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Nanocellulose and Proteins: Exploiting Their Interactions for Production, Immobilization, and Synthesis of Biocompatible Materials

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Abstract Nanocellulose has been used with promising results as reinforcement material in composites, many of which include hydrophobic polymers. However, the hydrophilic nature of nanocellulose can be better exploited in composites that incorporate high surface energy systems as well as in applications that can benefit from such properties. In fact, proteins can be ideal components in these cases. This paper reviews such aspects, which are based on the remarkable mechanical properties of nanocellulose. This material also exhibits low density, high aspect ratio, high surface area, and can be modified by substitution of its abundant hydroxyl groups. It also shows biocompatibility, low toxicity, and biodegradability. Convenient biotechnological methods for its production are of interest not only because of the possible reduction in processing energy but also because of positive environmental aspects. Thus, enzymatic treatments are favorable for effecting fiber deconstruction into nanocellulose. In addition to reviewing nanocellulose production by enzymatic routes, we discuss incorporation of enzyme activity to produce biodegradable systems for biomedical applications and food packaging. Related applications have distinctive features that take advantage of protein–cellulose interactions and the possibility of changing nanocellulose properties via enzymatic or protein treatments.

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Contents

1 Enzymatic Production of Nanocellulose

The production of nanocellulose through mechanical treatments requires high energy consumption [1], therefore a combination of different treatments has been suggested. One strategy to reduce the energy needed during these processes involves the use of different types of enzymes to improve accessibility and cellulose hydration and swelling. Also, reduction of the degree of polymerization of cellulose in fibers has been attempted by using cellulolytic enzymes. Specifically, endoglucanase enzymes are of interest because they preferentially attack the less crystalline regions within the fiber cell walls and cause their swelling and softening [2]. There are several studies highlighting the advantage of using enzymatic treatments for nanocellulose production (Table 1). In some cases, a reduction in yield as a result of cellulose loss is an important issue, for example, as reported in the case of fungal treatments [13].

An environmentally friendly method was developed by Henriksson et al. [3], who obtained microfibrillated cellulose (MFC) or nanofibers from bleached fibers after enzymatic hydrolysis with endoglucanases, followed by mechanical refining. The main advantage of this treatment compared with acid hydrolysis is the high aspect ratio of the nanofibers obtained after disintegration as a result of a decrease in the degree of polymerization of cellulose and an increase in swelling caused by endoglucanase action. These results were confirmed by another study that used a combination of high pressure shear forces and mild enzymatic hydrolysis to prepare MFC [4]. The material that resulted from using only mechanical shearing was not homogenous, in part because of blockages within the system. In contrast, when enzymatic hydrolysis steps were used between mechanical refining stages, the MFC obtained displayed a more uniform and smaller characteristic width and a high aspect ratio. This effect was mainly ascribed to cell wall delamination promoted by enzymatic action. The resulting material had higher elastic modulus than the material obtained using acid hydrolysis. Another interesting finding was the more

Table 1 Summary of some reported approaches to produce nanocellulose by using cellulolytic enzyme systems

Material	Pre/post-treatment	Enzymes used	Enzymatic hydrolysis conditions	Reference
Bleached wood sulfite pulps (*Picea abies*)	PFI-mill before and after enzymatic hydrolysis, mild acid hydrolysis (50°C, 1 h), stronger acid hydrolysis (NaOH 50°C, 10 min plus HCl 90°C, 2 h	Endoglucanase (commercial enzyme)	3% pulp, pH 7, 50°C, 2 h	[3]
Bleached sulfite softwood pulp	Refining before and after enzymatic hydrolysis plus homogenization	Endoglucanase	4% pulp, pH 7, 50°C, 2 h	[4]
Microcrystalline cellulose from cotton fibers	Hydrochloric acid hydrolysis (4 N)	*Trichoderma reseei* cellulases	5% inoculum, 1% MCC, 25 and 30°C, 150 rpm, 5 days	[5]
Recycled pulp (1% lignin)	Conventional and microwave heating after enzymes addition	Endoglucanase	1% pulp, 50°C, 60 min	[6]
Microcrystalline cellulose from cotton fibers	Hydrochloric acid hydrolysis (4 N)	Anaerobic microbial consortium (*Clostridium* sp. and coccobacillus)	1% MCC, 35°C, 5–15 days	[7]
Microcrystalline cellulose from *Cladophora* sp.		Exoglucanase	0.1% MCC, 38°C, pH 4.8, 2–3 days	[8]
Bacterial cellulose from *Acetobacter xylinum*		*Trichoderma reseei* cellulases	10% cellulose, pH 5, 50°C, 24 h	[9]
Bleached kraft eucalyptus pulp	Mechanical homogenization (microfluidizer) after enzymatic hydrolysis	Endo- and exoglucanase (commercial enzymes)	10% pulp, 5 and 10 FPU, 50°C, pH 4.8, 48 h	[10]
Bleached native sisal fibers	Mechanical shearing before and after enzymatic hydrolysis followed by mild acid hydrolysis	Endo- and exoglucanase (commercial enzymes)	2 and 5% pulp, 0.5 and 1% enzymes, 50°C, 2 h	[11]
Bleached native sisal fibers	Mechanical shearing before or after enzymatic hydrolysis followed by mild acid hydrolysis	Endo- and exoglucanase (commercial enzymes)	0.1% enzymes, 50°C, 2 h	[12]

entangled network formed by cellulose fibrils obtained enzymatically compared with those obtained by acid hydrolysis, which showed little or no entanglement. Siqueira and coworkers [12] took advantage of the combination of enzymatic hydrolysis followed by a mechanical shearing to produce nanocomposite films with good thermomechanical properties. A comparative study between commercial endo- and exoglucanases was performed earlier by same authors [11]. The enzymes were responsible for a much higher reduction in the degree of polymerization because they attacked specific sites on the chain and released small moieties in the form of nanoparticles, the morphology of which depended on the treatment used.

Fungi such as *Trichoderma reseei* have been used to prepare cellulose nanocrystals (CNC) from microcrystalline cellulose (MCC) from cotton [5], which was prepared by a conventional method employing hydrochloric acid. After controlled enzymatic hydrolysis, the slurry was subjected to additional fermentation stages to obtain CNC. It was found that the fungus consumed significant amounts of MCC for its own growth, as expected from the fact that cellulose was the only carbon source available for the microorganisms. In contrast to materials obtained after acid hydrolysis, fungal treatment produce no significant changes in surface chemistry. In fact, enzymatic or fungal methods do not install negatively charged groups on the surface (e.g., sulfate half ester groups from sulfuric acid hydrolysis) and result in material with negative zeta potential, less than −15 mV, making the material suitable for biomedical and related applications.

An integrated production of both cellulose nanofibrils (CNF) and bioethanol was developed by Zhu and coworkers [10]. The cellulosic material presented a decreased degree of polymerization after enzymatic hydrolysis, as found by other researchers, which facilitated the production of CNF by subsequent mechanical methods (microfluidization). The fiber length was significantly affected by cellulases, as observed in Fig. 1. The opacity and mechanical properties of nanopapers made from CNFs were better than those obtained from eucalyptus fibers. Moreover,

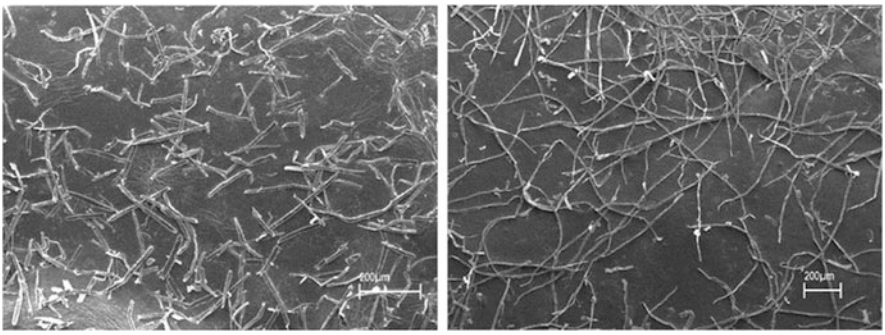

Fig. 1 SEM image of cellulosic material resulting from 48 h of enzymatic hydrolysis under enzyme loading of 5 FPU/g cellulase (*left*), and the original bleached Kraft eucalyptus fibers (*right*). Reproduced from Zhu et al. [10] with permission of The Royal Society of Chemistry (RSC)

and as a side advantage, the residual sugar stream was fermented by typical microorganisms to produce bioethanol with an efficiency of 92%.

Recently, Satyamurthy and Vigneshwaran [7] produced spherically shaped nanocellulose particles by using MCC subjected to degradation by an anaerobic microbial consortium of *Clostridium* sp. and coccobacillus. The nanocellulose obtained preserved its structure without any chemical modification, which makes it suitable for applications that demand minimum chemical changes to cellulose, such as biomedical, drug delivery and other applications requiring biocompatibility.

A major drawback of most methods for producing nanocellulose materials is the characteristic low yield. Satyamurthy and coworkers prepared CNC with a yield of 22% [5], whereas the same group reported a maximum yield of ~12% using an anaerobic microbial consortium [7]. In contrast, Filson et al. [6] studied the enzymatic hydrolysis of recycled paper using endoglucanases, following by microwave or conventional heating to produce related materials. The presence of nanocrystals was confirmed by flow birefrigerence and it was demonstrated that the heating method gave a higher yield (~38%) than conventional methods giving typical yields of ~ 29%. The authors highlighted the stability of the obtained crystals as nanofillers for reinforced polymer composites. They attributed the high negative zeta potential to the long-term stability of aqueous dispersions of CNC.

Although the production of nanocellulose from lignocellulosic materials has been heavily studied, other sources of cellulose could be useful. An exoglucanase (CBH I) was applied to produce shortened MCC from algal cellulose of *Cladophora* sp. [8]. These short elements exhibited high crystallinity because the cellulose allomorph I_α was preferentially degraded by the enzymes, leaving the highly ordered crystalline I_β domains unaffected. As an application, the authors indicated that the short elements could act as nano-ordered bioparticles.

Bacterial cellulose (BC) is a promising source for producing CNC. George et al. [9] prepared CNC from *Acetobacter xylinum* using cellulases from *Trichoderma reseei*. The amorphous domains were removed, whereas the crystalline portion was unaltered, in part because of better stability of this nanomaterial compared with material obtained by acid hydrolysis. Moreover, nanocomposites were produced using poly(vinyl alcohol) matrices. It was found that, even at low loading of CNC from BC (1 wt%), the mechanical and thermal stability was favorably affected.

Having discussed several prominent methods for producing CNF, MFC, and CNC, the following sections evaluate the functionality and application of these biobased nanomaterials. Not only does nanocellulose possess outstanding thermal and mechanical properties, it is also naturally biocompatible, which gives it tremendous potential in biomedical applications. Considered together, the mechanical properties, malleable nature, and biocompatibility render CNF, MFC, and CNC exceptional candidates in related fields.

2 Nanocellulose, Proteins, and Enzymes: Interactions and Immobilization

Nanocellulose is suitable for immobilization of different proteins. An inexpensive, simple, and direct immobilization method is desirable so that the nanocellulose can display its promising features [14]. Immobilization can be carried out by different mechanisms, involving covalent or noncovalent attachment, biochemical affinity, and physical adsorption (van de Waals forces, hydrogen bonds, electrostatic and hydrophobic interactions).

The immobilization of enzymes onto a material can help to increase their thermal and pH stability and provide relative longevity and reusability [15]. This could also allow substrates to be modified for biosensors, industrial applications, and continuous catalytic processes [15–17], as discussed in the next sections.

Ong et al. [14] demonstrated as early as 1989 that cellulosic materials offer a strong and stable noncovalent binding capacity for the carbohydrate binding domains (CBD) of certain cellulase enzymes, simplifying their immobilization onto the substrate. This technique was shown to extend enzyme activity (although decreased to 42% by immobilization) and helped to stabilize it against thermal and pH fluctuations [14]. Since the undertakings of Ong et al. [14], other successful studies utilizing covalent attachment have also been conducted [15, 18, 19]. Arola et al. [15] used CNF to covalently immobilize two types of proteins (alkaline phosphatase and anti-hydrocortisone antibody). Specialized techniques were utilized to conjugate the proteins to three CNF-derived substrates based on their prominent functional groups (epoxy, amine, and carboxylic acid) [15]. The study concluded that hydrophilic substrates can support immobilization better than their hydrophobic counterparts, and that certain kinds of covalent immobilization have a distinct advantage over nonspecific adsorption of proteins. Using this covalent approach, Mahmoud and coworkers [18] were able to attach an enzyme to a CNC matrix conjugated with gold particles. In this system, the specific enzyme activity and stability were improved [18]. Incani et al. [19] have similarly produced materials for use in biosensor applications by covalently immobilizing glucose oxidases (GOx) to CNC that had been previously modified with gold nanoparticles (AuNP), with their deposition being controlled using cationic polyethylenimine (PEI) at various pH levels [19] (see Fig. 2).

Adsorption interaction has been studied on cellulose-based aerogels with promising results [20–23]. The immobilized proteins tended to show increased thermal stability, probably as a result of noncovalent interactions. As a consequence, storage stability was improved [22]. Drug delivery based on nanocellulose has been studied [24]. The relative size of the drugs compared with the porous nature of CNF substrates was crucial, and electrostatic forces were found to be a primary mechanism of interaction. Such interactions were studied in the case of soybean protein adsorption on cellulose [25]. The storage proteins in soybean, glycinin, and β-conglycinin were found to interact with cellulose surfaces by different mechanisms (see Fig. 3). For instance, the adsorption of glycinin increased with

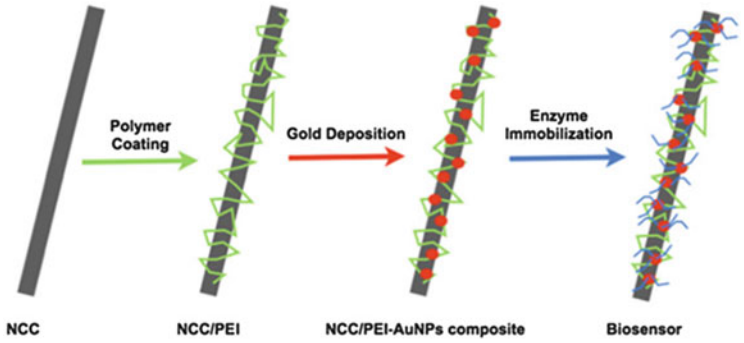

Fig. 2 Synthesis of a biosensor based on cellulose nanocrystals (here denoted as NCC) by modification with polyethylenimine and thiol-functionalized gold that is conjugated to glucose oxidase. Reprinted from reference [19], with kind permission from Springer Science and Business Media

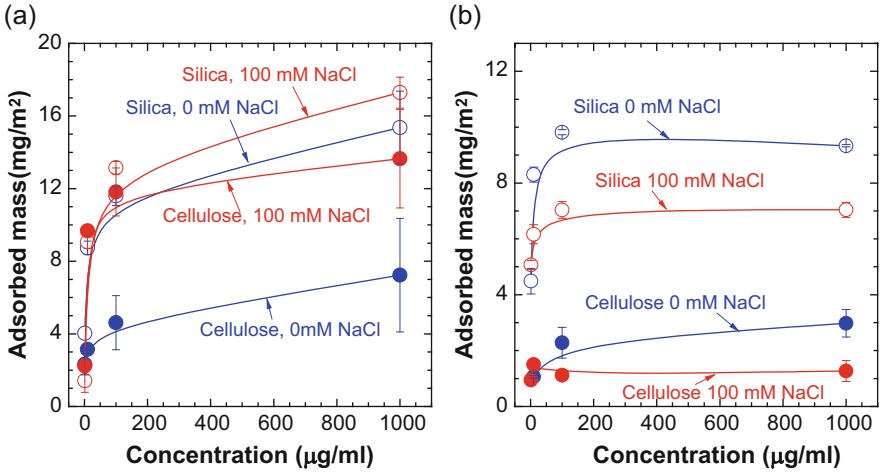

Fig. 3 Adsorption isotherms for (**a**) soy glycinin and (**b**) β-conglycinin on cellulose, as determined from quartz crystal microbalance measurements. Note the contrasting adsorption behavior of each protein as a function of ionic strength. Silica surfaces were used as reference, as indicated. Adapted from Salas et al. [25]. Reproduced with permission. Copyright © 2012 American Chemical Society

ionic strength but β-conglycinin adsorption was reduced. In addition, changes in pH and the use of a reducing agent (2-mercaptoethanol) were found to significantly reduce the adsorption of both proteins. For instance, 2-mercaptoethanol, a reducing agent of the disulfide bonds in proteins, unfolds the protein to expose their hydrophobic groups. The results highlight the fact that protein–cellulose interactions can be tuned by considering the protein structure and its response to physicochemical changes in the surrounding environment.

3 Nanocellulose–Protein Hybrids in 3D Structures: Gels/Hydrogels and Fibers

CNF surface modification via electrostatic interaction, adsorption, bioconjugation, or enzymatic catalysis can increase the versatility of CNF applications and result in increased material benefits. Examples of this include the production of bioinert or biospecific surfaces [26], cell adhesion on scaffolding [27], immobilization of proteins and enzymes for increased stability [14, 15, 19, 22], or production of novel nanocomposites for thin films, aerogels, and fibers.

Interactions between proteins and nanocellulose have been exploited in the development of hydrogels, which can also serve as template material for the preparation of aerogels (e.g., for drug encapsulation). One approach included coating of drug nanoparticles with hydrophobic proteins and embedding them in hydrogels that were subsequently freeze-dried into aerogels [28].

Although CNF is biocompatible, aerogels produced for cell scaffolding tend to have a relatively low affinity for cell attachment and require some protein-based modification to enhance this feature [27]. For instance, fibronectin and collagen type I were conjugated onto the surface of BC using 1-cyano-4-dimethylamino-pyridinium (CDAP) as crosslinking agent. This approach enhanced the adhesion and growth of human umbilical vein endothelial cells and mouse mesenchymal stem cell line C3H10T1/2 on bacterial nanocellulose [27].

CNF hydrogels can serve as three-dimensional (3D) cell culture scaffolds for the growth of human hepatic cells (HepaRG and HepG2). The approach included culturing the cells on the hydrogels. Evaluation of injectability of CNF hydrogels indicated that they can flow, even through very small needles, without damaging the cells. In addition, cell viability on CNF hydrogels was similar to that using conventional cell cultures, although cell growth was different for each type of cell studied. For example, HepG2 exhibited nonexponential growth and HepaRG showed less proliferation. Both cell types showed 3D multicellular spheroids (see Fig. 4) [29]. More recently, Lou et al. [30] used CNF hydrogels to create a 3D environment for proliferation and differentiation of human pluripotent stem cells (hPSCs). This new flexible culture system was able to maintain the pluripotency of hPSCs for up to 26 days, demonstrating that it could be a useful approach for research and regenerative medicine.

In related efforts, the ability of BC for cartilage regeneration was evaluated. Bovine cartilage samples were punched and BC inserted inside the cartilage cavity, followed by immersion in culture media for 8 weeks. The results indicated that cartilage cells still exhibited vital morphology after that period, with growth of chondrocytes on the surface of BC but not inside the pores. The chondrocytes at the nanocellulose surface showed successful re-differentiation [31].

BC nanofiber 3D networks, with pore sizes between 150 and 500 μm, were prepared by culturing *Gluconacetobacter xylinus* on medium containing paraffin beads that helped to create a uniform porous structure. These 3D networks served as

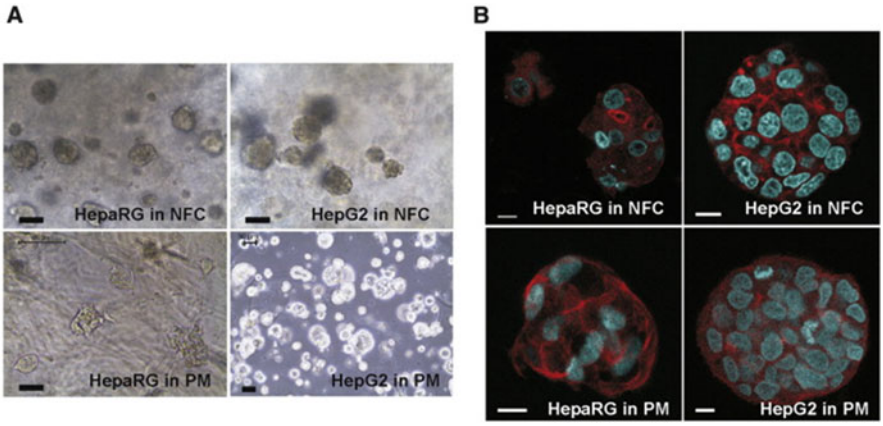

Fig. 4 Evidence of HepaRG and HepG2 cell spheroid formation in cellulose nanofibril (CNF) and peptide nanofiber (Puramatrix™, PM) hydrogel cultures. (**a**) Phase contrast microscopy and (**b**) confocal microscopy with structural staining of filamentous actin (*red*) and nuclei (*blue*). Reproduced with permission from reference [29]. Copyright © Elsevier

scaffolds for culture of human nasal and auricular chondrocyte cells and produced cartilaginous matrix protein for cartilage tissue engineering applications [32].

A different approach used unidirectional and 3D laser perforation with a CO_2 laser system to produce uniform, round-shaped pores (pore size ~220 μm) on never-dried BC hydrogels. The method included production of rectangular 3D porous structures that were used to grow bovine (24 h) and human (7–21 days) chondrocytes. The results indicated colonization of the BC nanofiber surface and of the laser-perforated channels with vital cells, with both unidirectional and 3D perforated channels, and allowed the re-differentiation of chondrocytes (see Fig. 5). The mechanical properties of the hydrogels were not significantly different from those of nonperforated hydrogels [33].

CNF hydrogels were used to culture HepaRG liver progenitor cells, which induced formation of 3D multicellular spheroids structures. Compared with hyaluronan gelatin hydrogels, the CNF hydrogels proved to be more effective for cell growth of undifferentiated cells and for maintaining differentiation of cells [34]. Likewise, BC hydrogels with high protein loads were prepared using a vortexing method, which took less time (10 min, uptake capacity of $8.4 \pm 0.1\%$) than the adsorption method (24 h, uptake capacity of $7.9 \pm 0.7\%$). The hydrogels produced by the faster method exhibited a denser fiber network morphology, slower protein release, and lower water holding capacity than conventional BC hydrogels [35].

Composites of BC with fish gelatin were prepared by immersing alkali-treated-BC pellicles in gelatin solutions and crosslinking the gels with different chemical agents (transglutaminase, genipin, and 1-ethyl-3-(3-imethylaminopropyl) carbodiimide hydrochloride, EDC). The results indicated an enhancement of the gel elastic behavior with increased protein content. In addition, the morphology of the

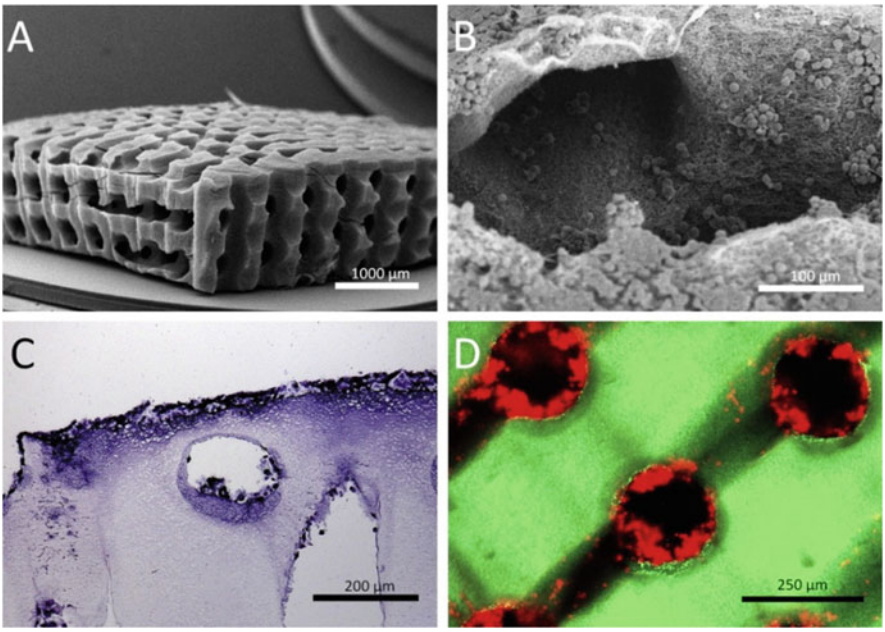

Fig. 5 (**a**, **b**) Scanning electron micrographs, (**c**) histological cross-section, and (**d**) laser scanning micrograph of 3D modified BC hydrogels (stained with 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein hydrochloride, DTAF) seeded with bovine chondrocytes. (**a**) View of 3D hydrogel channels. (**b**) Side view of channels showing chondrocytes (labeled with CellTracker Orange CMRA) adhered to inner surface after 24 h of culture. (**c**) Histological sections and (**d**) laser scanning micrographs of BC nanofiber surface and laser channels after seeding with cells of round morphology. Reproduced with permission from reference [33]. Copyright © Elsevier

composites indicated the formation of a dense porous network with gelatin covering the nanocellulose fiber network; after crosslinking, the gelatin improved the rehydration capacity of the material [36].

Widely available and inexpensive proteins from soy bean have been utilized to produce hydrogels and, subsequently, aerogels. For instance, soy protein isolate with a high protein content was used to prepare CNF–soy protein aerogels. The results indicated good synergy between the proteins and nanocellulose in the porous aerogels, which displayed mechanical properties comparable to those of aerogels obtained from pure nanocellulose. In addition, because of the hydrophilic nature of cellulose, these materials showed enhanced water absorption and, interestingly, similar absorption of nonpolar fluids [37]. Similarly, biocomposite porous scaffolds of ovalbumin/poly(vinyl alcohol) reinforced with CNC were prepared recently [38]. The addition of CNC as reinforcement increased the strength and flexibility of the porous scaffolds. The changes were explained by the different morphology of the aerogels obtained after addition of nanocrystals.

In addition to hydrogels, nanocellulose/protein composite fibers have been developed. CNC was used to reinforce prolamin protein (hordein/zein) electrospun

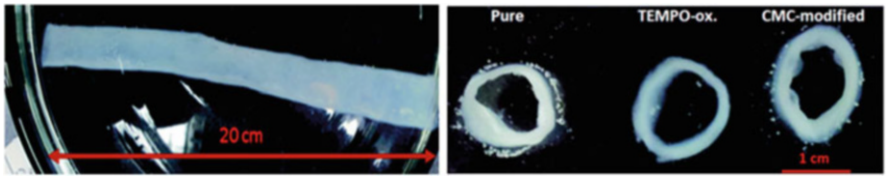

Fig. 6 Longitudinal (*left*) and cross-section (*right*) images of bacterial cellulose (BC) tubes. Tubes of pure BC, TEMPO-oxidized BC, and carboxymethyl cellulose (CMC)-modified BC were produced. Adapted from Orelma et al. [40] with permission from The Royal Society of Chemistry

nanofibers. The addition of cationically modified (using phenyltrimethylammonium chloride) CNC helped to increase the tensile strength, Young's modulus, water resistance, and alignment of the fibers. In addition, these fibers were encapsulated with a model drug (riboflavin) and were found to be effective for controlled release within a period of 24 h [39].

The potential of BC for selective biofiltration of blood proteins has been explored [40] by growing and modifying BC (from *Gluconacetobacter medellinensis*) in the presence of CMC. Such CMC-modified BC was used to synthesize tubules of given sizes. Synthesis was carried out using a silicon tube template through which air was supplied for bacterial growth. Also, 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO) oxidation was used to produce TEMPO-oxidized BC tubes. The CMC-modified BC tubes exhibited thicker walls than tubes of pure BC or TEMPO-oxidized BC (see Fig. 6). In addition, CMC not only reduced the irreversible structural changes in BC that occur upon drying but also facilitated the immobilization of anti-human serum albumin (anti-HSA) Affibodies via EDC-NHS conjugation. Interestingly, the CMC-modified BC carrying anti-HSA had better affinity for HSA than TEMPO-oxidized BC.

4 Biocompatible CNF/Polymer Systems

Nonspecific protein adsorption begins instantly after introduction of intracorporeal implants, marking the implant as a foreign or invasive entity needing to be destroyed or isolated [26, 41]. In either event, the effectiveness of the implant is obstructed by layers of protein or a fibrous avascular capsule growth that completely isolate it from the rest of the tissue [26]. Therefore, it is of extreme interest in the field of bionics to develop materials that are biocompatible. "Biocompatibility" can be described as the property of a material that provides an explicit purpose within an organism while, or by, suppressing or expressing all, most, or specifically few natural immunological or foreign body reactions by the organism [26]. Figure 7 illustrates a simplified model of immunoresponsive protein marking on bio-incompatible surfaces through adsorption and denaturation, and how polymer-coated surfaces can intervene in this interaction by making the

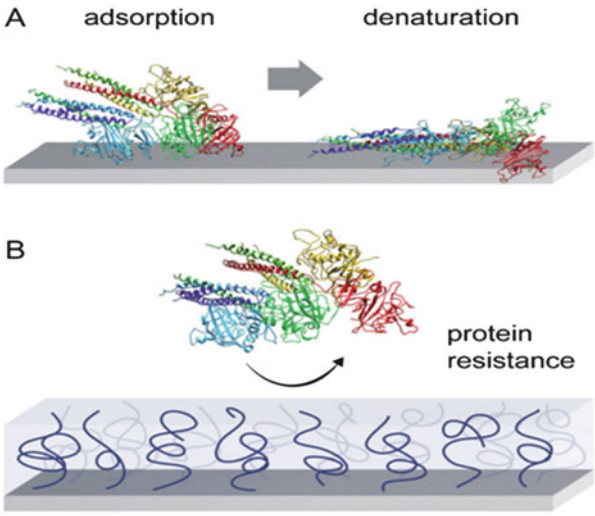

Fig. 7 (**a**) Dynamic adsorption and denaturation of proteins on a nonbiocompatible surface. (**b**) Protein resistance of a biocompatible polymer-coated surface. Reproduced from reference [26] by permission of John Wiley & Sons Ltd

surface more biocompatible [26]. The prevention of nonspecific protein adsorption is the key factor for biocompatibility of a material [26]. The surface characteristics responsible for preventing protein adsorption, referred to as "Whitesides rules," involve both the presence of polar and H-bond acceptor groups as well as the absence of net charge or H-bond donor groups [26]. This rule applies to cellulosic materials given their extreme hydration properties, which roughly compensate for the presence of H-bond acceptors in the form of hydroxyl groups [26].

In practice, CNF has been shown to be biocompatible [42–45]. Helenius et al. [46] demonstrated that subcutaneous implants of BC nanofibrils developed very little, if any, inflammation (both acute and chronic) and induced no foreign body responses, such as capsule formation or fibrosis [46]. CNF has been used in cell growth scaffolding for surrogate carotid arteries [42], tissue engineered blood vessels [44], cartilage [43], and burn tissue regeneration [45], to give a few examples.

Table 2 shows techniques that have been used for protein and enzyme immobilization on various cellulose–polymer matrices. Kuzmenko et al. [27] showed that cell adhesion to CNF scaffolds can be increased through bioconjugation of fibronectin and collagen proteins to its surface; these proteins are responsible for cell interactions [48]. This technique modifies CNF surfaces by adhering the cell binding domains of these proteins to the hydroxyl groups extending from the polysaccharides [27]. Crosslinking to produce an intermediate radical, followed by a nucleophilic substitution reaction with the protein amine groups, resulted in a stable, covalently bound protein. This, in turn, improved cell culture binding to the scaffolding, a crucial aspect for healthy tissue development [27]. Others have developed similar methods for different tissues, as each tissue type requires specific proteins for proper tissue adhesion and development [48–50].

Table 2 Techniques for protein/enzyme immobilization on nanocellulose/polymer systems

Protein(s)/ enzyme(s)	Polymer system	Method	Reference
Pancreatic serine protease trypsin	Poly(acrylic acid)-modified poly(glycidylmethacrylate)-grafted nanocellulose (PAPGNC)	PAPGNC-protein adsorption	[22]
Hemoglobin	Poly(methacrylic acid-*co*-vinyl sulfonic acid)-grafted–magnetite nanocellulose composite (P(MAA-*co*-VSA)-*g*-MNCC)	(P(MAA-co-VSA)-*g*-MNCC)-protein adsorption	[23]
Alkaline phosphatase/ anti-hydrocortisone antibody	TEMPO-/amine-/epoxy-functionalized CNF	Bioconjugation	[15]
Glucose oxidase	Nanocrystalline cellulose adorned with gold nanoparticles	Carbodiimide coupling	[19]
β-Casein	Nanocrystalline cellulose with functionalized reducing end	Click chemistry	[47]
Fibronectin and collagen type I	Bacterial nanocellulose	Bioconjugation using 1-cyano-4-dimethylaminopyridinium (CDAP) tetrafluoroborate as intermediate catalyst	[27]
Exoglucanase (from *Cellulomonas fimi*)	Cellulose material	Adsorption via cellulose-binding domain	[14]
Bovine serum albumin/antihuman IgG	TEMPO-oxidized CNF films	EDC/NHS activation	[16]

Protein adsorption has been studied on nanocellulose-based aerogels, and promising results for biomedical applications have been obtained [20, 22, 23]. Anirudhan and Rejeena [22] immobilized pancreatic serine protease trypsin (TRY) on a composite nanocellulose-based aerogel matrix through adsorption, resulting in increased thermal stability of the protein [22]. Storage shelf-life of the material was also improved by protein immobilization [22]. TRY is a protease enzyme used industrially for various applications and is notoriously unstable, making the enhancement of thermal and storage stabilities perspicuous advantages in the biomedical and food industries [22].

CNF has been investigated for the immobilization of proteins and subsequent film formation. In one study, nanofibers were first functionalized using different chemistries (amination, epoxydation, and TEMPO oxidation) then, alkaline phosphatase (AP) was conjugated in solution to each of the modified CNF, followed by spin-coating of these solutions onto silicon surfaces. Multiple layers of spin-coating

gave an increased amount of AP-conjugated CNF on the surface, as revealed by atomic force microscopy (AFM) imaging, which supports the hypothesis of higher enzyme immobilization. The results also indicated an increased stability of the AP-conjugated CNF at temperatures of 21°C and 37°C within a period of 168 h, which indicated biocompatibility for proteins [15].

The development of paper-based biosensors has been investigated. Orelma et al. [16] used TEMPO-oxidized CNFs, activated by EDC/NHS treatment, to conjugate antibodies and proteins. The adsorption of a human blood protein (human immunoglobulin G, hIgG) and bovine serum albumin was tested, demonstrating the use of this surface for the detection and diagnosis of biomolecules. Similarly, Zhang et al. [17] immobilized acetylated HWRGWVA peptide onto bioactive cellulose nanofibrils using the copolymer poly(2-aminoethyl methacrylate hydrochloride-*co*-2-hydroxyethylmethacrylate) as spacer and support layer. This modified-CNF network exhibited a high specific binding capacity for hIgG and high nonspecific protein resistance.

5 Enzymatic Modification of CNF

The natural hydrophilicity and ability of CNF to hydrogen bond into agglomerates makes it difficult to evenly disperse them amid nonpolar polymers in composites without some previous surface modification [51]. One technique uses TEMPO-mediated oxidation to increase the electronegative charge of CNF through the addition of anionic carboxylate groups [51]. Although TEMPO and its derivatives work as catalysts, they are continually reoxidized by primary oxidants, such as NaBr/NaClO or NaClO/$NaClO_2$ reagents in alkali conditions [52]. Oxidative enzymes have shown promise in replacing these primary oxidants while simultaneously maintaining milder reaction conditions. TEMPO-mediated oxidation using laccase enzymes with high oxidation rates was studied for the benefits of milder conditions and potential economic and ecological soundness [52, 53]. It was discovered that when using laccases as the primary oxidant, the percentage of aldehydes produced during oxidation increased between three- and fivefold compared with the chemical system. There were insignificant changes to the nanofibrillar structures, which could prove useful for various composites [52]. The investigation also revealed that site-specific surface modification produced a unique nanocellulose-derived product that could be of use in a number of novel nanocomposites [52]. Application of enzymatic modification of TEMPO-oxidized materials was investigated by Li et al. [54], who prepared nanocomposites through polymerization of phenol enzymatically in the presence of TEMPO-oxidized CNF. Polyphenols formed globular clusters on the nanocellulose, which improved the thermal stability and toughness of the composites and decreased their solubility in organic solvents [54].

Other work utilizing laccases has also been conducted. Garcia-Ubasart et al. [55] showed that the hydrophobicity of nanofibrillated cellulose can be controlled

through laccase-mediated coupling of different short, hydrophobic chains to its surface. The coupling reaction, catalyzed by laccase, showed that hydrophobization could be maximized by coupling dodecyl 3,4,5- trihydroxybenzoate (HB-C12) with flax fiber-based nanofibrillated cellulose [55]. The resulting water contact angles of the fiber webs were found to be 80–96° degrees, significantly greater than those of the control [55]. Cusola et al. [56] also manipulated surface hydrophobicity through applying a novel, multicomponent colloidal system comprised of laccase, hydrophobic dodecyl 3,4,5-trihydroxybenzoate (LG), and dispersant (sulfonated lignin) to couple LG onto the surface of CNF. It was observed that the low surface energy of LG was imparted to the composite and that the surface roughness greatly diminished, as shown by a 90° increase in water contact angle and AFM imaging on spin-coated thin films, respectively [56]. These reports concluded that laccase is capable of modifying the surface of cellulose-derived materials through the coupling of hydrophobic materials.

6 Final Remarks

The production of nanocellulose materials from lignocelluloses or other sources is still at the developmental and demonstration scale. According to the discussion presented here and literature on the subject, there is a large interest in the incorporation of biologically derived macromolecules. Examples include enzymes to decrease the energy demand of nanocellulose production, proteins and other molecules for development of bioactive cellulose, and novel materials. In these fields, chemical stability, (anti)fouling properties, swelling, and water resistance are central aspects that affect the full realization of these approaches involving proteins in their various forms.

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