

Sources and Isolation Methods for Nanocellulose Materials

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Nanocelluloses (NCs) are appealing nanomaterials that have experienced rapid development, with great potential in the biomedical field. This trend aligns with the increasing demand for sustainable materials, which will contribute both to an improvement in wellbeing and an extension of human life, and with the demand to keep up with advances in medical technology.

- nanocellulose
- biological properties
- tissue engineering
- drug delivery
- wound dressing

1. Introduction

Nanocelluloses (NCs) represent a unique category of engineering materials made from cellulose and are considered one of the most promising green resources of today due to their particular properties [\[1\]](#). NCs are attractive for a wide range of applications, including biomedical engineering (e.g., medical implants, wound healing, skin sensors) [\[2\]](#), smart packaging materials [\[3\]](#), environmental remediation (e.g., water and air filtration membranes, photocatalysis, flocculation) [\[4\]](#) or energy harvesting and storage (e.g., fuel cell membranes, stretchable and flexible electrodes) [\[5\]](#). Widespread interest in NCs-based materials in medical applications has been centered on their availability, sustainability, low cost, biodegradability, biocompatibility, outstanding mechanical properties and low cytotoxicity [\[6\]](#).

NCs include all cellulose-based particles (i.e., at least one dimension in tens of nanometers) having various shapes, sizes, surface chemistries and properties [\[7\]](#). Considering their sizes and functions, which in turn depend on the source and processing conditions, NCs can be classified into three main subcategories (**Table 1**): short, rigid cellulose nanocrystals (CNCs); long flexible cellulose nanofibrils (CNFs); and highly crystalline pure bacterial nanocellulose (BNC) [\[8\]\[9\]\[10\]\[11\]](#).

Table 1. Sources, preparation techniques and special characteristics of NCs.

Type	Sources	Preparation Methods	Special Characteristics	References
CNCs	Wood, cotton, hemp, wheat straw, tunicin, algae, bacteria	Acid hydrolysis	Short, rigid nanocrystals; woods (W/L): 5–40 nm/100–300 nm;	[12][13][14][15] [16][17][18]

Type	Sources	Preparation Methods	Special Characteristics	References
			non-woods (W/L):	
			7–25 nm/84–800 nm (cotton, wheat);	
			12–21 nm/107–215 nm (ramie);	
			25–30 nm/300–400 nm (BNC)	
CNFs	Wood, sugar, beet, potato tuber, hemp, flax	Delamination	Long, flexible nanofibers	[11] [19] [20] [21] [22]
		before/after chemical	with significant amorphous content;	
		or enzymatic treatment	web-like structures; W/L: 20–100 nm/several μm.	
BNC	Low-molecular-weight sugars and alcohols	Bacterial synthesis	Highly crystalline 3D network, high purity;	[20] [23] [24] [25]
			aggregated into nanofibrillar bundles;	
			W = 50–150 nm; Static fermentation: uniaxially oriented ribbons; agitated fermentation: disordered, overlapping ribbon-like morphology.	

These three types of NCs resemble each other by having a relatively close chemical compositions, but they clearly differ in particle size, degree of crystallinity and morphological properties [\[26\]](#). These specific characteristics, which distinguish them from one another, crucially depend on the source of origin and the processing technique of the materials. Thus, considering the essential importance of these two parameters, they are presented below, detailed for each type of nanocellulose.

2. Sources for Nanocellulose Materials

One of the main parameters that impact the special characteristics of cellulose nanomaterials, presented in **Table 1**, is their original source [\[27\]](#)[\[28\]](#). There are four main sources of nanocellulose: plants (trees, shrubs and herbs),

bacterial species (*Acetobacter*, *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium* or *Sarcina*), algae (*Phaeophyta*, *Chlorophyta*, *Rhodophyta*, etc.) and animals (Tunicata) [29].

Cellulose nanocrystals (CNCs), also referred to as nanocrystalline cellulose, cellulose nanowhiskers or cellulose crystallites, are extracted from wood or non-woody biomass (agricultural residues and annual plants) by chemically removing (i.e., acid hydrolysis) the lignin, hemicellulose and the amorphous regions of cellulose [30]. Compared to wood, the non-woody biomass contains low amounts of lignin and hemicelluloses, which allows easier access to the cellulose without the use of severe chemical treatments. Another source of CNCs extraction is industrial biowaste, a cheap source with low or even negative costs, which can be a solution to the current environmental problems regarding their elimination from industries [28]. Tunicates are considered the only animal source of nanocellulose, and these are synthesized by some specific enzyme complexes from the plasma membrane of epidermal cells [22]. Tunicate cellulose aggregates are composed of nearly pure cellulose I β allomorph [31], and after acid hydrolysis, these yield long nanoparticles with a high aspect ratio, high crystallinity and good mechanical strength [32][33].

Cellulose nanofibrils (CNFs), also known as microfibrillated cellulose, microfibrils or nanofibrillated cellulose, are extracted from wood, sugar beet, potato tuber, hemp, or flax by delamination of the pulp through mechanical treatments (under high pressure), usually after enzymatic prehydrolysis or chemical treatments [10]. CNFs can be biosynthesized in small quantities by brown (*Phaeophyta*), green (*Chlorophyta*), red (*Rhodophyta*), blue–green (*Cynophyta*) or golden algae (*Ochromophyta*) [34][35][36][37], and their structure varies depending on the algae specie. Algae have the advantage of growing faster than terrestrial counterparts, and in addition, they have a low lignin content, which represents an advantage. Besides this, algae are obtained in large quantities as waste from agar production; thus, they can be considered an alternative source for the production of nanocellulose to meet future demands [9].

Bacterial nanocellulose (BNC), also known as microbial cellulose or biocellulose, is a pure component of the biofilm resulting from the activity of aerobic bacteria, such as those belonging to the genus *Gluconacetobacter* [38].

3. Isolation Methods

Different isolation procedures are used to obtain NCs, depending on their source. These procedures are presented in detail below for each type of nanocellulose discussed: CNCs, CNFs and BNC.

3.1. CNCs

NCs in nanocrystalline form are one of the most studied types of nanocellulose in term of production methods and their properties [29]. The common method of separating CNCs is controlled hydrolysis of cellulose using mineral acids [12][39][40]. However, this method is part of a multi-step procedure that begins with alkali and bleaching pretreatments and is followed by acid hydrolysis, washing, centrifugation, dialysis, and ultrasonication to form a suspension that may be further subjected to lyophilization [19]. The isolation of CNCs involves turning large pieces

of starting material into fine nanoparticles. Thus, at the macroscopic or microscopic level, a transverse cleavage occurs along the amorphous regions of the cellulose, resulting in a rod-like material, as shown in **Figure 1** [40][41].



Figure 1. Scheme for obtaining CNCs by acid hydrolysis of cellulose, with the removal of the amorphous region and leaving only the crystalline region.

The main sources of CNCs and preparation conditions are presented in **Table 2**. The isolation of CNCs involves the use of various acids such as sulfuric, hydrochloric, hydrobromic and phosphoric acid, but of these, sulfuric acid is overwhelmingly the most used [29][42][43][44]. The use of different acids produces CNCs with different properties, among which is notably the ability to disperse in aqueous medium, rheological behavior, morphology or crystallinity degree [40]. Besides acid type, the reaction time is also an important parameter with great influence on the crystallinity degree. The longer the contact time, the greater the removal of the amorphous regions within the sample. However, a long contact time can degrade cellulose or even break it down into its precursor sugars. Considering the fact that short reaction times are not enough to obtain nanocrystals, it is obvious that it is necessary to find a balance in order to obtain the final product with the desired properties [39][43].

In the past few years, new routes have emerged regarding CNCs preparation, namely esterification using concentrated organic acids [36], periodate oxidation [13], TEMPO-mediated oxidation [45], reductive amination [46], and microbial or enzymatic hydrolysis [31][47]. The preparation conditions using these types of reactants are also presented in **Table 2**.

Table 2. CNCs sources as well as the preparation techniques and conditions.

Source	Preparation Technique	Preparation Conditions	References
Bleached hardwood pulp	Acid hydrolysis	75% PTA/90 °C/30 h	[12]
Black spruce	Periodate oxidation	NaIO ₄ /room T/105 rpm/96 h	[13]
Whatman ashless filter paper	Acid hydrolysis	85% H ₃ PO ₄ /50 min/100 °C	[44]
	Acid hydrolysis	64 wt% H ₂ SO ₄ /45 °C/45 min	[14]

Source	Preparation Technique	Preparation Conditions	References
Tunicates	Acid hydrolysis	2.5 M HCl/70 °C/2 h	[15]
	TEMPO-oxidation	TEMPO/NaClO	
	Enzymatic hydrolysis	Novozym 476 (20 FPU/g); 50 °C/2 h	[31]
	TEMPO-oxidation	TEMPO/NaBr/NaClO	
Red algae	Acid hydrolysis	55 wt% H ₂ SO ₄ /60 °C/20 min	[36]
	Acid hydrolysis	64 wt% H ₂ SO ₄ /45 °C/45 min	
Barley straw	Acid hydrolysis	64% H ₂ SO ₄ /50 °C/75 min	[16]
Ramie fibers	Acid hydrolysis	16 M H ₃ PO ₄ /150 °C/90 min	[48]
		41–50% H ₂ SO ₄ /45 °C/30 min	[17]
Bacterial cellulose	Acid hydrolysis	50% H ₂ SO ₄ /50 °C/40 min	[18]

Abbreviations: H₃PO₄—Phosphotungstic acid; Na₂O₄—Sodium (meta) periodate; H₃PO₄—Phosphonic acid; H₂SO₄—Sulfuric acid; TEMPO—(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl.

3.2. CNFs

CNFs are the second type of nanocellulose and have a web-like network structure. These are commonly derivatives from natural sources such as wood pulp by mechanical processes (high-pressure homogenization, grinding and refining) preceded or followed by chemical or enzymatic treatments [28][41]. Therefore, the process of obtaining CNFs results in nanofibrils built up by alternating amorphous and crystalline regions (Figure 2) whose cross-sections measure from tens to several hundreds of nanometers, while their length can reach several micrometers [30][39][49].



Figure 2. Scheme for obtaining CNFs through a mechanical process of cleaving the cellulose fiber to the nanometric size.

High-pressure homogenization (HPH) is the most widely used technique for both the laboratory and industrial-scale production of CNFs. However, other strategies are also applied, such as micro-fluidization, micro-grinding, cryo-crushing and ultrasonication [22][30][40]. In addition, different pre-treatments can be utilized before mechanical processes in order to reduce energy consumption as well as to make the surface hydrophobic, such as TEMPO oxidation [50][51], acetylation [52], carboxymethylation [53], alkali pretreatment [54] and enzymatic pretreatment [55]. For instance, an environmentally friendly process of preparing CNFs was reported by Mhlongo and coworkers [56] using industrial hemp (*Cannabis sativa* L.) bast fibers. The process combines the acid hydrolysis treatment with ultrasonication. Besides the fact that low-cost and sustainable industrial waste fibers are used, the obtained CNFs have superior crystallinity and thermal stability.

CNFs were first isolated in 1983 by Turbak and coworkers from bleached softwood fibers using high-pressure homogenization [57]. Additionally, cellulose nanofibers have been obtained from pear [58], *Helicteresisora* plant [59], oil palm tree [60], banana [61][62][63][64], *Citrullus colocynthis* seeds [65], cassava peel [66]; hemp [67][68], kenaf [69], wheat straw [70], bagasse [55][69], carrots [71], etc. The main sources of CNFs and their preparation conditions are presented in **Table 3**.

Table 3. CNFs sources, preparation techniques and conditions.

Source	Preparation Technique	Preparation Conditions	References
Bagasse	a. Enzyme pretreatment	a. Novozymes endoglucanase/50 °C/12 h	[55]
	b. Mechanical grinding	b. Ultrafine grinder, 10–15 J/1500 rpm	
Cassava roots	a. Alkaline treatment	a. 5% KOH/25 °C/14 h	[66]
	b. Acid hydrolysis	b. 30% SA/90 min/60 °C	
	a. Alkaline treatment	a. 5% KOH/25 °C/14 h	

Source	Preparation Technique	Preparation Conditions	References
	b. TEMPO-oxidation	b. TEMPO/NaBr/NaClO	
	a. Alkali/bleaching treatment	a. 2 wt/V% NaOH/50 °C/3 h; NaClO	[67]
	b. Acid hydrolysis	b. 64% wt/wt SA/45 °C/30 min	
Waste hemp	a. Alkali/bleaching treatment	a. 4 wt% NaOH/80 °C/2 h; 1.7 wt% NaClO ₂ /ABS (pH 4.8)/1 h/100 °C;	[56]
	b. Acid hydrolysis	b. 45%, 64% SA, FA, MA/60, 90 min/45°, 65 °C;	
	c. Ultrasonication	c. 4 min/low speed.	
Kenaf	a. Formic acid/acetic acid;	a. 85% FA/AA/110 °C/2 h;	[70]
	b. Peroxyformic acid/ peroxyacetic acid	b. 35% H ₂ O ₂ with 85% FA/AA/80 °C/2 h;	
Wheat straw	c. Bleaching treatment	c. 35% H ₂ O ₂ /NaOH/80 °C/2 h;	
	d. Ball milling	d. 30, 60, 90, 120 min.	
Carrots residue	a. Blanching	a. 80 °C/1 h;	[71]
	b. Refining	b. PFI mill to 10,000 revolutions;	
	c. Homogenization	c. Homogenizer: 2 wt%/5 passes/1000 bar.	
Sugar beet	a. Steam Explosion	a. 220 °C/35 min/2.4 MPa;	[72]
	b. Bleaching	b. 6 wt% H ₂ O ₂ /80 °C/24 h;	
	c. Ultrasonication	c. Ice/water bath/30 min/1000 W.	

PAA—peroxyacetic acid; TEMPO—2,2,6,6-tetramethylpiperidin-1-oxyl; ABS—acetate buffer solution.

3.3. BNC

BNC is produced by some bacteria in the form of an extracellular material, which is a direct response to their exposure to ultraviolet light, for example, or when defending against fungi, yeasts and other organisms [22]. These bacteria are able to directly produce cellulose microfibrils through microbial fermentation, but without the hierarchical order found in plant cell walls [42].

Some microorganisms with the ability to produce BNC have been reported, namely *Acetobacter (A.) xylinum*, *Salmonella* spp. and *Escherichia coli*. In addition to these, nanocellulose fibers produced by the interaction of acetic acid bacteria and yeast through the kombucha fermentation process, known as Symbiotic Culture of Bacteria and Yeast (SCOBY), can also be mentioned here [73][74][75]. SCOBY fibers have significant potential and are suitable for various applications (i.e., food, pharmaceutical, textiles, cosmetics) due to their special characteristics, such as strong gel film, high elasticity, and optimal deformation and comfort properties. For instance, SCOBY fibers are considered a potential substitute for cotton, a raw material for fabrics, because they have a flexible texture and are brown like synthetic leather. Moreover, they can be considered cheap and environmentally friendly fibers due to their high degradation rate in the environment [74].

However, the bacterium *A. xylinum* continues to be the highest producer of BNC so far [76]. *A. xylinum* is unique in its family for being able to convert carbohydrates into acetic acid during bacterial growth and cellulose production [41][77]. These acetic acid bacteria secrete through their tiny pores located on the cell membrane an abundant 3D network of cellulose fibrils under aerobic conditions, using glucose as a carbon source [78]. A single *A. xylinum* cell may polymerize up to 200,000 glucose molecules per second, with cellulose synthase or terminal complexes presented in pores on the cell surface and then extruded into the surrounding medium [79]. The cellulose excreted by *A. xylinum* has a chemical structure identical to plant cellulose [23], but it has the advantage of being pure cellulose and therefore does not require rigorous processing (i.e., chemical treatments) to eliminate undesired contaminants or impurities such as pectin, hemicellulose and lignin, as is the case with plant cellulose [40].

Most bacterial cellulose is produced via the conventional static fermentation technique, whereby the bacteria can grow in shallow containers of semi-defined growth medium, in a static incubator at 30 °C, for 7 to 14 days. BNC accumulates at the air–liquid interface as a thick, leather-like, white pellicle that can be easily harvested from the liquid surface interface [80]. Another fermentation technique is the dynamic one (with continuous stirring), in which BNC is obtained dispersed in the culture medium in the form of irregular pellets or suspended fibers [81].

The production parameters, including temperature, pH, culture medium, inoculum ratio and incubation time, should be optimized using readily available and cheap raw materials for the production of high-quality and cost-effective BNC with high yield [82]. Besides the selection of microorganisms and the fermentation method (static or dynamic), the choice of culture conditions has a high impact on BNC production [83]. Studies have shown that up to 30% of the cost of the fermentation bioprocess is due to the fermentation/culture medium, whose composition (i.e., glucose, fructose, etc.) influences the efficiency of bacterial nanocellulose production. Therefore, a high concentration of sugars is necessary for the culture media to improve productivity, which ultimately increases the overall bioprocess cost [84]. Residual products from the dairy industry, wheat straw, fruit juices, rotten fruit, molasses, wine fermentation broth and others have also been used as a source of nutrients for low-cost BNC

production [85]. Thus, several researchers have evaluated new sources of carbon and cultivation conditions to optimize BNC production and achieve a more significant yield with reduced costs and production time, and some of the results obtained are presented in **Table 4**.

Table 4. Optimization of BNC production using different bacteria strains, fermentation techniques and carbon sources.

Bacteria Strain	Fermentation Technique	Carbon Source	Optimal Fermentation Conditions	Productivity, g/L/day	Ref.
<i>G. xylinus</i> ATCC 700178	Static	Carob/Haricot bean	2.5 g/L carbon, T = 30 °C, pH = 5.5, t = 9 days	0.19	[82]
<i>G. xylinus</i> KCCM 41431	Static	Residual crude glycerol	20 g/L glycerol, pH = 5, t = 7 days	0.99	[86]
<i>G. xylinus</i> PTCC 1734	Static	Beet molasses/Cheese whey	T = 28 °C, pH = 5.5, t = 14 days	0.32	[18]
<i>G. sucrofermentans</i> B-11267	Dynamic	Wheat vinasse /Cheese whey	T = 28 °C, pH = 3.95–4.96, t = 3 days, 250 rpm	2.06	[85]
<i>A. xylinum</i> ATCC 23767	Static	Waste extract tobacco	T = 30 °C, pH = 6.5, t = 7 days	0.32	[87]
	Dynamic		T = 30 °C, 150 rpm	0.74	
<i>K. europaeus</i> SGP37	Static batch/Static	Sweet lime pulp waste	T = 30 °C, pH = 6, t = 16 days;	0.40	[88]

Bacteria Strain	Fermentation Technique	Carbon Source	Optimal Fermentation Conditions	Productivity, g/L/day	Ref.
	intermittent fed-batch		Addition every 48 h and 96 h		
<i>K. xylinus</i> PTCC 1734	Static	Vinasse	40% vinasse, T = 30 °C, pH = 6, t = 10 days	0.18	[89]
<i>K. xylinus</i> PTCC 1734	Dynamic	Date syrup/Cheese whey	Date syrup: cheese whey ratio = 50:50, T = 28 °C, pH = 4.48, t = 10 days	0.19	[84]

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Abbreviations: *G. xylinus*—*Glucanacetobacter xylinus*, *A. xylinum*—*Acetobacter xylinum*, *K. xylinus*—*Komagatacibacter xylinus*, *K. europaeus*—*Komagatacibacter europaeus*.
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