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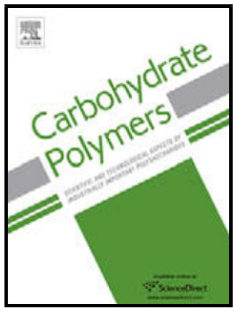

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Structural modification of Bacterial Cellulose fibrils under ultrasonic irradiation

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Highlights

- Short US treatment leads to a decrease in BC fibril dimensions
- Increase in stability, viscosity and thixotropy of BC suspensions
- Improvement of the physical properties of BC suspensions

Abstract

In the present study we investigated ultrasounds as a pretreatment process for bacterial cellulose (BC) aqueous suspensions. BC suspensions (0.1-1% wt) subjected to an ultrasonic treatment for different time intervals. Untreated BC presented an extensively entangled fibril network. When a sonication time of 1 min was applied BC fibrils appeared less bundled and dropped in width from 110 nm to 60 nm. For a longer treatment (3-5 min) the width of the fibrils increased again to 100 nm attributed to an entanglement of their structure. The water holding capacity (WHC) and ζ-potnential of the suspensions was proportional to the sonication time. Their viscosity and stability were also affected; an increase could be seen at short treatments, while a decrease was obvious at longer ones. Concluding, a long ultrasonic irradiation led to similar BC characteristics as the untreated, but a short treatment may be a pre-handling method for improving BC properties.

Keywords

Bacterial; cellulose; suspension; ultrasounds; fibrils; rheology

1. Introduction

Cellulose is a linear biopolymer of glucose that mainly exists in plants as a structural component of cell walls. Cellulose consists of an amorphous and a crystalline portion. While crystalline cellulose consists of long chains bound together by strong hydrogen bonds, amorphous cellulose is made up of shorter and weaker chains(Türünç & Meier, 2012).

BC and plant derived cellulose have the same chemical structure, but BC is obtained from bacterial species, such as *Komagataeibactersucrofermentans,* which have the ability to synthesize pellicles of cellulose, when placed in a culture medium(Martinez-Sanz, Lopez-Rubio & Lagaron, 2011; Okiyama, Shirae, Kano & Yamanaka, 1992). This pellicle consists of a bundle of fibrils of about 4 μm wide, which are composed of random nanofibrils less than 100 nm wide (Okiyama, Motoki & Yamanaka, 1993).

BC has unique physicochemical properties such as higher water holding capacity, higher crystallinity and higher purity as it does not associate with lignin and hemicelluloses, in contrast to plant derived cellulose (Iguchi, S. & A., 2000; Martínez-Sanz et al., 2013; Salas, Nypelö, Rodriguez-Abreu, Carrillo & Rojas, 2014). Thanks to these properties, BC has been receiving increased attention and has been used in various areas such as biomedicine, cosmetics, paper industry and many others (Iguchi, S. & A., 2000). Although not extensively used in food yet, BC has great potential as a food ingredient, changing the rheological profile of a food, as it serves as thickening, stabilizing or gelling agent. Recently, BC has been shown to act as a stabilizer in emulsions(Kalashnikova, Bizot, Cathala & Capron, 2011; Paximada, Koutinas, Scholten & Mandala, 2016;Paximada, Tsouko, Kopsahelis, Koutinas & Mandala, 2016).

One of the reasons why BC is not systematically used in the food industry is its low ability to be dispersed into water (Agoda-Tandjawa et al., 2010; Lowys, Desbrières & Rinaudo, 2001). In the food industry the thickeners have to be well-dispersed in order to be more acceptable by the consumers(McClements, 2005), while BC suspensions present pronounced particle aggregation due to Van der Waals attractions and hydrogen bonds (Kuijk et al., 2013).

A number of technological approaches have been developed to enhance the physical properties of the colloidal suspensions of polymer fibrils. The most commonly used method is to submit polymer to controlled acid hydrolysis conditions (Hirai, Inui, Horii & Tsuji, 2009; Martinez-Sanz, Lopez-Rubio & Lagaron, 2011;Olsson, Kraemer, Lopez-Rubio, Torres-Giner, Jose Ocio & Maria Lagaron, 2010).

However, this is of high energy and cost process that causes intense degradation of the polymer and hence the industry would have had benefit from cheaper alternative methods.

Chemically less aggressive concepts could be the mechanical treatment of cellulose, such as a high pressure homogenization which is used to treat microfibrillated cellulose (MFC) resulting in changes in the microstructure of the cellulose (Agoda-Tandjawa et al., 2010; Saito, Nishiyama, Putaux, Vignon & Isogai, 2006).

What is more, high-intensity ultrasound (16–100 kHz, 10–1000 W cm^{-2}) has immense potential for structural and functional properties of cellulose modification. By this method, the energy of ultrasound is transferred to the polymer chains through a process called cavitation, which is the formation, growth and violent collapse of cavities in the water. Therefore, the effect of ultrasound is related to cavitation, heating, dynamic agitation, shear stresses, and turbulence (Vilkhu, Mawson, Simons & Bates, 2008). Recently, structural and functional changes in ultrasound irradiated plant cellulose, have been reported by (Dehnad, Emam-Djomeh, Mirzaei, Jafari & Dadashi, 2014; Liu & Yang, 2008;Wang & Cheng, 2009). These authors reported that the controlled depolymerization of plant cellulose can be achieved by employing suitable ultrasonication settings.

However, to the best of our knowledge, the literatures about structural modification of bacterial cellulose under high-intensity ultrasound were limited, and the effects of ultrasound irradiation on the physical properties of bacterial cellulose nanofibrils (BCN) aqueous suspensions of cellulose have not been reported to-date.

2. Materials and methods

2.1 Bacterial cellulose production

Bacterial cellulose was produced as described previously (Tsouko et al., 2015). Briefly, bacterial cultivations (*Komagataeibactersucrofermentans* DSM 15973) were carried out using a synthetic medium as described by (Hestrin & Schramm, 1954) containing a carbon source (20 g/L), yeast extract (5 g/L), peptone (5 g/L), Na_2HP0_4 (2.7 g/L) and citric acid (1.15 g/L). The inoculum was prepared by growing the microorganism at 30 °C and 100-120 rpm during 2 days, in Hestrin and Schramm liquid medium. Fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of synthetic medium and were inoculated with 10% v/v inoculums volume. All shake flasks were incubated at 30 °C in static mode for 15 days.

After cultivation, bacterial cellulose (BC) was removed from the cultures and rinsed with tap water to remove any residual media. Next, it was treated with 2 M NaOH to eliminate bacterial cells and then washed repeatedly with tap water until the BC dispersions obtained a neutral pH.

2.2 Treatment of BC

The purified BC pellicles were cut into small pieces with scissors and mixed with deionized water to prepare a BC suspension (4% wt concentration). The BC pieces were further disintegrated with a high shear blender for 10 min (13500 RPM, Ultra Turrax T25, IKA, Germany) which led to the formation of a white precipitate. High shear was used as a first step in order to cut off the cellulose matrices into smaller pieces (0.5cm thickness). However, small fibrils cannot be obtained only with the high shear blender and ultrasonication was further used.

A dilution of the suspensions with deionized water took place leading to a final concentration of 0.1, 0.5 and 1% wt respectively. The suspensions was then submitted to ultrasonic treatment by an ultrasonic homogenizer model Sonopuls 3200 (Bandelin Electronic Gmbh& Co, Berlin) equipped with a 3 mm in diameter microtip (MS 73, 284 μm_{ss} peak-to peak amplitude). Ultrasonication carried out at a frequency of 20 kHz, while the processing time was 1 (BC1), 3 (BC3) and 5 min (BC5) and the final nominal power added to each sample was 82 W. The temperature was maintained at 25 (± 1)^{o}C by circulating cold water with a pump. All samples were prepared in triplicate.

2.3 Water holding capacity

To determine the water holding capacity of BCN suspensions, they were centrifuged at 5000 RPM for 15 min. After the removal of the supernatant, the sediment was weighed and dried at 60 ^{o}C in order in order to ensure complete drying. WHC was calculated by the following equation:

$$WHC = \frac{W_r}{W_c} \quad (Eq.1)$$

WhereW_r is the mass of the removed water during drying and W_c is the dry content of cellulose. The results are reported as the average of at least three samples.

2.4 Transmission electron microscopy (TEM)

The microstructure of cellulose aqueous suspensions was determined using a JEOL 100s equipped with an image acquisition system. Samples of freshly prepared suspensions were diluted 20 times with deionized water, freeze-dried, stained with PTA that is commonly used for staining cellulose fibrils

(Colvin & Sowden, 1985) and placed on the grid. After drying at room temperature, several pictures were taken from random sample positions representing the overall structure of the suspensions. These pictures were analyzed with an image analysis software (Image-Pro Plus 7.0, Media Cybernetics, Rockville USA) in order to measure the fibrils' width.

2.5 ζ-potential measurements

ζ-potential measurements were carried with Dynamic Laser Light Scattering (ZetasizerNano ZS, Malvern Instruments, Worcestershire, UK) at 25°C. As the ζ-potential is related to the electrophoretic mobility of the particles, the ζ-potential is calculated from the measured velocity using the Smoluchowski equation. The samples were previously diluted (1:100) with deionized water to avoid multiple scattering effects. The measurements are reported as the mean of at least two differently prepared injections, with five readings per injection.

2.6 Stability of BCN suspensions

The gravitational stability of suspensions upon storage was followed by measuring the backscattering (BS) intensity along the height of an optically transparent tube using a Turbiscan MA 2000 apparatus (FormulAction, Toulouse, France). Suspension samples (approximately 6 mL) were brought into test tubes, sealed with a plastic cap and stored at 20°C. Measurements were performed with intervals of 24 hours and a total period of 20 days. The stability is presented as the phase separation (PS), which is calculated as:

$$PS\,\% = \frac{H_p}{H_t} * 100 \quad (Eq.2)$$

where H_p is the height of the serum layer and H_e is the total height of the suspension. A lower PS therefore represents a more stable suspension. The results are reported as the average of at least three samples.

2.7 Rheological properties

Rheological measurements of the suspensions were performed on a stress-controlled rheometer (Discovery HR-3, TA Instruments, New Castle, DE, USA) equipped with a concentric cylinders geometry (30mm cup diameter, 28mm bob diameter). Temperature was kept constant (25.0 ± 0.1 °C) using a water bath and the gap was set at 0.1 cm.

The apparent viscosity was determined versus the imposed shear rate from 0.01 to 1000 s^{-1}. 10 points per decade were measured while the whole measuring time was 10 min. Solvent evaporation was negligible within this period.

Moreover, the same rheometer was used in controlled shear mode to test the shear rate/ time dependency of the suspensions. The used conditions were the same as the viscosity measurements. The shear rate increased from 0.01 to 1000 s^{-1}, followed by a pause at 1000 s^{-1} for 10 min and by a deceleration of shear rate from 1000 to 0.01 s^{-1}.

Oscillatory measurements were also performed on the BCN suspensions. Before the dynamic viscoelastic measurements, the linear viscoelastic region (LVR) was determined by strain sweep experiments with the strain varied from 0.01 to 10% at a fixed frequency of 10 rad/s. The region, where the G' and G'' values were parallel was characterized as LVR and in this case was 0.3% of strain. Subsequently, a dynamic frequency sweep was conducted by applying a constant strain, with a frequency range between 0.1-100 rad/s. All the rheological measurements are reported as the average of at least three different samples in order to assure reproducibility.

2.8 Statistical analysis

Statistical analysis of the results was performed with Statgraphics Centurion XV (Statgraphics, Rockville, MD, USA) and a F-test was applied in order to compare the mean values of selected properties at a 95% level of confidence.

3. Results and discussion

3.1 Morphological characterization of BCN fibrils

The morphology of a polysaccharide is a fundamental factor to its applications in the industry. As it is already known, the microstructure of BCN consists of a dense reticulated structure with widths varying from 1 to 9 μm, which is formed by ultrafine microfibrils with widths from 6 to 15 nm connected in between with hydrogen bonds (Iguchi, S. & A., 2000).

This morphology can be altered when a treatment is applied to the system and hence it is an essential property to understand the underlying mechanisms that occur during US treatment. It is of interest to note that 1min of ultrasonication treatment yield to energy up to 5 kJ in the BCN suspension, while 3 and 5 min yield to energies up to 15 and 25 kJ respectively. Therefore, the structure of the BCN fibrils after the ultrasonic treatment is presented in Fig 1A-D. Ultrasounds do not have a significant effect on

the fibrils' length, as it was between 2.9 and 3.1 μm (data not shown), but there was a predominant effect on the fibrils' width. The TEM micrographs of untreated BCN suspensions (BC0) showed an extensively entangled fibril network with irregular fibril and void arrangement (Fig 1A). The width of the BCN fibrils is presented in Table 1 and for the BC0 is found to be 114 nm. The highly bundled network as well as similar values of BCN width was previously reported byLin, Li, Lopez-Sanchez & Li, (2015b), who found a range of 95 nm for their BCN fibrils' width.

In the BC1, a significant decrease in fibrils' width occurs; lowering the width to roughly 60 nm. Moreover, the morphology of the fibrils also changed (Fig. 1B). The entangled network became less bundled and smaller fibrils are present. However, longer sonication time (BC3) causes an increase in the width to roughly 100 nm and changes in the structure as well (Fig. 1C). The main structure is individual ribbons as shown in the micrograph. The BCN fibrils consist of smaller fibrils, which associate together by hydrogen bonds. Previous studies have already evidenced a structure composed of nanofibrils, attached together through interconnecting amorphous cellulose chains in order to produce fibrils (around 100 nm width) that are the basic units of network of BCN (Klemm, Heublein, Fink & Bohn, 2005; Nishiyama, 2009).

Even higher sonication time (BC5) lead to a more pronounced increase of the width to 135 nm and to a reformation of the entangled network (Fig. 1D) of the untreated samples. This unexpected trend of fibrils' width to increase could be attributed to the ultrasonic treatment effect on the structure of the fibrils; it is tried to be explained below.

It is well known that due to the linearity of the cellulose backbone, adjacent chains of cellulose form a framework of water-insoluble aggregates of varying length and width. These microfibrils consist of both highly ordered (crystalline- cellulose I) and less ordered (amorphous) regions (Moon, Martini, Nairn, Simonsen & Youngblood, 2011). Studies revealed that ultrasounds could increase the crystallinity of BC membranes(Tischer, Sierakowski, Westfahl & Tischer, 2010). They attributed this increase in crystallinity index change because of the conversion of the amorphous cellulose into cellulose I (crystalline). The energy required for this process is provided by the cavitation from the ultrasonic treatment. This process primarily occurs in the amorphous regions. Therefore, when ultrasounds are applied in the suspensions, the amorphous parts of the bundled fibril transform into crystal and fuse the neighboring nanofibrils which lead to higher width values. From our results, it can be seen that this phenomenon happens only when longer sonication time are applied (3-5 min).

All in all, the ultrasonic treatment could change the microfibrillar arrangement, leading to suspensions with different nanostructure.

3.2 ζ-potential of BCN suspensions

The role of electrostatic interactions in stabilizing suspensions is examined by measuring the electrical charge of the BCN suspensions. The effect of sonication time on the charge of the fibrils is presented in Table 1. It can be clearly seen that BC bears a negative chargein all systems. This is in accordance with studies showing that BC fibrils are negatively charged independently of their pH, as most of the celluloses (Paximada, Koutinas, Scholten & Mandala, 2016).

When increasing the concentration of BCN in the suspensions, the ζ-potential values remain negative and increased in value and can be attributed to the amount of BCN that is added. Similar findings of the effect of concentration of polysaccharides on the charge have been previously reported (Winuprasith & Suphantharika, 2013).

The short treatment (1 min) increases the overall charge of the suspensions. For example, at 0.1% BCN suspensions, the overall charge increased (in absolute values) from -13 mV (BC0) to -16 mV (BC1). However, prolonged treatments (BC3, BC5) cause a decrease in ζ-potential values regardless of the BCN concentration. For instance, at 0.1% BCN suspensions, the overall charge dropped from -16 mV (BC0) to roughly -15 mV (BC3) and to -13 mV (BC5). This variation in the overall charge of the BCN suspensions is consistent with the idea that prolonged sonication yields aggregated fibrils with larger width, an outcome which tends to have a relatively lower charge than fibrils that are not aggregated. This is in agreement with works showing that larger droplets have lower charges in comparison with smaller droplets which tend to have higher charge (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny & Martín-Belloso, 2015). The results obtained in the present study showed that WHC follow the same trend as the fibrils' width (Fig.1).

3.3 Water holding capacity (WHC)

In Table 1 the WHC of BCN suspensions treated at different sonication time intervals (0-5 min) with varying BC concentrations (0.1-1% wt) is depicted.

Increasing BCN concentration resulted in higher WHC values from 63-77 % for suspensions containing 0.1% BCN up to 102-140 % for suspensions containing 1% BCN. BC fibrils are negatively charged independently of their pH, as most of the celluloses (Martinez-Sanz et al., 2011). This

meansthat by increasing the concentration on an anionic biopolymer in a suspension, such as BCN, a bigger matrix of fibrils is created which has the ability to retain more water into it and thus increase the WHC. This phenomenon is previously reported (Everett & McLeod, 2005). Specifically, they showed that an increase in the added concentration of anionic polysaccharides (λ-carrageenan or LM pectin) on yogurt, causes an increase in the WHC.

Tested materials that have been subjected to a short period of treatment (BC1) show an increase in WHC regardless of BCN concentration. For instance, the WHC increases up to 140% (BC1) when ultrasonic irradiation is applied in suspensions containing 1% wt BCN. On the other hand, a significant reduction in WHC is observed when longer treatments occur. WHC of suspensions containing 1% wt BCN decrease accordingly to 116% (BC3) and to 102% (BC5). The variation in the WHC of these BC samples can be attributed to their respective porosity and surface areas. The water molecules are trapped physically on the surface and inside the BCN matrix consisting of reticulated fibrils(Watanabe, Tabuchi, Morinaga & Yoshinaga, 1998). If there are plenty of empty spaces among the BCN fibrils then more water can penetrate and adsorb onto the material. Thus, the greater the surface area and the larger the pore size, the greater will be the WHC of the BC sample (Guo & Catchmark, 2012; Meftahi et al., 2010). BC has a wide range (100–200 times its dry weight) of WHC values (Lin, Li, Lopez-Sanchez & Li; Schrecker & Gostomski, 2005). This variation may be due to differences in the fibril arrangement, surface area, and porosity of different BCN samples. The results obtained in the present study showed that WHC follow the same trend as the fibrils' width (Fig.1).

3.4 Phase separation of BCN suspensions

The time during which the biopolymer is stable depends mainly on its nature. Hence, phase separation (PS) for all suspensions was recorded for a 20-day period and presented in Fig. 2.

As it can be seen, the higher the BCN concentration, the lower the phase separation and hence the higher the stability is. The increase in stability by increasing the polymer concentration is a well-known behavior, extensively studied. By the addition of higher amounts of BC a network is formed decreasing phase separation(Paximada, Tsouko, Kopsahelis, Koutinas & Mandala, 2016).

What is more, ultrasonic treatment bears an effect on the stability profile of BCN suspensions. Reduction of phase separation has been observed (Fig. 2) in a short period of time (1 min) in the current study, meaning higher stability for the suspensions. By way of explanation, the PS of the BC1 samples containing 0.5% wt BC decreases up to 9%. In spite of that, further sonication treatment leads

306 to an increased PS, meaning a decrease in suspensions' stability. The PS of the samples containing
307 0.5% wt BC decreases up to 12% (BC3) and to 18% (BC5). Clearly, this trend is in accordance with
308 the previous mentioned results (size, WHC, ζ-potential) and could be attributed to the structural
309 changes that ultrasounds have on the fibril. A reduction in the PS of BCN suspensions from 20% to 7%
310 has previously reported using high pressure (Lin, Li, Lopez-Sanchez & Li, 2015b). Also, ultrasounds
311 are known to be an efficient method to increase the stability of suspensions(Price, West & Smith, 1994;
312 Trzciński & Staszewska, 2004).

313 *3.5 Rheological measurements*

314 *3.5.1 Viscosity*

315 The viscosity of the suspensions as a function of different sonication time intervals and BCN
316 concentrations is presented in Fig.3. It is obvious that viscosity depends on the concentration of the
317 polymer, as an increase in the fibril concentration results in a significant increase in the viscosity from
318 roughly 10 Pas for 0.1% BCN (Fig. 3A) to 150 Pas for 1% BCN (Fig. 3C). This viscosity increase
319 could be attributed to the fact that by adding more fibrils into the system, they create bonds between
320 each other, leading to the formation of a stronger network (Iotti, Gregersen, Moe & Lenes, 2011). The
321 apparent viscosity of our sample is similar to literature findings (Lin, Li, Lopez-Sanchez & Li, 2015b).
322 Besides the large increase in viscosity, the suspensions also exhibit a pronounced shear thinning
323 behavior, like most suspensions containing MFC (Iotti, Gregersen, Moe & Lenes, 2011; Kuijk et al.,
324 2013).

325 Worth noting is the fact that all the suspensions show a three-region behavior with viscosity showing
326 shear-thinning behavior at low rates, a Newtonian-plateau region and then a precipitous drop in
327 viscosity. Researchers have previously reported similar shear thinning regions in MFC (Jia et al., 2015;
328 Karppinen, Saarinen, Salmela, Laukkanen, Nuopponen & Seppala, 2012; Naderi, Lindstrom &
329 Sundstrom, 2014). It is obvious that the shear rate at which the plateau is present, is concentration
330 dependent (Fig. 3). The explanation for such behavior is that at a critical shear rate, the fibrils align due
331 to their rod-like nature, greatly easing their flow. Under enough shear the chirality of the suspensions
332 breaks down in favor of a simple structure(Iotti, Gregersen, Moe & Lenes, 2011).

333 At rest, the BCN fibrils are flocculated in the aqueous phase. The first shear thinning region observed
334 at low shear rates (γ= 0.01 – 1 s-1), where the applied force is low but sufficient enough to disrupt the
335 flocculated fibril network. At intermediate shear rates (γ= 1 – 10 s-1), a viscosity plateau is observed.

An explanation for this plateau in the flow curve was previously proposed for MFC(Karppinen, Saarinen, Salmela, Laukkanen, Nuopponen & Seppala, 2012). They suggest that the Newtonian network is related to a significant increase in BCN floc size and homogeneity. In the second shear thinning region (γ= 10 – 100 s-1), the higher shear rate disrupts the network structure of the BCN flocs again and hence the structure becomes rather uniform once more. This indicates that the contacts between the fibrils are reversible. Due to less connection between the BCN they orientate themselves in the direction of flow thereby causing highly shear thinning behavior at the high shear rates (Barnes, 1997).

What is more, the duration of the sonication treatment significantly changes the viscosity of the BCN suspensions. As it can be observed in Fig. 3, the viscosity of the BC0 is the lowest. While in BC1 the suspensions' viscosity increased, in BCN, the viscosity of all samples decreased significantly. This tendency is the same as in the width results and can be attributed also to the structural changes that ultrasounds exert in the BCN fibrils. This unusual tendency of viscosity with sonication time was also reported on suspensions containing chitosan (Baxter, Zivanovic & Weiss, 2005). Specifically, their untreated chitosan dispersions had lower viscosity than dispersions submitted to US for few minutes, while in prolonged treatments, the viscosity fell again.

3.5.2. Thixotropic behavior

Thixotropy is a term used in rheology which means that the viscosity of a material decreases significantly with the time of shearing and then, increases significantly when the force inducing the flow is removed (Whelan, 1994). Time dependency of polysaccharides is fundamental to understand possible utilizations in food industries (e.g. the flow in mixers or pipes, coating applications, as thickening agent, in extrusion processes).

Hence, the effect of the sonication time on the thixotropic behavior of BCN suspensions has been evaluated and selected aqueous suspensions containing 0.5% BCN and treated for0 (◆), 1 (●) or 3 min (■) at ultrasounds are depicted in Fig. 4. As it can be seen, all samples exhibited typical thixotropic behavior, which is usually associated with systems containing flocculated particles or aligned fibrils(Barnes, 1997). Similar thixotropic behavior was found for MCC as well as for MFC (Jia et al., 2014). Researchers revealed a thixotropic behavior for amorphous cellulose suspensions at concentrations of 0.77% and 2.33% w/v, while (Araki, Wada, Kuga & Okano, 1998)found the same behavior for MCC prepared by HCl hydrolysis at concentrations >0.5% w/v.

It is also interesting to focus on the size and the shear rate that the hysteresis loop appears in relation to the sonication time. As it can be seen, there is a shift of the shear rate values that the loop is depicted by imposing sonication for 1 min (Fig. 4B), while for longer US treatment of 3 min, it returns to similar shear rates of the untreated samples (Fig. 4C). The reason for that phenomenon probably stems from the structural changes that take place in the BCN fibrils during the US treatment.

An explanation for the thixotropic behavior of MFC was previously reported (Iotti, Gregersen, Moe & Lenes, 2011). We assume that this is the case for our samples as well. BCN suspensions exhibit a structural breakdown. As the shear rate increases, shear thinning is the dominating effect. In the area that the loop takes place the shear rate velocity does not allow the preservation of the high shear structure that has been formed. Thus, the high shear structure breaks down and a formation of a new low shear structure is present. In this case (Fig. 4B), the high shear structure seems present until the drop in viscosity measured around $1s^{-1}$, where the curve suggests the end of the high shear structure and the reorganization in a different low shear organization.

3.5.3 Viscoelastic properties

The elastic modulus *G'* (solid symbols) and loss modulus *G''* (open symbols) as a function of frequency for the BCN suspensions at various total BCN concentrations and US treatments are shown in Fig. 5.

As it can be seen, increasing the BCN concentration leads to an augmentation in both moduli. For example, the storage modulus at 1Hz frequency increases from roughly 1 Pa for 0.1% BCN (Fig. 5A) to 13 Pa for 0.5% BCN (Fig. 5B) and to 40 Pa for 1% BCN (Fig. 5C). Moreover, all suspensions exhibit G' values higher than G'' (G' > G'') at all frequencies. The previous mentioned rheological measurements confirm the viscoelastic character of the suspensions already reported in the literature(Iotti, Gregersen, Moe & Lenes, 2011; Rezayati Charani, Dehghani-Firouzabadi, Afra & Shakeri, 2013).

Moduli as a function of ultrasounds treatment has the same trend as the viscosity: the moduli of the untreated suspensions are the lowest. In BC1, the moduli increased, while in BC3 the moduli decreased significantly, regardless of the concentration of BCN. The augmentation of hydrogen bonds and non-freezable bound water in suspensions by increasing the treatment has been brought forward earlier as explanation for this behavior(Kunzek, Opel & Senge, 1997). Specifically, studies found good correlation between the water retention capacities and the rheological properties for swollen cell wall

material originating from apples. In summary, the viscoelastic properties of the fibril suspensions altered significantly with progressive modification. This is in relation with the viscosity and the morphology results.

4. Conclusions

An extensive study of the effect of ultrasonic treatment on the physical properties of bacterial cellulose (BC) aqueous suspensions has been conducted, focused on their rheological behavior. BCN suspensions (0.1-1% wt) were treated with ultrasounds under various periods (0-5 min). Sonication was proved to be an appropriate method for the pre-treatment of BC. The time of the treatment is critical. Longer times (5 min) are not recommended, because the crystallinity of cellulose is increased and entangled fibrils are created. On the contrary, a short treatment (1 min) is beneficial for BC suspensions pretreatment. Fibrils break down, their width is reduced to the half of the initial value, the WHC is increased as also the viscosity and the solid-like character of the samples. The thixotropic character also changes as hysteresis loop observed moves to lower shear rates and a clear structural breakdown and reformation due to time effect is observed. BC suspensionultrasonication could be used for BC pretreatment before its addition in several foodstuffs, enhancing BC applications.

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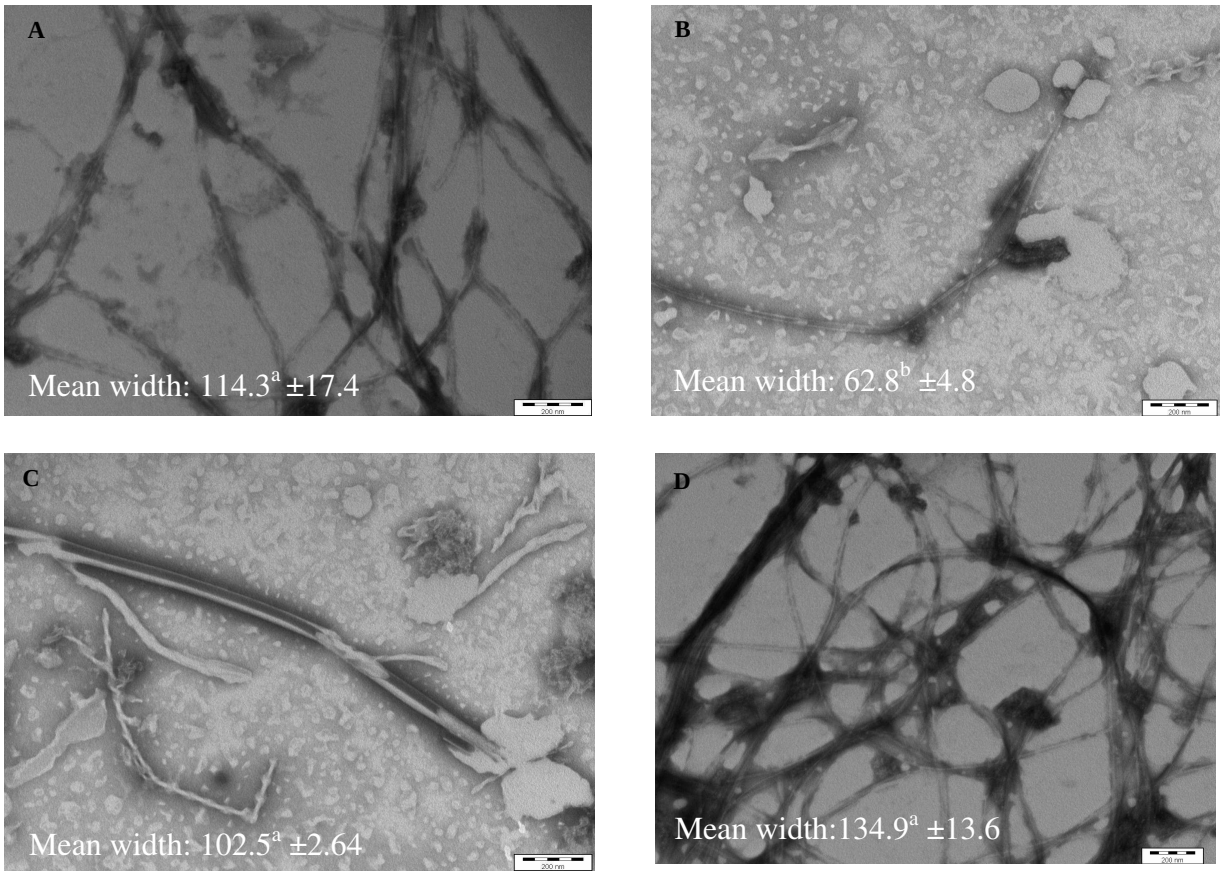
533 Caption of figures

534 **Fig. 1.** Typical TEM micrographs and the fibres' mean width of the untreated BCN suspensions (A)
535 and the ultrasonicated BCN suspensions at different time intervals: 1 min (B), 3 min (C) and 5 min (D).
536 In parenthesis standard deviation values. Mean values followed by the same letters are not significantly
537 different ($P > 0.05$).

538

539 **Fig. 2.** Phase separation (PS) as a function of BCN concentration and ultrasonic treatment for the
540 aqueous suspensions. PS was determined after 20 days of storage at 25°C. Bars indicating standard
541 deviations.From lighter to darker grey, sonication times: untreated, 1 min, 3 min and 5 min
542
543 **Fig. 3.**Viscosity curves of suspensions treated at different time intervals: 0 min (A), 1 min (B), 3 min
544 (C) and containing 0.1% (◆), 0.5% (●), 1% (■) wt BC. Bars indicating standard deviations.
545
546 **Fig. 4.** A typical hysteresis loop test of BC aqueous suspensions: 0.5% treated for for 0 (◆), 1 (●) or 3
547 min (■). Bars indicating standard deviations. treated
548 **Fig. 5.** Storage modulus and loss modulus as a function of frequency for suspensions treated at
549 different time intervals: 0 min (A), 1 min (B), 3 min (C) and containing 0.1% (◆), 0.5% (●), 1% (■)wt
550 BC. G' filled symbols, G'' empty symbols. Bars indicating standard deviations.
551
552
553 Fig. 1.



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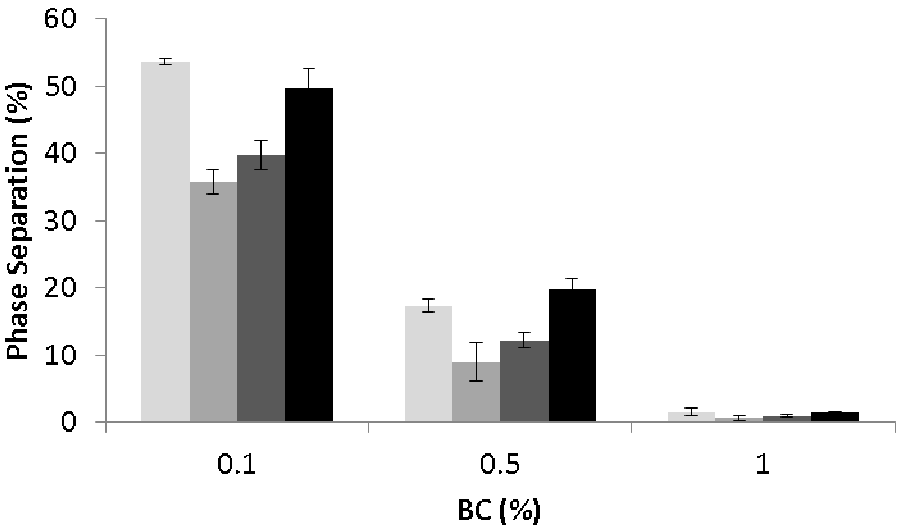
555 Fig. 2.



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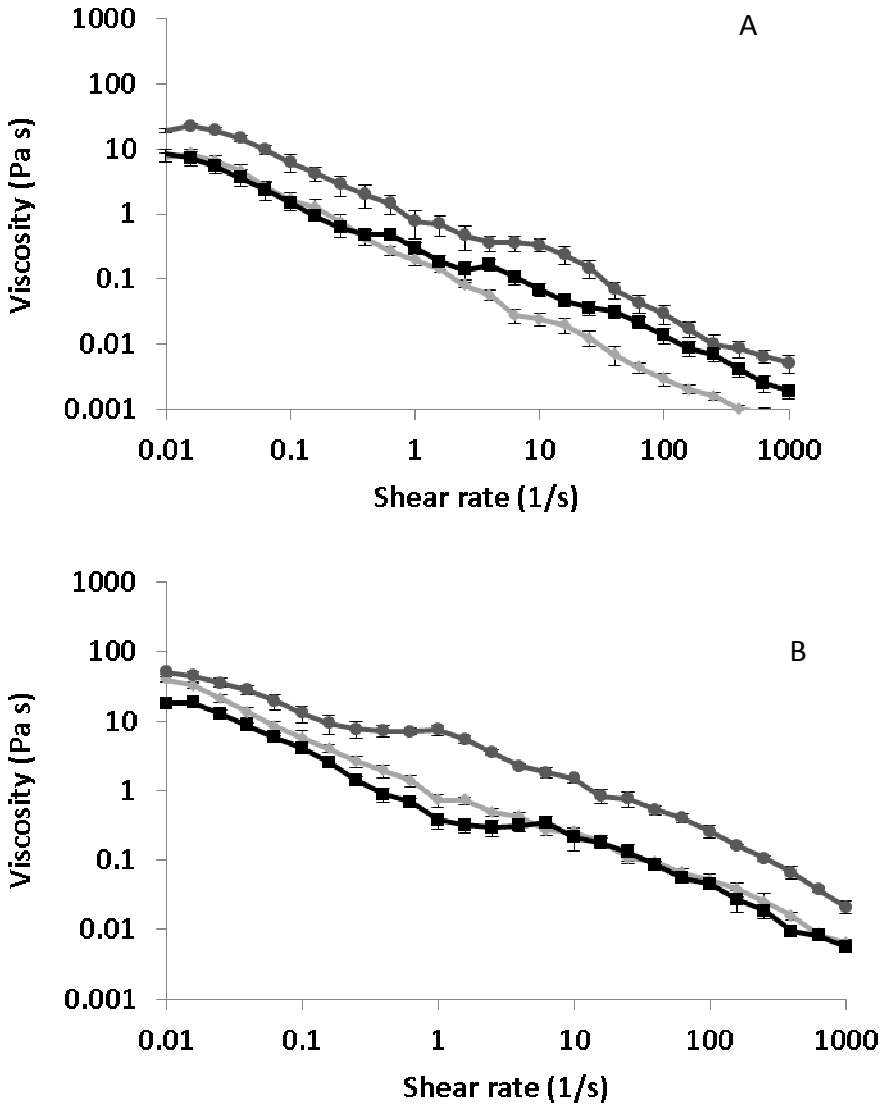
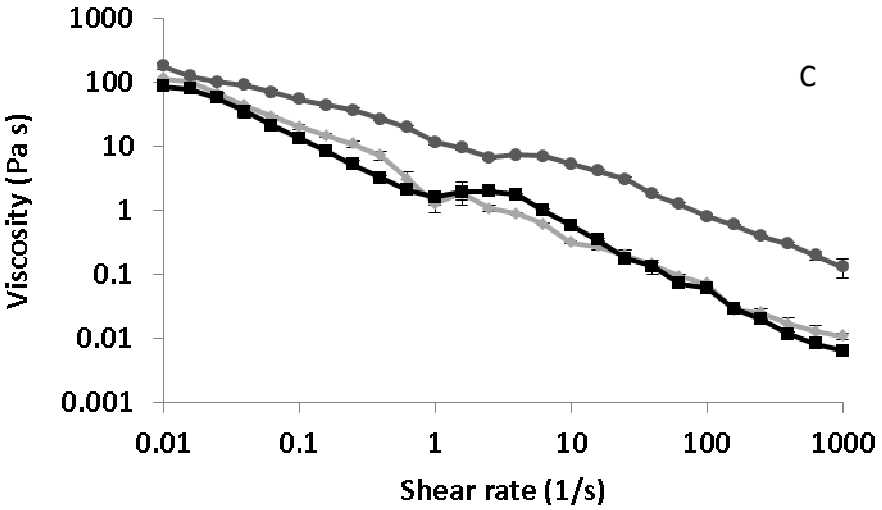
558 Fig. 3.





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560 Fig. 4.

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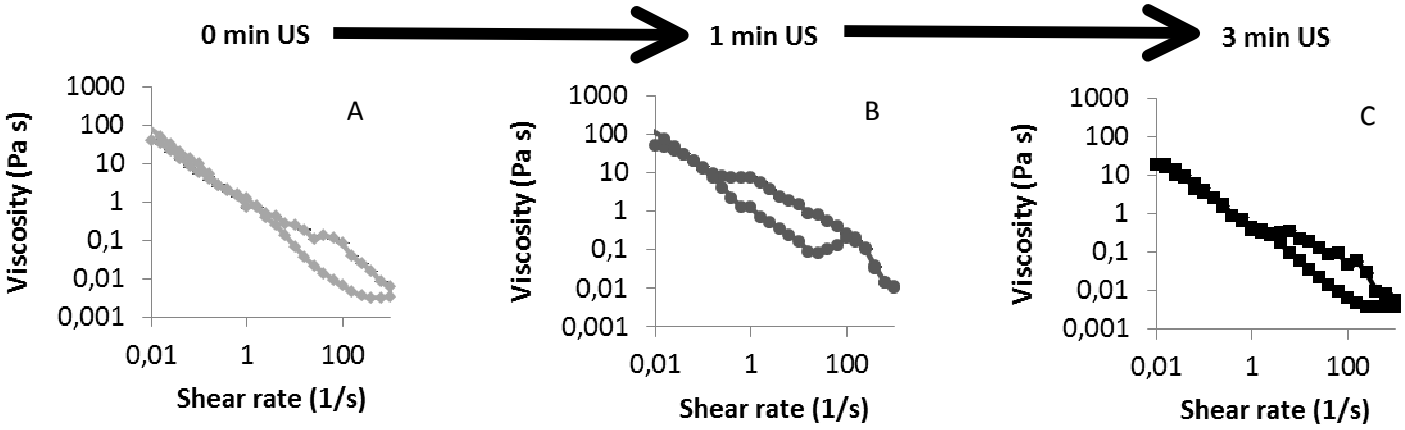

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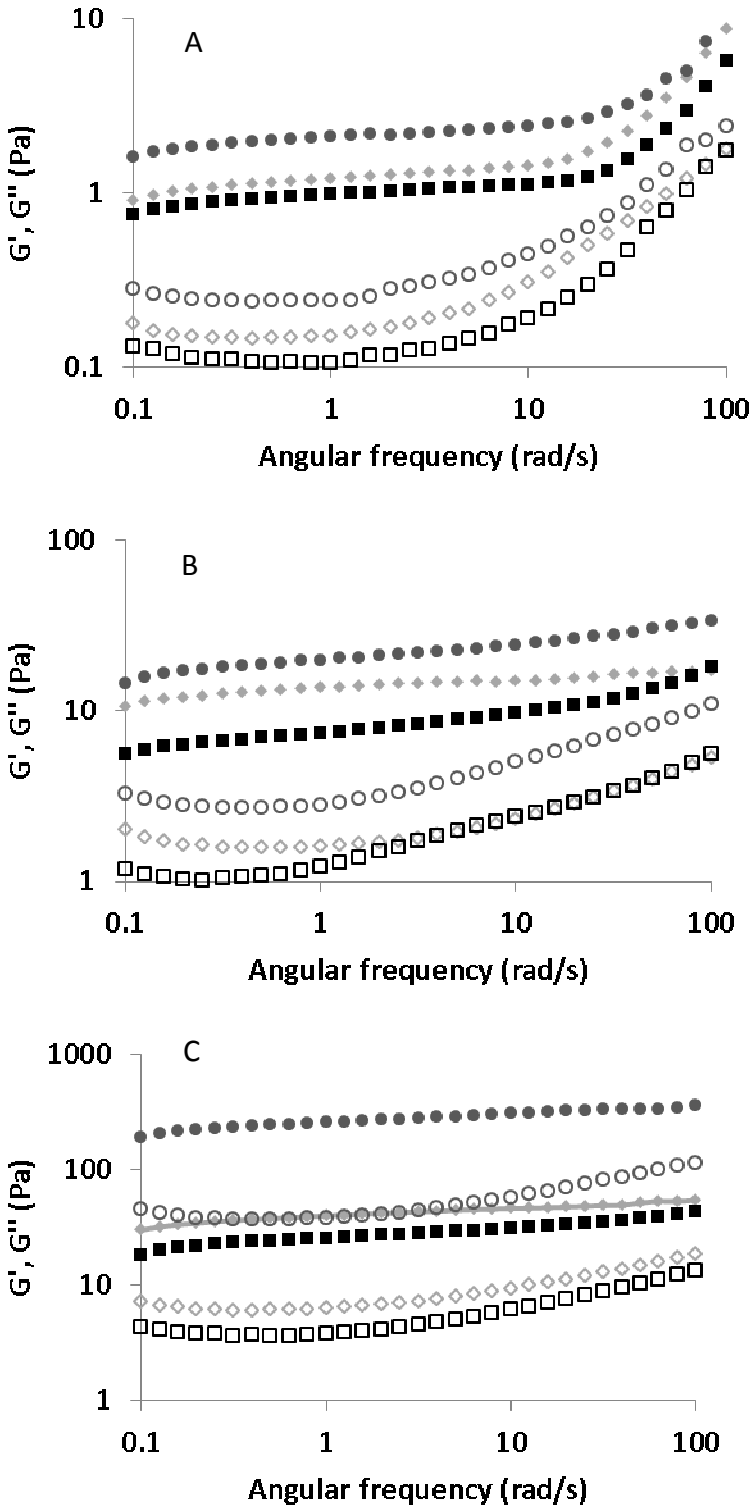
562 Fig. 5.



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567 Tables

568 **Table 1** Physical properties (ζ-potential and WHC) of 0.1-1% wt BCN suspensions treated by 0-5 min

569 at ultrasounds.

BC treatment	% wt	ζ-potential (mV)	WHC (%)
BC	0.1	-12.9^{g} ±1.3	62.5^{a} ±2.2
	0.5	-17.7^{cd}±2.5	83.4^{b} ± 5.1
	1	-23.2^{ab}±2.1	109.0^{cd} ±7.6
BC1	0.1	-16.1^{def} ±0.8	77.1^{b} ±3.0
	0.5	-20.3^{bc}±1.3	104.9^{c} ±6.6
	1	-25.8^{a} ±1.6	140.0^{e}±8.8
BC3	0.1	-14.7^{efg}±1.0	64.7^{a}±2.1
	0.5	-19.3^{bc} ±0.8	86.8^{b} ±5.7
	1	-24.8^{a} ±1.4	116.6^{d} ±7.6
BC5	0.1	-13.4^{fg}±1.4	61.4^{a} ±2.3
	0.5	-18.23^{bcd}±1.9	81.4^{b} ±5.7
	1	-23.6^{ab}±2.5	102.5^{c}±7.7

570 In parenthesis standard deviation values.

571 Mean values followed by the same letters are not significantly different (P > 0.05).