Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BACTERIAL NANOCELLULOSE AND ITS APPLICATION FOR MUCOADHESIVE FORMULATIONS

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Abstract

Bacterial nanocellulose (BNC) is a fibrillar nanomaterial composed of β-(1 → 4) glucan chains, with <100 nm widths. Usually, the BNC is produced by mechanical disintegration of the cellulose fibrils network biosynthesized by several bacterial species, both gram-negative bacteria such as acetic acid bacteria, agrobacteria, rhizobia, and gram-positive bacteria from Sarcina and Bacillus genera. One dimension of BC is still micrometric; therefore, it is considered a 1D nanomaterial. BNC presents suitable mucoadhesive formulation features – biocompatibility and biodegradability, water retention, shear-thinning, good interaction with mucin. In this review, we focus mainly on the non-Newtonian behavior / shear-thinning characteristic of the BNC hydrogel. Due to this characteristic, BNC could be used as an in-situ thickener for the mucoadhesive formulations, which generate low viscosity gel and droplets.

Key words*: bacterial nanocellulose, mucoadhesive formulations, in situ thickener, shear-thinning.*

INTRODUCTION

Nanocellulose is a fibrillar (cylindrical) nanomaterial composed of β-(1 → 4) glucan chains (Thomas *et al.*, 2020). Nanocellulose is considered a 1D-nanomaterial because one of its dimensions, the length, is usually outside of the upper limit of the nano-range, i.e., 200 nm (Fang *et al.*, 2019).

Nanocellulose is usually prepared from two approaches. The top-down process involves: (i) separation of cellulose fibrils from the other biopolymers associated with the plant lignocellulose matrix, majors (hemicelluloses, lignin) and minor (pectin / pectic fractions and glycoproteins such as arabinogalactan), and (ii) conversion of the separate cellulose fibers into nanocellulose (Pirich *et al.*, 2020). The nanocellulose produced from the top-down process is classified as cellulose-nanofibrils (CNF) and cellulose nanocrystals (CNC) (Gupta & Shukla, 2020). CNF diameter is from 5 to 60 nm, and its length is in the micron range (Oberlintner *et al.*, 2021). The average dimension of CNC depends on the preparation methods, from 3 to 35 nm in diameter and from 20 nm to 1000 nm in length (Clemons, 2016; Nechyporchuk *et al.*, 2016).

The bottom-up process is a biosynthetic one. The resulting nanomaterial is called bacterial nanocellulose because it is produced by converting the cellulose fibrils produced by bacteria (de Amorim *et al.*, 2020). Various bacterial species, both gram-negative bacteria such as acetic acid bacteria, agrobacteria, rhizobia, and gram-positive bacteria from *Sarcina* and *Bacillus* genera, produce cellulose (Jozala *et al.*, 2016; Romling & Galperin, 2015). Bacterial cellulose is free of lignin, hemicellulose, pectin, glycoproteins. Usually, bacterial cellulose is produced as a membrane/ pellicle, including a network of β-(1 → 4) glucan chains (Sharma & Bhardwaj, 2019). The conversion of bacterial cellulose ribbon to bacterial nanocellulose (fibrils) is usually done by applying high-shear forces intended to de-fibrillate the fibrils network. Such high-shear forces are typically provided by microfluidization equipment (Dima *et al.*, 2017; Salimi *et al.*, 2019), ball milling (Piras *et al.*, 2019), or colloidal milling (Panaitescu *et al.*, 2016).

Bacterial nanocellulose (BNC), prepared by a bottom-up approach, has several advantages comparing to plant nanocellulose. These advantages result from the absence of other biopolymers and the more porous structure and larger surface area (de Amorim *et al.*, 2020).
The absence of other associated biopolymers (and/or their degradation products formed during the preparation process) reduces the risk for adverse biological reactions. The porous structure promotes hydrogel formation and enhances the capacity of loading with bioactive substances. Due to these characteristics, bacterial nanocellulose gathered more interest in the biomedical field than plant nanocellulose (Jozala *et al.*, 2016; Sharma & Bhardwaj, 2019). Various biomedical applications were reported for nanocellulose: formulation agent for drug/ bioactive compounds delivery ((Pötzinger *et al.*, 2017); support and enhancer for wound-dressing formulation (Liyaskina *et al.*, 2017); scaffold for cell and tissue culture (Sämfors *et al.*, 2019), carriers for 3D bio-ink formulations used in 3D bioprinters (Apelgren *et al.*, 2019).
In this review, we focus on the BNC utilization for mucoadhesive formulations. We will describe several features essential for mucoadhesive formulations, such as non-Newtonian behavior/shear-thinning and mucin interaction of the BNC hydrogel.

NANOBACTERIAL CELLULOSE PREPARATION

As we already mentioned, bacterial cellulose (BC) is produced by bacterial biosynthesis. Several bacterial strains produce cellulose as the main component of a biofilm with specific functions. Such BC biofilms facilitate bacteria interactions with other microorganisms (in the mixed biofilms consortia) or with their metazoans hosts and promote survival in extreme environments.
In the case of Proteobacteria, BC facilitates the establishment of beneficial (i.e., symbiotic) or deleterious (i.e., pathogenic) relationships with plant, fungal or animal hosts (Augimeri *et al.*, 2015). Rhizobia produce BC during their early legume root colonization (Poole *et al.*, 2018). *Pseudomonadaceae* use their BC to promote phyllosphere colonization (Arrebola *et al.*, 2015). Enterobacteriaceae produce BC to facilitate their adherence to fresh vegetables and further intestinal colonization (Yu & Shi, 2021). In other bacteria, BC biofilm promotes their development in an environment with extreme conditions. Acetic acid bacteria produce BC pellicle, which allows them to survive at a pH lower than 1 (Qiu *et al.*, 2021). Thermophilic *Bacillus licheniformis* strain ZBT2 produces bacterial cellulose biofilm at 50°C temperature (Bagewadi *et al.*, 2020). Cellulose is the main component of the sulfur-turf bacterial mat developed in Yellowstone hot springs (Ogawa & Maki, 2003; Romling & Galperin, 2015).
The scheme of the BC production and conversion to BNC is presented in Figure 1.

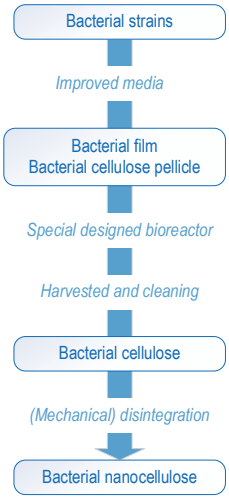

Figure 1. Scheme of bacterial nanocellulose (BC) production and its conversion to bacterial nanocelllulose (BNC)

Because BC is associated with biofilm formation, BC biosynthesis is challenging. The formation of the large pellicle, including strong cellulose chains/fibrils, require static conditions. However, BC producers are aerobes needing large O_2 amounts, challenging to be supplied in static conditions. BC production in submerged cultivation was also tested. In submerged fermentation, BC in the form of small pellets was produced, instead of BC membrane/pellicle BC prepared from pellets present worst mechanical properties, due to a lower degree of polymerization and reduced crystallinity, an, compared to BC prepared from pellicle produced by the same strains, in the same cultivation media, in static conditions. These

worst mechanical conditions, associated with a less-organized form of BC, can result from the shear stress resulting during the agitation for aeration of the submerged biosynthesis.

Various bioreactor designs were explored to increase BC production, improving the ratio between the oxygen-rich surface and total bioreactor volume. Such design included: cylindrical silicon vessel, aerated from bellow (Yoshino *et al.*, 1996); rotating disk reactors (Serafica *et al.*, 2002); rotary drum bioreactor (G. Chen *et al.*, 2019); moving bed biofilm reactor (Cheng *et al.*, 2009), static, intermittent fed-batch (Sharma & Bhardwaj, 2019).

Another challenge to producing bacterial cellulose/nanocellulose is related to the composition of the media used to make cellulose by cultivating the acetic acid bacteria, including the most known producer of bacterial cellulose - *Komagataeibacter xylinus*. The initial growing media was a complex media, including glucose, yeast extract, and peptone (Hestrin & Schramm, 1954). Other complex media were developed, replacing the peptone with corn steep liquor (Zhou *et al.*, 2007) or with ammonium sulfate (Yamanaka *et al.*, 1989). Various by-products from the bioeconomy were used as ingredients of such complex media to produce bacterial cellulose: raw glycerol, a by-product from the production of the biodiesel from soybean oil (Jung *et al.*, 2010; Tsouko *et al.*, 2015); the wastewater from acetone - butanol - ethanol fermentation (Huang *et al.*, 2015); spent beer yeast ((D. Lin *et al.*, 2014); vinasse from the production of (bio)ethanol from molasses (Barshan *et al.*, 2019); cheese whey and sugar beet molasses (Salari *et al.*, 2019); kitchen waste (M. Wu *et al.*, 2019); sugar cane molasses (Abol-Fotouh *et al.*, 2020).

Chemically defined media were also developed almost twenty years ago (Heo & Son, 2002), replacing bacterial growth factors from yeast extract with inositol and nicotinamide. Further developments lead to delivering a minimal defined media (de Souza *et al.*, 2019). Table 1 summarizes the composition of several complex media and the chemically defined and minimal defined media developed in the last years.

Table 1. Composition of the various media used to produce bacterial nanocellulose

Components	Complex Media (g/L) (Hestrin & Schramm, 1954)	Complex Media (g/L) (Yamanaka *et al.*, 1989)	Complex Media (g/L) (Zhou *et al.*, 2007)	Chemically defined medium (g/L) (Heo & Son, 2002)	Minimal defined medium (g/L) (de Souza *et al.*, 2019)
Glucose	20	50	40	40	4.5
Ethanol	-	-	-	11.05	
Corn steep liquor	–	–	20	-	-
Yeast extract	5	5	–	-	-
Peptone	5	–	–	-	-
Na_2HPO_4	2.7	–	–	3.0	2.32
Citric acid. H_2O	1.15	–	–	-	-
Inositol				0.0025	-
Nicotinamide				0.0006	-
$(NH_4)_2Cl$				-	0.5448
$(NH_4)_2SO_4$	–	5	4	2.0	-
KH_2PO_4	–	3	2	2.5	1.2
$MgSO_4.7H_2O$	–	0.05	0.4	0.05	0.1971
$CaCl_2x_2H_2O$	-	-	-	-	0.0294
H_3BO_3	-			0.0025	-
$FeSO_4x7H_20$	-	-	-	0.002	-

Bacterial nanocellulose was used for several biomedical applications. Several of these applications are listed in Table 2.

Essential BNC characteristics for biomedical applications are, besides those already mentionned, higher surface area and higher porosity, are water retention/thickener, and shear-thinning. Our group developed a process for preparing bacterial nanocellulose from the Kombucha pellicle, a side-stream of the production of a healthy beverage - tea fermented with a SCOBY consortium (Dima *et al.*, 2017).

Table 2. Biomedical applications of bacterial nanocellulose

Application	Other ingredients	References
Wound dressing	silver nanoparticles	(J. Wu *et al.*, 2014)
	chitosan	(W.-C. Lin *et al.*, 2013)
	montmorillonite	(Ul-Islam *et al.*, 2013)
Drug delivery	tizanidine, famotidine	(Badshah *et al.*, 2018)
	tetracycline	(Shao *et al.*, 2016)
Scaffold for cell culture	bone morphogenetic protein-2	(Shi *et al.*, 2012)
Bio-ink for the 3D bioprinter	alginate	(Jessop *et al.*, 2019)
Facial mask	essential oil	(Indrianingsih *et al.*, 2020)
Mucoadhesive formulation	curcumin, gelation	(Chiaoprakobkij *et al.*, 2020)

We should mention here that the bacterial pellicle protects the SCOBY consortium from extreme conditions similar to Mars (Orlovska *et al.*, 2021).

We develop the process involving an alkaline cleaning process (intended to remove both biomass and melanoidins formed during SCOBY fermentation) and a two-step defibrillation process - Figure 2. The resulting BNC exhibit higher swelling/higher water retention characteristics than the BNC produced by the pure culture of acetic acid bacteria. This higher porosity is also because the pellicle should accommodate a consortium that includes yeast, larger than acetic acid bacteria.

The nanocellulose produced from Kombucha has better water retention and shear thinning characteristics compared to nanocellulose made by pure acetic acid bacteria.

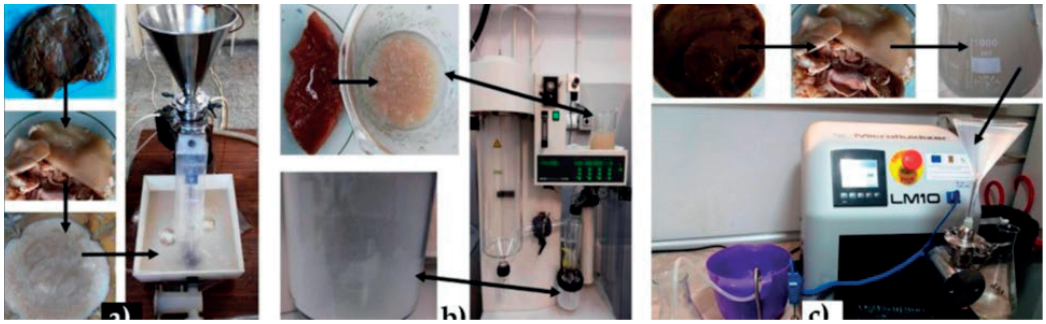

Figure 2. Purification and preparation of bacterial nanocellulose (BNC) from Kombucha pellicles: (a) purification, grinding with a blender, and deep grinding with a colloidal mill; (b) purification by alkaline treatment and drying by using a spray-dryer; (c) purification by alkaline treatment and defibrillation by microfluidization at pressures higher than 1300 bar. From Dima *et al.* (2017).

Never dried bacterial nanocellulose

For mucoadhesive formulations, one of the most efficient forms of BNC is the never-dried BNC. Never-dried BNC (NDBNC) has specific properties that result from its biosynthesis peculiarities. Clusters of the water molecules adsorbed by the nascent β-(1 → 4) glucan chains are kept in their initial, native stages. During the drying process, the structure of BNC collapse (S.-Q. Chen *et al.*, 2020), and the loading capacity for the bioactive ingredients significantly decreases.

BNC has a wide palette of properties, like biocompatibility, high hydrophilicity, flexibility, transparency, high mechanical strength, chemical stability, high surface area, and rich surface chemistry. NDBNC also has a higher purity compared to plant cellulose and a high degree of polymerization (up to 8000), presents a 3D network of nanofibrils of 40-60 nm in diameter and has a crystallinity up to 90% and a water content up to 99% (Muller *et al.*, 2013). Water vapor permeability of NDBNC varies from 1 x 10^{-13} g m^{-1} s^{-1} Pa^{-1} at 31% humidity to 1.1 x 10^{-12} g m^{-1} s^{-1} Pa^{-1} at 82% humidity. Moreover, NDBNC showed biocompatibility and favorable adhesion and growth of tissue cells such as chondrocytes on the BNC matrix (Ahrem *et al.*, 2014). Unmodified NDBNC showed a three-layer structure with a heterogeneous pore distribution and a relatively low diffusion of bovine chondrocytes through the outer layers, of a maximum 70 μm depth. From the rheological point of view, NDBNC showed

an elastic behavior, but not a visco-elastic one, and also presents a strain-independent elastic modulus, without meaningful changes after 26% cellulose loss by laser perforation. NDBNC has a superior mechanically resistance and a higher adsorption capacity for proteins, like cellobiose dehydrogenase, for peptides and chimeric proteins. (Muller *et al.*, 2013). Compared to freeze-dried BNC who presented a 7.2% uptake capacity and 12.5-128.3% bovine serum albumin loading, never-dried BNC showed a 9.8% uptake capacity and 15.5-132.2% protein loading. Also, NDBNC has a lower susceptibility towards esterification with organic acids than freeze-dried BNC. NDBC has a lower susceptibility to chemical functionalization in general, maybe because of the residual, intermolecular water molecules that block a part of the -OH groups (Lee & Bismarck, 2012).

NDBNC low susceptibility to chemical functionalization makes it more attractive to physical modification and blending. Nanocellulose blending is largely used to obtain nanocomposites, aerogels, hydrogels, and other nanomaterials. Still, the majority of the studies are using nanocellulosic materials (cellulose nanocrystals – CNC, cellulose nano-fibers – CNF) derived from wooden plants (Abitbol *et al.*, 2016; De France *et al.*, 2017; Dumanli, 2017; Zubik *et al.*, 2017). NDBNC can be physically functionalized in micro-channels creation using 3D laser perforation and cutting by pulsed CO_2 (Ahrem *et al.*, 2014). The 300 µm laser beam performed uniform 200 µm channels inside the BNC matrix. Channels proved to increase the porous hydrogel's homogeneity without changes in the elastic modulus. This finding means that, without losing biomechanical resistance, although an estimated 26% BNC is lost by laser perforation. Moreover, FT-IR and XPS analyses evidenced no chemical changes in the hydrogel's structure after laser contact and no undesirable effect on the vitality of bovine chondrocytes. Instead, a stimulated growth of vital cells inside the laser-perforated hydrogel, making the BNC hydrogels more appealing in tissue engineering as an implant material for *in situ* cellularizations (Ahrem *et al.*, 2014). The porosity of NDBNC can also be modified during the biosynthesis process using water-soluble and insoluble additives like β-cyclodextrin, poly(ethylene glycol), or carboxymethylcellulose (Muller *et al.*, 2013). Further physical modification can be performed by coating BNC with polymers, metals, metal oxides (Muller *et al.*, 2013). Chemical functionalization of NC includes phosphorylation, amidoximation, carboxy-methylation (Muller *et al.*, 2013), functionalization with carboxylic acids, acrylamide, xyloglucan, alkyl chains (Mondal, 2017), or with trimethylsilane (Grunert and Winter, 2002). NDBNC was less studied as nanofiller or structural nanomaterial. Some blends of NDBNC are reported to be performed using poly(vinyl alcohol) (Tan *et al.*, 2015) or chitosan (Cabañas-Romero *et al.*, 2020).

MUCOADHESIVE FORMULATIONS

Biocompatible drug-delivery systems are of high interest for the medical and pharmaceutical fields, particularly in developing new alternative products for adjusting vaginal microflora imbalances. Some contain probiotic lactic strains (Liu et al., 2012), but in the case of lactobacilli presence in the treated vagina, the products have limited efficacy due to intra-specific competition. Moreover, they can induce adverse effects, such as urinary tract infections (Czaja et al., 2007).

Various mucoadhesive compositions are known to be desirable for re-balancing vaginal microflora. The normal vaginal microflora in women at the age of reproduction is characterized by the dominance of lactic acid-producing bacteria from the *Lactobacillus* group. They maintain an acidic pH of the vaginal fluids and represent a biomarker of the health status (Palmeira-de-Oliveira et al., 2015).

More than 70% of adult women had vaginal problems and used vaginal products to treat infections. Recent studies have demonstrated the antimicrobial, antifungal, and immunomodulatory properties of lactic acid produced by lactobacilli and have evaluated the use of lactic acid or probiotic lactobacilli in the prevention or treatment of bacterial vaginitis (Tachedjian et al., 2017).

An alternative to probiotics is represented by the new generation of prebiotics such as polyphenols from plant extracts, which specifically stimulate the growth of microbial populations from healthy human microbiocenosis. In some

compositions, prebiotics compounds are the only active ingredients. In others, they are associated with estrogenic plant extracts that favor the trophism of vaginal mucosa (Bou A.S., 2010) or with anti-Candida thiazole antibiotics (Dikovskiy et al., 2015).

Essential oils have a significant capacity to remove biofilms formed by pathogenic microorganisms in the vagina (Bogavac et al., 2015) and are considered an important component of new strategies for local treatment of intestinal microflora imbalances. However, essential oils may inhibit the development of lactobacilli (Dunn et al., 2016). To increase the selectivity of essential oils towards beneficial vaginal microflora, new technical solutions are required to protect lactobacilli and stimulate their development.

Another essential characteristic of the (ND)BNC for the mucoadhesive formulation is related to the non-Newtonian behavior, i.e., shear-thinning. Such behavior means that when a force is applied to the (ND)BNC hydrogel, the viscosity of the solution is significantly decreesing. Such a feature allows the development of a formulation that is a robust and stable gel (keeping uniformly distributed low soluble ingredients), easy generate low-viscosity droplets, which reconstitute stable, adhesive droplets on the target organs - Figure 3.

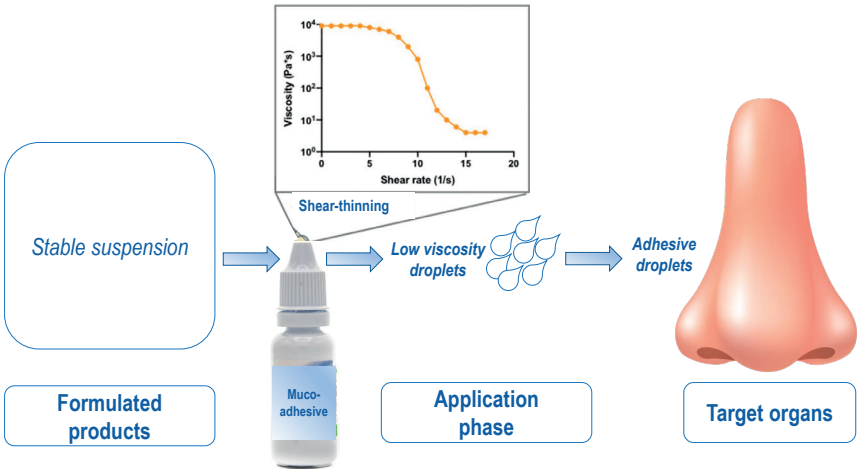

Figure 3. Illustration of the importance of the shear-thinning feature for the formulation of stable, easy to apply and efficient mucoadhesive product

Such a shear-thinning characteristic is essential also for the development of the bio-ink used for 3D-bioprinting. Stable suspension should be converted in low viscosity droplets, which produce stable and adhesive additive layers (Wilson *et al.*, 2017).

Another characteristic which is necessary for the mucoadhesive formulation is thermogelling. Thermogelling systems have been developed to improve vaginal drug delivery due to their liquid form at room temperature and the sol-gel transition at physiological temperature. Additionally, after gelling, these systems may exhibit mucoadhesion improving retention in the vaginal cavity. The gelling temperature (T_{gel}) specific to each termogelling system is crucial for their performance and ranges between 25-37°C. Termogelling properties of the vaginal formulations rely on the use of some specific polymers, among which poloxamers were the most studied. At physiological temperature in suitable interactions, poloxamers solutions change from micellar properties and hydrophobic interactions, leading to a reversible sol-gel transition. (Palmeira-de-Oliveira et al., 2015).

To develop such formulation, additional polymers are needed. Pharmaceutical poloxamers are available under the trade name Lutrol® (Europa) and Pluronic® (BASF), Pluronic® F127 (poloxamer 407, P407) and Pluronic® F68 (poloxamer 188, P188) (SUA) (Palmeira-de-Oliveira et al., 2015). The role of mucoadhesive polymers like poloxamer, chitosan, gelatin

cellulose derivatives, and the combination thereof is essential in obtaining various efficient product for the treatment of urogenital infections, especially vaginal infections.

CONCLUSIONS

Bacterial nanocellulose (BC) and never dried cellulose (NDBNC) presents essential features for mucoadhesive formulation features – biocompatibility and biodegradability, water retention, shear-thinning, good interaction with mucin. Shear-thinning is essential for a formulation that is a robust and stable gel (keeping uniformly distributed low soluble ingredients), easy generate low-viscosity droplets, which reconstitute stable, adhesive droplets on the target organs.
Additional biopolymers and active ingredients are necessary to optimise others characteristic, such as thermogelling, important for formulation of the efficient mucoadhesive products.

ACKNOWLEDGEMENTS

This work was supported by European Regional Development Fund (ERDF), the Competitiveness Operational Programme (POC), Axis 1, project POC-A1-A1.2.3-G-2015-P_40_352, My_SMIS 105684 SECVENT.

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