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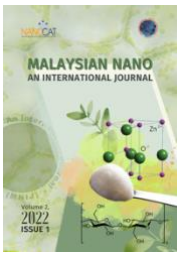

Review Article

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Applications of nanocellulose as biosensing platforms for the detection of functional biomacromolecules: A Review

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Abstract

Cellulose is a group of materials that can be made into low-cost devices because they are the most common biomaterials in nature. Cellulose-based polymers are flexible, biocompatible, biodegradable, and easy to functionalise and mass produce. Cellulosic substrates are attractive biosensing platforms because of their unique properties, exceptional simplicity, and compatibility with standard technologies. Furthermore, cellulose-based biosensing approaches can meet the following criteria for optimal diagnostic assays or devices: real-time connectivity; simplicity of specimen collection; affordability; specificity; sensitivity; user-friendliness; speed and robustness; and deliverability to end-users. As a result, cellulose is suitable for constructing novel analytical devices in the biosensing community. The use of cellulose as a nano-engineered matrix material has enabled recent advancements in biosensors. Several methodologies for producing cellulose-based composites for the fabrication of various biosensors have been described and reviewed. Biological macromolecules have immense importance in genetic and pathogenicity detection. Likewise, there are many research reports, but there is a gap regarding review in this area of biological macromolecule detection like nucleic acids and proteins. This study looked at this previously unexplored area as well as the unique features that make it a good choice for biosensing applications and the engineering features of cellulose-based biosensors. It also looked at how different analytical systems have used such matrices to detect biological macromolecules (DNA, proteins, and RNA) in different samples.

Keywords*:* Biomolecules; Biosensor; Functional; Macromolecules; Nanocellulose

1. Introduction

Cellulose is a common natural linear biopolymer made up of a hierarchical arrangement of cellulose nanofibrils [1, 2]. Nanocelluloses, in particular, has a lot of potential as a low-cost advanced material with applications ranging from material science to biomedical engineering. For many reasons, together with its natural abundance on the planet, superior mechanical and optical properties, strong biocompatibility, minimal cytotoxicity, and the appealing ability to undergo surface chemical changes [1, 3]. They can also be easily manipulated to produce useful products due to their chemical activity. Compared to synthetic materials, nanocellulose has been demonstrated to be an ecologically benign material with low density, Young's modulus, high specific strength, and a surface-to-volume ratio. It may also be isolated from many plants and microbial sources using a number of easy and quick processes [4]. Green composites based on nanocellulose are currently undergoing intensive investigation and have gained favour among scientists because to their lightweight, low density, low cost, and good physical and mechanical properties [2].

These qualities are also customisable, and they are mostly dependent on the chemical conversion methods and the sort of nanomaterials incorporated into the cellulose matrix. Chemical activation, coating, grafting, implantation, covalent binding, microfluidisation, cryocrushing, super ultrasonication, and other wet chemistry techniques can all be used to develop these nano-cellulosic systems [3]. Cellulose nanocrystals (CNC), cellulose nanofibres (CNF), and bacterial nanocellulose (BNC) are examples [5]. Nanocellulose's extraordinary qualities make it one of the most exciting and innovative materials with applications in a variety of disciplines [6] including biosensing. Because of its full decomposition and biocompatible qualities, cellulose is also known as "green cellulose" [7] and is used to make disposable and biodegradable biosensors. In addition, the hydrogen bonding in cellulose's core structure gives both mechanical strength and flexibility; this unique trait aids in the creation of an adaptable matrix for sensors [8-10]. Cellulose has been regarded as one of the top exciting and commonly applied resources for developing biosensors due to its multifaceted features and cost-effectiveness. Nanocellulose-based materials have a thin fibrous matrix that allows immobilisation of receptor or transducer elements like nanoparticles. To improve the electric and magnetic conductivity of native nanocellulose, conducting elements such as carbon nanotubes (CNTs), graphene oxide (GO), gold nanoparticles (AuNPs), gold-nanorods, platinum (Pt), and palladium (Pd) nanoparticles and other nanocomposites can be used to enhance the sensitivity of the cellulose matrix [11-13].

Biological macromolecules are big, and life-sustaining compounds made up of smaller organic components. Proteins, lipids, carbohydrates, and nucleic acids are the 4 major types of biological macromolecules; each is a crucial cell element and plays a variety of functions. Biological macromolecules are huge cellular components found in abundance in nature that perform a number of vital activities in the growth and survival of living organisms. Biological

macromolecules have the ability to influence the pathophysiology of living organisms, including diseases such as neurodegenerative disorders [14]. As a result, gene, protein, and metabolite analysis have long been a part of genetic and disease biomarker identification efforts. When physiological status becomes pathogenic, gene transcription and downstream protein expression can be drastically changed, getting gene transcripts and proteins valuable indicators. Likewise, because metabolic pathways can vary in disease conditions, basic metabolites can be employed as disease detection markers [15]. That is why nucleic acids (DNA and RNA) and proteins-based biomarkers have significant roles in diagnosis [16-19]. For the detection and treatment of diseases, clinical diagnostic procedures should be fast, accurate, simple to perform, and economical. Biosensors have drawn the attention of both scientists and end-users in this field due to their new features. They are efficient, reliable, and reasonably priced. Point-of-care (POC) monitoring, forensics, and biomedical research are all applications for biosensors in medical diagnosis [16].

In current decades, there has been a lot of attention to combining nanocellulose and other nanomaterials in detection aspects with electronic components to make novel electrochemical biosensors [20-22]. Electrochemical sensing systems rely on electrode materials to support high-performing electrochemical detecting platforms that can sensitively detect targets using numerous analytical methods. Nanocellulose and nanomaterials in electrochemical detection interfaces enable the insertion of unique functions and, as a result, high sensitivities [23, 24]. Porous noble metal nanostructures with large surface areas and pore volumes, in particular, can have a synergistic effect on conductivity, catalytic efficiency, and biocompatibility. Furthermore, because porous noble metal nanostructures are highly connected and do not need any support, corrosion can be prevented greatly. These nanostructure-enhanced electrochemical detection platforms can significantly speed up signal transduction, subsequently increasing sensitivity and reducing the limit of detection. Biological macromolecules such as proteins and DNA-based biosensors are an emerging field of research and have been investigated recently using cellulose-based nanostructures in electrochemical biosensors [23, 24]. But there is no review available in this area.

We have critically reviewed the cellulose-based biosensors that have been created using various modification procedures, as well as their various uses for analysing various sample types, such as proteins, enzymes, DNA, and RNA macromolecules, in this review. The basic features of nanocellulose, their manufacture and properties, and composite synthesis are listed in the following sections, followed by their nano-bioengineering design aspects for detecting various macromolecules in numerous real sample matrices.

2. Features of Nanocellulose

The most prevalent carbohydrate on the planet is cellulose. Nanocellulose is a natural nanofiber that may be separated from biomass resources such as herbs, wood, plants, and

creatures via defibration of cellulose. From the macroscale to the nanoscale, a tree's hierarchical structure can be defined as follows (Figure 1): A tree's cross section can be up to 100 metres long. The cross-section contains centimetre-scale structures, growth rings are millimetres, cellular anatomy is tens of micrometres, hemicellulose and lignin configuration in cellulose microfibrils is measured in tens of nanometres, and cellulose molecular structure is nanometric [25]. The three categories of nanocellulose are CNC, CNF, and BNC.

As shown in Figure 1a, CNF is a bundle of stretched CNF. The cellulose chains are intertwined and have a huge surface area. CNF is also known as CNF, nanofibrillar cellulose, and cellulose nanofibrils. Amorphous regions with sizes ranging from tens to hundreds of nanometres, as well as soft and lengthy chains with micrometre-scale lengths, make up CNF. Based on natural resource, CNF can have various mechanical properties [26, 27]. CNC, also known as nanowhiskers, has the shape of an extended crystal rod, as seen in Figure 1b, and is stiffer than NFC. CNC has a breadth of 3 to 50 nm and a length of 50 to 500 nm [28]. CNC has high Young's modulus (20-50 GPa) [29], high axial rigidity (105-168 GPa), a low coefficient of thermal extension (about 0.1 ppm/K) [30], low density (1.5-1.6 g/cm^3) [31], high thermal stability (up to about 260 °C) [32], and thixotropy with time-reliant shear thinning characteristics [33]. The *Gluconoacetobacter xylinus* family produces and secretes BNC. Other bacterial species that generate BNC include Pseudomonas, Agrobacterium, Rhizobium, and Sarcina. BNC has a higher molecular weight up to 8000 Da, high Young's modulus (79-88 GPa) [34], and high water retention capacity [35]. BNC can be generated from low molecular mass biomass by increasing the growth environment of bacteria that make cellulose, as opposed to CNF and CNC, which are made from the top-down approach by mechanical and chemical treatments.

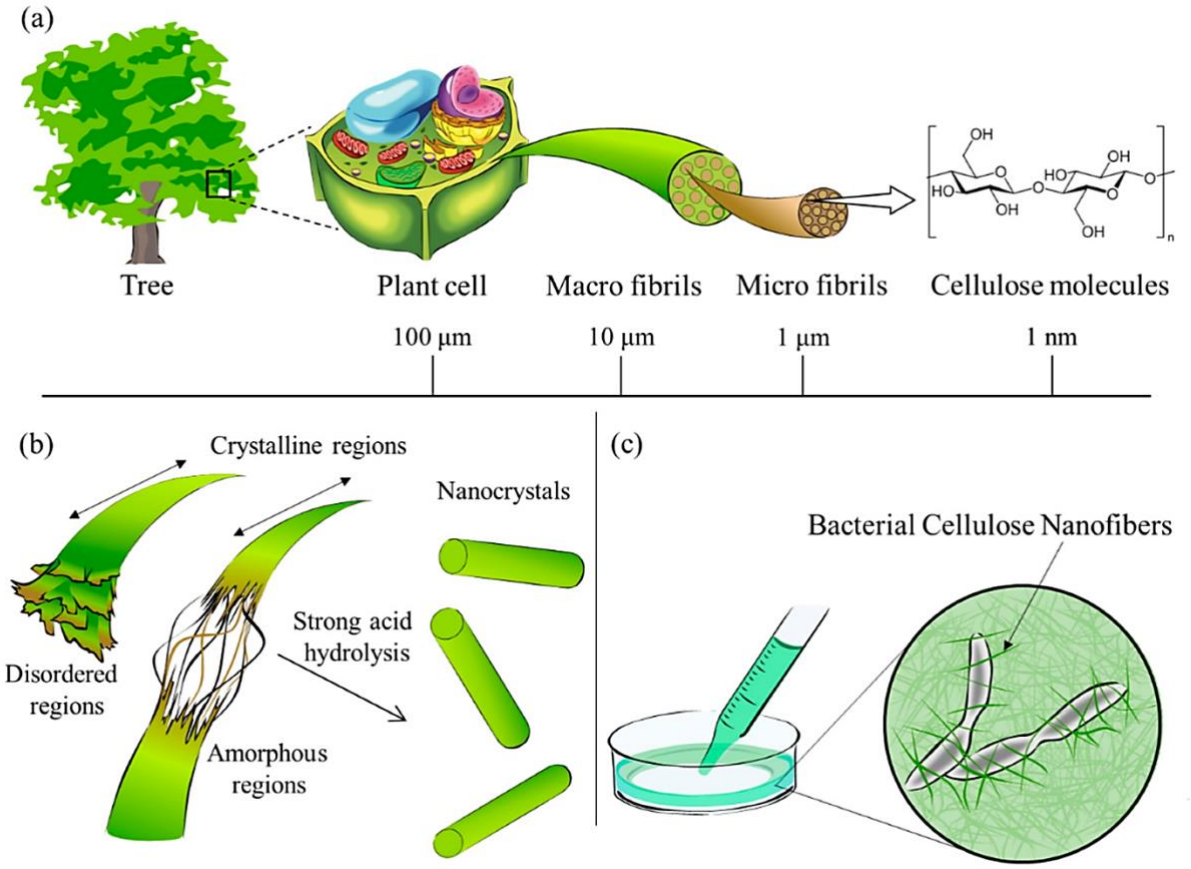

Figure 1: From the metre to the nanoscale scale, cellulose in plants and trees has a hierarchical structure, as seen in (a). The reaction of cellulose with intense acid to produce nanocellulose is depicted in a schematic diagram (b). In this image, bionanocellulose is produced from cellulose-producing bacteria (c). Reprinted with permission from [36] under Creative Commons Attribution license.

Nanocellulose has sparked a lot of attention and interdisciplinary study on cellulose-based materials and goods because of these features. Its solubility is governed by a number of factors, together with its molecular weight, source, and structure. Polysaccharides, the primary source of nanocellulose, have a great attraction for accumulation or low solubility due to the formation of hydrogen bonds, which is one of the top important factors influencing its physical and chemical properties. Both intramolecular and intermolecular bonding patterns can affect crystalline characteristics, the solubility, and reactivity of the hydroxyl functional units on cellulose polymer chains (Figure 2). Depending on the preparation process, these qualities alter after nanocellulose preparation [37].

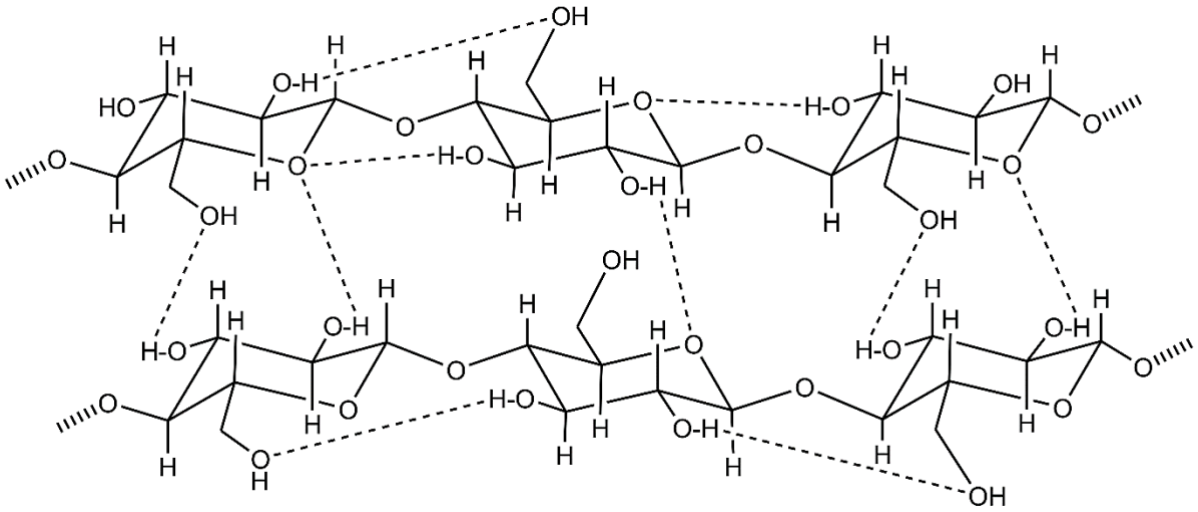

Figure 2: The patterns of intra-and intermolecular hydrogen bonding in the structure of cellulose. Reprinted with permission from [37] under Creative Commons Attribution (CC BY) license.

3. Preparation methods and properties of nanocellulose

Nanocellulose is divided into three categories based on its form and source: CNCs, NFC, and BNC. Plant CNCs and NFCs are created via a top-down strategy that involves chemical or mechanical disintegration of plant matter [38]. Plants biosynthesize cellulose, which results in partly crystalline fibres. To obtain the desired nanocellulose, mechanical trimming or acid hydrolysis will first destabilise and destroy the least crystalline areas. The amorphous portions have distinct physical characteristics than crystalline cellulose, and by splitting the fibrils at amorphous sites, cellulose nanoparticles are formed. Mechanical trimming or regulated acid hydrolysis can be employed to deconstruct cellulose fibres, generating varied architectures depending on the method [39]. CNCs are nanometre-long, extremely crystalline rod-like pieces produced by acid hydrolysis. Mechanical shearing methods degrade cellulose fibres into their substructural nanoscale elements, yielding in micrometric NFCs [5]. BNC is made utilising a bottom-up technique in which bacteria cultures are used to synthesis the material [35].

In the literature, cellulose nanofibers, cellulose nanocrystals, cellulose whiskers, cellulose nanowhiskers, cellulose nanofibrils, microfibrillated cellulose, nanofibrillated cellulose, and nanocrystalline cellulose are all terms for nanocellulosic materials. Fibrils are usually used to characterise cellulose with a larger aspect ratio than crystals or whiskers, while there is no formal definition for each one. Likewise, there is no universally accepted term for "nanoscale cellulose." We'll go over how the three types of nanocellulose are made, as well as their distinct features [35].

3.1 Cellulose Nanocrystals (CNCs) preparation and properties

CNCs, also termed as nanowhiskers, have extended crystalline rod-like forms and are more rigid than NFC owing to the elimination of a greater fraction of the amorphous regions [40]. The crystallinity of CNCs varies between 54 and 88 percent. Although enzymatic hydrolysis can be used to make CNCs [41], the most frequent preparation method is strong acid hydrolysis, notably sulfuric acid treatment [39]. A typical CNC production technique begins with alkali and bleaching pre-treatments, followed by acid treatment, rinsing, centrifugation, dialysis, and ultrasonication to create a suspension that can then be lyophilised (spray-drying or freeze-drying) as needed. Several of the acquired features of CNCs are affected by both the reaction settings and the cellulose supply, including morphology, crystallinity, aspect ratio, and dimensional dispersion [28]. CNCs are whiskers or rods that range in breadth from 3 to 50 nm and in length from 50 to 500 nm. CNCs have low density (1.5−1.6 g/-cm^3) [33], high Young's modulus (20-50 GPa) [42], high axial stiffness (105-168 GPa) [29], high tensile strength (~9 GPa) [30], high thermal stability (~260 °C) [43], low coefficient of thermal expansion (~0.1 ppm/K) [32], high aspect ratio (~10−70) [31], lyotropic liquid crystalline behaviour, and shear thinning rheology [44, 45].

Malucelli and colleagues have published a detailed analysis of the origin, preparation, characteristics, and potential prospects of CNC derived from agro-industrial waste [46]. CNC aqueous solutions were discovered to have liquid crystal qualities [47], and outstanding photonic properties [48] by Marchessault and colleagues in 1950. They will produce chiral nematic structures above a critical concentration (~4.5 wt%) [28, 49], which can be seen using polarised optical microscopy [49]. The phase behaviour of lyotropic CNC suspensions of sulfonated CNCs generated from cotton was studied by Ureña-Benavides *et al*. [45]. A phase partition appeared in the suspension between 3.07 and 10.4 vol %, giving liquid crystalline and isotropic domains. The isotropic phase vanished at 12.1 vol %, leaving a characteristic texture that is typical of cholesteric liquid crystals. As concentrations were increased, the characteristic texture of the liquid crystal phase vanished, and the suspension began to behave like a rheological gel. Functional composites are created by combining CNCs with other synthetic or natural polymers. Reviews by J. Moon [6] and A. Dufresne [50] provide a detailed examination of several procedures for preparing CNC composites. Surface modification methods are also applied to regulate the interfacial characteristics of composites and affect the self-assembly behaviour of CNCs in suspensions. CNCs give composite materials improved mechanical qualities, a low density, and a large surface area. Figure 3a displays 3D and cross-section shape of the molecular assembly of a single CNC particle, which is generated by hydrogen bonding (h-bonding) of numerous cellulose chains (β-D glucopyranose), with unit cell parameters of a = 7.784 Å, b = 8.201 Å, c = 10.380 Å, α = 90°, β = 90°, γ = 96.55°, at 293 K reported [6].

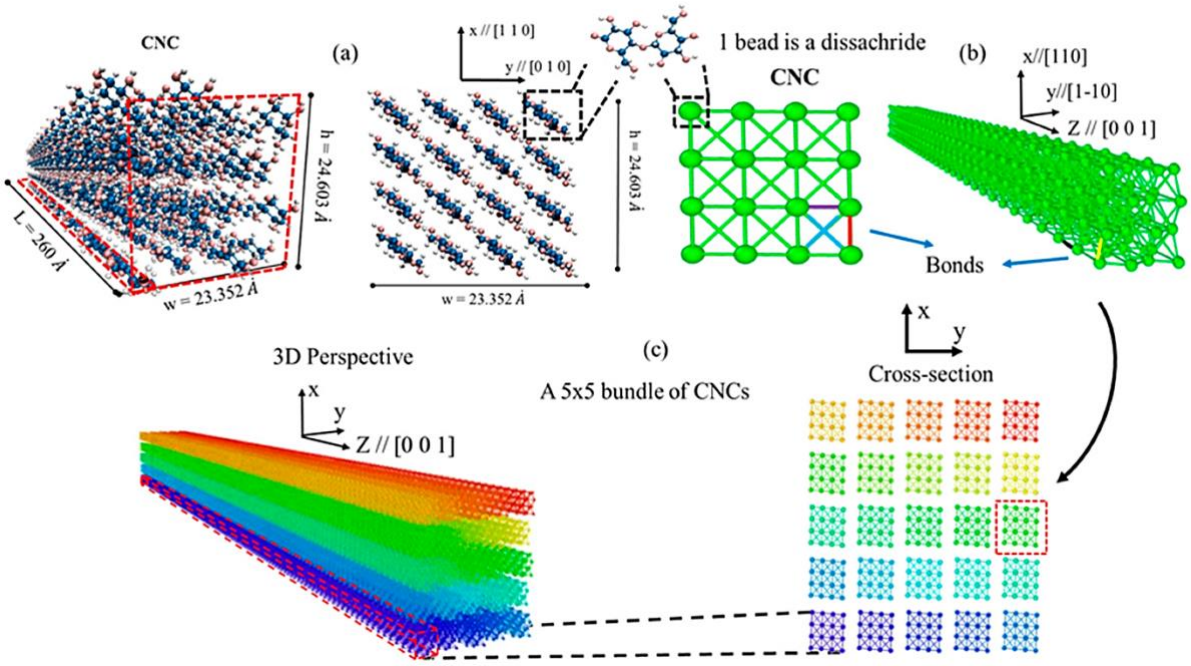

Figure 3: (a) 3D and cross-section landscapes of CNC' atomic structure of CNC. Oxygen atoms are represented by pink spheres, hydrogen atoms by white spheres, and carbon atoms by blue spheres. (b) The coarse-grained (CG) style of CNC is illustrated with different colours, where every bead signifies a disaccharide and is coupled with five distinct interior linkages. (c) A 3D and cross-section image of a 5×5 CNC bundle, with distinct colours indicating different particles. Reprinted with permission from [10] under Creative Commons Attribution license.

3.2 Nanofibrillated Cellulose (NFC) preparation and properties

An NFC is a cellulose nanofiber bundle that has been stretched [51]. The cellulose chains are intertwined and have a huge surface space. NFC is also well-known as nanofibrillar cellulose [52], cellulose nanofibrils [53], and cellulose nanofibers (CNF) [54]. NFCs, unlike CNCs, have large amorphous areas with soft, lengthy chains with breadths spanning from 10 to a few 100 nanometres and lengths in the micrometre range [55]. Physical, biochemical, and biological methods have all been utilised to separate NFCs from a variety of resources, with physical/mechanical treatments being the most popular one. Mechanical methods used to remove CNF include high stress homogenization, cryocrushing, and grinding [56-58]. Alkali treatments are used in chemical treatments, while enzymatic therapies are used in biological treatments [59, 60]. Researchers frequently use a combination of these strategies to achieve the desired result. The essential features of NFCs, like CNCs, vary depending on the raw resource and the exact extraction procedure used. The shape, extent of fibrillation, morphology, and characteristics of the produced NFC might vary substantially depending on the treatment procedure. Desmaisons *et al*. devised an eight-criteria quality index to compare the variety of

reported NFCs [61]. An SEM image of a bleached Northern red oak (Quercus rubra) sample exhibiting mechanical fibrillation morphology was shown in Figure 4.

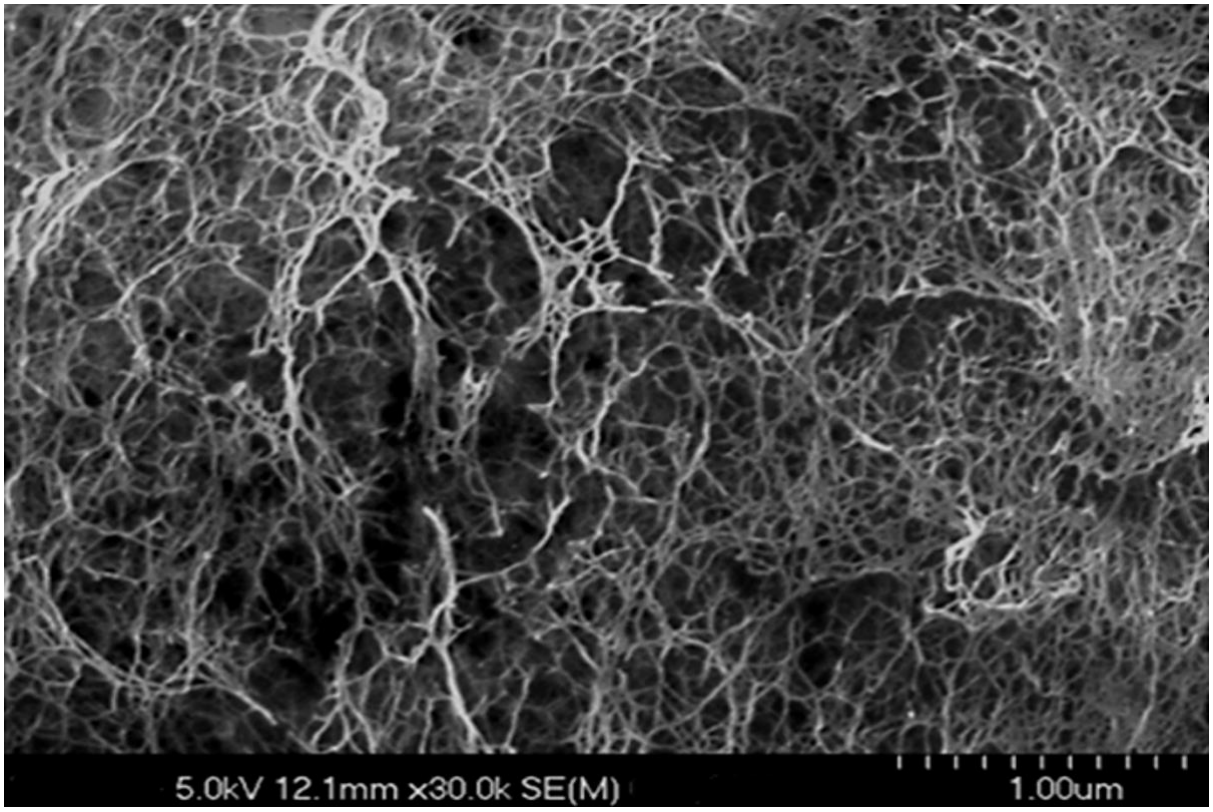

Figure 4: FESEM micrographs of Northern red oak (Quercus rubra) samples that were bleached and then freeze-dried after a solvent exchange (E/tert-B-FD treatment). Reprinted with permission from [62] under Creative Commons Attribution License.

3.3 Bacterial Nanocellulose (BNC) preparation and properties

The *Gluconoacetobacter xylinus* family synthesises and secretes bacterial nanocellulose [5]. Aerobacter, Acetobacter, Azotobacter, Alcaligenes, Agrobacterium, Pseudomonas, Salmonella, Rhizobium, and Achromobacter are among the cellulose-producing bacteria, with *Acetobacter xylinum*, a gram-negative acetic acid bacterium, being the most well-known. Acetic acid bacteria have been reclassified, with Acetobacter being classed as Gluconacetobacter and lately as a new kind of Komagataeibacter [63, 64]. BNC is made by culturing bacteria in a liquid culture medium including glucose, phosphate, nitrogen, and carbon sources for a few days. Cultivation variables like nutrition source, bacterial strain type, oxygen ratio, incubation duration, and culture in a bioreactor can all affect the structure and characteristics of BNC tubes [65-67]. The influence of culture parameters on the characteristics of BNC tubes have lately been investigated in detail [67]. In silicone tube bioreactors, *Gluconacetobacter xylinus* CGMCC No. 1186 was cultured with either glucose or fructose. The nanocellulose yield was enhanced by using fructose. The amount of dissolved oxygen in the reactor altered, and hence the shape of the nanocellulose tubes produced. The cell membrane of the microbes that make BNC is made up of a cellulose

network construct of ribbon-shaped filaments that are less than 100 nm thick and are made up of bundles of much finer nanofibrils [68] with diameters of 2-4 nm. In terms of size, the fibrils are reasonably straight and continuous, with little polydispersity. Because of their high crystallinity (84-89%), the bundles have outstanding intrinsic characteristics [69]. BNC has a molecular weight of up to 8000 Da, and a high-water retention capacity and as well as an elastic modulus of 78 GPa [70]. Due to their high surface area and low apparent density, BNCs have the potential to seem in functional materials as scaffolds with good magnetic, optical, and mechanical properties, such as in situ Fe_3O_4 nanoparticle implantation to produce a magnetic BNC or employed in order to prepare of ferromagnetic cobalt ferrite nanoparticles [71-73].

BNC has a long history of use in biomedical applications [74]. BNC encompasses great physical strength, a very hydrophilic surface, and a permeating structure, making it ideal for biomedical applications [75], including tissue engineering implants and scaffolds, artificial skin, and wound dressings [76] as well as drug delivery transporters [77]. They can also shield neurons and substitute tiny blood vessels [77]. BNC membranes could also be employed in regenerative medicine, such as guiding tissue restoration, treating periodontitis, and replacing membranes. Figure 5 displays a FESEM image of dried BNC networks with native BNC morphological features.

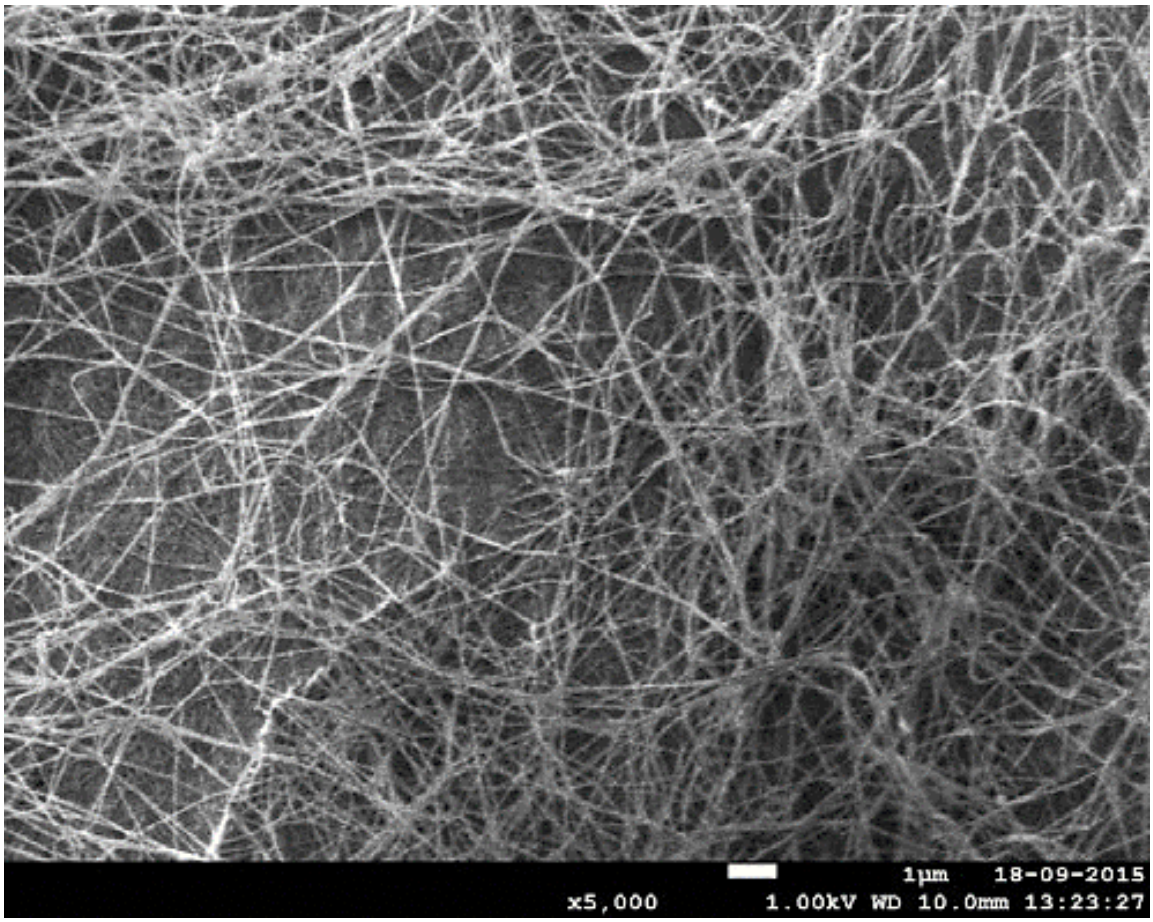

Figure 5: A FESEM image of a BNC sample shows a coherent 3-D network formed by cellulose fibres. Reprinted with permission from [78] under the Creative Commons Attribution license.

4. The synthesis of nanocellulose composites enables sensing

The cellulose surface chemistry can be easily altered. Cellulosic nanomaterials with surface modifications are an excellent substrate for specific applications. Nanocellulose's extensive use is limited by its poor miscibility in nonpolar solvents and incompatibility with hydrophobic matrices, as well as weak interfacial adherence. Researchers have tried a variety of surface and nanocellulosic structural alterations to overcome this problem. Pendant surface hydroxyl groups, effectively the principal alcohol group, are used to chemically modify the surface of cellulose nanoparticles (-CH_2OH). On nanocellulosic materials, various chemical modification strategies were used to (1) improve the efficiency of the separation process and (2) change the surface hydrophobicity, which enhances the compliance and dispersibility of nanocelluloses in certain solvents. [35]. Different surface modification techniques illustrated in figure 6.

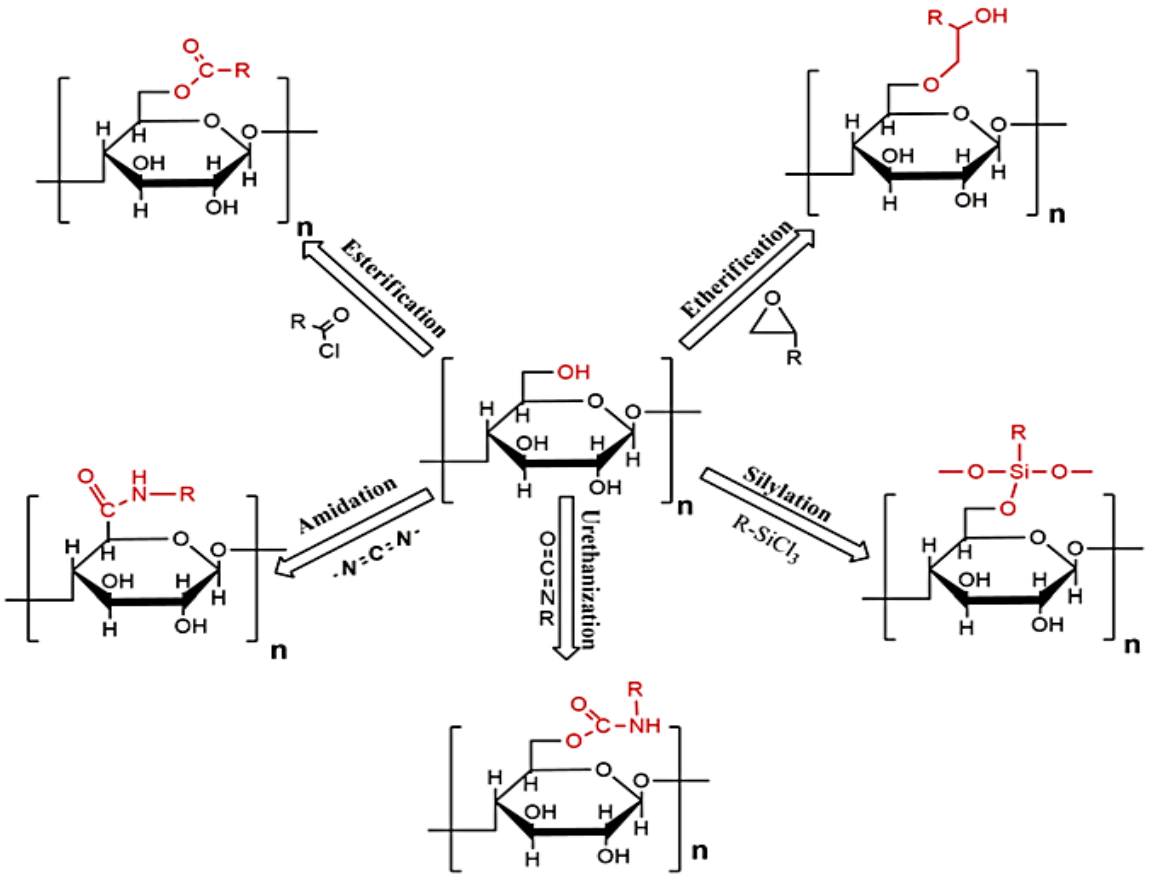

Figure 6: Different surface modification procedures described in the literature to offer hydrophobicity to the nanocellulose surface. Reprinted with permission from [35]. Copyright (2018) American Chemical Society.

Bacterial cellulose composites can be made utilising a bottom-up strategy since these bacteria manufacture cellulose by forming nanofibril bundles [79]. Bacterial cellulose composites have been created by introducing a second phase into the bacteria's growth

environment. One of the benefits of adding a second phase is that it can improve or change the properties of native bacterial cellulose. Bacterial cellulose is extremely pure, as it contains no other polymers or functional groups save alcohol [38, 80]. According to Gatenholm and Klemm [81], cellulose production in a conventional bacterial cellulose method can take up to two weeks. Moreover, nanocellulose made from bacterial cellulose has a better crystallinity than nanocellulose made from plants.

The production of nanocellulose from cellulose usually involves two steps [82-84]. The first stage focuses on pre-treatment of feedstocks to achieve pure cellulose, while the second phase focuses on cellulose conversion to nanocellulose. Extractives (monomers, dimers, and polymers of fatty acids, tannins, fat, resin, flavonoids, terpenoids, rosin, waxes, terpenes, free sugar, and so on), lignin, and hemicelluloses must be partially or completely removed from the feedstocks during the first step using particular pre-treatment procedures [85, 86]. The second stage, on the other hand, is commonly used to make CNCs. This guarantees that amorphous domains are removed from virgin cellulose, allowing CNCs to be produced [87, 88]. The disordered regions spread as chain dislocations on segments along the primary fibril are susceptible to hydrolytic action due to lower steric hindrance and kinetic considerations, but the ordered domains, which have better resistance to hydrolysis process, remain intact. The cellulose fibrils are then divided transversely, generating short CNCs with good crystallinity. To recover CNC products after the hydrolysis process, more processes are required. Washing, removing the solvent, neutralising, separation, purification, centrifugation, dialysis, sonication, fractionation, changing the surface, stabilising, and drying are some of the steps involved (spray-drying, freeze-drying). [82].

The functionality of nanocellulose is improved when they are combined with other elements such as noble metal nanoparticles. The characteristics of noble metal particles, such as Au, Ag, Pt, and Pd, can be altered by altering their size and shape [89-92]. Different techniques have been devised to attain this particular structure with adjustable morphology, porosity, and dimension in porous noble metal nano/microparticles. The template process, which can be divided into two primary stages: the synthesis of the template and the expansion of target materials, is very prevalent. Any substance containing nanostructured characteristics, such as metals or metal oxides, can be used as a potential template in realistic synthesis. Because pre-grown templates permit the morphology and sponginess of the generated particles to be manipulated easily, the template approach is a simple and successful approach for the synthesis of porous units with regulated size and morphology [93]. For the direct synthesis of target materials on the template, an appropriate reaction must be designed. Then, if necessary, a proper approach for removing the initial template without disrupting the product's structure must be followed. In template methods, the main ways that noble metal porous units are made

are through the Kirkendall effect in galvanic replacement reactions, the volume reduction caused by the formation of pores, and the growth of crystals along the 3D microporous structure made up of channels and voids in polymers or zeolites [94]. Colloidal synthesis is another way to make noble metal nanoparticles. A 'colloid' is a two-phase system in which insoluble particles are disseminated in water or, more broadly, in solvents [95]. Using a colloidal technique to make porous noble metal NPs of certain sizes has been proven to be beneficial. Compared to the template approach, which requires numerous steps of painstaking synthesis, colloidal synthesis is more akin to a "one-pot" procedure that can give a simple route for large-scale synthesis. But nanosynthesis still faces difficulties in precisely controlling the porosity and shape of colloidal solutions. A typical approach for generating noble metal nanoparticles in colloidal solutions is to reduce ionic noble metal precursors with reducing agents in the presence of capping agents. To avoid solid nanoparticle formation in porous noble metal nanoparticles, the nucleation and growth processes of the crystals must be meticulously managed. In general, dendritic growth behaviour or the aggregation of isolated crystals through a random or oriented attachment process is required to develop holes in nanoparticle growth [94].

Chanzy *et al*. tagged 1,4 β-D-glucan cellobiohydrolase I (CBHI) from *Trichoderma reesei* with sphere-shaped AuNPs (4–6 nm) in 1984, which was one of the earliest instances of cellulose–gold composite [96]. By adsorbing the enzyme to the surface of cellulose microfibrils and microcrystals, they investigated how the enzyme digests the semi-crystalline material. Using an acidomechanical treatment, the microcrystals of *V. macrophysa* cellulose and microcrystalline bacterial cellulose were discovered. This treatment was performed on algal cell walls and *Acetobacter xylinum* cellulose. To produce the Au-CBHI combination, a colloidal gold solution was mixed with a CBHI solution in a sodium acetate buffer (pH 4.8). Following that, aqueous NaCl and polyethylene glycol (PEG) solutions were added. A crimson mobile phase was produced after centrifugation and pipetted into a citrate phosphate buffer (CPB, pH 4.8) having 0.1 percent PEG. To generate a stable Au-CBHI complex, the centrifugation procedure was repeated numerous times. First, the cellulose microcrystals were suspended in a CPB buffer having 0.1 percent PEG. After adding Au-CBHI to the cellulose suspension, the Au-CBHI complex adsorption was accomplished. After 30 minutes of mixing, electron microscopy revealed that 100% of the gold complex was attached to the cellulose microcrystal surface. Although few clusters were identified, the gold nanoparticles primarily appeared as single individuals. The Au-BCHI complex was found to have a predilection for binding along the margins of cellulose microfibrils and microcrystals, and the Au-CBHI combination adsorbed on the surface of bacterial cellulose preserved 60% of its initial CBHI activity [96].

Treatment of gold salts with reducing elements in the existence of cellulose is the most prevalent approach for cellulose–gold composite production. CNCs, in general, enhance the nucleation of monodisperse nanoparticles while preventing agglomeration, resulting in extremely thin particle size distributions [97]. In 2003, Evans *et al*. was among the first to apply nanocellulose as a template for the production of AuNPs [98, 99]. They used no external reducing agent to precipitate gold from their metal sources onto bacterial CNFs [98]. Four main cellulose-gold composite production methods are available: in situ gold nanoparticle formation in a cellulose matrix, combining of preformed AuNPs with cellulose, dip coating, and layer-by-layer assembly. Seeded growth, micro-patterning, solid grinding, electrospinning, e-beam evaporation, and inkjet printing are some of the less prevalent preparation procedures [100].

5. Applications of biosensors for the detection of bio-functional Macromolecules

Macromolecules are polymers with a high molecular mass and hundreds of monomeric components. Macromolecules also have peculiar features that are rarely seen in tiny molecules. In living creatures, there are primarily three types of macromolecules that aid biological functions: DNA, RNA, and proteins, all of which are monomer units, as well as additional non-polymer macromolecules, including lipid moieties and macrocycles. This section discusses the cellulose-based sensors developed to detect various functional biological macromolecules.

5.1 Applications for the detection of nucleic acid

The nucleic acid (DNA, RNA, PNA) biosensor, the most recent innovation in this sector, unites the sensitivity of analytical procedures with nucleic acids' inherent bioselectivity [16]. Jirakittiwut *et al*., 2015, developed a cellulose paper-based biosensor for the optical recognition of DNA. The biosensor was created on cellulose paper by divinyl sulfone-mediated conjugation of acpc PNA (D-prolyl-2-aminocyclopentane-carboxylic acid PNA) [101]. PNA is a synthetic DNA mimic that can be used to detect DNA. The connection between DNA and PNA is predicated on the charges on these molecules being different. Because DNA is a negatively charged molecule, and PNA is a neutral molecule, they have a one-of-a-kind possibility of interacting. The created biosensor is quite specific, and it can even distinguish between genes with single base-pair mutations. Human leukocyte antigen and the 26th and 27th thalassemia mutations are used to test the biosensor's functionality. The biosensor's signal detection has been combined with the cationic dye Azure A, which reduces the LOD. Mohanraj *et al*., 2020, developed another paper-based biosensor to investigate dsDNA.

The sensor was made out of graphene nanosheets obtained by electrochemical exfoliation of corncob biomass [102]. This graphene sensor made of paper can detect electrolytes without sample pre-treatment. The oxidation of guanine and adenine within the recognition scale of 2×10^{-4} ng.mL^{-1} to 50×10^{-4} ng.mL^{-1} and the LOD of 0.68 pg.mL^{-1} were

used to detect dsDNA. For *Mycobacterium tuberculosis* detection, a team of researchers developed a new PNA electrochemical sensor based on reduced graphene oxide (NH2-rGO)/2,2,6,6-tetramethyl piperidine-1-yl) oxyl nanocrystalline cellulose (TEMPO-NCC) functionalised with reduced graphene oxide. Applying methylene blue as the electrochemical indicator, the PNA probe-modified (NH_2-rGO)/TEMPO-NCC response indicated that the constructed biosensor could differentiate between complementary, one-base mismatch and noncomplementary DNA sequences with high sensitivity and specificity [103]. Figure 7 shows a schematic diagram of the approach as an example of the detecting mechanism. More information about various nucleotide-detection biosensors can be found in Table 1.

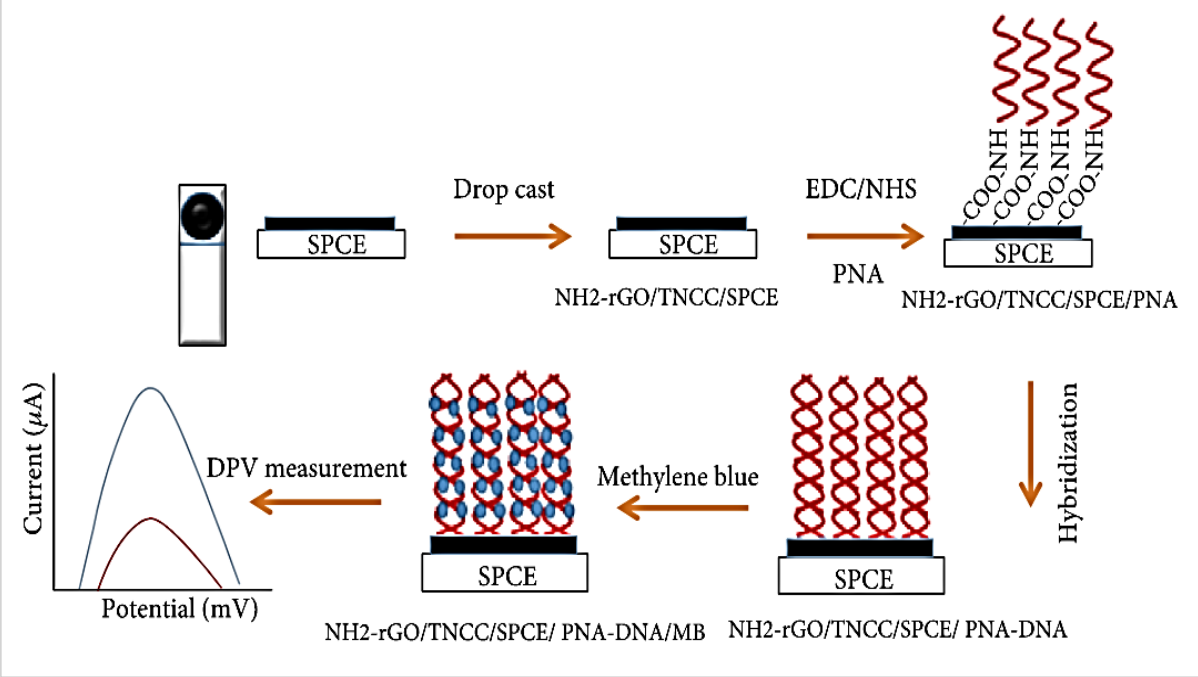

Figure 7: The step-by-step investigational procedure for assembling the PNA electrochemical biosensor based on NH_2-rGO TEMPO-NCC for the detection of *M. tuberculosis*. TNCC: TEMPO-nanocrystalline cellulose. Reprinted with permission from [103] under the Creative Commons Attribution license.

Table 1: Biosensors based on (nano)cellulose platforms for the detection of nucleic acids

Sl.	Analyte	Sensor design	Detection method	Sample source	Detection range	LOD	References
1	DNA	Acpc PNA on cellulose paper by divinyl sulfone (DVS) conjugation	Colorimetric	Human leukocyte antigen alleles	low as 200	-	[101]
2	dsDNA	Paper-based modified electrode sensos	CV, EIS	-	0.2 $pg.mL^{-1}$ to 5 $pg.mL^{-1}$	680 $fg.mL^{-1}$	[102]
3	miRNA	PNA-based paper biosensor	Voltammetric	Serum	up to 100 nm	6 nm	[104]
4	miR-21	Cerium dioxide-Au@ glucose oxidase paper-based sensor	Voltammetric	Serum	0.001 pm to 1 pm	0.434 fm	[105]

Note: dsDNA- Double stranded DNA; miRNA – MicroRNA; miR-21- microRNA 21; PNA - Peptide nucleic acid; CV -Cyclic Voltammetry; EIS- electrochemical impedance spectroscopy; Acpc PNA - D-prolyl-2-aminocyclopentane-carboxylic acid PNA.

5.2 Applications for the detection of proteins

Cellulose-based biosensors have been created to detect a variety of proteins in addition to nucleotides. A new sensor technology has been used to detect many proteins in this direction. One such example is the discovery of transcription factor (TF), a DNA-binding protein required for gene regulation. Lin *et al*., 2019, reported a paper-based biosensor for the visual recognition of TF, which was made by coating dopamine onto cellulose paper and employed to produce analytical signals [106]. Characterisation of the constructed biosensor was done using FTIR and other methods. The response of the created biosensor to the target NF-κB p50, which is built on the Exo III-mediated cycle amplification reaction, was examined through a colour change. It was also speculated that the suggested sensor is generic and may be expanded to new biomolecules by modifying the identification system, perhaps resulting in a low-cost, disposable, and portable biosensing device. Alkaline phosphatase (ALP), a metalloprotein available naturally in milk and used as a biomarker in pasteurised milk research, is another type of protein molecule. Mahato *et al*., 2019 created a paper-based sensor for visual detection of ALP that was coupled with a smartphone system [107]. The biosensor was made by immobilising the ALP antibody on the surface of the paper. In the presence of 5-bromo-4-chloro-3-indolyl phosphate, an immunological complexation reaction between the ALP and probe creates a blue-green precipitate, which was used to detect the ALP. The quantification was done with a digital image colorimetry approach, and the recognition range was determined to be between 10 and 1000 $U.mL^{-1}$, with a LOD of 0.87 (±0.07) $U.mL^{-1}$. This research contributes to the development of a low-cost biosensor for evaluating milk quality in miniaturised and individualised settings.

Applying cerium dioxide-Au@glucose oxidase (CeO_2-Au@GOx) as an electrochemical probe for signal magnification, an electrochemical biosensor relying on Au nanorods (NRs) adapted microfluidic paper-based analytical devices (μPADs) was built for sensitive detection of microRNA. [105]. Table 2 contains more information on developed biosensors for protein detection.

Table 2: Biosensors based on (nano)cellulose platforms for the detection of nucleic acid

Sl.	Analyte	Sensor design	Detection method	Sample source	Detection range	LOD	References
1	Transcription factor	Dopamine coated on the surface of cellulose paper	Colorimetric	20 sNF-κB p50 in biological fluids	-	-	[106]
2	Glycoprotein	Paper-based sensor for glycoprotein based on boronate affinity tag	Voltammetric	Ovalbumin	0.001 $ng.mL^{-1}$ to 1 $\mu g.mL^{-1}$	870 $fg.mL^{-1}$	[108]
3	Bilirubin	BC nanopaper-based sensor through implanting of carbon dot sensing probes	Photoluminescence	Infant's blood	2 to 20 mg dL^{-1}	-	[109]
4	Interleukin-6	Paper sensor for IL-6 detection in COVID-19 patients	Colorimetric	Respiratory	up to 10^{-1} $ng.mL^{-1}$	1 $fg.mL^{-1}$	[110]
5	Suppression of Tumorigenicity 2	Graphite paper-based disposable sensor through modification of fullerene C_{60}	CV, EIS	Serum	as low as 414 $ag.mL^{-1}$	124 $ag.mL^{-1}$	[111]
6	Bovine haptoglobin	AuNP/MWCNT-anti-Hpnanobioconjugate paper-based biosensor	Colorimetric	Serum	0.01 to 0.9 $mg.mL^{-1}$	28 $\mu g.mL^{-1}$	[112]

In addition to these applications, cellulose-based techniques have been investigated to detect glycoproteins, which are important in cell signalling, cell division, and cell migration, as well as as a marker for many illness diagnoses [113]. Sun *et al*., 2019 designed yet another paper-based electrochemical sensor for the ultra-sensitive detection of ovalbumin (OVA) glycoprotein. The biosensor was made by embedding Au nanorods in cellulose paper, which served as a platform for the fabrication of boronate-based molecularly imprinted polymers (MIPs) [105]. AuNPs were implanted on the surface of SiO_2 nanoparticles for biosensor synthesis, and the resulting SiO_2@Au was affixed with dsDNA to boost the signal. CeO_2 nanoparticles were employed as a binding indication for dsDNA, resulting in the development of the SiO_2@Au/dsDNA/CeO_2 signal tag. The boronate affinity-based MIPs were immobilised on a paper template to detect the target OVA glycoprotein by forming a covalent connection between the OVA and the boronic acid. The

developed biosensor's detection range was calculated to be 0.001 ng.mL^{-1} to 1 μg.mL^{-1}, with a LOD of 0.87 pg.mL^{-1}. Other proteins, such as bilirubin, linked to jaundice and other clinical problems, have been identified using cellulose-based sensors. Tabatabaee *et al*., 2019, reported a photoluminescent nanopaper-based biosensor for detecting bilirubin in infant blood samples in order to diagnose jaundice early. This creates a simple, effective, non-toxic, disposable, and low-cost biosensor that can be read on a smartphone [109]. POC and point-of-need platforms can benefit greatly from smartphone readout systems [114]. The photoluminescent carbon dot sensing probes were inserted in a bacterial cellulose nanopaper substrate to create it. Upon availability of bilirubin, which functions as a quencher, photoluminescence was quenched and selectively regained after exposure to blue light (λ= 470 nm). With a recognition scale of 2 to 20 mg.dL^{-1}, the biosensor's intensity was shown to be linearly proportional to the quantity of bilirubin content in the samples.

5.3 Applications for the detection of enzymes

In addition, the cellulose-based matrix is being investigated for the fabrication of biosensors for the detection of a variety of enzymes. Ling *et al*., 2019, reported a cellulose-based calorimetric sensor for detecting the human neutrophil elastase (HNE) enzyme [115]. HNE is a serine protease released by neutrophils in response to chronic wounds that causes the degradation of healing proteins. The HNE peptide was immobilised on cotton and wood nanocellulose to create the biosensor. Cotton CNCs are more sensitive to light than wood cellulose nanofibrils. The cotton CNCs colorimetric biosensor was found to have a sensitivity of less than 0.005 U.mL^{-1}. Aside from HNE enzyme, cellulose-based biosensors have been created for additional enzymes like acetylcholinesterase (AChE), which is involved in the breakdown of the neurotransmitter acetylcholine into acetic acid and choline. AChE is found in the junction of neuromuscular and the chemical synapse, where synaptic transmission is terminated [116]. Wang *et al.*, 2021, created a cellulose nanofiber-based sensor to detect AChE by grafting the DNA aptamer onto CNF. CNF-DNA was coupled with silver to create CNF-DNA-AgNCs to measure AChE activity, and chemical characterisation was done using FTIR, XPS, SEM, and TEM analyses [117]. AChE uses acetylthiocholine (ATCh) as a substrate, causing ATCh to hydrolyse and convert to thiocholine, which interacts with CNF-DNA-AgNCs. This overall reaction created the diagnostic signals that were calibrated to identify the target molecule. The constructed biosensor was shown to have a sensitivity of 0.053 mU.mL^{-1} for AChE concentration. These and the cellulosic matrix were created to detect some macromolecules, as shown in Table 3 with their design patterns and diagnostic results. Figure 8 shows a cellulose-based enzyme sensor with a surface associated with biomolecules and a surface related to a fluorogenic moiety.

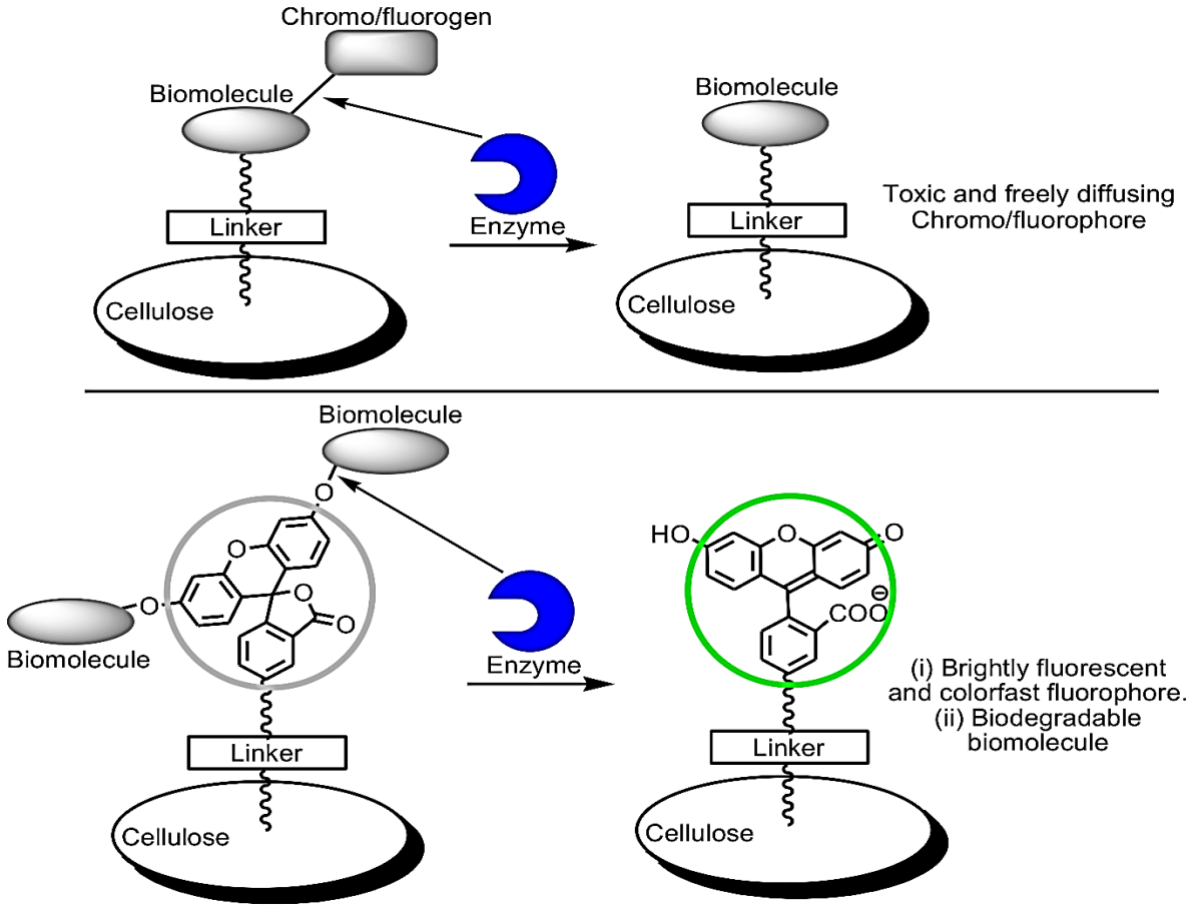

Figure 8: A cellulose-based enzyme biosensor comprises a surface connected with biomolecules (top) and a surface connected with a fluorogenic moiety (bottom). Reprinted with permission from [37] under Creative Commons Attribution license.

Table 3: Biosensors based on (nano)cellulose platforms for the detection of enzymes

Sl.	Analyte	Sensor design	Detection method	Sample source	Detection range	LOD	References
1	ALP	Paper-based visual detection	Colorimetric	Milk	10 to 1000 $U.mL^{-1}$	0.87 (±0.07) $U.mL^{-1}$	[107]
2	Esterase	Chemoenzymatic technique used for modification of cellulose matrix	Fluorescence	-	-	-	[118]
3	Human neutrophil elastase (HNE)	Immobilising HNE peptide to the cotton and wood nanocellulose	Colorimetric	Chronic wound fluid	Less than 0.005 $U.mL^{-1}$	-	[115]
4	Acetylcholinesterase (AChE)	DNA aptamer immobilised on the surface of cellulose nanofiber	Fluorescence	-	-	-	[117]

Nanocellulose can be used to generate enzymes as covalent connections, salt bridges, or physical inclusion complexes [119-125]. The hydrophilic surface features of cellulose make a biocompatible matrix that allows for specific action. The activity of glucose oxidase immobilised on nanocrystalline cellulose with AuNPs attached via thiols of polyethylenimine derivatives varied with the thiol linker [119]. Dendrimers were used as linkers to connect antigens to nanocellulose, then coupled with zeolite to increase particular antibody detection [120]. Glucose oxidase, useful in glucose checking for diabetes management, was also discovered in the first sensor [121]. Glucose oxidase was also immobilised on electrodes made from BNC-filtered carbon nanotube composites. The sensor demonstrated direct electron transport between glucose and glucose oxidase [122]. Quarternised cellulose nanoparticles were mixed with acetylene black and applied to physically embed glucose oxidase on the electrode surface, resulting in excellent glucose and H_2O_2 sensitivity [126]. Edwards *et al*. disclosed very high lysozyme action when the hydrolase was affixed to cotton CNCs [123], suggesting that anti-microbial enzyme activity can be boosted when immobilised on cotton CNCs, and this strategy may have further consequences for the detection and inhibition of microbial biofilm formation [124]. Finally, cellulases have been employed in an attempt that illustrates how enzymatic cellulose digestion combined with soft lithography can be applied to produce striped surfaces [125].

6. Conclusions

Cellulose is a common material that has several advantages over other materials. Characteristics of cellulose include mechanical strength, flexibility, biocompatibility, biodegradability, electrical qualities, and cost-effectiveness. Meanwhile, plant cellulose and bacterial cellulose have more desirable qualities such as mechanical strength, water-retaining capacity, and biocompatibility. Because of these benefits, cellulose has become a popular matrix material for biosensor construction in recent years. Some applications of cellulose-based sensing of functional biological macromolecules relevant to various illness states have been reported. Due to substantial advancements, numerous strategies for modifying cellulose into nanocomposite materials with unique structures and desirable features have been investigated. The numerous modifications required for cellulose matrix synthesis, as well as the engineering features of cellulose-based biosensors for their applications, have been comprehensively discussed.

We attempted to include all the biosensors for functional biomacromolecules that have been manufactured to date for various types of molecules, as well as the methodologies and methods utilised to modify them. Despite the significant development, there are still some challenges to be resolved. Recent improvements in cellulose-based biosensors in this industry often only have one

function type and lack multi-functionality, limiting their functionality and negatively impacting performance and customer satisfaction. A variety of novel technologies could be used in order to produce cost-effective multifunctional cellulose-based biosensors.

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Conflict of interest

The authors declare no conflict of interest.

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