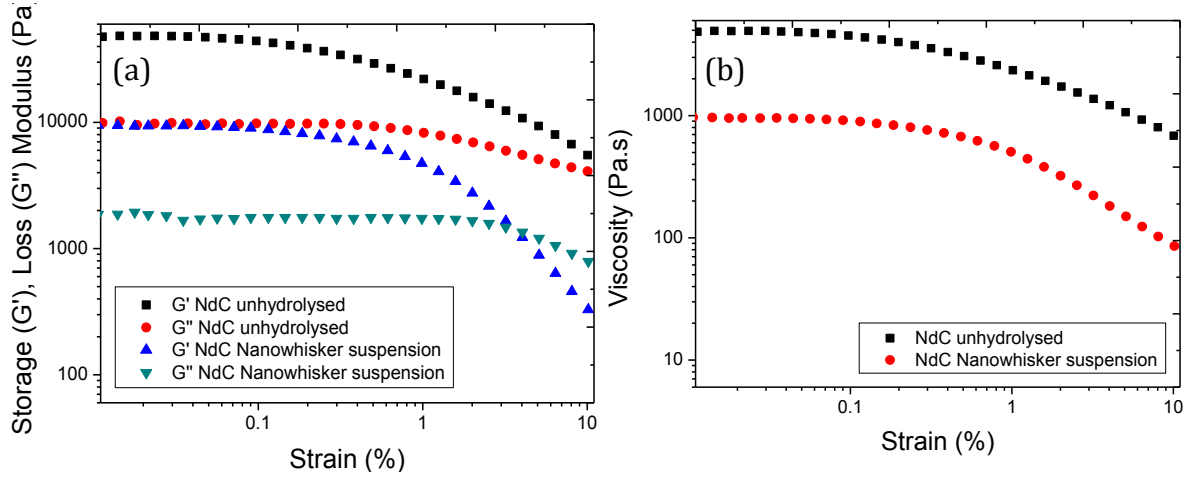


Figure 6.1: Variation in a) viscosity with strain rate b) elastic and loss modulus with strain for NdC before hydrolysis and 10 wt% nanowhiskers suspensions

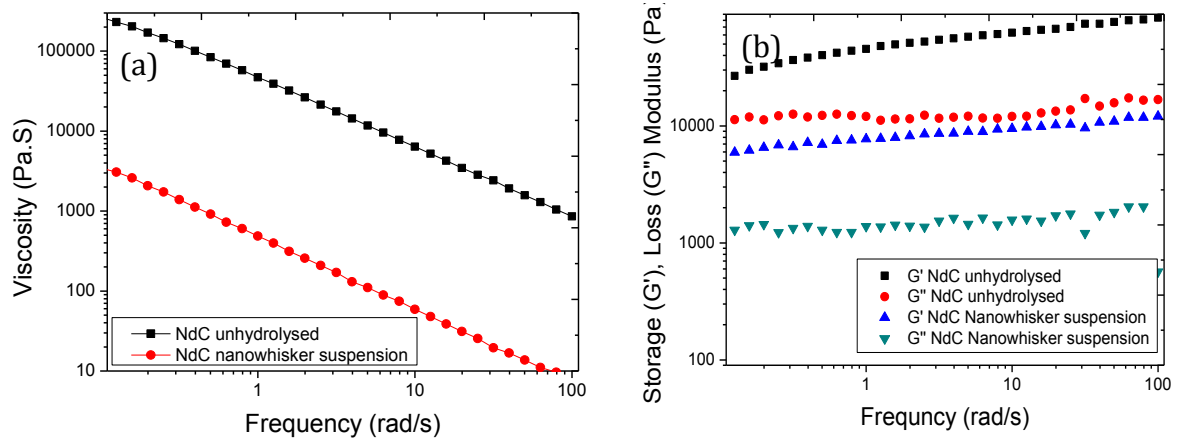


Figure 6.2: Variation in a) viscosity with frequency at 0.03% strain b) storage and loss modulus with frequency at 0.03% strain for NdC before hydrolysis and 10 wt% nanowhiskers suspensions

b) Variation with concentration

Figures 6.3 (a, b) shows the variation of viscosity, G' and G'' of BC nanowhiskers suspensions (5 wt% and 10 wt%) with frequency in linear viscoelastic region. Both, G' and G'' are higher for 10 wt % as compared to the values for 5 wt%, as would be expected owing to higher nanowhisiker content, forming a better network. Similar

observation can be made for the viscosity values, which decreased from 10000 Pa.s to about 300 Pa.s for 10 wt% and from 2000 Pa.s to below 100 Pa.s for 5 wt%.

This trend is consistent for NdC and TC and the relevant data are shown in Appendix C. As the viscosity was found to increase with increase in concentration, a concentration suitable for spinning can be obtained. The spinnable concentration range is investigated in the next section. However, suspensions from all of these different sources would have their own unique spinnable concentrations, according to their rheological behaviour. The rheological behaviour of suspensions from all the sources used in this work is compared in the next section.

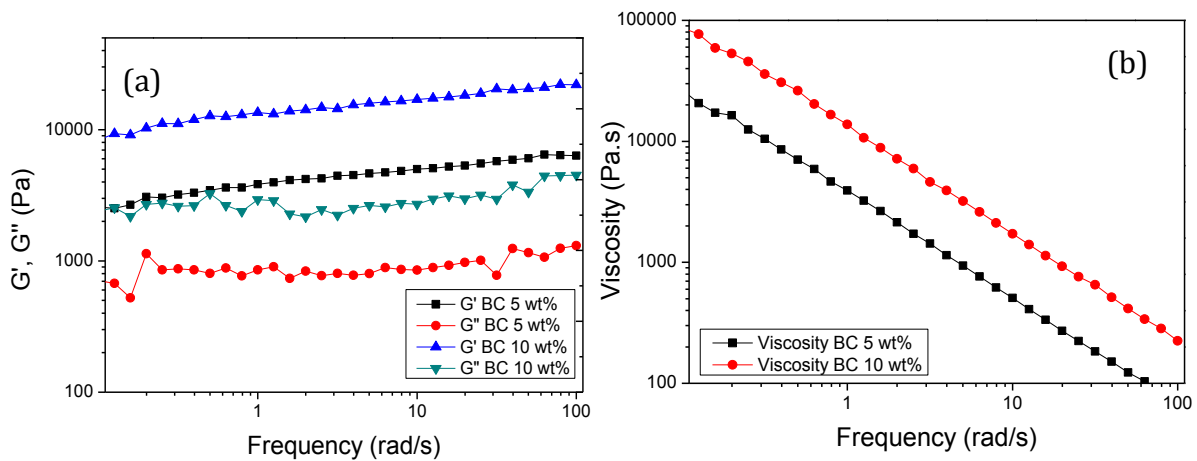


Figure 6.3: Variation in a) viscosity with frequency at 0.03% strain b) Storage and loss modulus with frequency at 0.03% strain for 5 wt% and 10wt% BC nanowhiskers suspensions

c) Variation with cellulose sources

Figures 6.4 and 6.5 shows a comparison of the rheological parameters for 5 wt% nanowhiskers suspensions obtained from BC, NdC and TC. The NdC and TC nanowhiskers suspensions have similar viscosity and lower than that for the BC suspensions. The shear thinning starts at a lower strain for TC and BC nanowhiskers suspensions as compared to the NdC nanowhiskers suspensions. The G' and G'' are higher for BC suspensions than for NdC and TC suspensions.

As explained before the frequency sweep demonstrates the decrease in viscosity with increase in frequency due to breaking of the network. The viscosity decay followed similar behaviour for all the suspensions. The viscosity of BC suspensions was about 5-10 times higher than the viscosities exhibited by NdC and TC nanowhisker suspension. The values of G' and G'' were higher for BC as compared to those for NdC and TC at all frequencies, implying a higher resistance to deformation.

These investigations reveal that the viscosity and moduli are higher for BC nanowhisker suspension. This is because the BC nanowhiskers are longer than TC and NdC nanowhiskers, as shown by the AFM data in the section 5.2.3, which causes more interaction between nanowhiskers and thus higher viscosity. Similar effect of aspect ratio has been reported by Liu [239] and Tatsumi [238].

Similar rheological parameters were observed for NdC and TC nanowhisker suspensions. The isotropic to liquid crystalline phase transition concentrations were found to be similar. This shows, in spite of different aspect ratio distribution, NdC and TC nanowhisker suspensions exhibit similar bulk properties.

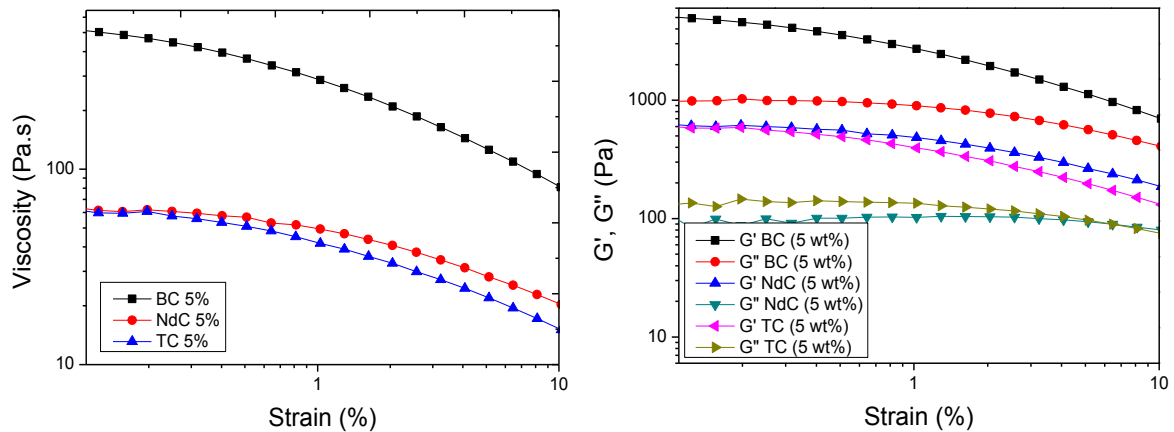


Figure 6.4: Variation in a) viscosity and b) storage (G') and loss (G'') modulus with strain for 5 wt% BC, NdC and TC nanowhiskers suspensions

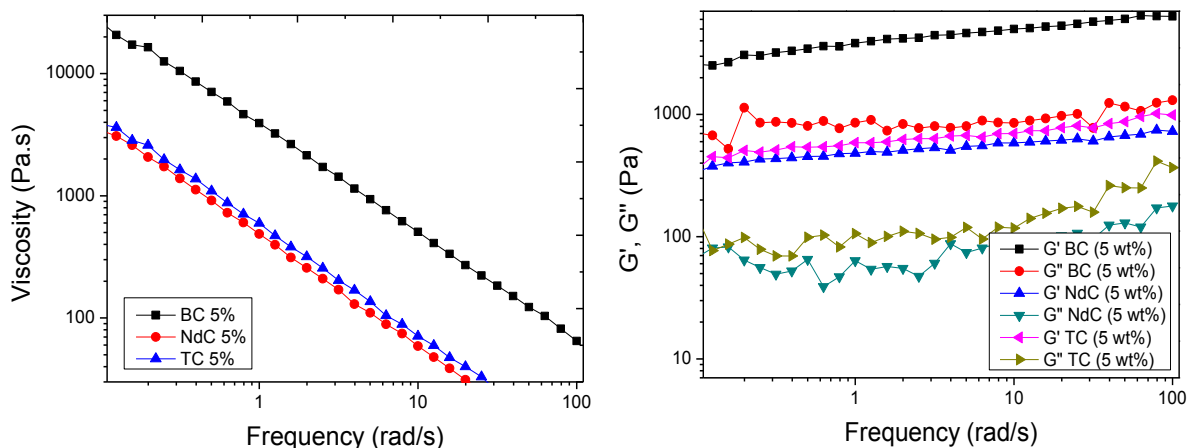


Figure 6.5: Variation in a) viscosity and b) storage (G') and loss (G'') modulus with frequency for 5 wt% BC, NdC and TC nanowhiskers suspensions

6.2.2 Liquid crystallinity

It was shown in section 5.3 that the suspensions of the nanowhiskers obtained from the three sources undergo transition from isotropic to liquid crystalline phase via a biphasic region which starts at about 0.5 wt% and ends at 4 wt%, 8-10 wt% and 11-12 wt% for BC, TC and NdC, respectively. This implies that the nanowhiskers are locally aligned and result into a poly-domain structure. The transition from isotropic to liquid crystalline phase is dependent on the aspect ratio, poly-dispersity and the surface charge of the nanowhiskers. The nanowhiskers with smaller aspect ratio undergo the transition to liquid crystalline phase at a higher concentration, in comparison to the ones with higher aspect ratio. Therefore the NdC nanowhiskers suspensions with aspect ratios from below 50 to just over 100 undergo transition at 12 wt% in comparison to the BC nanowhiskers suspension at 4 wt% with aspect ratios of below 50 to much over 150. The poly-dispersity contributes to the width of the biphasic concentration range between the onset and completion of the transition as is observed for all suspensions. A completely liquid crystalline phase ensures local alignment, but results into a microstructure composed of domains with different directors.

Apart from being responsible for the different transition concentrations, the aspect ratio of the nanowhiskers also determines the size of the locally ordered domains. Intuitively, larger domains will be formed if the aspect ratio is larger, given the flexibility and stiffness are constant. Thus, the domain size formed by BC

nanowhiskers is larger than those formed by the TC and NdC nanowhiskers, as has been discussed before in section 5.4. As the domains are present in various directions, an external force is needed to orient all the directors in the same direction. This is required in order to extract full advantage of spinning from liquid crystalline phase.

6.3 Spinnability

Different researchers have used various definitions of spinnability. However, in this work, spinnability has been considered as a property of combination of suspension properties (concentration, viscoelastic properties) and processing conditions (extrusion diameter, speed of extruding, speed of winding, length of air gap, temperature, humidity) under which long filaments (>1 m for reference) can be extruded uniformly and continuously. However, the fibre properties are dependent to an equally large extent on post spinning factors such as coagulation, temperature of drying and so on.

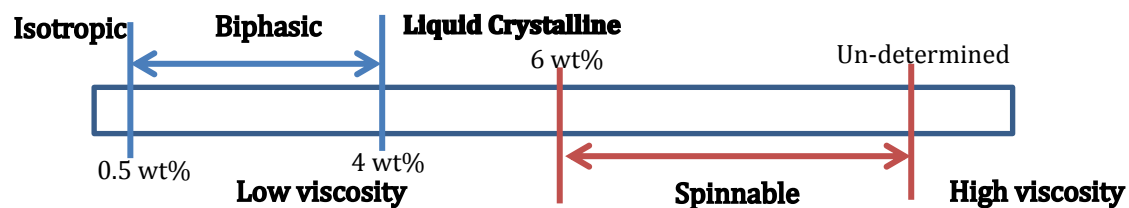
The spinning setup used in this work is briefly recapitulated here and discussed in detail in section 3.3.3. A 5 mL syringe was used to extrude filaments through a 22 G needle, through an air gap of 5 cm, on a conveyer belt whose speed could be controlled used a voltage supplier. Thus, the process was a dry spinning process with no post spinning treatment. The work was done only to obtain preliminary parameters for fibre productions and prove the advantages of using a liquid crystalline dope for spinning.

A suspension is spinnable if a continuous filament can be made. This requires a consistency in terms of viscosity and homogeneity of the suspension. A range of concentrations were investigated for spinnability and a suitable concentration range was determined. The NdC nanowhisiker suspensions are spinnable above 10 wt%. The TC nanowhisiker suspension failed to form a continuous filament at any concentration for TC, due to inhomogeneity. BC suspension achieved sufficient viscosity for spinning at a low concentration of about 6 wt%. The upper limit of spinnable concentration was not determined. The spinnability was reported at a lower concentration in this work than those reported in literature for liquid crystalline suspensions of cellulose chains [6], but higher than that for nanofibrillated suspension of tunicate cellulose [240] and wood pulp [241] made by TEMPO mediated oxidation. The later can be explained on the basis lower aspect ratio of nanowhiskers in this work as compared to the aspect ratio of nanofibrillated cellulose in the literature.

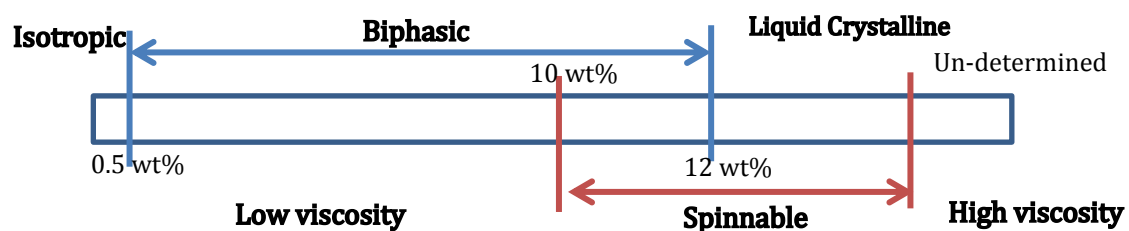
Below the concentrations mentioned as the lower limit of the spinnable concentration range, blobs are formed and the suspension fails to form a continuous fibre. On the other hand, if the concentration and hence the viscosity is too high, the extrusion becomes difficult, as the needle hole is blocked frequently. The spinnable concentrations for NdC and BC with respect to the isotropic to liquid crystalline phase transition are schematically represented in Figure 6.6.

For the sake of simplicity and comparison, same concentration was chosen for all the suspensions. The combinations of spinning and winding speeds were determined. The winding speed ranges were determined for three extrusion flow rates 20, 40 and 60 mL/hour which corresponded to the speeds of 0.05, 0.09 and 0.14 m/s for a 22 G needle and 11 mm diameter BD syringe. The voltage was varied to manipulate the speed of the conveyer belt. First, the speed of the conveyer belt was determined by measuring time taken to complete one rotation that is one belt length. Initially lower speed is required to form a continuous filament. Once continuity is established, the speed of winding can be increased. At too low winding speed, the winding cannot cope up with spinning volume input and thus forms wavy filament. Above upper limit of winding speed, the pull cannot be handled by the inherent viscoelasticity of the suspension at that concentration and filaments of very short length are formed. Thus, for a given concentration and speed, there is a range of winding speeds, depending on the stretchability of the suspension. For 20, 40 and 60 mL/hour, the suitable winding speeds were found to be 0.025-0.045, 0.055-0.075 and 0.085-0.15 m/s (Figure 6.7). These values of winding speed are lower than the extrusion speed except that for 60 mL/hour.

The difference between the winding speed and the spinning speed determines the amount of extensional forces applied on the extruded filament. The filaments made out of the cellulose nanowhisiker suspensions, in this work, could not bear any degree of extensional force under the given setup. Since the extensional force is important in order to produce orientation in fibres, the inability to apply extensional force would limit the orientation in the fibre.



Concentration of suspension (Weight %)



Concentration of suspension (Weight %)

Figure 6.6: Schematic showing transition concentrations and relative position of the spinnable concentration for BC and NdC

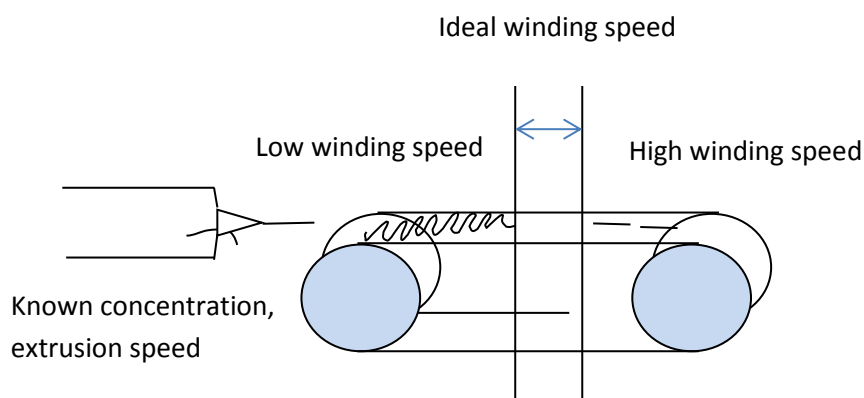


Figure 6.7: Schematic representation of product of various combinations of spinning and winding speeds in fibre formation

6.4 Characterisation of fibres from various cellulose sources

Development of fibre technology has witnessed many high performance fibre making processes out of which spinning from a liquid crystalline suspension is one of the most promising one. The spinning from a liquid crystalline suspension has benefited natural fibre silk [12], the man-made fibres Kevlar [242], and also has shown to be beneficial in field of regenerated cellulose fibres [243, 244]. The properties of fibres made from the liquid crystalline suspensions obtained in this work are reported in the following section. For the sake of comparison, 10 wt% suspensions were used for all materials. All fibres were spun at 40 mL/h and wound at 0.6 m/s.

6.4.1 Optical Microscopy

Figure 6.8 shows the just-spun fibres before drying and after drying between crossed polars with a full wavelength retardation plate. The fibres possess a poly domain structure, but are more oriented than the poly-domain suspensions before spinning.

The optical micrographs of NdC and BC fibres are shown in Figure 6.9. It is seen that the suspensions produced fibres with almost uniform diameter. Considering the material was extracted naturally and not in synthetic way from well controlled low molecular weight petrochemical derivatives, the macroscopic quality of fibre remarkable.

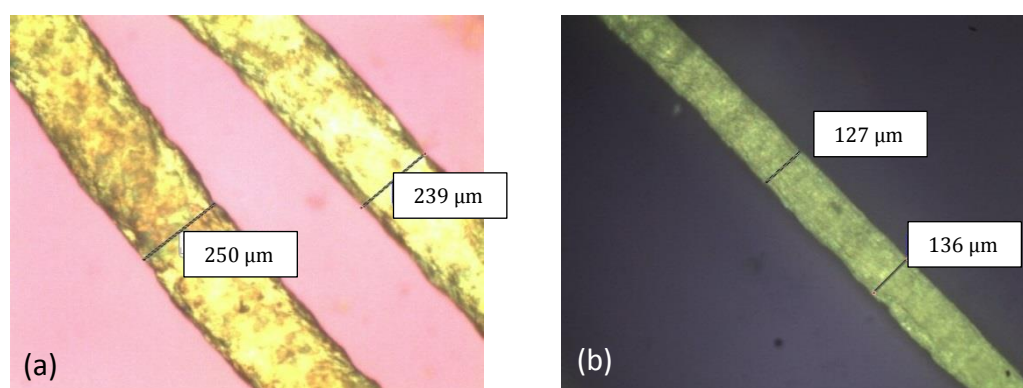


Figure 6.8 Optical micrograph of fibres between crossed polar a) as spun (before drying) and b) after drying

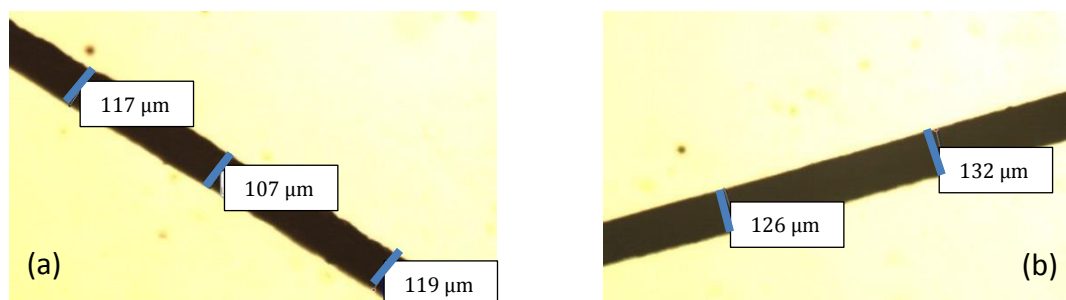


Figure 6.9 Optical micrograph showing a) BC and b) NdC fibres with measured values of diameter at different locations

The diameter of fibres produced from the two cellulose suspensions has a large scatter. The scatter in the diameter measurement can be related to the variation in the drying conditions (as a result of surrounding humidity and temperature) and variation between suspension batches. The average diameter of cellulose fibres made from NdC and BC nanowhisiker suspensions (10 wt%) are 111 ± 6 and 137 ± 11 μm , respectively.

The difference in diameter may be because of various reasons such as difference in interaction between nanowhisikers of different aspect ratios, different amount of porosity and so on. In comparison to the diameter of the needle used to spin fibre, the fibre diameters are smaller by 50%. The concentration used is 10% which implies about 7% volume and thus in ideal 100% packing should shrink the fibre to 7% of the needle diameter. However that is obviously not the case here. The difference from the expected is due to the trapped air causing porosity and thus increased volume. This may be due to insufficient coagulation or difference in coagulation speed between the interior and exterior of the fibre.

6.4.2 Mechanical properties

The tensile properties were measured for BC and NdC fibres in order to assess the mechanical properties. A set of tensile test measurements are shown in Figure 6.10 and Figure 6.11 for BC and NdC fibres, respectively, where strain was measured for applied stress. The curves reflect the typical stress-strain behaviour for a fibre put under tensile stress. A wide variation was observed in the tensile properties for both the samples. The tensile strength for the BC fibres was about 10.6 ± 3.3 MPa and NdC fibre was about 9.9 ± 6.4 MPa. The elongation was found to be 1.5 ± 0.5 % and 1.05 ± 0.5 % for BC and NdC fibres, respectively. The Young's modulus was measured from the initial elastic part of

the curves. The modulus values were calculated to be 1.5 ± 0.4 GPa and 2.4 ± 0.9 GPa for BC and NdC fibres.

Fibres from both the sources, considering the wide variation, exhibited similar tensile properties. The average tensile strength, elongation and modulus are slightly higher for BC fibres as compared to NdC fibres. However, these values are lower than any of the reported measurements for commercial [245, 246] or laboratory fibres [240, 247]. Tensile strength and other parameters are dependent on intrinsic properties of the element making the fibre (cellulose nanowhiskers in this case), orientation and the fracture mechanism. It is also worth mentioning that according to the observation made in the previous section, the fibres made in this work possessed high porosity owing to poor coagulation. Some of these aspects are investigated by microscopy and diffraction as discussed in the following sections.

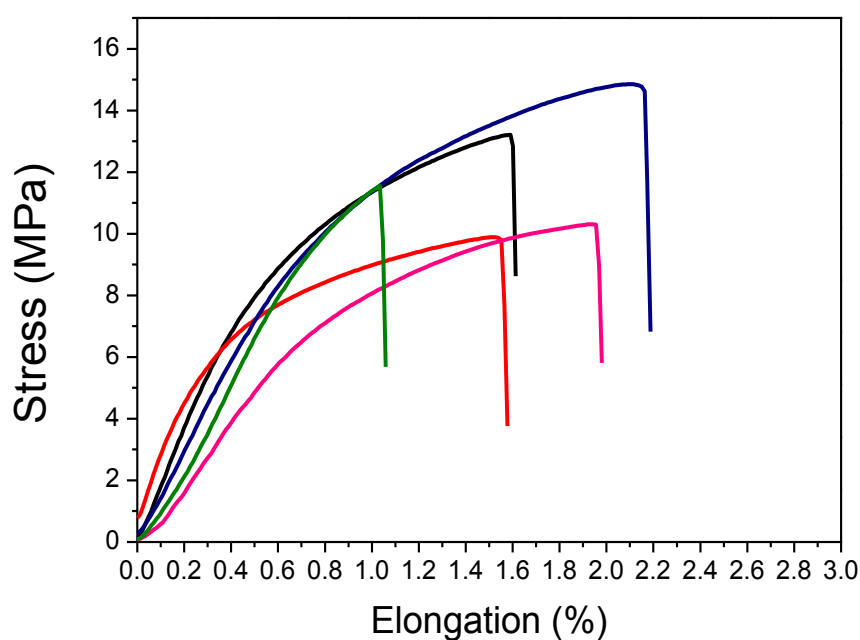


Figure 6.10: Stress (MPa) vs elongation (%) for 5 representative BC fibres

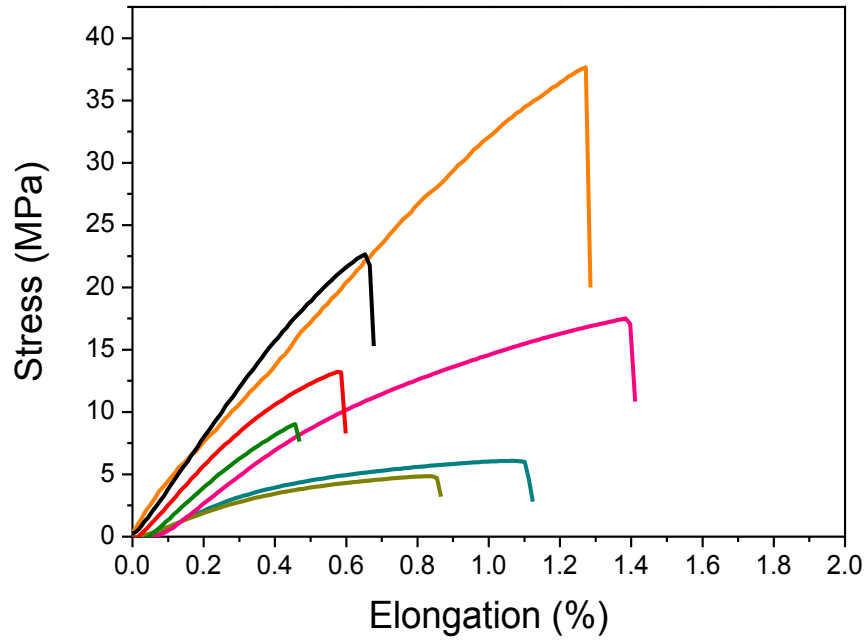


Figure 6.11: Stress (MPa) vs elongation (%) for 7 representative NdC fibres

6.4.3 Wide angle X-ray diffraction investigation

Wide angle X-ray diffraction was performed for two purposes a) to confirm the crystal structure b) to quantify the orientation of the nanowhiskers within the fibre.

Figure 6.11 shows the WAXS pattern for the starting material (BC, NdC) and of the fibres obtained from pilot trials. The three main reflections corresponding to the planes (110), (1-10) and (200) can be identified, which are characteristic peak for cellulose I crystal structure. This confirms that the native crystal structure was not disturbed during the process of making fibre in this work.

In the WAXS pattern for a non-oriented material uniform rings are seen. In case of some orientation, arcs are obtained instead of rings. The length of the arc gives a measure for orientation. The orientation was measured for two set of planes namely 200, 110. This is measured by the formula

$$f_c = \frac{180 - FWHM}{180} \quad (6.1)$$

The average width of the arc obtained was 84° and 70° for NdC and BC fibres, respectively (Figure 6.12). This implies an orientation of about 50% and 60% in NdC and BC fibres, respectively. Better orientation implies more efficient transfer of

properties from nanowhiskers to fibres. As there was no post spinning treatment done and not much tension was introduced while winding, the fibre owes its orientation to liquid crystalline phase. The orientation of the nanowhiskers depends on the aspect ratio and the forces responsible for orienting them. The orientation attained is in line with the fibre work reported by cold drawing of surface oxidised nanofibrillated cellulose [248], a multifilament fibre made by dissolution in sodium hydroxide and urea solution [247] and even viscose fibres [245]. This work shows that even without post treatment, a decent fibre orientation can be obtained.

Various forces act on a fibre during the process of fibre production. The poly-domain structure of the liquid crystalline spinning dope undergoes constant transformation resulting from the net effect of forces that change it and the recovery due to its own elasticity. In the present setup, there is shear force from the walls of the syringe, and the needle.

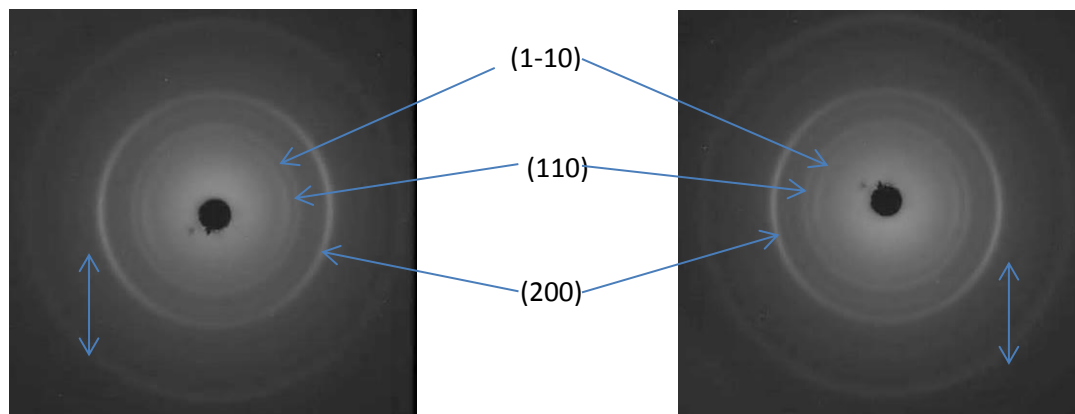


Figure 6.12: WAXS photograph of fibre obtained from NdC and BC (fibre axis shown by arrow) Peaks indexed according to $I\beta$

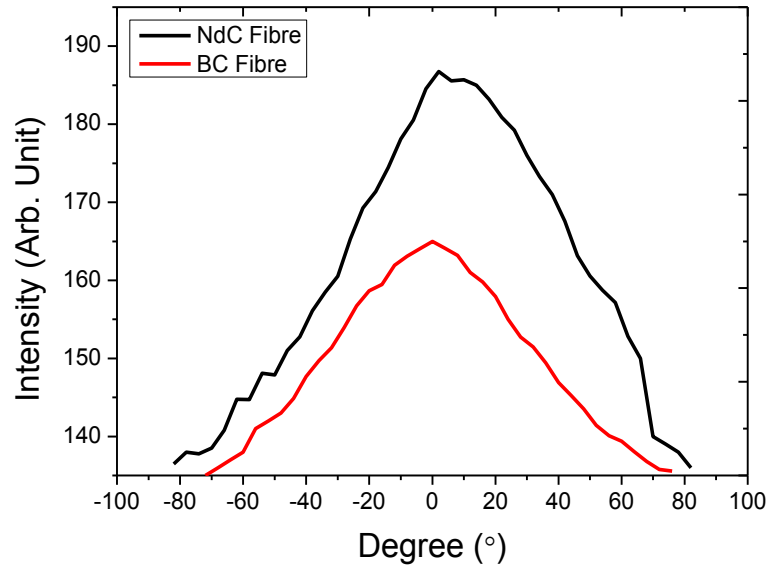


Figure 6.13: Intensity distribution along the circumference of the ring corresponding to the 200 plane in Figure 6.12 (—) NdC (—) BC

6.4.4 Scanning Electron Microscopy

The structure of fibre surface and cross section, made from liquid crystalline suspensions, was observed under high resolution SEM. Figure 6.14 (a) and (b) shows the image of the fibre surface and cross section of the fibre, respectively. The fibres were sometimes found to be more flat than round due to the effect of non-uniform drying on a substrate. The surface of the fibre was found to be wavy or crimped. When the cross section is seen with the microscope, it appears like stacked crimped sheets rolled together.

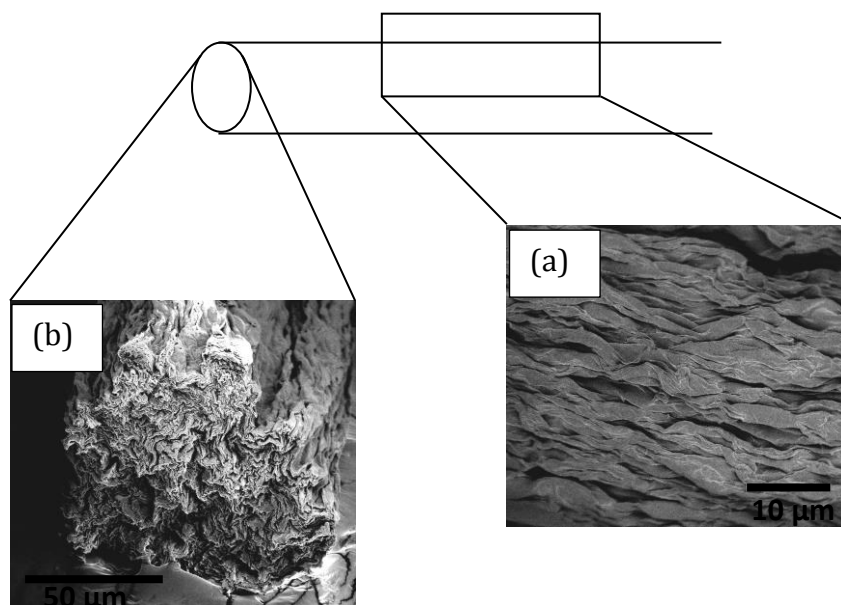


Figure 6.14: SEM image of a) cross section (scale bar 50 μm) and b) surface (scale bar 10 μm) of a BC fibre produced from a liquid crystalline suspension

In 100 μm wide fibres, 15-20 distinct layers can be spotted along a radial direction from surface to the centre, with increasing orientation of nanowhiskers (Figure 6.15). The structure has a similarity to cellulose film cast in general from the liquid crystalline suspension of cellulose nanowhiskers (Figure 6.16 (a)) [203]. Formation of a similar morphology has also been reported by Liu *et al.* (Figure 6.16 (b)) [239]. In case of a fibre the additional effect is of the circular cross section, which stacks the sheet in a circular fashion.

These fibres are made by extrusion of a liquid crystalline suspension. The shearing force exerted by the walls of the extruding unit causes some alignment of the randomly oriented domains as is seen from the images shown in Figure 6.17. However, this force competes against the relaxation. Some areas are well oriented while most of the regions are combination of various domains with directors in different directions. Shear force exerted by the walls of the needle, may also lead to shear induced phase separation, which might lead to layered structured exhibited in this work. The effect of shear on nanowhisker suspensions needs to be studied in future.

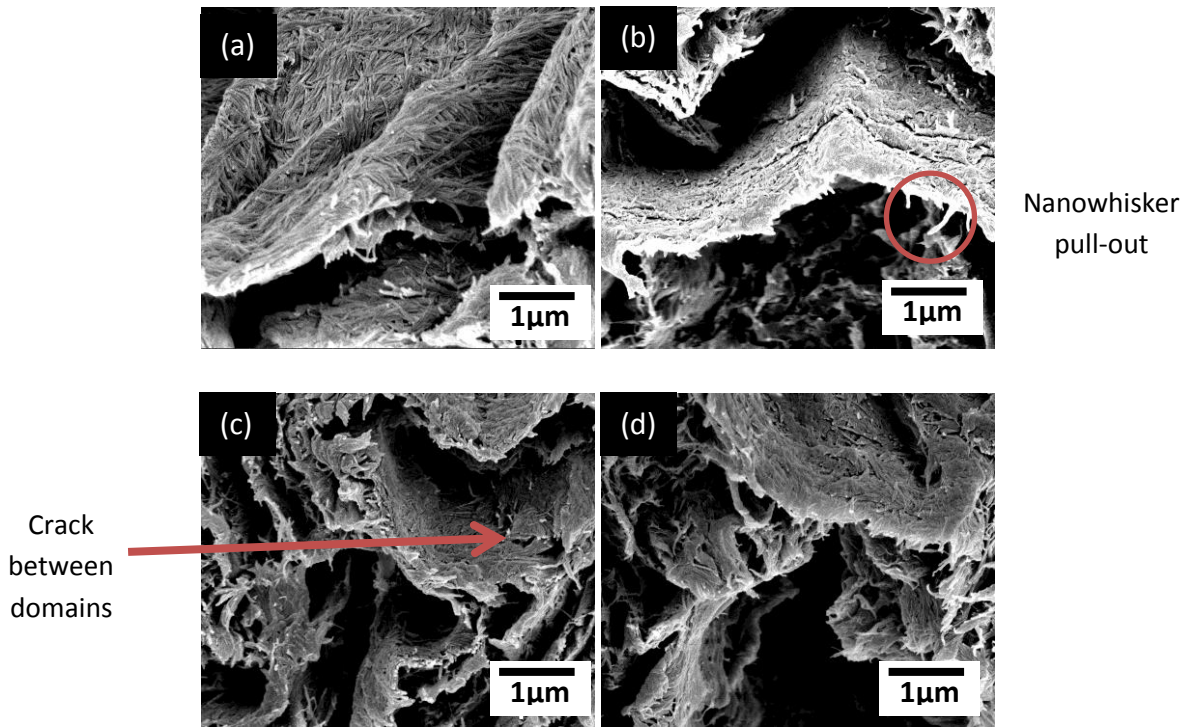


Figure 6.15: High Resolution SEM image of the concentric layers seen in the cross section of fibres in Figure 6.14 a) being the outer most and d) being the innermost (scale bar 1 μm), showing nanowhisker pull out and crack between domains

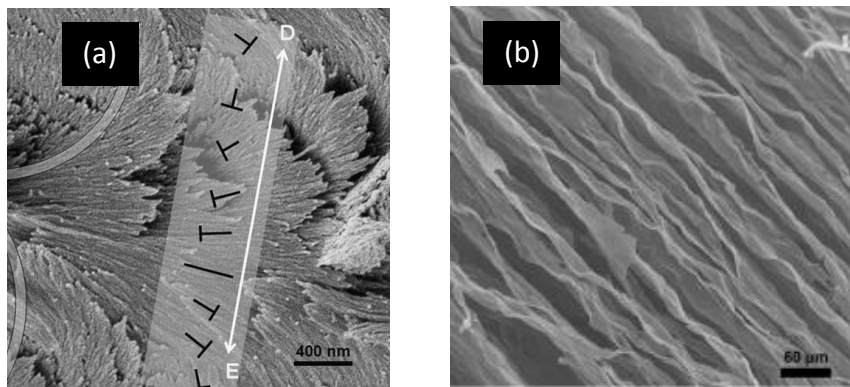


Figure 6.16: a) Film made by drying a chiral nematic liquid crystalline suspensions of cellulose nanowhisker showing layered structure [203] b) SEM micrographs of nanofibril foam prepared from the freeze-dried cellulose nanowhisker along the fibre alignment [239]

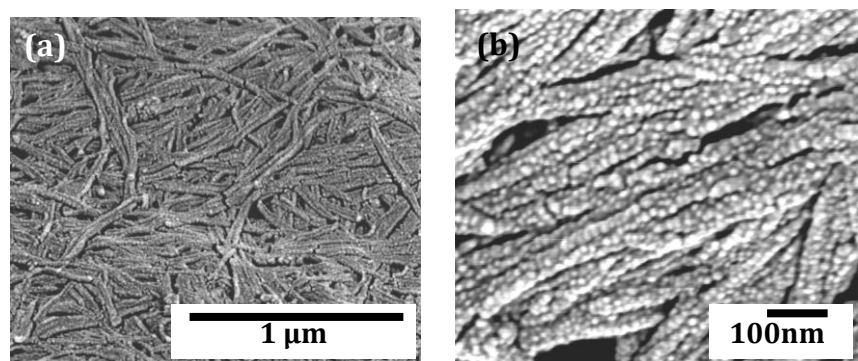


Figure 6.17: SEM image of fibre surface showing some oriented domains formed by nanowhiskers at a) low resolution (scale bar 1 μm) b) high resolution (scale bar 100 nm)

The detailed analysis of the cross section reveals information about the fracture mechanism. Normally, the fibre fracture modes are fibre-break, fibre pull-out and matrix de-cohesion. Investigating the fracture mechanism in the fibres, in this work, it can be seen that the mechanism of fracture is the fibre pull-out and may be de-cohesion of the liquid crystal domains (indicated in Figure 6.15). The fibre pull out mechanism of fracture is affected by the length of the entity comprising the fibre. The disadvantage lies in the reduction of the overall length of the microfibrils during acid hydrolysis and also the labile surface charges, which might affect the interaction between nanowhiskers. The properties of fibres, made from smaller fibrillar entities, depend on the length of the constituting entities. Also, a good orientation would minimise the chances of de-cohesion between oriented domains. Moreover, the poor performance of the fibres here may also be attributed to surface defects originating from usage of non-sophisticated spinning setup in this work.

6.5 Summary

The rheological behaviour of the celluloses and nanowhiskers suspensions were characterised. It was found that the as-received hydrated NdC, BC and TC behaved like a tough hydrogel due to the long entangled microfibrils, which made them unsuitable for spinnable. The elastic modulus for both, the as-received hydrated (un-hydrolysed) materials as well as suspensions, was higher than the viscous modulus which implies a gel-like behaviour. The BC nanowhiskey suspensions were observed to be more viscous than the NdC and TC suspension, as the BC nanowhiskers were longer than the NdC and TC nanowhiskers.

Fibres were successfully spun from the liquid crystalline suspension of BC and NdC. The TC suspensions were not found suitable for spinning due to inhomogeneity. Suitable spinning concentration range was determined for NdC and BC suspensions to be above 10 wt% and 6 wt%, respectively. The fibres were spun from 10 wt% suspensions of both the materials. The mechanical properties of the fibres produced were not significantly high but were found to possess a good overall orientation 0.5, without the aid of any post-spinning treatment. An interesting cross section morphology comprising of concentric sheet-like structure, was observed. The examination of the fracture surface revealed the fibre pull-out mechanism involved in the fracture.

The properties of the fibres can be improved by employing ways to increase aspect ratio of the cellulose nanowhisiker or retaining the length of the microfibrils. The length of the nanowhiskers in our work was up to 3 μm for BC and 1.5 μm for NdC. The properties of the fibres are directly related to the the length of the elements making up the fibre. A more sophisticated and a more controlled spinning set up would definitely improve the quality of fibres.

This is the first report of cellulose fibre production from bacterial source, where cellulose I native crystal structure was retained as well as a liquid crystalline phase was utilised to obtained orientation. This chapter establishes a promising novel method for cellulose production. One of the possible uses of the fibres made in this work may be as a biocompatible carrier for functional materials owing to their high porosity.

Chapter 7

In situ Bacterial Cellulose Modification

7.1 Background

In chapter 4, the hierarchical organisation of cellulose from bacteria and tunicate was elucidated and the formation of liquid crystal phase was reported in chapter 5. It has been identified that the phase transition of cellulose nanowhisker suspension, from an isotropic to a liquid crystalline phase, is dependent on the morphological features of nanowhisker (such as length, aspect ratio) and these features in turn are dependent on the source of cellulose. For example, the formation of lyotropic liquid crystalline phase was observed at the lowest concentration in the case of the laboratory grown bacterial cellulose (BC) owing to the largest aspect ratio of cellulose nanowhiskers in comparison to the nanowhiskers from the other sources, namely, nata-de-coco (NdC) and tunicate cellulose (TC).

In the vast literature on cellulose nanowhisker production, properties and applications, a wide range of geometrical dimensions and aspect ratio of nanowhiskers has been reported. The wood cellulose nanowhiskers are 3-5 nm in width and 100-200 nm in length [122, 140, 141], cotton derived nanowhiskers are 5-10 nm wide and 100-300 nm long [144, 145], while tunicate cellulose nanowhiskers are about 10-20 nm in width and 500-2000 nm in length [142, 143]. This leads to the question as to which properties of cellulose determine the geometrical features of nanowhiskers. It has been discussed in section 2.5-2.7 that the arrangement of the cellulose synthesizing terminal complexes (TCs) determines the cross section of the microfibrils. In fact different arrangements of TCs have been reported across various cellulose producing organisms [1]. However, the factors determining the nanowhisker length remains unexplored. There is a need to fill this gap as a thorough understanding of this may provide a tool to tune the nanowhiskers. In section 4.3, a correlation between the aspect ratio of nanowhiskers and the distance between microfibrils branching points and the crystalline content of the material was noted. In this chapter, in order to investigate the dependence of the nanowhiskers, obtained after acid hydrolysis, on the microstructure

of the parent cellulosic material, various morphologies were produced by *in situ* modifications.

The modifications in culture medium and growth conditions during bacterial cellulose production, led to the formation of bacterial cellulose with different microstructures. A substantial literature exists on the *in situ* modification of bacterial cellulose primarily with the aim to understand cellulose biosynthesis and the hierarchical organisation of cellulose [16, 103, 109, 110, 126]. For example, the effect of addition of calcofluor (now called Tinopol) and carboxy methyl cellulose during the bacterial cellulose synthesis has been studied to determine the levels of hierarchical organisation in cellulose [16, 103, 107]. Other sets of experiments have been reported with various other polymeric additives such as polyethylene glycol, in which the effect on the overall morphology in terms of pore size and microfibril width distribution has been studied [110]. Further work could provide the capability to tune the cellulose microstructure which would facilitate newer applications [249]. For example, a control over pore structure and microfibrils dimensions would allow better membrane design or facilitate tissue scaffolding.

The early part of this chapter is dedicated to the effect of various modifications in the culture media on the morphology of cellulose and the later part to the nanowhiskers obtained from their subsequent hydrolysis. For this work, first of all, a suitable strain of bacteria was chosen, a brief account of which is presented in the section 7.1. The effect of additives in terms of the width distribution of the microfibrils, branching pattern, crystallite size and shape has been studied using scanning electron microscopy (SEM) and X-ray diffraction (XRD) in section 7.2. Finally, the geometrical features of the nanowhiskers obtained from the hydrolysis of the various modified and unmodified bacterial cellulose are compared in section 7.3.

7.2 Selection of strain

Many strains and species of bacteria are known to produce cellulose [1, 249]. Cellulose was produced using three different strains of bacteria, ATCC, AX and AY (strain details included in section 4.1). They were all cultured in the same medium and the cellulose pellicles were collected at the end of 14 days (Figure 7.1). The strain AX formed a thick tough pellicle while the strain ATCC produced a much thinner cellulose pellicle. On the

other hand, the strain AY failed to form a pellicle and instead formed an entangled mass. The wet and dry weights of the cellulose produced by all strains in 14 days were measured and are listed in Table 7.1. It can be seen from the table that the strain AX produced the maximum amount of cellulose (dry weight), almost twice of that produced by the strain ATCC and AY, under the same culture condition and time of cultivation. Thus, the strain AX was found to be the most efficient in cellulose production or in other words it exhibited highest glucose to cellulose conversion efficiency, amongst the species of bacteria studied here and was used for further work. Understanding the reason behind different glucose conversion is beyond the scope of this work. However, it may be added that different biosynthetic pathways may lead to different efficiencies.



Figure 7.1: Cellulose pellicles collected at the end of 14 days of culture using Hestrin-Schramm medium at 25°C inoculated with the strains a) AX, b) AY and c) ATCC, cleaned by boiling in 1M NaOH solution and repeated washing with distilled water

Table 7.1: Wet and dry weight of cellulose pellicles obtained in 14 days from the three strains

Strain	Pellicle weight		Weight %
	Wet (g)	Dry (g)	
AX	101±10	0.55±0.15	0.5
AY	No pellicle formation		0.4
ATCC	25±2.5	0.21±0.02	1

7.3 Modification by additives

It has been mentioned earlier that cellulose synthesis by bacteria provides opportunities for *in situ* modifications. It is this advantage of bacterial cellulose that has been extensively exploited to understand cellulose biosynthesis with bacteria as the model system for last five decades. A detailed account of modification work reported in

the literature has been presented in chapter 3. The following section discusses the effect of chosen additives on bacterial cellulose micro-macro-structure, observed in this work.

7.3.1 Additives

In the current work, four chemicals, namely, carboxy methyl cellulose (CMC), poly ethylene glycol (PEG), calcofluor (CF) and nalidixic acid (NA) have been used as additives, a brief description of these are given below:

- PEG4000(PEG): PEG is a water soluble high molecular weight polymer of ethylene oxide. In literature, various molecular weights of PEG have been used to modify the pore structure of bacterial cellulose [5]. The maximum productivity was seen at 4 wt% and thus same concentration has been used here.
- Calcofluor (CF): It is a dye used to locate cellulose owing to its ability to bond with β 1-4 and 1-3 linkage in polysaccharides. It bonds with cellulose chains and thus disrupts the assembly into microfibrils. The CF has been instrumental in separating the processes of crystallization and polymerization in cellulose as well as chitin [250, 251]. About 0.1 % concentration of CF was used on the basis of a previous work [16].
- Carboxy Methyl Cellulose (CMC): CMC is cellulose with one hydroxyl group substituted with the carboxy methyl group. It has a capability to bind with cellulose and also increase the viscosity of the media. Several reports exist on effect of CMC addition to bacterial cellulose synthesis, suggesting interference with crystallization [110]. 2 wt% CMC was used as a maximum yield was seen at that concentration.
- Nalidixic acid (NA): NA is an antibiotic, which is bacteriostatic (stops bacteria from reproducing, while not necessarily harming them otherwise) at low concentration and bacteriocidal (kills bacteria) at high concentration. An earlier report has indicated branching pattern in microfibril is a result of bacterial cell division while producing a microfibril [48]. Thus, the effect of an antibiotic has also been investigated in this work. Only one previous report exists on the effect of addition of NA, which has shown increase in microfibril width due to elongation of bacterial cell but without any comment on its effect on microfibril branching pattern [106]. The same concentration 0.1mM, as also used in the previous work, has been used here.

The modified cellulose was collected in 14 days and cleaned by following the routine protocol to remove media and bacterial cell debris discussed in section 4.1. The bacterial cellulose modified by adding CMC, PEG, NA and CF will be referred to as BC_CMC, BC_PEG, BC_NA and BC_CF.

7.3.2 Characterisation by PXRD

The X-ray diffraction patterns for cellulose formed in all modified media (BC_CMC, BC_PEG, BC_NA and BC_CF) and the unmodified cellulose (BC-control) are shown in Figure 7.2. The background was subtracted from the patterns and the data was analysed considering amorphous contribution as described in section 3.3.1. All the diffraction patterns, both for BC and modified BC, exhibited the 3 main reflections at 2-theta values of 14.4°, 16.7°, 22.5° for BC, 14.3°, 16.6°, 22.45° for BC_PEG, 14.32°, 16.62°, 22.46° for BC_CF, 14.69°, 16.73°, 22.73° for BC_CMC and 14.2°, 16.57°, 22.36° for BC_NA corresponding to the typical d-spacing values for planes of cellulose I crystal structure [54]. Therefore, it is evident that the *in situ* modifications performed in this work do not affect the resulting crystal structure of cellulose. This is consistent with the observations reported for CF and CMC, however the previous reports on PEG and NA modification did not comment on the effect on cellulose crystal structure [16, 103, 106, 107, 110].

Along with the small shift in the peak positions seen for the modified cellulose with respect to the unmodified BC, there was also a change in the relative intensity with respect to the amorphous peaks and the width of peaks, which indicates a deviation in the crystallinity and the crystallite size.

a) Crystallinity: The crystallinity for all the samples was calculated as discussed in section 3.3 and are listed in Table 7.2. The crystallinity of unmodified bacterial cellulose was almost 85%. In case of all the modified bacterial cellulose, the crystallinity invariably decreased, with the largest decrease observed for BC_CF to about 54%. The crystallinities for BC_CMC, BC_PEG and BC_NA were calculated to be 71, 85, and 79%, respectively. The values of crystallinities are consistent with those obtained in the previous studies [16, 103, 107, 109, 110]. The decrease in crystallinity on addition of modifiers suggests that the crystallisation is interrupted to different extent in presence

of these additives, which would depend on interaction between the additive and cellulose assembly.

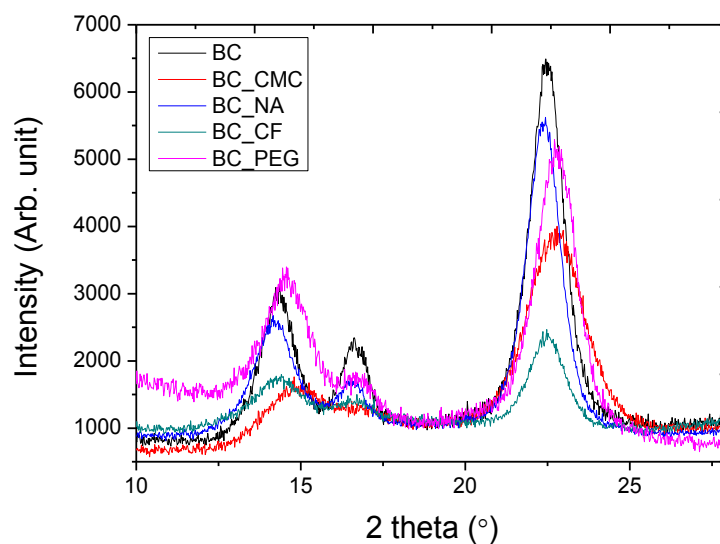


Figure 7.2: XRD pattern for unmodified BC, BC_CF, BC_CMC, BC_PEG and BC_NA showing 3 main reflections consistent with the pattern corresponding to cellulose I crystal structure

Table 7.2: Crystallinity, peak positions, d-spacing and crystallite size calculated from the XRD pattern

	Crystallinity(%)	Plane	2-theta [°]	d-spacing [Å]	Crystallite size [Å]
BC	85	1-10	14.4	6.19	65
		110	16.7	5.31	87
		200	22.5	3.96	64
BC_PEG	85	1-10	14.3	6.19	64
		110	16.62	5.33	85
		200	22.45	3.95	65
BC_CF	54	1-10	14.32	6.18	39
		110	16.62	5.32	81
		200	22.46	3.95	58
BC_CMC	71	1-10	14.69	6.02	45
		110	16.73	5.29	22
		200	22.73	3.91	36
BC_NA	79	1-10	14.2	6.24	49
		110	16.57	5.35	75
		200	22.36	3.97	62

b) Crystallite size: Using Sherrer's equation, discussed in section 3.3, the crystallite sizes were calculated and are listed in the Table 7.2. Similar to the observations on crystallinity, a general decrease in crystallite size was observed for modified cellulose as compared to unmodified BC, but with the exception of the BC_PEG where the crystallite size remained the same. The decrease in crystallite size can be attributed to the interference in the assembly and the crystallisation of poly-glucan chain by the additives. However, it must be remembered that contribution to the peak broadening due to the paracrystalline distortion has not been accounted and thus all crystallite sizes calculated here are an under-estimation of the actual crystallite sizes. It is also possible that the all the modified celluloses might possess different degree of paracrystalline distortion.

Still, following the work by Elazzouzi *et al.* [121], and similar to the analysis performed in section 4.3, the crystallite cross-sections were constructed from the crystallite sizes and are furnished in Figure 7.3. It can be seen that the crystallite sizes are affected to different extents in various crystallographic directions. This implies the additives target the different levels of bonding involved in crystallisation and assembly of cellulose (Figure 7.4).

i) Effect of PEG

The crystallite sizes calculated in the three directions ($[010]_t$, $[100]_t$, $[110]_t$) corresponding to the first three intense peaks in the XRD pattern, for BC and BC_PEG are (65, 87, and 64 Å) and (64, 85 and 65 Å) respectively. Thus for PEG as an additive, no change in crystallite sizes was seen, indicating PEG does not interfere with the van der Waal interaction or the H-bonding during the cellulose assembly and crystallization. Therefore, PEG does not affect crystallisation and its effect on microstructure is studied by SEM in the next section.

ii) Effect of CF and CMC

The crystallite sizes obtained for BC_CF and BC_CMC are 39, 81, 52 Å and 45, 22, 36 Å in the crystallographic directions $[010]_t$, $[100]_t$, and $[110]_t$, respectively. In both the cases, the crystallite sizes are smaller than that of unmodified BC. However, a larger decrease is observed in case of BC_CMC, especially the crystallite size in $[200]$ direction. This may suggest that the van der Waals interaction as well as H-bonding is affected. A

larger decrease in the crystallinity was seen as the crystallinity was calculated to be 54% and 71% for BC_CF and BC_CMC, respectively. This suggested crystallisation kinetics is affected to a larger extent in presence of CF. The higher relative crystallinity in case of CMC as compared to CF but smaller crystals imply more number of smaller crystals in case of CMC and less number of larger crystals in case of BC_CMC.

CMC, being a cellulose derivative, has the capability to interact with the nascent cellulose chains. Thus, it can cause disruptions in the assembly process by getting incorporated in the system [103], which has been investigated in the next section by SEM. Once CMC coats the cellulose microfibrils it prevents further H-Bonding. CF also penetrates between poly-glucan aggregates synthesised and extruded at closely spaced sites, before they intersect and crystallise into microfibrils. Because of its ability to bond at crystallization level it acts as an agent to separate polymerisation from crystallisation [16, 250]. The effect of CF decreases as the dye is consumed and the normal biosynthesis is resumed. In previous report it was seen that 69 Å crystallites are obtained if 0.025% of CF is used and 28 Å crystallite is obtained for larger concentrations. CF has also been reported to affect assembly in other cellulose-producing organisms [107]. Haigler has explained the effect of CF and CMC by emphasising on the hierarchical assembly process and that these agents interfere at different sites to cause effect on the different hierarchical levels [16, 103]. CF interferes at the glucan aggregation level while CMC interferes in later assembly in fasciation of bundles of fibrils. However, in this work effect of CMC has been observed at the same level as CF.

iii) Effect of NA

A decrease in crystallite size is seen, especially in the H-Bonding direction. It is difficult to comment on the mechanism by which, NA causes this effect. It has been investigated only once before [106], where the antibiotics caused elongation of the bacterial cells and thus an increase in the number of cellulose producing sites.

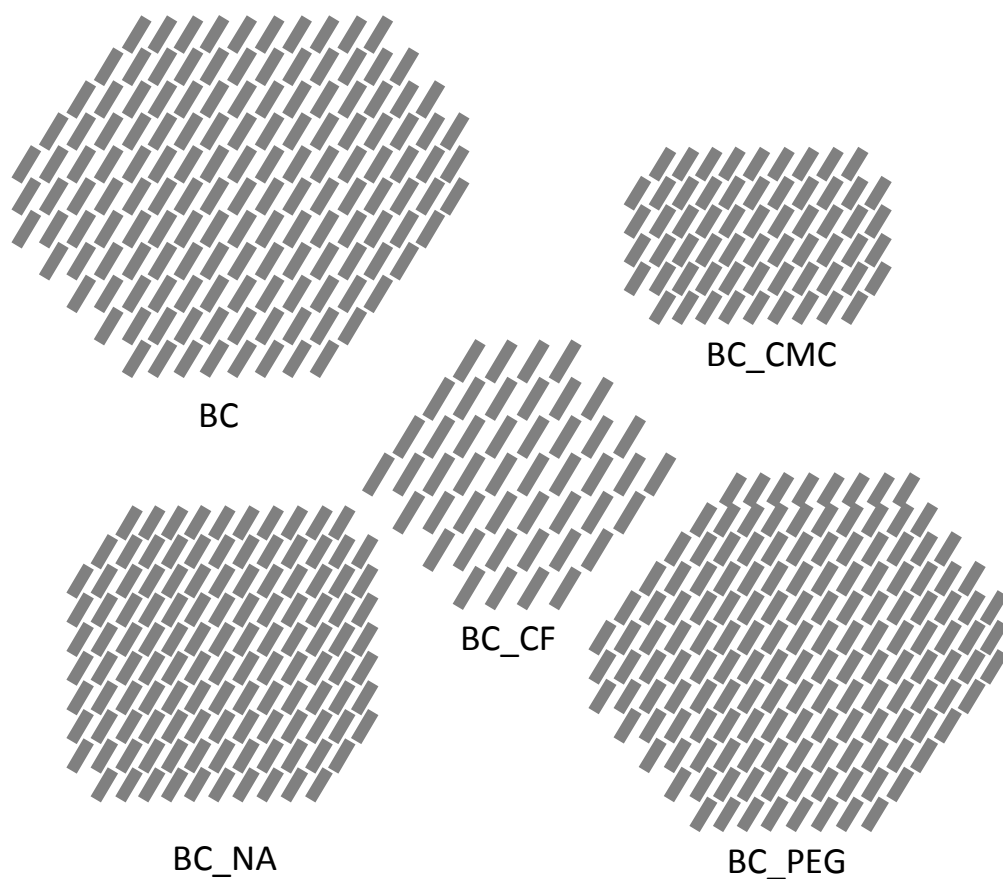


Figure 7.3: Crystallite cross-section for BC, BC_PEG, BC_CMC and BC_CF developed using the crystallite sizes calculated from their XRD patterns and listed in Table 7.2

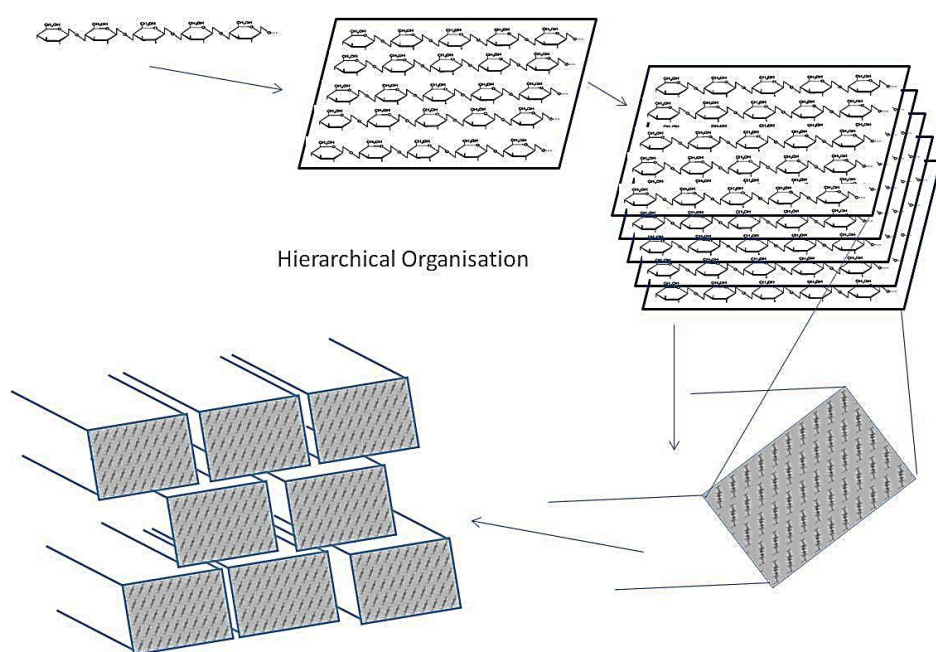


Figure 7.4: Schematic representation of hierarchical organisation of cellulose: chains into mini sheets, stacking of mini sheets, formation of mini crystals and microfibrils

7.3.3 Characterisation by SEM

The SEM images of BC, BC_PEG, BC_CMC, BC_CF and BC_NA are furnished in Figure 7.5, 7.7 and 7.10. The uniform long microfibrils are seen in all samples. As discussed in chapter 5, a wide distribution of microfibril width was seen due to a variable degree of aggregation of microfibrils into ribbons, however the width distribution was found to be different for the various samples. Similar branch-like pattern was seen in all the samples of modified cellulose; however the extent of branching or in other words the distribution of segment lengths between branching points was different for all modifications.

i) Effect of PEG

The microfibrils width distributions obtained from the SEM images (Figure 7.5 (a,b)) are shown in Figure 7.6 (a). The average microfibril width of BC and BC_PEG were 65 ± 36 nm and 67 ± 62 nm. Therefore no significant change in the width distribution was seen using PEG as a modifier. This reconfirms the observation from PXRD that PEG does not interfere with the assembly or crystallisation process. The separation between branching points of microfibril is quantified in Figure 7.6 (b). It was observed that the average separation between branching points reduced in case of BC_PEG as compared to unmodified BC. In a previous work by Heßler *et al.* [110], it was shown that PEG with different degree of polymerisation could be used to obtain different pore sizes in the BC pellicle. However, the explanation behind this observation and those in the current work remains unexplained.

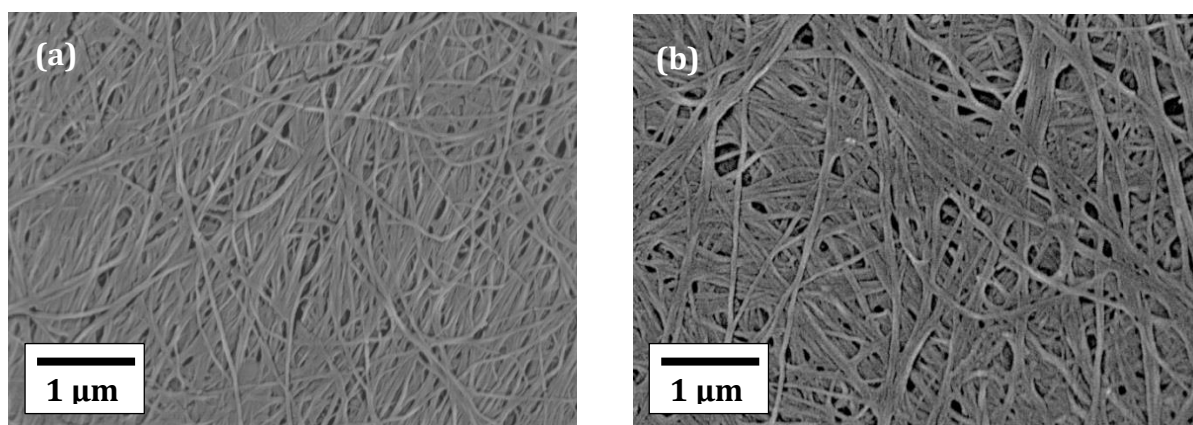


Figure 7.5: SEM image of a) BC and b) BC_PEG (scale bar 1 μm)

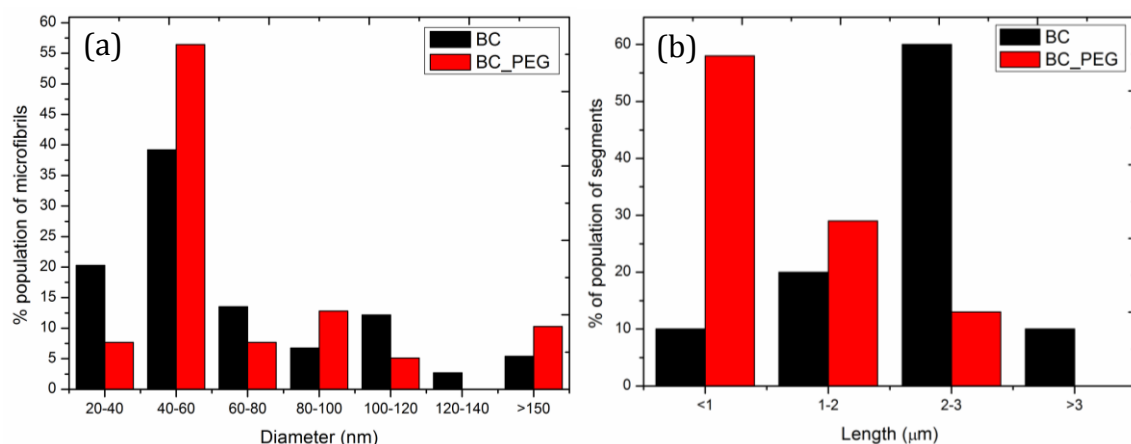


Figure 7.6: Distribution of a) microfibril width and b) separation between branching points for BC and BC_PEG obtained from SEM images from 200 measurements

ii) Effect of CF and CMC

The SEM images are presented in Figure 7.7 (a) and (b) for BC_CMC and BC_CF, respectively. It is seen that in the presence of CMC as well as CF the width distribution, shown in Figure 7.8 (a) and (b), shifts to lower width value with average width of 32 ± 22 nm and 49 ± 24 nm in BC_CMC and BC_CF, respectively. This is line with the observations by XRD, where it was inferred that CF and CMC disrupts the assembly process at the crystallisation state and would obviously affect the microfibril and ribbon formation and thus the width distribution. Earlier reports also exist on the ability of calcofluor and CMC to disrupt the assembly process [16]. The SEM images were also used to produce a distribution of microfibril length between branching points, which is illustrated in Figure 7.9 (a) and (b). The separation between branching reduced in both the cases, and the decrease was more in case of BC_CF as compared to BC_CMC.

Most of the additives used here are water-soluble and ideally would be removed during the washing of the pellicles. However, the pellicles produced with CMC as the modifier had a different texture in comparison to all other. They were slimier and more stretchable. The cross-section of BC_CMC was studied with the SEM (Figure 7.7 (c)). As discussed in section 4.2.1, bacterial cellulose is produced as layered deposition. A similar feature was seen in the cross section, in addition to the inclusion of CMC in the system. Amongst all the additives used here, CMC is least soluble with water and leads to a medium of high viscosity on autoclaving before the culture. The viscosity and

relatively less water solubility lead to the incorporation of CMC into the pellicle. Previous work on BC modification by CMC has also reported incorporation of CMC in the system [110].

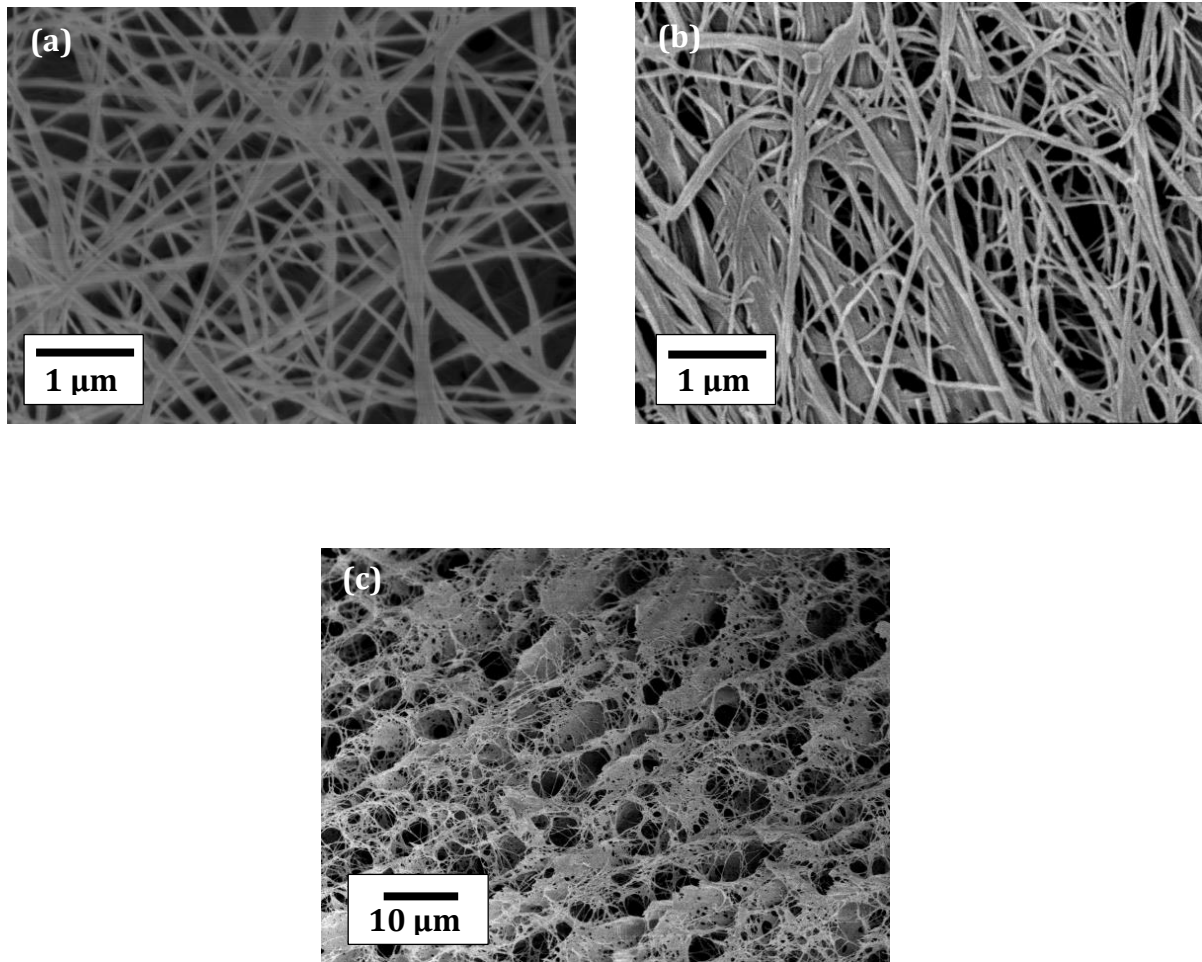


Figure 7.7: SEM image of a) BC_CMC (scale bar 1 μm) b) BC_CF (scale bar 1 μm) c) cross section BC_CMC showing CMC inclusion (scale bar 10 μm)

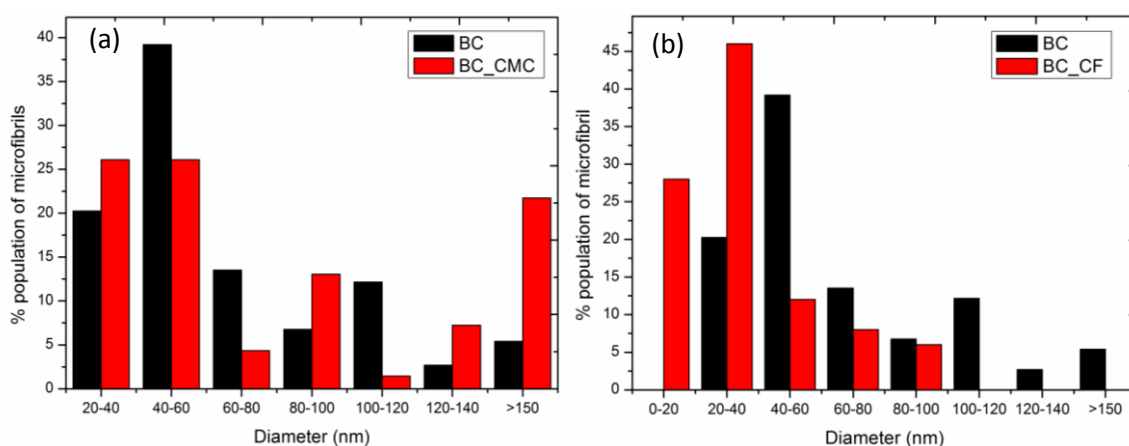


Figure 7.8: Distribution of microfibril width for a) BC and BC_CMC, b) BC and BC_CF, obtained from SEM images from 200 measurements

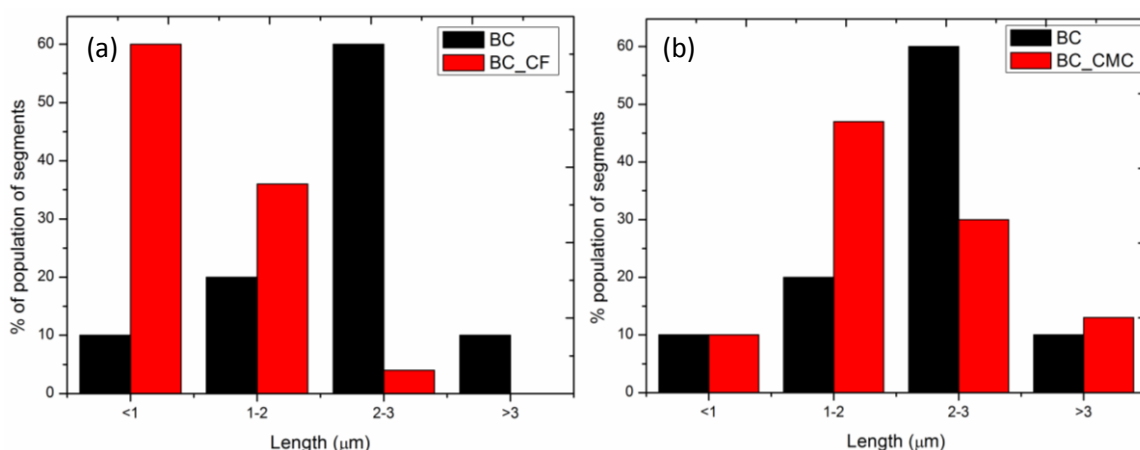


Figure 7.9: Distribution of separation between branching points for a) BC and BC_CMC b) BC and BC_CF obtained from SEM images from 200 measurements

iii) Effect of NA

The SEM image and the microfibril width distribution of BC_NA are shown in Figure 7.10 and Figure 7.11(a), respectively. In presence of NA, the average width of the microfibril increases from 65 ± 36 nm to 82 ± 62 nm. Similar observation has been reported before, which suggested that in the presence of NA (bacteriostatic in low concentration), the bacterial cell grows longer in length and fails to divide. Owing to larger bacterial cell length, which would possess more cellulose chain extrusion ports, the wider microfibrils are produced [106]. The separation between branching reduced, as is evident from the distribution obtained from SEM images and presented in Figure 7.11 (b).

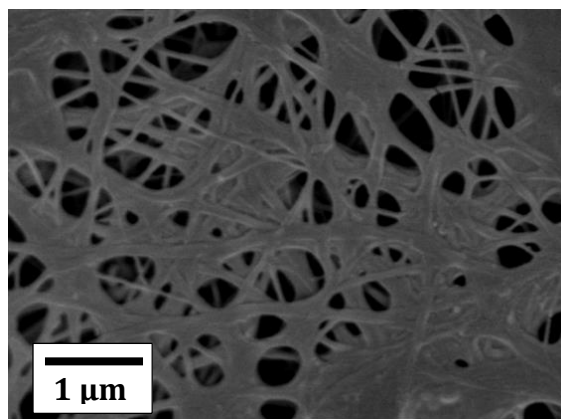


Figure 7.10: SEM image of BC_PEG (scale bar 1 μm)

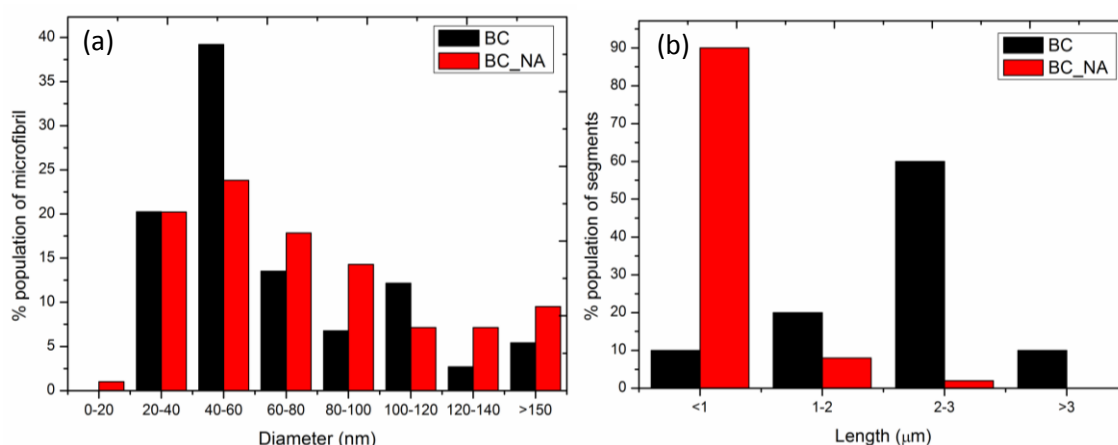


Figure 7.11: Distribution of a) microfibril width and b) separation between branching points for BC and BC_NA

7.3.3 Classification of additives

The addition of PEG to BC production did not cause a large change in the crystallite size and crystallinity, as it did not interfere with the crystallisation and the assembly process. The width distribution of microfibrils was also not affected. It does affect the pore size of the material by affecting the later assembly of microfibrils into ribbons. In case of CMC and CF, crystallite sizes were reduced. They both possess the capability to bond with cellulose, affecting intra and inter-chain cellulose bonding and also the crystallisation. Thus, they interfered very close to cell surface at the site of polymerisation as well as crystallisation. NA also led to a decrease in crystallite size. However the idea behind using NA was to check the dependence of microfibril

branching on the bacterial cell reproduction. The observations were contrary to the bacterial cell division resulting into microfibril branching hypothesis and thus the branching is not due to cell division but due to splaying/bundling of microfibrils. This is in line with the conclusion drawn in chapter 4.

On the basis of crystallinity, crystallite sizes, incorporation into the system, width of the ribbons, role of the additives can be deciphered and can be classified. Additives influence on the specific stage of assembly. The ability of different additives to affect different levels of organisation is evidence to multi-level hierarchical organisation. PEG acts much later after crystallisation and the assembly, while CMC and CF act very close to the site of synthesis and organisation. Role of NA is not clear but on the basis of its effect on the crystallite size, it can be said it does interfere with the poly-glucan chain assembly process.

7.4 Nanowhiskers from modified bacterial cellulose

The preceding sections have illustrated various possible microstructural changes in bacterial cellulose by introducing additives such as PEG, CF, NA and CMC, in the culture media. The variation was observed in terms of various morphological features such as crystallinity, crystallite size, pore size, microfibril width, and degree of association. In the vast literature on cellulose nanowhisker production, properties and utilisation, the relationship between the cellulose micro-macrostructure to the properties of nanowhiskers obtained from its acid hydrolysis has not been investigated. In this section, the variation in microstructure and crystallinity obtained by modifications investigated in the previous sections has been exploited to understand this relationship. This study has been carried out using three sets of variations: commercial (NdC) and laboratory (BC_AX) produced bacterial cellulose, bacterial cellulose produced in laboratory with two different bacterial strains (AX and AY) and two variations in microstructure obtained using CF and CMC as media modifying agent.

7.4.1 Nanowhisker variation between NdC and BC

The bacterial cellulose produced from the strain AX (BC_AX) and commercially produced NdC, was hydrolysed using the protocol described in section 3.2.1, to produce nanowhiskers. The AFM images of the nanowhiskers obtained from BC_AX and NdC, are shown in Figure 7.12 and the nanowhiskers length distribution for both samples

obtained from AFM is furnished in Figure 7.13. The average length of the BC_AX nanowhiskers was about $2.42 \pm 0.52 \mu\text{m}$, which is almost twice the average length of the NdC nanowhiskers ($1.13 \pm 0.35 \mu\text{m}$). The length of NdC nanowhiskers varied from below $0.5 \mu\text{m}$ to just over $2 \mu\text{m}$ while the maximum population had a length of 1-1.5 μm . On the other hand, BC_AX nanowhiskers possessed length below $0.5 \mu\text{m}$ to over $3 \mu\text{m}$ with maximum population with length in the range of 2-3 μm .

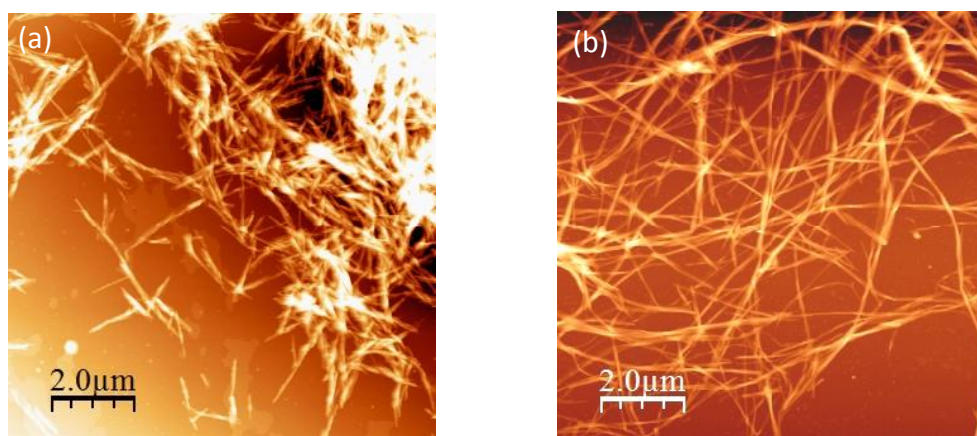


Figure 7.12: AFM images of nanowhiskers obtained by acid hydrolysis of a) NdC and b) BC_AX (scale bar $2\mu\text{m}$)

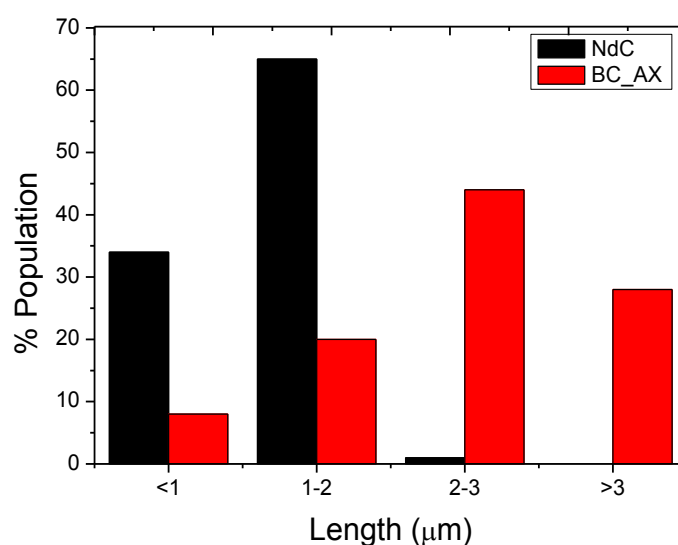


Figure 7.13: Distribution of nanowhiskers length for NdC and BC_AX

The width of the microfibrils of BC_AX and NdC before hydrolysis remained the same as can be seen in the SEM images represented in Figure 7.14 (a, b). The individual microfibrils in both cases were about 20-30 nm, which aggregated to form wider ribbon like structures. This aggregation results in a branch like morphology of cellulose fibrils including bundling of microfibrils and splaying of ribbons.

In the case of polymers, the degree of flexibility is critical, which is expressed in terms of the Kuhn length and the persistence length, which determine the stiffness and straightness of the chain. Similarly, in the case of cellulose, a supra-molecular polymer, equivalent of Kuhn length may be considered similar to the distance between the branching or splaying points mentioned above. The length distribution of these segments was obtained from SEM images of each material and is presented in Figure 7.15 along with the length distribution of nanowhiskers obtained in each case. The average length of segment between branching points was larger for lab-cultured BC_AX ($2.20 \pm 0.65 \mu\text{m}$) than that for NdC ($0.98 \pm 0.45 \mu\text{m}$), similar to that observed for the length distribution of nanowhiskers. Moreover, the distribution trends of length segments and length of nanowhiskers were found to be similar for both the materials (Figure 7.15).

According to the popular belief, the nanowhiskers are obtained from the removal of amorphous region [74]. It may be recollected that in this work or any reported before, no microscopic evidence of amorphous region has ever been seen. However, on the basis of the observation reported in section 5.2.1 that the acid hydrolysis led to an increase in the crystallinity, it does indicate removal of non-crystalline regions by hydrolysis. Moreover, according to a study, it has been found that the hydrolysis depends on the crystallinity of the starting materials [139]. Therefore, the crystallinity is an important parameter determining the nanowhiskey production. The XRD patterns, as shown in Figure 7.2, were used to calculate the crystallinity and it was found that the crystallinity of BC_AX is about 85% and that of NdC is about 65%. As expected, the length of nanowhiskers obtained decreased with decrease in crystallinity and thus the nanowhiskers length was smaller for NdC, which also has lower crystallinity than BC_AX. The difference in the dimensional distribution of nanowhiskers obtained from two grades of bacterial cellulose is due to different periodicity of defects which in turn is determined by differences in the cultivation conditions. Laboratory cultured bacterial

cellulose was produced in small amount in static culture conditions with a known species of bacteria, on the other hand, NdC is produced on a large scale with a different strain, medium and conditions. These differences lead to difference in morphologies and different crystallinity.

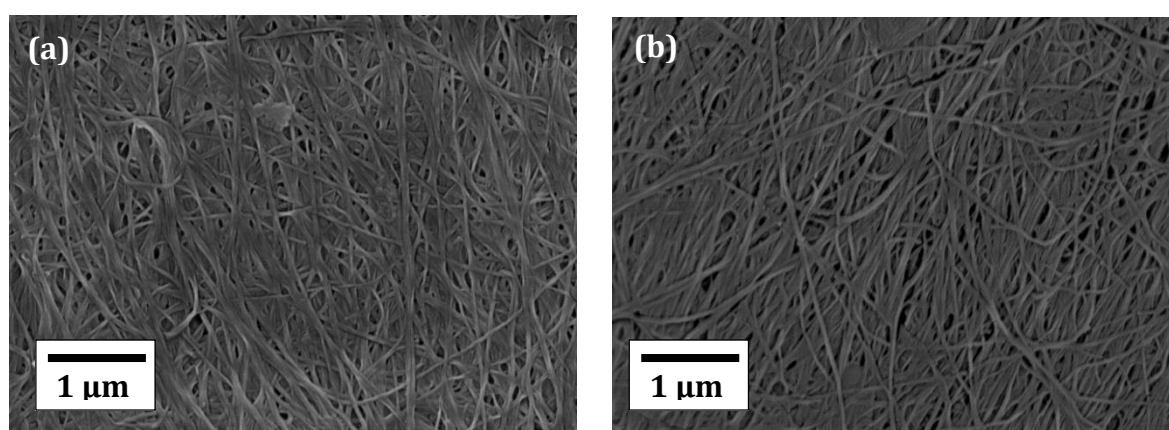


Figure 7.14: SEM images showing microstructure of NdC and BC_AX (scale bar 1 μm)

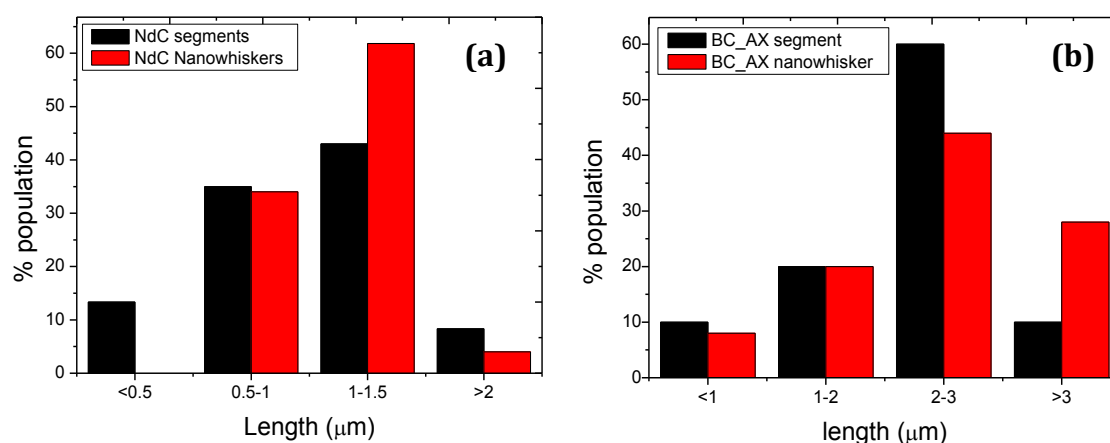


Figure 7.15: Distribution of nanowhiskers length and microfibril segment length between branching points for a) NdC (average nanowhisker length $1.13 \pm 0.35 \mu\text{m}$ and average microfibril segment length $0.98 \pm 0.45 \mu\text{m}$) and b) BC_AX (average nanowhisker length $2.42 \pm 0.52 \mu\text{m}$ and average microfibril segment length $2.20 \pm 0.65 \mu\text{m}$)

This is the first evidence of the possible correlation between the morphology of cellulose and the length distribution of nanowhiskers obtained after the hydrolysis. The effect of culture condition on the morphology of cellulose has been studied in the past,

but the relationship between morphology and periodicity of defects or nanowhiskers obtained by elimination of these defects remains unexplored.

7.3.2 Nanowhisiker variation with strains

The different strains produce cellulose with different efficiency, morphology and crystallinity as discussed in section. It may be recollected that the strain AX produced a tough thick pellicle of cellulose while the other strain AY failed to produce a pellicle. Celluloses obtained from both the strains were treated with dilute sulphuric acid according to the protocol described in section 3.2.1 to produce nanowhiskers. The AFM image and the length distribution of BC_AX and BC_AY nanowhiskers are presented in Figures 7.16 and 7.17, respectively. The nanowhiskers obtained from BC_AY were much shorter with average length of $1.22 \pm 0.48 \mu\text{m}$ as compared to the BC_AX nanowhiskers with the average length of $2.42 \pm 0.52 \mu\text{m}$.

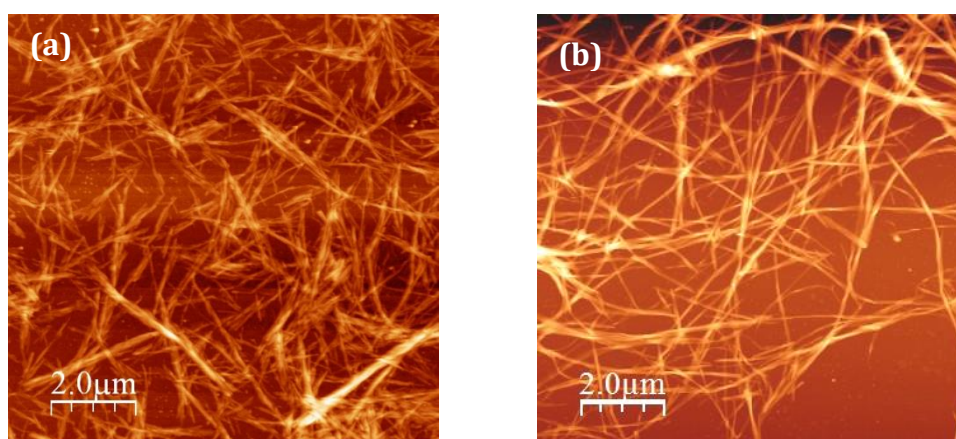


Figure 7.16: AFM images of nanowhiskers obtained from a) BC_AY and b) BC_AX (scale bar 2 μm)

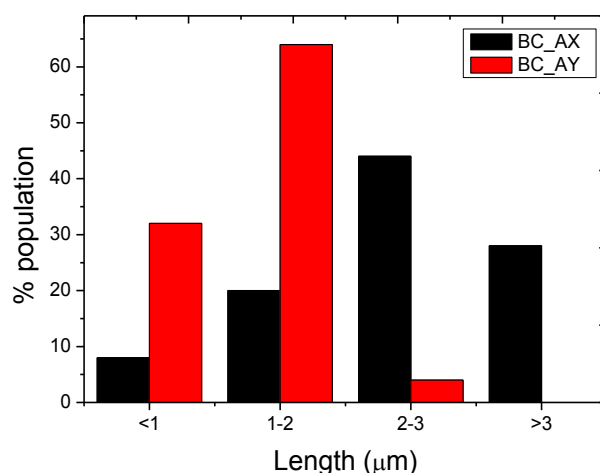


Figure 7.17: Distribution of nanowhiskers length for BC_AX and BC_AY

The SEM images of celluloses obtained from both the strains are shown in Figure 7.18 and the distributions of length segment between branching points derived from the SEM images are presented in Figure 7.19. Similar to the above observation about NdC and BC, the length distributions of nanowhiskers length here are consistent with the trends of the length segment distribution in the un-hydrolysed material. The average unbranched microfibril length of BC_AY and BC_AX are $0.86 \pm 0.12 \mu\text{m}$ and $2.20 \pm 0.65 \mu\text{m}$, respectively, which are in close agreement with the average nanowhisker lengths.

The crystallinities of the cellulose produced by two strains were calculated to be 85% for BC_AX and 67% for BC_AY, from the XRD pattern shown in Figure 7.2. Again a decrease in the length of nanowhiskers was seen with the decrease in crystallinity.

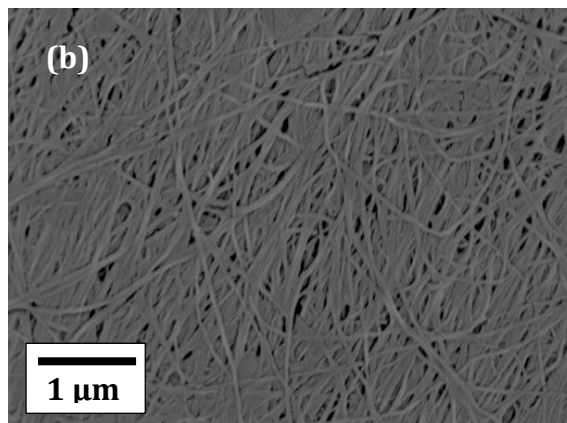
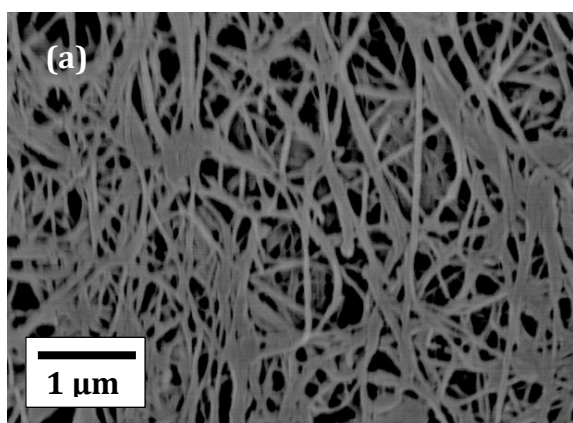


Figure 7.18: SEM image of BC_AY and BC_AX (scale bar 1 μm)

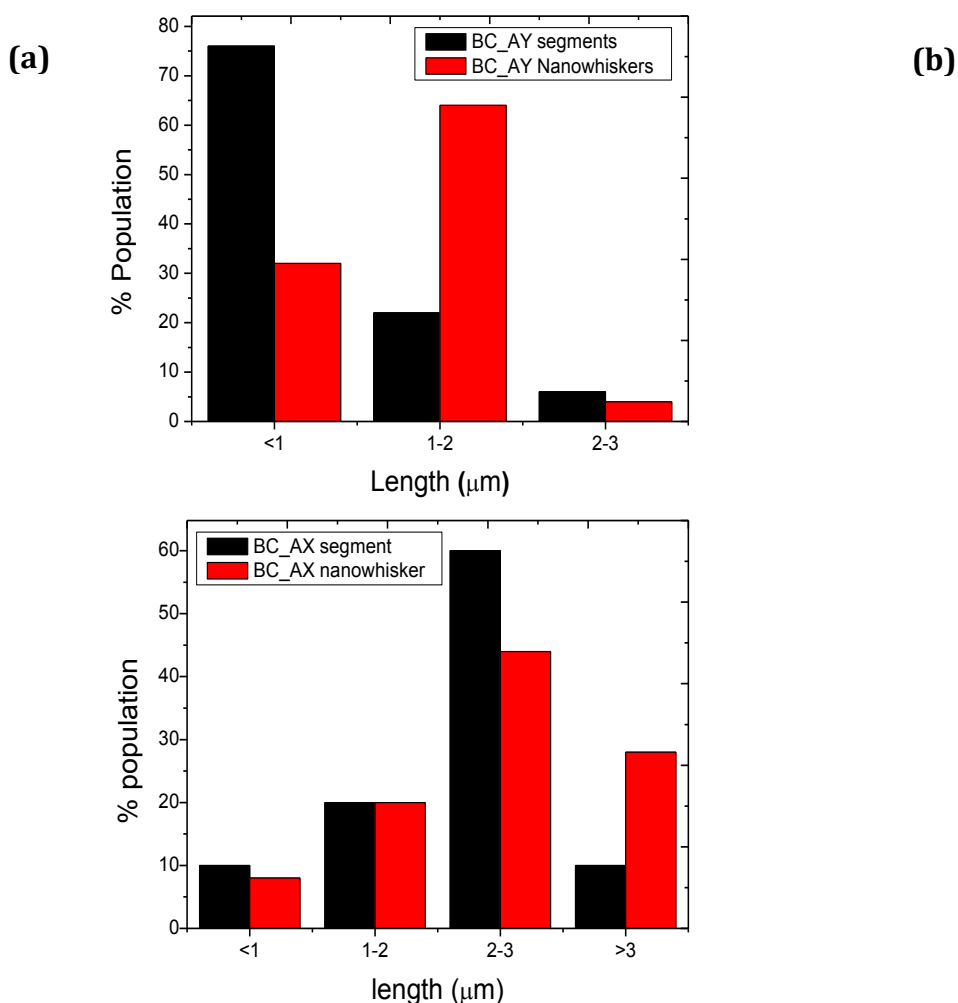


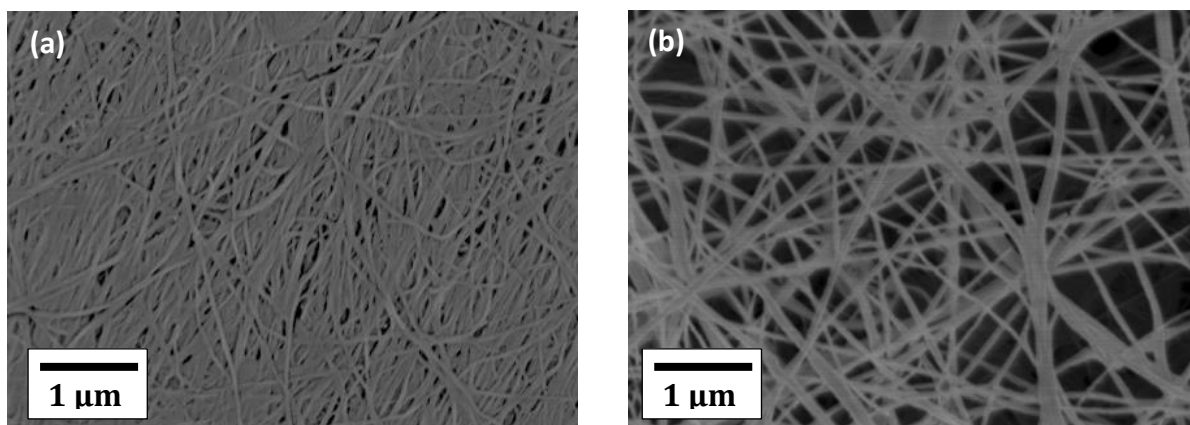
Figure 7.19: Distribution of nanowhiskers length and segments between branching points for a) BC_AY (average nanowhisiker length $1.22 \pm 0.48 \mu\text{m}$ and average microfibril segment length $0.86 \pm 0.12 \mu\text{m}$) b) BC_AX (average nanowhisiker length $2.42 \pm 0.52 \mu\text{m}$ and average microfibril segment length $2.20 \pm 0.65 \mu\text{m}$)

7.4.3 Additives

From the above two studies on three kinds of bacterial cellulose grown with different strains and under different conditions, there is an evidence of correlation between the morphology of the cellulose and the length of nanowhiskers obtained after acid hydrolysis. It was found that the length of nanowhiskers is dependent on the crystallinity and the Kuhn equivalent length segment of microfibrils, that is, the length between bundling or debundling points. As the crystallinity and the spacing between branching points increased, the length of resulting nanowhiskers was found to increase.

On the basis of this observation, in this section, predictions about the nanowhiskers length were made by analysing the microstructure of the parent cellulose material and validated this with the experimental results. In order to obtain different bacterial cellulose morphologies, bacterial cell culture was modified by CMC and CF. CMC and CF penetrate between glucan aggregates synthesised and extruded at closely spaced sites, before they intersect and crystallise into microfibrils. Thus, the crystallinity was found to be less in both the cases in comparison to the unmodified bacterial cellulose.

The SEM images of unmodified bacterial cellulose, BC_CMC and BC_CF are shown in Figure 7.20. The distribution of length segment between branching points is also presented in Figure 7.21, and it is seen that the average length segment for BC_CMC and BC_CF are 1.93 ± 0.3 and 0.94 ± 0.22 μm , which are smaller than that for unmodified BC_AX with average length segment of 2.20 ± 0.65 μm . As far as crystallinity is concerned, the crystallinity of BC_CMC and BC_CF were 72% and 54%, both of which are less than that of the unmodified cellulose (85%). Therefore according to the hypothesis, on hydrolysis with acid, BC_CMC and BC_CF nanowhiskers should be smaller in length than the nanowhiskers from unmodified bacterial cellulose, with BC_CF nanowhisker being the smallest.



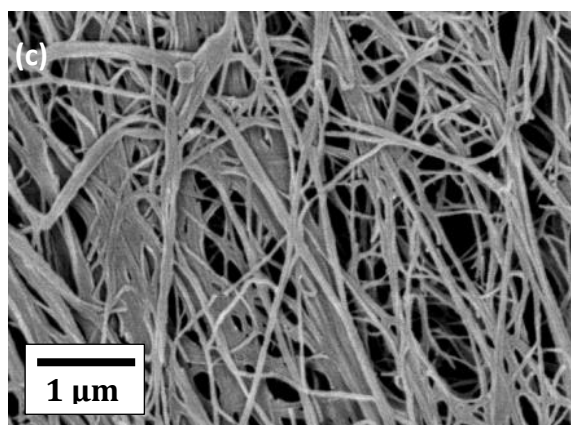


Figure 7.20: SEM images of a) BC_AX, b)BC_CMC and c) BC_CF (scale bar 1 μm)

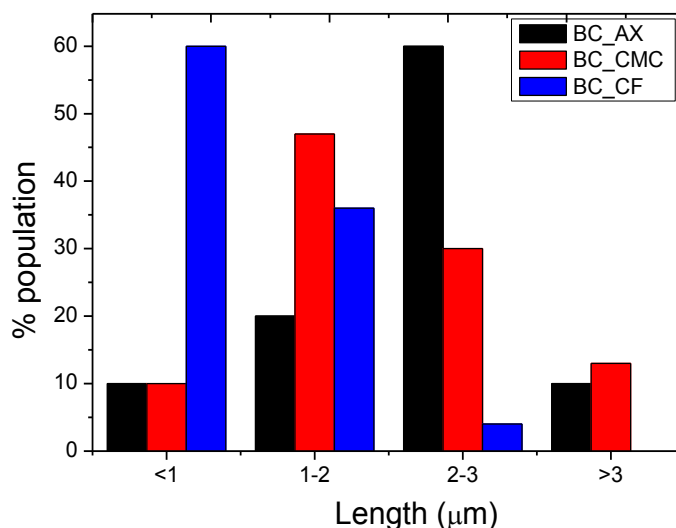
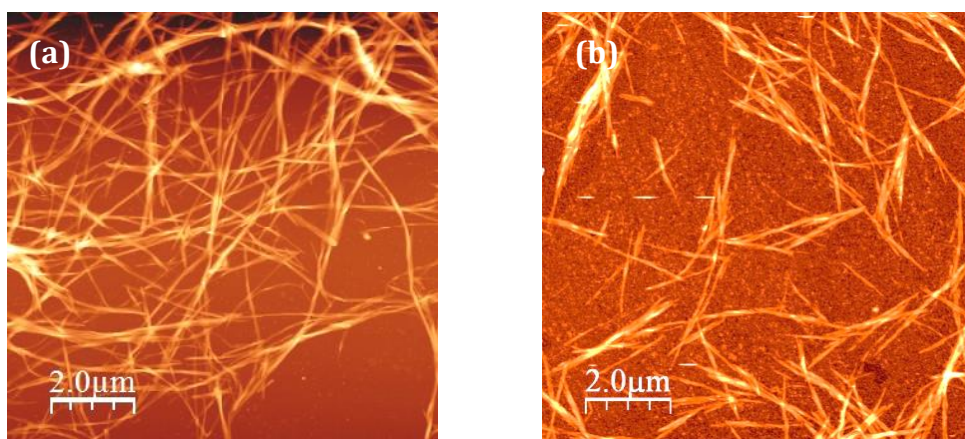


Figure 7.21: Distribution of segment length obtained from hydrolysis of BC_AX, BC_CF and BC_CMC

BC_CMC and BC_CF were hydrolysed to produce nanowhiskers. From AFM images shown in Figure 7.22, the average lengths of nanowhiskers were found to be $1.04 \pm 0.36 \mu\text{m}$ and $0.86 \pm 0.12 \mu\text{m}$ for BC_CMC and BC_CF, respectively. These lengths are smaller than the nanowhiskers from unmodified bacterial cellulose, as predicted on the basis of the hypothesis. The length distribution of nanowhiskers was again found to follow the distribution of length segments as shown in Figure 7.23.

The above examples prove that there is a relation between microstructure (branching pattern), crystallinity and nanowhiskers that are obtained after hydrolysis. The dependence of the nanowhisker length on the crystallinity can be explained as the

lower crystallinity implies higher amount of less dense, more acid prone amorphous regions. As the amount of amorphous material increases, following the widely accepted model of microfibril, where crystalline and amorphous regions are arranged in series, along the length of microfibrils, crystalline regions would be more frequently intercepted by amorphous regions or longer amorphous regions leading to shorter crystalline segments and thus smaller nanowhiskers. However, the high resolution scanning electron microscopy failed to reveal presence of amorphous region. The dependence of nanowhisker length on separation between branching points cannot be completely explained at the moment. However, it may be speculated that the branching points are somehow acid prone regions, which would imply a direct correspondence between branching point separation and nanowhisker length. This would mean the branching or bundling occurs at less crystalline regions. One of the reasons could be the branching points may be a result of intersection of microfibrils during their crystallization which would leads to inclusion of defects or formation of non-crystalline regions. However, this hypothesis warrants further work to completely explain the observation.



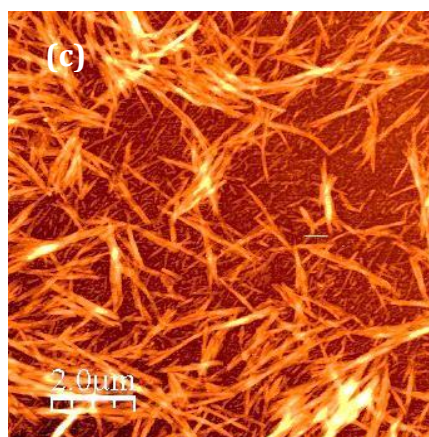


Figure 7.22: AFM image of nanowhisker obtained from a) BC_AX, b) BC_CMC and c) BC_CF

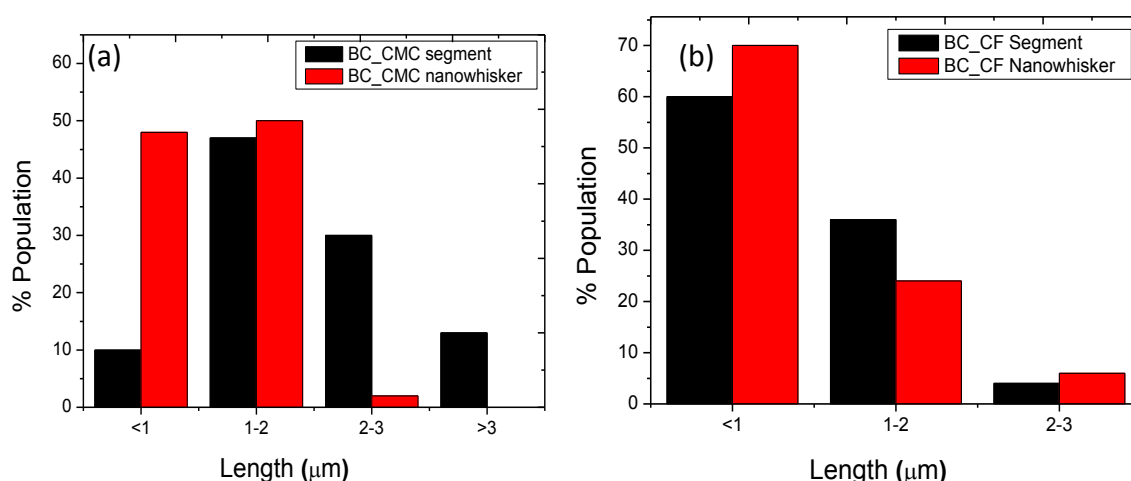


Figure 7.23: Comparison of length segment of microfibrils and length of nanowhiskers for a) BC_AX (average nanowhisker length $1.93 \pm 0.3 \mu\text{m}$ and average microfibril segment length $1.04 \pm 0.36 \mu\text{m}$), and b) BC_CMC (average nanowhisker length $0.94 \pm 0.22 \mu\text{m}$ and average microfibril segment length $0.86 \pm 0.12 \mu\text{m}$)

7.5 Summary

Different cellulose producing bacterial strains have been shown to possess different cellulose pellicle production efficiencies. In this work three strains have been used and the amount produced was found to be the maximum by the strain AX. The strains AX and ATCC formed a tough pellicle of bacterial cellulose while AY did not form a pellicle but just an entangled mass.

Effect of the additives namely CF, CMC, NA and PEG on macro-microstructure of bacterial cellulose has been studied by XRD and SEM. It is seen that crystallite size and

crystallinity were reduced due to interference with cellulose assembly and crystallisation by all additives, except PEG. CF and CMC interfered with the early assembly process and crystallisation. NA interfered with the bacterial cell division leading to elongation of cell and the wider microfibrils. PEG only modified the pore structure of cellulose pellicle, while the crystallite size and microfibrils width remained unchanged.

The correlation between the microstructure of cellulose and the length of nanowhiskers obtained from their acid hydrolysis has been studied in the later part of the chapter. It was found that the length of nanowhiskers varied with the branching pattern of cellulose microfibrils and the overall crystallinity of the material (findings have been summarised in Figure 7.24).

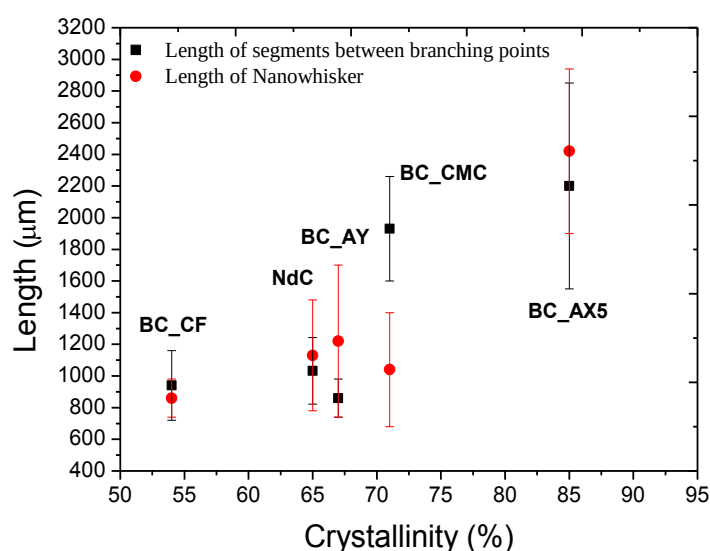


Figure 7.24: Variation in microfibril length segment between branching points and nanowhiskers length with crystallinity of the starting material (BC_AX, BC_AY, NdC, BC_CMC and BC_CF)

As the separation between microfibrils branching point and the crystallinity of the material increased, an increase in the average length of nanowhiskers was also seen. This has been shown in three cases: NdC and BC, BC produced by two strains and BC_CF and BC_CMC. The length distributions of nanowhiskers were found to closely follow the distribution of length of un-branched microfibril segment.

Chapter 8

Conclusions and Future work

This work was intended to harness the potential of cellulose: the most abundant biopolymer. An objective was set out to exploit the combination of the good mechanical properties of cellulose in native state (that can be ascribed to the intrinsic hierarchical architecture) and the possibility of liquid crystalline processing to produce high performance cellulose fibres. This chapter presents the main conclusions of the work and comprehensively elaborates on the new challenges for future work.

8.1 Conclusions

The main objectives of this work have been:

1. Architectural characterisation of cellulose produced by bacteria and tunicates
2. Formation of liquid crystalline phase from bacterial and tunicate cellulose
3. Pilot fibre spinning trials from the liquid crystalline cellulose suspensions
4. Exploratory study into *in-situ* modification of bacterial cellulose to tune micro-macro-structure of cellulose

The main accomplishments in each of these aspects are summarised below.

8.1.1 Architectural characterisation of cellulose

Bacterial cellulose (BC), nata-de-coco (NdC) (food grade commercially available bacterial cellulose) and tunicate cellulose (TC) have been characterised by scanning electron microscopy (SEM), atomic force microscopy (AFM), powder X-ray diffraction (XRD), and small angle X-ray scattering (SAXS) to unfold the hierarchical organisation of the poly-glucan chains. Interpretation of the data obtained by these techniques confirmed the following:

- 1) The cellulose from both the sources were present as long thin uniform microfibrils with cross section dimensions of 10-20 nm by 7-10 nm for TC and 20-30 nm by 6-10 nm for NdC and BC .

- 2) The microstructure of all the cellulose samples is characterised by cellulose microfibrils, which aggregate to variable extent to form ribbons and also give rise to branched morphologies (Figure 4.1-4.5).
- 3) The branching was quantified in terms of the distance between branching points and the average length was found to be the largest for BC with about $1.2 \pm 0.7 \mu\text{m}$ followed by TC and NdC with $1.08 \pm 0.8 \mu\text{m}$ and $0.9 \pm 0.8 \mu\text{m}$, respectively.
- 4) The branching of the microfibrils was concluded to be a result of aggregation of microfibrils produced by two or more different bacteria, as the observations in this work contradicts the theory of bacterial cell division leading to microfibril branching.
- 5) Another interesting concept that arose from SEM was that of a critical lateral size of microfibrils (20-30 nm) which retains a helical twist, possibly originating from the chirality of cellulose chains and beyond this size.
- 6) The microfibrils are semi-crystalline and the crystallinities measured by XRD were 85%, 75% and 65% for BC, TC and NdC, respectively. However, high resolution SEM failed to show any evidence for amorphous regions (section 4.2.3).
- 7) The crystallite sizes were calculated from the XRD peaks and determined to be 5.1, 8.6, and 5.2 nm for NdC, 6.4, 6.6 and 7.8 nm for TC and 6.4, 8.5, and 6.5 nm for BC in the crystallographic directions [1-10], [110] and [200]. These values were used to deduce the crystallite cross section. It was found to be rectangular or square-like with truncated ends (Figure 4.13). However, the broadening due to paracrystallinity could not be calculated and hence these crystallite sizes determined may be an under-estimation of the actual size.
- 8) SAXS data analysis confirmed the microfibril dimension obtained from the SEM and AFM and established that the crystallites were directly organised into microfibrils.
- 9) A bottom-up assembly model has been developed for the formation of bacterial cellulose using previous reports and from the data deduced in this work. Each pore on the bacterial cell surface produces bundles of cellulose chains of 1.5 nm diameter, and the cellulose chains from about 20-30 pores form a crystalline fibril. Subsequently, contribution from about 80 pores associate to form a microfibril. (Figure 4.20)

8.1.2 Liquid crystalline phase formation of cellulose

- 1) The cellulose formed by bacteria or tunicates are in the form of a dilute but tough hydrogels, which comprises of highly entangled microfibrils, that prevents orientation.
- 2) Cellulose nanowhiskers were obtained by acid hydrolysis which formed suspensions in water stabilized by electrostatic repulsion.
- 3) Owing to the geometric anisotropy and intrinsic rigidity of the cellulose nanowhiskers, the formation of a liquid crystalline phase was observed.
- 4) The phase transition diagram from isotropic to liquid crystalline phase has been established for nanowhiskers obtained from NdC, TC and BC, by phase separation studied and polarised optical microscopy. The completion of phase separation was aided by centrifugation.
- 5) The phase transition was characterised by a biphasic region with onset around 0.5 wt% for all the nanowhiskers suspensions and completion of transition at 12 wt%, 10 wt% and 4 wt% for NdC, TC and BC nanowhiskers, respectively.
- 6) The aspect ratio was found to be an important parameter that determines the phase transition - higher the aspect ratio, lower was the transition concentration, which is in agreement with the theoretical predictions.
- 7) This work reported formation of both nematic as well chiral nematic liquid crystalline phases.
- 8) The origin of the chiral interaction between cellulose nanowhiskers has been investigated. Various observations support origin of chirality from the twisted morphology of nanowhiskey, for example, SEM images showing twists in cellulose microfibrils and AFM images showing intertwining and twisting of nanowhiskers. The origin of chiral interaction has been postulated to be from random points of contact between nanowhiskers. This also gives rise to the possibility of contact of chiral surfaces leading to twist in the nanowhiskers. (section 5.5)

8.1.3 Tuning micro-macro structure of bacterial cellulose

- 1) Effect of various additives (polyethylene glycol, carboxy methyl cellulose, calcofluor and nalidixic acid) on the micro-macrostructure of bacterial cellulose has been analysed by SEM and XRD.
- 2) Polyethylene glycol was not found to interfere with the poly-glucan chain assembly and crystallisation while carboxy methyl cellulose and calcofluor interrupted the crystallisation, as indicated by the decrease in crystallinity and the crystallite sizes.
- 3) Carboxy methyl cellulose and calcofluor leads to an overall decrease in the average microfibril diameter, unlike poly ethylene glycol.
- 4) In the presence of antibiotic nalidixic acid, an increase in the microfibril diameter and a decrease in crystallinity and crystallite size were observed.
- 5) It has been shown with the help of these microstructural variations that the length of the nanowhiskers obtained after hydrolysis was dependent on the crystallinity of the parent material and the length of the un-branched microfibril.

8.1.4 Production of novel cellulose fibres

- 1) The celluloses as well as the nanowhiskey suspensions demonstrated a gel-like behaviour. The exceptionally high viscosities and moduli explain the non-spinnability of the cellulose hydrogels.
- 2) Fibres from liquid crystalline suspensions of bacterial cellulose nanowhiskers were spun.
- 3) The fibres retained the native crystal structure and a decent orientation (50-60%) was obtained without any post processing.
- 4) The cross section morphology of the fibres obtained in this work resembled concentric layers of sheets comprising of nanowhiskers.

8.2 New Challenges for Future Work

During the course of this study, some interesting challenges in field of cellulose have been unravelled, some of which are discussed below.

8.2.1 Chirality transfer

The observations of twisted microfibrils, twisted nanowhiskers, untwisting in ribbons, and chiral-nematic phase formations (section 5.2) drive the need to understand their origin and the correlation between them. One of the most accepted hypotheses on the origin of chiral interaction leading to the formation of a chiral-nematic phase is the presence of morphological twist in microfibrils and nanowhiskers [135], evidence to which has been presented in this thesis. Many suggest a sequential transfer of chirality across length scales by relating the chirality precipitated as helical twist in cellulose chains to the development of twist in simulated nanofibrils [197], and further to the twist often seen in trees. Understanding the exact nature of chirality transfer across these length scales is still an unconquered domain of research.

In addition, the nature of interaction between nanowhiskers in a chiral nematic phase needs attention. The question goes back to the basic research on lyotropic liquid crystals. Two new and interesting concepts have been introduced in this thesis: first is the concept of critical cellulose fibril size that can compromise on energy and retain a chiral twist and second is the concept of twist arising from the interaction of chiral surfaces of cellulose nanowhiskers. These concepts need to be further investigated with experiments and computational modelling.

8.2.2 Microfibril branching

The branching of ribbons or aggregation of microfibrils of these leads to a branched morphology as has been discussed in section 4.2.2. The possibility of origin of branching because of bacterial cell division has been contradicted in this work. A control over branching would allow a better control over the orientation of microfibrils which is useful in fibre like applications. One of the starting points would be produce cellulose microfibrils by a dilute bacterial cell population so that long un-branched cellulose microfibrils can be produced. This may be done by maintaining a suitable flow field. A better understanding and control of the branching phenomenon in a macromolecular polymer like cellulose would enhance the understanding in polymer science and provide a better control on the polymer microstructure.

8.2.3 Control of cellulose micro-macrostructure

The manipulation of micro-macro-structure of cellulose can produce material suitable for multifarious applications. For example, control over pore size can provide opportunity to produce efficient membranes or tissue scaffolds [249].

The control may be sought in various aspects such as the orientation and diameter distribution of microfibrils, pore size, compositing and so on. Of the various possible approaches, the simplest is using additives during culture that has been partially explored in this thesis (chapter 7). Other approaches include directing the cellulose producing bacterial cell under the influence of electromagnetic gradient [114] and nanopatterning [113]. Expanding the understanding in this avenue would provide opportunity to tune the cellulose micro-macro-structure according to applications and thus allow better utilisation of cellulose.

8.2.4 X-ray crystallography of cellulose

Although the structure of cellulose has gone multitudinous journey of discoveries, there is still a need for more focussed effort. The quantification of paracrystallinity in cellulose has not gained enough attention. There are some pieces of work where methods to determine the crystallinity index have been studied in details but paracrystallinity determination remains unexplored or inconclusive. The importance of paracrystallinity has been repeatedly emphasised in this work. Another observation reported in this work, is the shift in the peak positions in XRD pattern, especially in the samples obtained by *in situ* modifications, which needs further study.

8.2.5 Improvement of fibres

One of the reasons for the limited performance of fibres produced in this work is the limited length of the nanowhiskers. Solution of this problem can come from a multi-fold approach. Extensive amount of work is needed to choose and optimise treatment for cellulose microfibril to disentangle the microfibrils without compromising on the native crystal structure and length. It may be desirable to develop a strategy to orient microfibrils in their native state, without compromising on their length. This would be advantageous in developing better fibres. A few useful attempts in this direction could be inspired from the use of various *in situ* parameters to influence the orientation of microfibrils. Previous attempts have involved the application of silicone oil or nano-

patterning to obtain orientation in microfibrils [112, 113]. A more controlled sophisticated fibre spinning setup would also ensure better quality.

8.3 Concluding remarks

This report has established the basis for novel processing strategies for fibrous cellulose from bacterial and ascidian (tunicates) origin. Formation of liquid crystalline phases from these celluloses has been established. One of the applications of liquid crystalline phase processing is to produce fibres. The fibres production by liquid crystalline processing of bacterial cellulose has been demonstrated. A way towards developing a rationale to tune the bacterial cellulose micro-macro-structure has been shown by *in situ* modifications. Some new hypotheses have been developed to explain the twists observed in cellulose microfibrils and formation of chiral nematic phase. Some important challenges for future work have been identified, some of which include understanding the transfer of cellulose chirality across different length scales and production of un-branched cellulose microfibrils. Continuing a dedicated research on understanding the structure, properties and efficient utilisation of cellulose would ensure a sustainable technological advancement.

References

1. Brown, *The biosynthesis of cellulose*. Journal of Macromolecular Science, Part A, 1996. **33**(10): p. 1345-1373.
2. St. John Manley, *Molecular morphology of cellulose*. Journal of Polymer Science Part A-2: Polymer Physics, 1971. **9**(6): p. 1025-1059.
3. Diddens, Murphy, Krisch, and Müller, *Anisotropic elastic properties of cellulose measured using inelastic x-ray scattering*. Macromolecules, 2008. **41**(24): p. 9755-9759.
4. Moon, Martini, Nairn, Simonsen, and Youngblood, *Cellulose nanomaterials review: Structure, properties and nanocomposites*. Chemical Society Reviews, 2011. **40**(7): p. 3941-3994.
5. Woodings, *Regenerated cellulose fibres*. 2001: Woodhead Publishing.
6. Northolt, Boerstoeel, Maatman, Huisman, Veurink, and Elzerman, *The structure and properties of cellulose fibres spun from an anisotropic phosphoric acid solution*. Polymer, 2001. **42**(19): p. 8249-8264.
7. Kroon-Batenburg, Kroon, and Northolt, *Chain modulus and intramolecular hydrogen bonding in native and regenerated cellulose fibres*. Polymer communications Guildford, 1986. **27**(10): p. 290-292.
8. Gardner and Blackwell, *The structure of native cellulose*. Biopolymers, 1974. **13**(10): p. 1975-2001.
9. Northolt and De Vries, *Tensile deformation of regenerated and native cellulose fibres*. Die Angewandte Makromolekulare Chemie, 1985. **133**(1): p. 183-203.
10. Nishino, Takano, and Nakamae, *Elastic modulus of the crystalline regions of cellulose polymorphs*. Journal of Polymer Science Part B: Polymer Physics, 1995. **33**(11): p. 1647-1651.
11. Tanner, Fitzgerald, and Phillips, *The kevlar story—an advanced materials case study*. Angewandte Chemie International Edition in English, 1989. **28**(5): p. 649-654.
12. Vollrath and Knight, *Liquid crystalline spinning of spider silk*. Nature, 2001. **410**(6828): p. 541-548.
13. Kimura and Itoh, *Biogenesis and function of cellulose in the tunicates*. Cellulose: Molecular and Structural Biology, 2007: p. 217-236.
14. Iguchi, Yamanaka, and Budhiono, *Bacterial cellulose—a masterpiece of nature's arts*. Journal of Materials Science, 2000. **35**(2): p. 261-270.
15. Haigler, *Biogenesis of cellulose i microfibrils occurs by cell-directed self assembly i acetobacter xulinum*, in *Cellulose and other natural polymer system*, Brown, Editor. 1982, Plenum Press: New York.
16. Haigler, Brown, and Benziman, *Calcofluor white st alters the in vivo assembly of cellulose microfibrils*. Science, 1980. **210**(4472): p. 903-906.
17. Rehm, *Bacterial polymers: Biosynthesis, modifications and applications*. Nat Rev Micro. **8**(8): p. 578-592.
18. Dumitriu, *Polysaccharides: Structural diversity and functional versatility*. 2004: CRC.
19. Thomas, *Man's role in changing the face of the earth*. Chicago, London, 1956: p. 10-13.
20. Hon, *Cellulose: A random walk along its historical path*. Cellulose, 1994. **1**(1): p. 1-25.
21. Engelhardt, *Sources, industrial derivatives and commercial applications of cellulose*. Carbohydr. Eur, 1995. **12**: p. 5-14.
22. Reid and Dryden, *Anselme payen-discoverer of cellulose*. Textile Colorist, 1940. **62**: p. 43-45.
23. Payen, *Comptes rendus de l'académie des sciences*. C. r, 1839. **8**: p. 51.
24. Payen, *Mémoires sur les développements des végétaux*. 1842: Imprimerie royale.
25. Bolton, *The potential of plant fibres as crops*. Outlook on Agriculture, 1995. **24**(2): p. 85-89.
26. Eichhorn, Baillie, Zafeiropoulos, Mwaikambo, Ansell, Dufresne, Entwistle, Herrera-Franco, Escamilla, and Groom, *Review: Current international research into cellulosic fibres and composites*. Journal of Materials Science, 2001. **36**(9): p. 2107-2131.
27. loelovich, *Cellulose as a nanostructured polymer*. BioResources, 2008. **3**.

28. Jonas and Farah, *Production and application of microbial cellulose*. Polymer Degradation and Stability, 1998. **59**(1-3): p. 101-106.
29. Schramm and Hestrin, *Factors affecting production of cellulose at the air/ liquid interface of a culture of acetobacter xylinum*. J Gen Microbiol, 1954. **11**(1): p. 123-129.
30. Schramm and Hestrin, *Synthesis of cellulose by acetobacter xylinum*. 1. *Micromethod for the determination of celluloses*. Biochemical Journal, 1954. **56**(1): p. 163.
31. Hestrin and Schramm, *Synthesis of cellulose by acetobacter xylinum*. 2. *Preparation of freeze-dried cells capable of polymerizing glucose to cellulose*. Biochemical Journal, 1954. **58**(2): p. 345.
32. Schramm, Gromet, and Hestrin, *Synthesis of cellulose by acetobacter xylinum*. 3. *Substrates and inhibitors*. Biochemical Journal, 1957. **67**(4): p. 669.
33. Gromet, Schramm, and Hestrin, *Synthesis of cellulose by acetobacter xylinum*. 4. *Enzyme systems present in a crude extract of glucose-grown cells*. Biochemical Journal, 1957. **67**(4): p. 679.
34. Keshk and Sameshima, *Evaluation of different carbon sources for bacterial cellulose production*. African Journal of Biotechnology, 2005. **4**(6): p. 478-482.
35. Mikkelsen, Flanagan, Dykes, and Gidley, *Influence of different carbon sources on bacterial cellulose production by gluconacetobacter xylinus strain atcc 53524*. Journal of applied microbiology, 2009. **107**(2): p. 576-583.
36. Ramana, Tomar, and Singh, *Effect of various carbon and nitrogen sources on cellulose synthesis by acetobacter xylinum*. World Journal of Microbiology and Biotechnology, 2000. **16**(3): p. 245-248.
37. Krystynowicz, Czaja, Wiktorowska-Jezierska, Gonçalves-Miśkiewicz, Turkiewicz, and Bielecki, *Factors affecting the yield and properties of bacterial cellulose*. Journal of Industrial Microbiology and Biotechnology, 2002. **29**(4): p. 189-195.
38. Shoda and Sugano, *Recent advances in bacterial cellulose production*. Biotechnology and Bioprocess Engineering, 2005. **10**(1): p. 1-8.
39. Kralisch, Hessler, Klemm, Erdmann, and Schmidt, *White biotechnology for cellulose manufacturing—the holir concept*. Biotechnology and bioengineering, 2009. **105**(4): p. 740-747.
40. Saturnino-Dimaguila, *The "nata de coco" 2. Chemical nature and properties of nata*. Philippine agriculturist, Manila, 1967. **51**: p. 475-485.
41. Valera-Peters, *The "nata de coco" 1. Characterization and identity of the causal organism1*. The Philippine agriculturist, 1967. **51**(6-10): p. 462.
42. Richmond, *Occurrence and functions of native cellulose*. Biosynthesis and Biodegradation of Cellulose, 1991: p. 5-23.
43. Kimura, Ohshima, Hirose, Nishikawa, and Itoh, *Cellulose in the house of the appendicularianoikopleura rufescens*. Protoplasma, 2001. **216**(1): p. 71-74.
44. Berrill, *The tunicate*. The Ray Society, London, 1950.
45. Winterstein, *Zur kenntniss der thiercellulose oder des tunicins*. Hoppe-Seyler's Z. physiol. Chem, 1894. **18**: p. 43-56.
46. Iwamoto, Kai, Isogai, and Iwata, *Elastic modulus of single cellulose microfibrils from tunicate measured by atomic force microscopy*. Biomacromolecules, 2009. **10**(9): p. 2571-2576.
47. Štuncová, Davies, and Eichhorn, *Elastic modulus and stress-transfer properties of tunicate cellulose whiskers*. Biomacromolecules, 2005. **6**(2): p. 1055-1061.
48. Yamanaka, Watanabe, Kitamura, Iguchi, Mitsuhashi, Nishi, and Uryu, *The structure and mechanical properties of sheets prepared from bacterial cellulose*. Journal of Materials Science, 1989. **24**(9): p. 3141-3145.
49. Kimura and Itoh, *New cellulose synthesizing complexes (terminal complexes) involved in animal cellulose biosynthesis in the tunicatemetandrocampa uedai*. Protoplasma, 1996. **194**(3): p. 151-163.

50. Samir, Alloin, and Dufresne, *Review of recent research into cellulosic whiskers, their properties and their application in nanocomposite field*. *Biomacromolecules*, 2005. **6**(2): p. 612-626.
51. Haworth, Hirst, and Miller, *Cccxxvi.—the structure of the normal and γ -forms of tetramethyl glucose. Oxidation of tetramethyl δ -and γ -gluconolactones*. *Journal of the Chemical Society (Resumed)*, 1927: p. 2436-2443.
52. Haworth, Long, and Plant, *Ccclxxvi.—the constitution of the disaccharide. Part xvi. Cellobiose*. *J. Chem. Soc.*, 1927: p. 2809-2814.
53. Staudinger, *Die hochmolekularen organischen verbindungen, kautschuk und cellulose*. 1960: Springer.
54. Sugiyama, Vuong, and Chanzy, *Electron diffraction study on the two crystalline phases occurring in native cellulose from an algal cell wall*. *Macromolecules*, 1991. **24**(14): p. 4168-4175.
55. Cheng, Catchmark, and Demirci, *Enhanced production of bacterial cellulose by using a biofilm reactor and its material property analysis*. *J Biol Eng*, 2009. **3**: p. 12.
56. Horii, Hirai, and Yamamoto, *Microstructural analysis of microfibrils of bacterial cellulose*. *Macromolecular Symposia*, 1997. **120**(1): p. 197-205.
57. Manley, *Chain folding in amylose crystals*. *Journal of Polymer Science Part A: General Papers*, 1964. **2**(10): p. 4503-4515.
58. Mühlethaler. *Fine structure of natural polysaccharide systems*. in *Journal of Polymer Science Part C: Polymer Symposia*. 1969: Wiley Online Library.
59. Frey-Wyssling, *The fine structure of cellulose microfibrils*. *Science*, 1954. **119**(3081): p. 80-82.
60. Marx-Figini and Schulz, *Über die kinetik und den mechanismus der biosynthese der cellulose in den höheren pflanzen (nach versuchen an den samenhaaren der baumwolle)*. *Biochimica et Biophysica Acta (BBA)-Biophysics including Photosynthesis*, 1966. **112**(1): p. 81-101.
61. Newcomb and Bonnett Jr, *Cytoplasmic microtubule and wall microfibril orientation in root hairs of radish*. *The Journal of Cell Biology*, 1965. **27**(3): p. 575-589.
62. Heyn, *Small particle x-ray scattering by fibers. II. Radial distribution of microcrystallites*. *Journal of Applied Physics*, 1955. **26**(9): p. 1113-1120.
63. Peterlin and Ingram, *Morphology of secondary wall fibrils in cotton*. *Textile Research Journal*, 1970. **40**(4): p. 345-354.
64. Stöckmann, *Developing a hypothesis: Native cellulose elementary fibrils are formed with metastable structure*. *Biopolymers*, 1972. **11**(1): p. 251-270.
65. Nägeli, Cramer, and Wartmann, *Die stärkekörner: Morphologische, physiologische, chemisch-physikalische und systematisch-botanische monographie*. Vol. 2. 1858: bei F. Schulthess.
66. Seifriz, *The contractility of protoplasm*. *American Naturalist*, 1929: p. 410-434.
67. Meyer, *Räumliche vorstellungen über den bau der kohlenstoffverbindungen und ihre verwendung in der chemie der hochpolymeren*. *Colloid & Polymer Science*, 1930. **53**(1): p. 8-19.
68. Nishikawa and Ono, *Transmission of x-rays through fibrous, lamellar and granular substances*. *Proceedings of the Tokyo Mathematico-Physical Society*, 1913. **7**: p. 131-138.
69. Herzog, Jancke, and Polanyi, *Röntgenspektrographische beobachtungen an zellulose. II*. *Zeitschrift für Physik A Hadrons and Nuclei*, 1920. **3**(5): p. 343-348.
70. Mukherjee and Woods, *X-ray and electron microscope studies of the degradation of cellulose by sulphuric acid*. *Biochimica et Biophysica Acta*, 1953. **10**: p. 499-511.
71. Muhlethaler, *Die feinstruktur der zellulosemikrofibrillen*. *Beih. Z. schweiz. Forstver*, 1960(30): p. 55-64.
72. Rowland and Roberts, *The nature of accessible surfaces in the microstructure of cotton cellulose*. *Journal of Polymer Science Part A-1: Polymer Chemistry*, 1972. **10**(8): p. 2447-2461.

73. Nieduszynski and Preston, *Crystallite size in natural cellulose*. Nature, 1970. **225**(5229): p. 273-274.
74. Battista, *Hydrolysis and crystallization of cellulose*. Industrial & Engineering Chemistry, 1950. **42**(3): p. 502-507.
75. Preston, *The physical biology of plant cell walls*. 1974: London.: Chapman & Hall.
76. Rånby and Ribi, *Über den feibau der zellulose*. Cellular and Molecular Life Sciences, 1950. **6**(1): p. 12-14.
77. Sharples, *The hydrolysis of cellulose and its relation to structure*. Transactions of the Faraday Society, 1957. **53**: p. 1003-1013.
78. Hearle, *A fringed fibril theory of structure in crystalline polymers*. Journal of Polymer Science, 2003. **28**(117): p. 432-435.
79. Nishiyama, Kim, Kim, Katsumata, Roland, and Langan, *Periodic disorder along ramie cellulose microfibrils*. Biomacromolecules, 2003. **4**(4): p. 1013-1017.
80. RÅNby, *Recent progress on the structure and morphology of cellulose*, in *Cellulases and their applications*. 1969, AMERICAN CHEMICAL SOCIETY. p. 139-151.
81. Astley, Chanliaud, Donald, and Gidley, *Structure of acetobacter cellulose composites in the hydrated state*. International Journal of Biological Macromolecules, 2001. **29**(3): p. 193-202.
82. Kataoka and Kondo, *Ft-ir microscopic analysis of changing cellulose crystalline structure during wood cell wall formation*. Macromolecules, 1998. **31**(3): p. 760-764.
83. Hishikawa, Togawa, Kataoka, and Kondo, *Characterization of amorphous domains in cellulosic materials using a ftir deuteration monitoring analysis*. Polymer, 1999. **40**(25): p. 7117-7124.
84. Kondo, Togawa, and Brown Jr, *"Nematic ordered cellulose": A concept of glucan chain association*. Biomacromolecules, 2001. **2**(4): p. 1324-1330.
85. O'Sullivan, *Cellulose: The structure slowly unravels*. Cellulose, 1997. **4**(3): p. 173-207.
86. Roelofsen, *Cell-wall structure as related to surface growth. Some supplementary remarks on multinet growth*. Acta bot. neer, 1958. **7**: p. 77-89.
87. Preston, *Structural plant polysaccharides*. Endeavour, 1964. **23**: p. 153-9.
88. Zimmermann, *The formation of wood in forest trees*. The formation of wood in forest trees., 1964.
89. Brown Jr and Montezinos, *Cellulose microfibrils: Visualization of biosynthetic and orienting complexes in association with the plasma membrane*. Proceedings of the National Academy of Sciences, 1976. **73**(1): p. 143-147.
90. Brown, Willison, and Richardson, *Cellulose biosynthesis in acetobacter xylinum: Visualization of the site of synthesis and direct measurement of the in vivo process*. Proceedings of the National Academy of Sciences, 1976. **73**(12): p. 4565-4569.
91. Ross, Mayer, and Benziman, *Cellulose biosynthesis and function in bacteria*. Microbiol. Mol. Biol. Rev., 1991. **55**(1): p. 35-58.
92. Saxena and Brown, *Cellulose biosynthesis: Current views and evolving concepts*. Annals of Botany, 2005. **96**(1): p. 9-21.
93. Kimura and Itoh, *Cellulose synthesizing terminal complexes in the ascidians*. Cellulose, 2004. **11**(3-4): p. 377-383.
94. Okuda, Tsekos, and Brown, *Cellulose microfibril assembly in erythrocladia subintegra rosenv.: An ideal system for understanding the relationship between synthesizing complexes (tcs) and microfibril crystallization*. Protoplasma, 1994. **180**(1): p. 49-58.
95. Mizuta and Brown, *High resolution analysis of the formation of cellulose synthesizing complexes in vaucheria hamata*. Protoplasma, 1992. **166**(3): p. 187-199.
96. Sugiyama, Harada, Fujiyoshi, and Uyeda, *Lattice images from ultrathin sections of cellulose microfibrils in the cell wall of valonia macrophysa kütz.* Planta, 1985. **166**(2): p. 161-168.
97. Haigler, *The functions and biogenesis of native cellulose*, in *Cellulose chemistry and its applications*. 1985, Ellis Horwood Limited. p. 31-83.

98. Mueller and Brown Jr, *Evidence for an intramembrane component associated with a cellulose microfibril-synthesizing complex in higher plants*. The Journal of Cell Biology, 1980. **84**(2): p. 315-326.
99. Brown Jr and Saxena, *Cellulose biosynthesis: A model for understanding the assembly of biopolymers*. Plant Physiology and Biochemistry, 2000. **38**(1): p. 57-67.
100. Cousins and Brown, *Cellulose i microfibril assembly: Computational molecular mechanics energy analysis favours bonding by van der waals forces as the initial step in crystallization*. Polymer, 1995. **36**(20): p. 3885-3888.
101. Li and Renneckar, *Supramolecular structure characterization of molecularly thin cellulose i nanoparticles*. Biomacromolecules, 2011. **12**(3): p. 650-659.
102. Tischer, Sierakowski, Westfahl Jr, and Tischer, *Nanostructural reorganization of bacterial cellulose by ultrasonic treatment*. Biomacromolecules, 2010. **11**(5): p. 1217-1224.
103. Haigler, White, Brown, and Cooper, *Alteration of in vivo cellulose ribbon assembly by carboxymethylcellulose and other cellulose derivatives*. The Journal of Cell Biology, 1982. **94**(1): p. 64-69.
104. Zaar, *Visualization of pores (export sites) correlated with cellulose production in the envelope of the gram-negative bacterium acetobacter xylinum*. The Journal of Cell Biology, 1979. **80**(3): p. 773-777.
105. Brown, *The mechanism of cellulose biosynthesis by <i>acetobacter acetigenum</i>*. Journal of Polymer Science, 1962. **59**(167): p. 155-169.
106. Yamanaka and Sugiyama, *Structural modification of bacterial cellulose*. Cellulose, 2000. **7**(3): p. 213-225.
107. Quader, *Interruption of cellulose microfibril crystallization*. Naturwissenschaften, 1981. **68**(8): p. 428-429.
108. Cheng, Catchmark, and Demirci, *Effects of cmc addition on bacterial cellulose production in a biofilm reactor and its paper sheets analysis*. Biomacromolecules, 2011. **12**(3): p. 730-736.
109. Hirai, Tsuji, Yamamoto, and Horii, *In situ crystallization of bacterial cellulose iii. Influences of different polymeric additives on the formation of microfibrils as revealed by transmission electron microscopy*. Cellulose, 1998. **5**(3): p. 201-213.
110. Heßler and Klemm, *Alteration of bacterial nanocellulose structure by in situ modification using polyethylene glycol and carbohydrate additives*. Cellulose, 2009. **16**(5): p. 899-910.
111. Park, Kim, Kwon, Hong, and Jin, *Synthesis of bacterial celluloses in multiwalled carbon nanotube-dispersed medium*. Carbohydrate Polymers, 2009. **77**(3): p. 457-463.
112. Putra, Kakugo, Furukawa, and Gong, *Orientated bacterial cellulose culture controlled by liquid substrate of silicone oil with different viscosity and thickness*. Polym. J, 2009. **41**(9): p. 764-770.
113. Putra, Kakugo, Furukawa, Gong, Osada, Uemura, and Yamamoto, *Production of bacterial cellulose with well oriented fibril on pdms substrate*. Polym. J, 2007. **40**(2): p. 137-142.
114. Sano, Rojas, Gatenholm, and Davalos, *Electromagnetically controlled biological assembly of aligned bacterial cellulose nanofibers*. Annals of Biomedical Engineering. **38**(8): p. 2475-2484.
115. Frone, Panaitescu, and Donescu, *Some aspects concerning the isolation of cellulose micro- and nano-fibers*. UPB Buletin Stiintific, Series B: Chemistry and Materials Science, 2011. **73**(2): p. 133-152.
116. Klemm, Heublein, Fink, and Bohn, *Cellulose: Fascinating biopolymer and sustainable raw material*. Angewandte Chemie International Edition, 2005. **44**(22): p. 3358-3393.
117. Bondeson, Mathew, and Oksman, *Optimization of the isolation of nanocrystals from microcrystalline cellulose by acid hydrolysis*. Cellulose, 2006. **13**(2): p. 171-180.
118. Turbak, Snyder, and Sandberg. *Microfibrillated cellulose, a new cellulose product: Properties, uses, and commercial potential*. in J. Appl. Polym. Sci.: Appl. Polym. Symp.:(United States). 1983: ITT Rayonier Inc., Shelton, WA.

119. Saito, Nishiyama, Putaux, Vignon, and Isogai, *Homogeneous suspensions of individualized microfibrils from tempo-catalyzed oxidation of native cellulose*. *Biomacromolecules*, 2006. **7**(6): p. 1687-1691.
120. Hanley, Revol, Godbout, and Gray, *Atomic force microscopy and transmission electron microscopy of cellulose from micrasterias denticulata; evidence for a chiral helical microfibril twist*. *Cellulose*, 1997. **4**(3): p. 209-220.
121. Elazzouzi-Hafraoui, Nishiyama, Putaux, Heux, Dubreuil, and Rochas, *The shape and size distribution of crystalline nanoparticles prepared by acid hydrolysis of native cellulose*. *Biomacromolecules*, 2007. **9**(1): p. 57-65.
122. Beck-Candanedo, Roman, and Gray, *Effect of reaction conditions on the properties and behavior of wood cellulose nanocrystal suspensions*. *Biomacromolecules*, 2005. **6**(2): p. 1048-1054.
123. Bai, Holbery, and Li, *A technique for production of nanocrystalline cellulose with a narrow size distribution*. *Cellulose*, 2009. **16**(3): p. 455-465.
124. Helbert, Nishiyama, Okano, and Sugiyama, *Molecular imaging of halocynthia papillosa cellulose*. *Journal of structural biology*, 1998. **124**(1): p. 42-50.
125. Hanley, Giasson, Revol, and Gray, *Atomic force microscopy of cellulose microfibrils: Comparison with transmission electron microscopy*. *Polymer*, 1992. **33**(21): p. 4639-4642.
126. Tokoh, Takabe, Fujita, and Saiki, *Cellulose synthesized by acetobacter xylinum in the presence of acetyl glucomannan*. *Cellulose*, 1998. **5**(4): p. 249-261.
127. de Souza Lima and Borsali, *Rodlike cellulose microcrystals: Structure, properties, and applications*. *Macromolecular Rapid Communications*, 2004. **25**(7): p. 771-787.
128. Peng, Dhar, Liu, and Tam, *Chemistry and applications of nanocrystalline cellulose and its derivatives: A nanotechnology perspective*. *The Canadian Journal of Chemical Engineering*, 2011. **89**(5): p. 1191-1206.
129. Ranby, *Aqueous colloidal solutions of cellulose micelles*. *Acta Chemica Scandinavica*, 1949. **3**(5): p. 649-650.
130. Nickerson and Habrle, *Cellulose intercrystalline structure*. *Industrial & Engineering Chemistry*, 1947. **39**(11): p. 1507-1512.
131. Dong, Revol, and Gray, *Effect of microcrystallite preparation conditions on the formation of colloid crystals of cellulose*. *Cellulose*, 1998. **5**(1): p. 19-32.
132. Martínez-Sanz, Lopez-Rubio, and Lagaron, *Optimization of the nanofabrication by acid hydrolysis of bacterial cellulose nanowhiskers*. *Carbohydrate Polymers*, 2011. **85**(1): p. 228-236.
133. Eichhorn, *Cellulose nanowhiskers: Promising materials for advanced applications*. *Soft Matter*, 2011. **7**(2): p. 303-315.
134. Elazzouzi-Hafraoui, Putaux, and Heux, *Self-assembling and chiral nematic properties of organophilic cellulose nanocrystals*. *The Journal of Physical Chemistry B*, 2009. **113**(32): p. 11069-11075.
135. Hirai, Inui, Horii, and Tsuji, *Phase separation behavior in aqueous suspensions of bacterial cellulose nanocrystals prepared by sulfuric acid treatment*. *Langmuir*, 2008. **25**(1): p. 497-502.
136. Jiang, Esker, and Roman, *Acid-catalyzed and solvolytic desulfation of h2so4-hydrolyzed cellulose nanocrystals*. *Langmuir*. **26**(23): p. 17919-17925.
137. Habibi, Lucia, and Rojas, *Cellulose nanocrystals: Chemistry, self-assembly, and applications*. *Chemical Reviews*. **110**(6): p. 3479-3500.
138. van den Berg, Capadona, and Weder, *Preparation of homogeneous dispersions of tunicate cellulose whiskers in organic solvents*. *Biomacromolecules*, 2007. **8**(4): p. 1353-1357.
139. Zhao, Kwak, Wang, Franz, White, and Holladay, *Effects of crystallinity on dilute acid hydrolysis of cellulose by cellulose ball-milling study*. *Energy & fuels*, 2006. **20**(2): p. 807-811.

140. Araki, Wada, Kuga, and Okano, *Influence of surface charge on viscosity behavior of cellulose microcrystal suspension*. Journal of Wood Science, 1999. **45**(3): p. 258-261.
141. Araki, Wada, Kuga, and Okano, *Flow properties of microcrystalline cellulose suspension prepared by acid treatment of native cellulose*. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1998. **142**(1): p. 75-82.
142. Angles and Dufresne, *Plasticized starch/tunicin whiskers nanocomposites. 1. Structural analysis*. Macromolecules, 2000. **33**(22): p. 8344-8353.
143. Kimura, Kimura, Tamura, Hirai, Ikuno, and Horii, *Magnetic alignment of the chiral nematic phase of a cellulose microfibril suspension*. Langmuir, 2005. **21**(5): p. 2034-2037.
144. Araki and Kuga, *Effect of trace electrolyte on liquid crystal type of cellulose microcrystals*. Langmuir, 2001. **17**(15): p. 4493-4496.
145. Heux, Chauve, and Bonini, *Nonflocculating and chiral-nematic self-ordering of cellulose microcrystals suspensions in nonpolar solvents*. Langmuir, 2000. **16**(21): p. 8210-8212.
146. Revol, *On the cross-sectional shape of cellulose crystallites in *Valonia ventricosa**. Carbohydrate Polymers, 1982. **2**(2): p. 123-134.
147. Kvien, Tanem, and Oksman, *Characterization of cellulose whiskers and their nanocomposites by atomic force and electron microscopy*. Biomacromolecules, 2005. **6**(6): p. 3160-3165.
148. Fleming, Gray, Prasannan, and Matthews, *Cellulose crystallites: A new and robust liquid crystalline medium for the measurement of residual dipolar couplings*. Journal of the American Chemical Society, 2000. **122**(21): p. 5224-5225.
149. Habibi, Goffin, Schiltz, Duquesne, Dubois, and Dufresne, *Bionanocomposites based on poly(ϵ -caprolactone)-grafted cellulose nanocrystals by ring-opening polymerization*. J. Mater. Chem., 2008. **18**(41): p. 5002-5010.
150. Bras, Dufresne, and Siqueira, *Cellulose whiskers vs. Microfibrils: Influence of the nature of the nanoparticles and its surface functionalization on the thermal and mechanical properties of nanocomposites*. Biomacromolecules, 2009. **10**(2): p. 425-432.
151. Grunert and Winter, *Nanocomposites of cellulose acetate butyrate reinforced with cellulose nanocrystals*. Journal of Polymers and the Environment, 2002. **10**(1): p. 27-30.
152. Saïd Azizi Samir, Alloin, Paillet, and Dufresne, *Tangling effect in fibrillated cellulose reinforced nanocomposites*. Macromolecules, 2004. **37**(11): p. 4313-4316.
153. Eichhorn, Dufresne, Aranguren, Marcovich, Capadona, Rowan, Weder, Thielemans, Roman, and Renneckar, *Review: Current international research into cellulose nanofibres and nanocomposites*. Journal of Materials Science, 2010. **45**(1): p. 1-33.
154. Roman and Winter, *Effect of sulfate groups from sulfuric acid hydrolysis on the thermal degradation behavior of bacterial cellulose*. Biomacromolecules, 2004. **5**(5): p. 1671-1677.
155. Araki, Wada, and Kuga, *Steric stabilization of a cellulose microcrystal suspension by poly(ethylene glycol) grafting*. Langmuir, 2001. **17**(1): p. 21-27.
156. Dong, Kimura, Revol, and Gray, *Effects of ionic strength on the isotropic-chiral nematic phase transition of suspensions of cellulose crystallites*. Langmuir, 1996. **12**(8): p. 2076-2082.
157. Lima, Wong, Paillet, Borsali, and Pecora, *Translational and rotational dynamics of rodlike cellulose whiskers*. Langmuir, 2003. **19**(1): p. 24-29.
158. Miller and Donald, *Imaging of anisotropic cellulose suspensions using environmental scanning electron microscopy*. Biomacromolecules, 2003. **4**(3): p. 510-517.
159. Roohani, Habibi, Belgacem, Ebrahim, Karimi, and Dufresne, *Cellulose whiskers reinforced polyvinyl alcohol copolymers nanocomposites*. European polymer journal, 2008. **44**(8): p. 2489-2498.
160. Li, Zhou, and Zhang, *Structure and properties of the nanocomposite films of chitosan reinforced with cellulose whiskers*. Journal of Polymer Science Part B: Polymer Physics, 2009. **47**(11): p. 1069-1077.

161. Capadona, Shanmuganathan, Trittschuh, Seidel, Rowan, and Weder, *Polymer nanocomposites with nanowhiskers isolated from microcrystalline cellulose*. Biomacromolecules, 2009. **10**(4): p. 712-716.
162. Habibi, Goffin, Schiltz, Duquesne, Dubois, and Dufresne, *Bionanocomposites based on poly(?-caprolactone)-grafted cellulose nanocrystals by ring-opening polymerization*. Journal of Materials Chemistry, 2008. **18**(41): p. 5002-5010.
163. Junior de Menezes, Siqueira, Curvelo, and Dufresne, *Extrusion and characterization of functionalized cellulose whiskers reinforced polyethylene nanocomposites*. Polymer, 2009. **50**(19): p. 4552-4563.
164. Terech, Chazeau, and Cavaille, *A small-angle scattering study of cellulose whiskers in aqueous suspensions*. Macromolecules, 1999. **32**(6): p. 1872-1875.
165. Matsuo, Sawatari, Iwai, and Ozaki, *Effect of orientation distribution and crystallinity on the measurement by x-ray diffraction of the crystal lattice moduli of cellulose i and ii*. Macromolecules, 1990. **23**(13): p. 3266-3275.
166. Guhados, Wan, and Hutter, *Measurement of the elastic modulus of single bacterial cellulose fibers using atomic force microscopy*. Langmuir, 2005. **21**(14): p. 6642-6646.
167. Hsieh, Yano, Nogi, and Eichhorn, *An estimation of the young's modulus of bacterial cellulose filaments*. Cellulose, 2008. **15**(4): p. 507-513.
168. Reiling and Brickmann, *Theoretical investigations on the structure and physical properties of cellulose*. Macromolecular Theory and Simulations, 1995. **4**(4): p. 725-743.
169. Eichhorn and Davies, *Modelling the crystalline deformation of native and regenerated cellulose*. Cellulose, 2006. **13**(3): p. 291-307.
170. Groom, Mott, and Shaler, *Mechanical properties of individual southern pine fibers. Part i. Determination and variability of stress-strain curves with respect to tree height and juvenility*. Wood and fiber science, 2002. **34**(1): p. 14-27.
171. Eichhorn and Young, *The young's modulus of a microcrystalline cellulose*. Cellulose, 2001. **8**(3): p. 197-207.
172. Rusli and Eichhorn, *Determination of the stiffness of cellulose nanowhiskers and the fiber-matrix interface in a nanocomposite using raman spectroscopy*. Applied Physics Letters, 2008. **93**(3): p. 033111-033111-3.
173. Lahiji, Xu, Reifengerger, Raman, Rudie, and Moon, *Atomic force microscopy characterization of cellulose nanocrystals*. Langmuir, 2010. **26**(6): p. 4480-4488.
174. Werbowyj and Gray, *Liquid crystalline structure in aqueous hydroxypropyl cellulose solutions*. Molecular Crystals and Liquid Crystals, 1976. **34**(4): p. 97-103.
175. Tseng, Valente, and Gray, *Cholesteric liquid crystalline phases based on (acetoxypentyl)cellulose*. Macromolecules, 1981. **14**(3): p. 715-719.
176. Bhadani and Gray, *Crosslinked cholesteric network from the acrylic acid ester of (hydroxypropyl)cellulose*. Molecular Crystals and Liquid Crystals, 1984. **102**(8-9): p. 255-260.
177. Giasson, Revol, Gray, and St.-Pierre, *Preparation of chiral nematic gels by radiation cross-linking*. Macromolecules, 1991. **24**(7): p. 1694-1696.
178. Bouligand, *Twisted fibrous arrangements in biological materials and cholesteric mesophases*. Tissue and Cell, 1972. **4**(2): p. 189-217.
179. Willison and Abeysekera, *A liquid crystal containing cellulose in living plant tissue*. Journal of Polymer Science Part C: Polymer Letters, 1988. **26**(2): p. 71-75.
180. Revol, Bradford, Giasson, Marchessault, and Gray, *Helicoidal self-ordering of cellulose microfibrils in aqueous suspension*. International Journal of Biological Macromolecules, 1992. **14**(3): p. 170-172.
181. Dogic and Fraden, *Ordered phases of filamentous viruses*. Current Opinion in Colloid & Interface Science, 2006. **11**(1): p. 47-55.
182. Livolant and Leforestier, *Condensed phases of DNA: Structures and phase transitions*. Progress in Polymer Science, 1996. **21**(6): p. 1115-1164.

183. Song and Windle, *Isotropic-nematic phase transition of dispersions of multiwall carbon nanotubes*. *Macromolecules*, 2005. **38**(14): p. 6181-6188.
184. Dong and Gray, *Effect of counterions on ordered phase formation in suspensions of charged rodlike cellulose crystallites*. *Langmuir*, 1997. **13**(8): p. 2404-2409.
185. Araki, Wada, Kuga, and Okano, *Birefringent glassy phase of a cellulose microcrystal suspension*. *Langmuir*, 2000. **16**(6): p. 2413-2415.
186. Fleming, Gray, and Matthews, *Cellulose crystallites*. *Chemistry – A European Journal*, 2001. **7**(9): p. 1831-1836.
187. Zanchetta, Giavazzi, Nakata, Buscaglia, Cerbino, Clark, and Bellini, *Right-handed double-helix ultrashort DNA yields chiral nematic phases with both right-and left-handed director twist*. *Proceedings of the National Academy of Sciences*, 2010. **107**(41): p. 17497-17502.
188. Grelet and Fraden, *What is the origin of chirality in the cholesteric phase of virus suspensions?* *Physical review letters*, 2003. **90**(19): p. 198302.
189. Tombolato, Ferrarini, and Grelet, *Chiral nematic phase of suspensions of rodlike viruses: Left-handed phase helicity from a right-handed molecular helix*. *Physical review letters*, 2006. **96**(25): p. 258302.
190. Orts, Godbout, Marchessault, and Revol, *Enhanced ordering of liquid crystalline suspensions of cellulose microfibrils: A small angle neutron scattering study*. *Macromolecules*, 1998. **31**(17): p. 5717-5725.
191. Colvin, *Twisting of bundles of bacterial cellulose microfibrils*. *Journal of Polymer Science*, 1961. **49**(152): p. 473-477.
192. Elazzouzi-Hafraoui, Nishiyama, Putaux, Heux, Dubreuil, and Rochas, *The shape and size distribution of crystalline nanoparticles prepared by acid hydrolysis of native cellulose*. *Biomacromolecules*, 2007. **9**(1): p. 57-65.
193. Hanley, REVOL, GODBOUT, and Gray, *Atomic force microscopy and transmission electron microscopy of cellulose from *micrasterias denticulata*; evidence for a chiral helical microfibril twist*. *Cellulose*, 1997. **4**(3): p. 209-220.
194. Haigler and Chanzy, *Electron diffraction analysis of the altered cellulose synthesized by *acetobacter xylinum* in the presence of fluorescent brightening agents and direct dyes*. *Journal of ultrastructure and molecular structure research*, 1988. **98**(3): p. 299-311.
195. Ritcey and Gray, *Induced cd provides evidence for helical solution conformation in cellulosic chains*. *Biopolymers*, 1988. **27**(3): p. 479-491.
196. Viswanathan and Shenouda, *The helical structure of cellulose i*. *Journal of Applied Polymer Science*, 1971. **15**(3): p. 519-535.
197. Paavilainen, Róg, and Vattulainen, *Analysis of twisting of cellulose nanofibrils in atomistic molecular dynamics simulations*. *The Journal of Physical Chemistry B*, 2011. **115**(14): p. 3747-3755.
198. Matthews, Skopec, Mason, Zuccato, Torget, Sugiyama, Himmel, and Brady, *Computer simulation studies of microcrystalline cellulose β* . *Carbohydr Res*, 2006. **341**(1): p. 138-52.
199. Bowling, Amano, Lindstrom, and Brown, *Rotation of cellulose ribbons during degradation with fungal cellulase*. *Cellulose*, 2001. **8**(1): p. 91-97.
200. Godinho, Canejo, Pinto, Borges, and Teixeira, *How to mimic the shapes of plant tendrils on the nano and microscale: Spirals and helices of electrospun liquid crystalline cellulose derivatives*. *Soft Matter*, 2009. **5**(14): p. 2772-2776.
201. Gray, *Chiral nematic ordering of polysaccharides*. *Carbohydrate Polymers*, 1994. **25**(4): p. 277-284.
202. Ruben, Bokelman, and Krakow. *Triple-stranded left-hand helical cellulose microfibril in *acetobacter xylinum* and in tobacco primary cell wall*. in *Plant Cell Wall. Polymers-Biogenesis and Biodegradation*. ACS Symposium Series. 1989: ACS Publications.
203. Majoinen, Kontturi, Ikkala, and Gray, *Sem imaging of chiral nematic films cast from cellulose nanocrystal suspensions*. *Cellulose*, 2012: p. 1-7.

204. Pan, Hamad, and Straus, *Parameters affecting the chiral nematic phase of nanocrystalline cellulose films*. *Macromolecules*, 2010. **43**(8): p. 3851-3858.
205. Roman and Gray, *Parabolic focal conics in self-assembled solid films of cellulose nanocrystals*. *Langmuir*, 2005. **21**(12): p. 5555-5561.
206. Godbout and Gray, *Solidified liquid crystals of cellulose with optically variable properties*. 1997, Google Patents.
207. Favier, Chanzy, and Cavaille, *Polymer nanocomposites reinforced by cellulose whiskers*. *Macromolecules*, 1995. **28**(18): p. 6365-6367.
208. Roman, Dong, Hirani, and Lee. *Cellulose nanocrystals for drug delivery*. in *ACS Symposium Series*. 2009: ACS Publications.
209. Dong and Roman, *Fluorescently labeled cellulose nanocrystals for bioimaging applications*. *Journal of the American Chemical Society*, 2007. **129**(45): p. 13810-13811.
210. Capadona, Shanmuganathan, Tyler, Rowan, and Weder, *Stimuli-responsive polymer nanocomposites inspired by the sea cucumber dermis*. *Science*, 2008. **319**(5868): p. 1370-1374.
211. Nogi and Yano, *Transparent nanocomposites based on cellulose produced by bacteria offer potential innovation in the electronics device industry*. *Advanced Materials*, 2008. **20**(10): p. 1849-1852.
212. Segal, Creely, Martin, and Conrad, *An empirical method for estimating the degree of crystallinity of native cellulose using the x-ray diffractometer*. *Textile Research Journal*, 1959. **29**(10): p. 786-794.
213. Thygesen, Oddershede, Lilholt, Thomsen, and Ståhl, *On the determination of crystallinity and cellulose content in plant fibres*. *Cellulose*, 2005. **12**(6): p. 563-576.
214. Patterson, *The scherrer formula for x-ray particle size determination*. *Physical Review*, 1939. **56**(10): p. 978.
215. Revol, Dietrich, and Goring, *Effect of mercerization on the crystallite size and crystallinity index in cellulose from different sources*. *Canadian Journal of Chemistry*, 1987. **65**(8): p. 1724-1725.
216. Satyamurthy, Jain, Balasubramanya, and Vigneshwaran, *Preparation and characterization of cellulose nanowhiskers from cotton fibres by controlled microbial hydrolysis*. *Carbohydrate Polymers*, 2011. **83**(1): p. 122-129.
217. Holt, Stoyanov, Pelan, and Paunov, *Novel anisotropic materials from functionalised colloidal cellulose and cellulose derivatives*. *Journal of Materials Chemistry*, 2010. **20**(45): p. 10058-10070.
218. Watanabe, Tabuchi, Morinaga, and Yoshinaga, *Structural features and properties of bacterial cellulose produced in agitated culture*. *Cellulose*, 1998. **5**(3): p. 187-200.
219. Van Daele, Revol, Gaill, and Goffinet, *Characterization and supramolecular architecture of the cellulose-protein fibrils in the tunic of the sea peach (< i> halocynthia papillosa</i>, ascidiacea, urochordata)*. *Biology of the Cell*, 1992. **76**: p. 87-96.
220. Jakob, Fengel, Tschegg, and Fratzl, *The elementary cellulose fibril in picea abies: Comparison of transmission electron microscopy, small-angle x-ray scattering, and wide-angle x-ray scattering results*. *Macromolecules*, 1995. **28**(26): p. 8782-8787.
221. Chen, Cho, and Jin, *Modification and applications of bacterial celluloses in polymer science*. *Macromolecular Research*. **18**(4): p. 309-320.
222. Tsekos, *The supramolecular organization of red algal cell membranes and their participation in the biosynthesis and secretion of extracellular polysaccharides: A review*. *Protoplasma*, 1996. **193**(1): p. 10-32.
223. Colvin and Dennis, *The shape of the tips of growing bacterial cellulose microfibrils and its relation to the mechanism of cellulose biosynthesis*. *Canadian Journal of Microbiology*, 1964. **10**(5): p. 763-767.

224. Focher, Palma, Canetti, Torri, Cosentino, and Gastaldi, *Structural differences between non-wood plant celluloses: Evidence from solid state nmr, vibrational spectroscopy and x-ray diffractometry*. Industrial Crops and Products, 2001. **13**(3): p. 193-208.
225. Iwamoto, Nakagaito, and Yano, *Nano-fibrillation of pulp fibers for the processing of transparent nanocomposites*. Applied Physics A, 2007. **89**(2): p. 461-466.
226. Eichhorn, Sirichaisit, and Young, *Deformation mechanisms in cellulose fibres, paper and wood*. Journal of Materials Science, 2001. **36**(13): p. 3129-3135.
227. Sakurada, Ito, and Nakamae, *Elastic moduli of polymer crystals for the chain axial direction*. Die Makromolekulare Chemie, 1964. **75**(1): p. 1-10.
228. Wagner, Raman, and Moon, *Transverse elasticity of cellulose nanocrystals via atomic force microscopy*. Cellulose, 2009. **7**: p. 27.
229. Chanzy, Nawrot, Peguy, Smith, and Chevalier, *Phase behavior of the quasiternary system n-methylmorpholine-n-oxide, water, and cellulose*. Journal of Polymer Science: Polymer Physics Edition, 2003. **20**(10): p. 1909-1924.
230. Kalashnik, Papkov, Rudinskaya, and Milkova, *Liquid crystal state of cellulose*. Polymer science USSR, 1991. **33**(1): p. 107-112.
231. Flory, *Phase equilibria in solutions of rod-like particles*. Proceedings of the Royal Society of London. Series A. Mathematical and Physical Sciences, 1956. **234**(1196): p. 73-89.
232. Ureña-Benavides, Ao, Davis, and Kitchens, *Rheology and phase behavior of lyotropic cellulose nanocrystal suspensions*. Macromolecules, 2011. **44**(22): p. 8990-8998.
233. Onsager, *The effects of shape on the interaction of colloidal particles*. Annals of the New York Academy of Sciences, 2006. **51**(4): p. 627-659.
234. Ballauff, *Liquid crystalline polymers*. By a. M. Donald and a. H. Windle cambridge university press, cambridge 1992, 310 pp., hardcover, \$ 100, isbn 0-521 -30666-3. Advanced Materials, 1992. **4**(10): p. 695-696.
235. Finkelzeller, *Liquid crystals. Nature's delicate phase of matter*. By p. J. Collings, adam hilger, bristol 1990, handbook, £ 25, 222 pp., isbn 0-7503-0054x. Advanced Materials, 1991. **3**(10): p. 518-519.
236. Zhang, Kinloch, and Windle, *Mesogenicity drives fractionation in lyotropic aqueous suspensions of multiwall carbon nanotubes*. Nano letters, 2006. **6**(3): p. 568-572.
237. Mazeau, *On the external morphology of native cellulose microfibrils*. Carbohydrate Polymers. **84**(1): p. 524-532.
238. Tatsumi, Ishioka, and Matsumoto, *Effect of fiber concentration and axial ratio on the rheological properties of cellulose fiber suspensions*. 日本レオロジー学会誌, 2002. **30**(1): p. 27-32.
239. Liu, Chen, Yue, Chen, and Wu, *Structure and rheology of nanocrystalline cellulose*. Carbohydrate Polymers, 2011. **84**(1): p. 316-322.
240. Iwamoto, Isogai, and Iwata, *Structure and mechanical properties of wet-spun fibers made from natural cellulose nanofibers*. Biomacromolecules, 2011. **12**(3): p. 831-836.
241. Walther, Timonen, Díez, Laukkanen, and Ikkala, *Multifunctional high-performance biofibers based on wet-extrusion of renewable native cellulose nanofibrils*. Advanced Materials, 2011. **23**(26): p. 2924-2928.
242. Srinivasan and Reneker, *Structure and morphology of small diameter electrospun aramid fibers*. Polymer International, 2003. **36**(2): p. 195-201.
243. Picken, *Applications of liquid crystal polymers: <i>part 1: Fibre spinning</i>*. Liquid Crystals Today, 1996. **6**(1): p. 12 - 15.
244. Chung, *The recent developments of thermotropic liquid crystalline polymers*. Polymer Engineering & Science, 2004. **26**(13): p. 901-919.
245. Gindl, Reifferscheid, Adusumalli, Weber, Röder, Sixta, and Schöberl, *Anisotropy of the modulus of elasticity in regenerated cellulose fibres related to molecular orientation*. Polymer, 2008. **49**(3): p. 792-799.

- 246. Röder, Moosbauer, Kliba, Schlader, Zuckerstätter, and Sixta, *Comparative characterisation of man-made regenerated cellulose fibres*. Lenzinger Berichte, 2009. **87**: p. 98-105.
- 247. Cai, Zhang, Zhou, Qi, Chen, Kondo, Chen, and Chu, *Multifilament fibers based on dissolution of cellulose in naoh/urea aqueous solution: Structure and properties*. Advanced Materials, 2007. **19**(6): p. 821-825.
- 248. Sehaqui, Ezekiel Mushi, Morimune, Salajkova, Nishino, and Berglund, *Cellulose nanofiber orientation in nanopaper and nanocomposites by cold drawing*. ACS applied materials & interfaces, 2012. **4**(2): p. 1043-1049.
- 249. Klemm, Schumann, Kramer, Heßler, Koth, and Sultanova, *Nanocellulose materials – different cellulose, different functionality*. Macromolecular Symposia, 2009. **280**(1): p. 60-71.
- 250. Herth, *Calcofluor white and congo red inhibit chitin microfibril assembly of poterioochromonas: Evidence for a gap between polymerization and microfibril formation*. The Journal of Cell Biology, 1980. **87**(2): p. 442-450.
- 251. ELORZA, RICO, and SENTANDREU, *Calcofluor white alters the assembly of chitin fibrils in saccharomyces cerevisiae and candida albicans cells*. Journal of general microbiology, 1983. **129**(5): p. 1577-1582.