Highly aligned bacterial nanocellulose films obtained during natural biosynthesis

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Article

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Abstract

Bacterial nanocellulose (BNC) is usually produced as films of randomly-organized highly pure cellulose nanofibers and its properties such as high water-holding capacity and porosity, tunable morphology, mechanical strength, and biocompatibility make it a unique material. As a result, BNC has attracted interest in the paper and food industry, biotechnology, photonics and optoelectronics. Ordered structures found in nature, as the reed leaf, and the potential properties appearing upon aligning polymers fibers (piezoelectricity, conductivity, optoelectronics, to name a few) inspired us to develop a straightforward method to achieve highly aligned BNC films (Herman's order S-parameter of 0.85) benefiting from the natural bacteria biosynthesis and extrusion of the cellulosic fibers. We locally confined the bacteria at the bottom of a tube containing liquid culture media and statically incubated them for a few days. Due to the oxygen requirements of the bacteria and the water insolubility of BNC, cellulose nanofibers were synthesized in a single direction without entanglement, which allowed obtaining band-chain aligned BNC in the liquid phase. The obtained film is highly oriented within the total volume of the film, as shown by polarization-resolved second-harmonic generation signal (P-SHG) and Small Angle X-ray Scattering (SAXS). Additionally, we proved an increase of hydrophilicity, suitable to tissue engineering scaffolds as human fibroblasts successfully adhered and grew on it. Finally, we evaluate the thermal conductivity by two independent approaches, i.e. using the well-known 3ω -method, as well as through a recently developed contactless thermorefectance approach, confirming a thermal conductivity of 1.63 W/mK in the direction of the aligned fibers in contrast with 0.3 W/mK in the perpendicular direction. The 5-fold increase in thermal conductivity of BNC in the parallel direction forecasts the potential of BNC-based devices outperforming some other natural polymer and synthetic materials.

Introduction

Polymers are exploited in a wide range of industrial applications ranging from structural materials to electronics due to their diverse functionality, lightweight, low cost, and excellent chemical stability. Among them, biopolymers use is expanding to offer sustainable solutions to current applications of petroleum-derived or synthetic polymers^{1,2}.

Cellulose is the most abundant biopolymer in nature³ and is commonly obtained from plants, even though several organisms also present the ability to produce it, such as algae⁴, fungi and bacteria⁵. The non-pathogenic bacteria *Komagataeibacter xylinus* (*K. xylinus*) produces bacterial nanocellulose (BNC) as an extracellular polymer at the liquid-air interphase⁶. BNC is a supramolecular hierarchical network with a semi-crystalline structure and high water holding capacity. The biomaterial is porous, mechanically strong and elastic, conferring high biocompatibility and bio-welding properties⁷. As a result, BNC has attracted interest in the paper and food industry, biotechnology, photonics and optoelectronics^{8,9}.

The BNC field is advancing in controlling the intrinsic properties of cellulose fibrils, such as the fibrils arrangement, to understand how they interact with proteins and solvents or add additives to endow novel properties to the material among others^{10,11,12}. Understanding the basic material and the novel

composites have allowed its uses in many fields, ranging from batteries^{13,14} up to skin or grafts^{15,16,17,18}, or even heart replacement pouches in regenerative medicine^{19,20}.

Anisotropic structures are ubiquitously found in nature²¹ and our tissues. For instance, aligned actin fibers confer strong resistance to muscles, highly aligned collagen fibers in the eye^{22,23} offer a highly transparent stroma tissue or in skin²⁴ and articular cartilages²⁵. Anisotropy often plays an essential role in biological systems carrying out particular functions, including mass transport, surface lubrication, cell adhesion and force generation, as well as the alignment of polymeric fibers usually confers novel properties such as piezoelectricity, conductivity, or light-harnessing properties^{21,26,27}.

Cellulose nanofibers and bacterial nanocellulose fibers are long and flexible and highly entangled as-obtained, which makes the alignment of cellulose nanofibers challenging²⁸. Fig. 1A-B summarizes reported top-down and bottom-up approaches achieving satisfactory results to align BNC fibers²⁶. Top-down methods, as uniaxial stretching of BNC wet hydrogels^{29,30} have obtained strong and tough aligned BNC films²⁹. The use of wet spinning of a BNC solution also produced aligned BNC fibers, with an Orientation Index (OI) of 0.69 and Herman's order parameter (S-parameter) of 0.63³¹ (values further explained in the methods section). On the other hand, bottom-up strategies produced oriented materials involving the bacteria biosynthesis. Honglin Luo et al. obtained oriented BNC fibers by forcing the bacteria to move in a single direction inside a bioreactor³². However, it produced bacteria mutations and the decrease of the BNC production yield. Ananda Putra et al. obtained aligned BNC tubular hydrogels using oxygen-permeable patterned silicone tubes inside the bacterial culture³³. Nevertheless, the fiber alignment of BC is not yet fully achieved. The orientation of cellulose nanofibers in polymer materials is scientifically and technically challenging²⁶ and other polymers (such as PVA or PLGA) or non-polymeric additives are often required to improve and stabilize the aligned material²⁹. For example, adding soy protein to uniaxially-stretched BNC films significantly improved the mechanical properties by 2.75 and 3-fold in tensile strength and tensile modulus, respectively³⁰.

The simplicity and beauty of the natural biosynthesis of polymers inspired us. Time-lapse videos of the bacteria producing cellulose fibers recorded by R. Malcolm Brown, JR. laboratory in 2013 showed that ordered cellulose fibrils are linearly extruded through the bacteria cell wall's pores^{6,34,35} (Fig. 1C). Recently this description was also presented by Caro-Astorga et al., where they again observed BNC-chains produced as band-like growth from one side of the bacteria longitudinal axis using microfluidic chips and the fluorescent *Brightener 28*⁷. Motivated by this biosynthesis process, we wondered if the restriction of the bacteria's movement could allow to retain the intrinsic parallel orientation of individual crystalline cellulose molecules produced by the bacteria³⁴.

In this work, we report a novel, reproducible and easy bio-fabrication method to obtain aligned BNC (A-BNC) hydrogels with high control avoiding complex equipment and additives. We produce A-BNC harnessing its natural biosynthesis. Additionally, we display the final properties of the material after the rearrangement of the BNC structural organization, including modifications in morphology, optical activity

and how the anisotropy of this material impacts its hydrophilicity, biocompatibility and thermal conductivity.

Results And Discussion

Aligned bacterial cellulose biosynthesis

Inspired by the biosynthesis process, we restricted the bacteria movement to control the alignment of individual cellulose nanofibrils. *K. xylinus* is an aerobic bacteria strain^{6,34}, which implies the microorganism requires oxygen when cultured. We retained the bacteria anchored on an agar surface at the bottom of a vessel, filled with fresh bacteria-free liquid media, and cultured the bacteria at their optimal cellulose production conditions: 30°C in static (Fig. 1A,B). After 5 days of culture, we obtained the typical circular hydrogel pellicle of BNC at the air-liquid interphase and we could also visualize in the liquid a translucent film of aligned BNC fibers, as shown in Fig. 2C,D. The water-insoluble and low dense BNC fibers facilitated this method. We separated the top part of the produced cellulose (BNC) and the aligned cellulose (A-BNC) in the middle from the agar substrate. Therefore, we obtained BNC of different morphologies in a straight-forward step.

We cleaned the films from bacteria detritus and culture media by the commonly described treatment³⁵. We obtained semi-transparent hydrogels of BNC of the shape of the vessel, in our case circles of Ø1.6 cm², and aligned bacterial nanocellulose (A-BNC) of 7-8 cm in length. BNC and A-BNC films were dried at 60°C for a minimum of 12 hours under 1 Kg weight and without weight, respectively. After drying A-BNC maintained the aligned structure and did not decrease in length. The thickness of the BNC films of 300 µm in wet decreased to ≈20 µm upon drying. A-BNC films were easily manipulable and flexible, with a final thickness of ≈10 µm after drying. Optical images of the A-BNC clearly show the alignment of the fibers (Fig. 2C,D); conversely, the random BNC does not show any orientation of its structure. Moreover, A-BNC did not disassemble upon different wetting and drying cycles indicating a stable bonding among cellulose fibers, conferred by hydrogen bonds between hydroxyl groups³⁶.

The process has been repetitively performed more than 200 times, obtaining aligned cellulose consistently. Many parameters control the BNC production and they have been extensively evaluated elsewhere^{10,11,37}. However, dissolved oxygen (DO) in the media is considered the main driver (Fig. 2B). We quantified the dissolved oxygen (DO) present in the solution at different time points using indigo-carmin reaction^{38,39} (Fig. S1). Leuco-indigo carmine reagent oxidizes under the presence of oxygen to keto-indigo carmine, which causes a color change from yellow to red upon oxidation (Fig. S1-A). A color-DO chart allows to qualitatively estimate the DO concentration (Fig. S1-B). As shown in Figure S1-C, the initial DO concentration at the top and bottom part of a non-oxygen permeable tube was ≈0.06 ppm. After 7 days of culture, the DO concentration at the top was maintained at 0.06 ppm while, at the bottom, it decreased to 0.03 ppm. We hypothesize that the deployment of oxygen at the bottom of the vessel over time promotes the movement of the bacteria to the surface, towards higher DO content. Bacteria produce low-

density cellulose fibers from the bottom of the vessel and uses them as “climbing ropes” (Fig. 2B) to reach the high oxygen-content surface where continue producing its exoproduct with no movement restriction, which allows us to obtain BNC as a random mesh. So, efficiently from the same biosynthesis process, we can obtain random BNC and A-BNC by restricting the initial movement of bacteria.

To further exploit the efficiency and yield of our new method we investigated the reuse of the bacteria. From the produced films we cut out the aligned BNC section and again confined it at the bottom of the tube, filled with fresh media: a) the previously used agar substrate (Fig. S2-A), and b) the formed BNC pellicle (Fig. S2-B) before cleaning it, both containing cultured “active” bacteria. We confirmed that we could use the same agar substrates with bacteria colonies (Fig. S2-A) for at least 7 cycles, obtaining BNC and A-BNC in only 3 days. After that, the bacteria decreased the speed of production. We also proved the use of the as-produced BNC as a substrate to grow A-BNC and BNC in, again, only 3 days, which we named film-to-film biosynthesis (Fig. S2-B). The reuse of those bacteria substrates in sequentially A-BNC production increased the number of films obtained exponentially. We hypothesize that the already used bacteria are acclimatized to the liquid culture, and thus, more “active”, which reduces the lag phase of a bacteria culture from the initial 5 days of the first culture to 3 days.

To increase the size of the A-BNC produced we evaluate the time, vessel and bacteria amount. Culturing the bacteria from 3 to 15 days did not increase the width or length of A-BNC produced, although it increased the thickness of the BNC film. On the other hand, longer and wider A-BNC films could be obtained using longer and wider tubes, respectively. Using tubes of 10 cm and 30 cm allowed us to obtain A-BNC 7-8 cm up to 20-23 cm respectively, however, the width of the A-BNC was not increased (3-5 mm). Finally, instead of using the typical streak plate (or zig-zag) method to obtain the initial agar substrate with bacteria colonies, we grew confluent agar Petri dishes to obtain the maximum number of bacteria per surface to obtain high-density A-BNC fiber films. This strategy allowed us to obtain A-BNC films with a thickness of $\sim 200\ \mu\text{m}$ in wet.

A-BNC Characterization

This biosynthesis process produced aligned BNC films, which possess different morphology and properties than traditional BNC films with randomly distributed fibers. Therefore, an extensive characterization of both A-BNC and BNC (considered as a control) was performed to evidence the change of morphology and evaluate the properties arising upon the alignment of the material.

After drying, A-BNC is more transparent than BNC, and this increase can be qualitatively appreciated by the naked eye, clearly seen in Fig. 3B and 3C, respectively, where pictures are taken with a micron-mesh under the films and top illumination. The scattering of light of BNC produced a haze and whitish color to the material that was not seen in the A-BNC. Moreover, under localized white light, the A-BNC films showed some iridescence indicating higher transparency and structuration of the material (Fig. 3D).

As seen in Fig. 3E, FTIR for both samples (BNC (blue) and A-BNC (black)) contained the cellulose characteristics peaks: hydroxyl groups are seen as a strong peak at $3000\text{-}3700\ \text{cm}^{-1}$, CH groups at 2800-

3100 cm^{-1} , and the internal deformation frequencies of CH_2 and CH_3 groups are seen in the region of 1340-1480 cm^{-1} , as well as other cellulose characteristics peaks. However, the A-BNC spectra shows more intense transmittance peaks in the OH and CH region, even though A-BNC films were thinner than the BNC.

To further investigate the structure of the material, the fiber alignment was also analyzed at the nano-scale (Fig. 4). At this scale the collapse of the structure upon drying made more difficult the observation of the alignment observed at the macroscale. Nevertheless, a higher significant degree of organization was observed in the A-BNC in comparison to BNC. Fig. 4-B,E show the Transmission Electron Microscopy (TEM) images and Scattering Angle Electronic Diffraction (SAED) patterns of BNC (Fig. 4-B) and A-BNC (Fig. 4-E). Although BNC is known to present high crystallinity (60-80%)³⁴ the random orientation of the fibers makes it difficult to analyze the crystallinity of the BNC pellicle by TEM. However, SAED analysis in A-BNC samples allowed visualizing the cellulose crystallinity lattice planes (indexed in Figure S3-A). We considered that this increase discretization of the lattice planes intensity (from diffuse bands to localized bright spots, Fig. S3) was due to the increased order orientation and different texture of A-BNC. SEM and AFM images show a similar compact structure for the BNC (Fig. 4-C,D) and A-BNC (Fig. 4-F,G) films, however, we could see an alignment of fibers in the latter. Diameter measurements of the nanofibers (2-4 nm) and microfibrils bundles (15-20 nm) from both BNC and A-BNC revealed that the modification in the culture to obtain aligned BNC did not impact the single fiber structure. Using the color analysis from the *OrientationJ* plugin for ImageJ we computed the local orientation as a color-survey HSB image, where hue represents the fiber orientation and saturation represents coherency of the orientation. These images (insets of Fig. 4-C and 4-F) indicated the preferential fiber orientation of the images in degrees. We can see that A-BNC had a predominance of colors in the green (Fig. 4-F) region, indicating a predominant orientation (pointed by white arrows for a clearer visualization) not appreciable for BNC samples (Fig. 4-C), where a variety of colors represent the different orientations within the image. The mean height of AFM images analyzed by MountainView8 was found 28.6 nm for BNC and 21.09 nm for A-BNC indicating a flatter surface. Additionally, we evaluated the isotropy of the samples as an insert in Fig. 4-D and 4-G, indicating higher values for BNC, 31.50%, in contrast with A-BNC, 18.81%, confirming that the latter was more aligned than BNC. Interferometry assisted us to visualize the alignment at a micron scale, obtaining volumetric images of the aligned cellulose BNC bundles (Fig. S4-A), in contrast with the non-aligned BNC (Fig. S4-B) where non-bundle ordering is appreciated.

To evaluate the alignment in the 3D volume of the material, we used interferometry, Small-angle X-ray scattering (SAXS) and polarization-resolved second-harmonic generation signal (P-SHG). The raw 2D scattering SAXS patterns of BNC and A-BNC fibers, respectively, recorded at the ALBA synchrotron facility, differ on the shape of the scattering pattern; being circular for randomly distributed fibers of the BNC (Fig. 5-A) and distorted (oval-shaped) for preferential orientated fibers of the A-BNC sample (Fig. 5-B), indicating a higher degree of alignment of the A-BNC fibers. To quantitatively evaluate differences in the orientation degree, the spectra were integrated as shown in Fig. 5-C, as an intensity vs azimuthal degree plot from -90° to 90° . A-BNC showed an intensity peak centered around 0° , whereas BNC gave no peak at

all. From the spectra, the widely accepted orientation quantitative parameters OI, FWHM, and S-parameter^{31, 26} are obtained (formulas and explanation in the methods section). FWHM stands for full width at the half-maximum intensity and the smaller this value is, the highest is the orientation. The orientation index (OI) is obtained from FWHM, ranging from 0 for randomly oriented structures to 1 for perfectly aligned fibers. Herman's order parameter, also called S-parameter, can be calculated from the intensity and azimuthal degrees values and its value also ranges between 0 to 1, being 1 a perfect orientation^{31, 26}. Thus, as shown in Fig. 5-D FWHM, OI, and S-parameter were computed, obtaining 47.18°, 0.74, and 0.85 for A-BNC, respectively. For BNC, S-parameter resulted to be much smaller than A-BNC, 0.16. FWHM and OI could not be determined due to the lack of a peak in the spectra, and thus, lack of internal orientation. The numerical results obtained corroborated the qualitative information extracted from the 2D images, obtaining a higher volumetric fiber orientation evaluation in the alignment of A-BNC films. The orientation values OI and S-parameter we obtained (0.74 and 0.85, respectively) were higher than previously reported values from SAXS analysis of aligned BNC fibers obtained with wet spinning; with OI of 0.69 and an S-parameter of 0.63³¹.

Secondary Harmonic Generation (SHG) is a nonlinear optical process used in biomedicine for imaging SHG active biological structures such as collagen nanofibers, rather than its bulk^{40,41}. Polarization-resolved SHG microscopy (PSHG) is a powerful tool that exploits the dependence of SHG signals on the polarization degree of the excitation beam. The SHG emission depends on the structuration of the imaged material and PSH includes an extra dimension that is used to probe the molecular organization. Here, PSHG was used to assess the bacterial nanocellulose intrinsic organization. Frames of the linear polarization of the incident beam from 0 to 180 degrees in steps of 10 degrees using a $\lambda/2$ plate were recorded for the whole volume of the samples. Fig. 6-A shows how the changes of light intensity in A-BNC from the minimal (70°) to the maximum projection (150°) could be appreciated, whereas in BNC, all projections remained unchanged, indicating no alignment. We evaluated the alignment coherency for each frame by the OrientationJ plugin for ImageJ and plotted it (Fig. 6-B), where random BNC has a high but invariable signal intensity (blue line) and a low alignment coherency (dotted blue line). Conversely, A-BNC alignment coherency (dotted black line) is even higher than the signal intensity (black line) at the maximum projection and lower in the minimum projection, confirming the alignment of the material. These features were observed for the whole thickness of the samples, confirming a volumetric alignment in A-BNC.

Optical properties

As we have presented, A-BNC films exhibit iridescence and strong light polarization properties. As shown in Fig. 7, the visualization of BNC fibers under a polarized light microscope with the same orientation as the incident light (0° angle) absorbed light and were not seen. Conversely, when the light source was located at 45° from the fiber orientation, the fibers are not seen. BNC did not exhibit any light polarization neither at 0° and 45° (Fig. 7-A,D), confirming the random distribution of the fibers. On the other hand, A-BNC films gave dark-light patterns of light when disposed at 0° and 45° from the incident polarized light

beam, respectively (Fig. 7-B,E), confirming the anisotropy of the material. Moreover, A-BNC is a flexible material and can be folded into any desirable shape, such as an “N”. As seen in Fig. 7-C,F, N-shaped films of A-BNC created a pattern of light polarization at different positions.

Hydrophilicity and cell substrates evaluation

Most of the electrospun polymers are rather hydrophobic, which is unfavorable from the point of view of tissue engineering. For instance, aliphatic polyesters such as PLLA or PCL show contact angles in the range of 116–135°, while for tissue engineering requirements, it ought to be below 100°^{42,43}. Niemczyk-Soczynska et al.⁴⁴ studied the highest level of fibroblasts cell attachment at hydrophilic surfaces, and the best results were observed for surfaces with contact angles in the range of 20–60°⁴⁵. As the use of cellulose, and specifically, bacterial nanocellulose drives many applications in medicine^{46–48} for their biocompatibility and hydrophilic character we wanted to analyze the impact of the fiber alignment in the hydrophilic character of bacterial cellulose. We deposited a blue colored drop of water over A-BNC film and we could clearly see a faster horizontally spreading of the liquid in comparison to BNC, indicating a different structure, Figure 8A. To quantify the hydrophilicity of both BNC and A-BNC we performed “apparent contact angle” (ACA) measurements. After 5 seconds of depositing a water drop over the surfaces, we observe an ACA of 0° for A-BNC (Fig. 8-B) whereas it remained 29.7° for BNC after 5 seconds (Fig. 8-C). These results indicated that the alignment of fibers seemed to increase the hydrophilicity of the films, which also it matches the lower roughness of A-BNC in AFM and the increased density of OH groups by FTIR.

This change of hydrophilicity was further explored *in vitro* in cell cultures. A human dermal fibroblast (hDF) cell line (1BR.3.G) was cultured on A-BNC and BNC films and the cell attachment and growth directionality was assessed visually and with orientation coherency measurements with the *OrientationJ* plugin of ImageJ. As seen in Fig. S5, cells attached and proliferated on A-BNC, BNC and control (glass slide) until reaching a 100% confluence at day 7 (Fig. S5, Fig. 8-D,E). As seen in Fig. 8-F, hDF cells cultured on aligned cellulose fibers tend to be more elongated (orientation coherency of 0.25) than on random fiber distributions (BNC film) (orientation coherency of 0.12) and on the slide (orientation coherency of 0.8) The increase of hDF directionality indicated that cells perceived at some extent the alignment of the cellulosic fibers.

A-BNC thermal conductivity

The advent of more sophisticated implants combining novel biomaterials, polymers, electrical components among others sustained that thermal management becomes critical⁴⁹. Most of the biomaterials have an amorphous structure meaning they are thermal insulators, however, in order to develop materials with enhanced thermal conductivity, self-assembled biomaterials with higher degree of crystallinity and/or higher anisotropy is desirable. BNC has already shown promising results as scaffold in regenerative medicine^{17,19,50}, therefore we studied the influence geometrical order on the thermal

conductivity of BNC fibers. In particular, we show that it is possible to enhance the thermal conductivity by directional alignment of the fibers, i.e. in the direction parallel to the alignment direction. The thermal conductivity of A-BNC was investigated by two independent approaches, i.e. using the well-known 3ω -method⁵¹, as well as through a recently developed contactless thermorefectance frequency-domain approach developed by L. A. Pérez, et al.⁵², and labeled after Anisotropic Thermorefectance Thermometry (ATT). The later methodology is particularly suitable to study thermally anisotropic materials (e.g. aligned BNC), since it delivers the angular distribution of the thermal conductivity perpendicular to the surface of the sample. In all cases the BNC fibers were deposited on silicon substrates, and the thickness of the BNC samples was $\sim 10\text{ }\mu\text{m}$. Fig. 9-A displays the 3ω measurements of A-BNC in perpendicular (red) and parallel (blue) to the alignment direction. Whereas the lower frequency range is dominated by the thermal response of the Si substrate, the higher range contains information on the thermal properties of the fibers. The thermal conductivity is estimated using the “slope method” ($\kappa \propto [\partial V_{3\omega} / \partial \log(f)]^{-1}$) in the higher frequency range. The lower frequency range is dominated by thermal signal from the substrate, and it is used as calibration for each measurement ($\kappa_{Si} = 150\text{ W/m}\cdot\text{K}$). In the parallel direction, A-BNC shows a larger thermal conductivity at $1.63 \pm 0.15\text{ W/m}\cdot\text{K}$, whereas in the perpendicular direction the thermal conductivity is $0.3 \pm 0.02\text{ W/m}\cdot\text{K}$, i.e. slightly lower to what we previously obtained for BNC, $0.5 \pm 0.05\text{ W/m}\cdot\text{K}$ ⁵³. The alignment of the fibers led to a 5-fold increase of the thermal conductivity of the BNC in the parallel direction, Fig. 9-A. Similar results were obtained with ATT, where thermal conductivity was measured almost continuously with 1° angular steps from the alignment direction, Fig. 9-B. The highest value for A-BNC, at its parallel direction, ($1.42 \pm 0.15\text{ W/m}\cdot\text{K}$), is similar to that observed using the 3ω -method in the same direction ($1.63 \pm 0.15\text{ W/m}\cdot\text{K}$), and it is on the same order as polyethylene (PE) ($1.33\text{ W/m}\cdot\text{K}$)⁵⁴, and larger than collagen ($0.5\text{ W/m}\cdot\text{K}$)⁵⁵, which are materials commonly used in implants. Therefore, geometrical alignment of the fibers offers the possibility to control at different planes the thermal conductivity just by tailoring the anisotropy of the biomaterial⁵⁴. Interestingly, the perpendicular component of the thermal conductivity from the ATT scans ($0.87 \pm 0.09\text{ W/m}\cdot\text{K}$) resulted twice as large as compared to the value obtained using the 3ω -method ($0.3 \pm 0.02\text{ W/m}\cdot\text{K}$). The origin of this different values in the perpendicular direction possibly arises from different thermal resistances between parallel fibers. From those results we foresee that the potential of natural-based materials, which may outperform in some properties to polymer and synthetic materials.

Conclusions

Inspired by the bacterial cellulose biosynthesis we presented a facile and reproducible method to obtain films of aligned cellulose fibers (A-BNC) and non-aligned cellulose (BNC) fibers in a single step. Optical, chemical, and physical techniques evaluated confirmed the alignment of the BNC fibers superficially and in the whole volume of the film. Cellulose fibers are strongly interacting creating an aligned film which are maintained upon different dry-wetting cycles and exhibit polarization of light and the thermal conductivity of this material is modified due to the novel structure. The thermal conductivity of A-BNC was thoroughly studied with innovative techniques which confirmed a 5-fold increase of the thermal conductivity in the parallel direction, $1.63 \pm 0.15\text{ W/m}\cdot\text{K}$, whereas in the perpendicular direction the thermal

conductivity is 0.3 ± 0.02 W/m. The properties risen by the alignment encompasses the properties already exploited for celluloses as their easy functionalization, biocompatibility and purity which bring cellulose to be exploited in an even larger number of fields, and even join the selective group of biomaterials to be part of our future and active implants.

Methods

Biosynthesis of aligned BNC, A-BNC, films and BNC

Biosynthesis of A-BNC and BNC. *Komagataeibacter xylinus* (*K. xylinus*) strain colonies (NCIMB 5346 from CECT, Valencia, Spain) were grown confluent on *Hestrin-Schramm* (HS) solid medium⁶ in Petri dishes. The media consists of 1.15 g/L of citric acid, 6.80 g/L of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 5.00 g/L of peptone, 5.00 g/L of yeast extract, 15.00 g/L of agar and 20.00 g/L of dextrose all from Condalab, dissolved and autoclaved in 1L Milli-Q water. *K. xylinus* strain were cultured in HS solid medium Petri plates for 15 days at 30 °C. Next, agar squares of 70 mm side with *K. xylinus* grown colonies were placed carefully facing up with a sterile spatula at the bottom of a sterile tube with oxygen-permeable tap filled with 10 mL sterile bacteria-free liquid HS media, which consists of the same compounds as the solid media without agar. The system was cultured for 5 days at 30°C under static conditions, until a layer of BNC hydrogel (random fiber organization) was detected at the air-liquid interface. The A-BNC films were obtained within the length of the tube.

Bacterial Nanocellulose Cleaning. Once produced, A-BNC and BNC films were separated by carefully cutting and pulling out from the extremes of the films using Teflon tweezers. After separating the films, the cleaning process took place separately. BNC films were soaked and stirred with a magnetic stirring plate in the following steps: (i) 1:1 Ethanol: Milli-Q water solution for 10 min, (ii) 40 min in boiling Milli-Q water, and (iii) two periods of 20 min in 0.1 M NaOH (Sigma-Aldrich) aqueous solution⁶. Finally, the BNC films were rinsed with Milli-Q water until reaching neutral pH, autoclaved at 121 °C for 20 min and stored suspended in water in glass vials at room temperature. To avoid entanglement of the aligned fibers, A-BNC films were gently cleaned with the same steps as BNC but without magnetic stirring.

Bacterial Nanocellulose Drying. BNC samples were placed between two Teflon plates and dried at 60 °C for at least 12 hours under a 1 Kg weight. A-BNC-films were dried on top of a single Teflon plate and without any applied weight, at the same conditions.

BNC and A-BNC Characterization

Atomic Force Microscopy (AFM). A-BNC and BNC films were dried on top of a thin conductive Si surface. $10 \times 10 \mu\text{m}^2$ micrographs were obtained using a modular PM/AFM (Keysight 5500 LS SPM/AFM), using tapping mode.

Contact Angle (CA). 3 mm width and $10 \pm 2 \mu\text{m}$ thick dry A-BNC and BNC films were fixed flat on a Teflon plate with tape. The surface wettability and capillarity of the materials were assessed by a contact angle

measurer (KRÜSS Drop Shape Analyzer DSA 100), using the sessile drop method. 2 μL of Milli-Q water were placed on the BNC surface. CA values were computed over 0.5 s for 10 s after depositing the drop.

Dissolved oxygen content. The reagent *Indigo-Carmine* was used to quantify the dissolved oxygen (DO) content in liquid HS culture media. The Indigo-Carmine method^{38,39} is a simple, rapid, and accurate colorimetric procedure for determining small amounts of DO in water (0 to 50 ppm). First, 9 mg of the powder reagent (Sigma-Aldrich) was hydrated with 2.5 mL of Milli-Q water, which contained 0.1 gr of dextrose (Condalab). At the same time, 10 mL of 37.5% of KOH (Sigma-Aldrich) was mixed with 37.5 mL of ethylene glycol (Sigma-Aldrich). Both mixtures were added together and refrigerated until the Indigo-Carmine reagent was completely reduced. This could be noted due to the color change: starting from blue to red, and finally turning yellow. To test the water sample (refrigerated in advance), 0.1 mL of the reduced solution of Indigo-Carmine was added to a 7.5 mL liquid sample inside a vacuum free container. The presence of any DO oxidized the reagent and made the solution change color. The variation in color of the dye is directly proportional to the amount of DO present in the sample, which can quantitatively be determined by using a color scale (Fig. S1-B).

Fourier-Transformed Infrared (FTIR) Spectrophotometry. Dry A-BNC and BNC films were analyzed with a FT/IR spectrophotometer (Jasco 4700LE). The transmittance spectra collection was performed at 2 cm^{-1} . For each spectrum, 32 scans were co-added over the measuring range $400\text{-}4000\text{ cm}^{-1}$. Air was used as blank (machine lid open). The spectra were processed with the Spectra Manager™ Suite software for the reduction of CO_2 and H_2O noise levels, baseline correction, smoothing and peak find.

Optical microscopy. The morphology and structure of A-BNC and BNC was assessed using a conventional optical microscope (Olympus RXSITRF 52787, MAB INDUSTRIAL). To obtain polarized microscopy images, a retardation slide accessory made of a birefringent material (Olympus U-PO3) was coupled to the optical microscope. For observing the change of light pass through the dry A-BNC and BNC films, the polarized light direction was kept constant and the sample was circularly rotated in the same plane within a turning center point for 360° . Photos were taken every 45° .

Fiber orientation analysis. The A-BNC fibers orientation was analyzed using the *OrientationJ*^{60,61} Java plugin for ImageJ/FIJI (D. Sage, EPFL, 2.0.5 version). The *OrientationJ* structure tensor computes the orientation and isotropy properties in a local window. The local window is characterized by a 2D Gaussian function of standard deviation σ , based on the evaluation of the structure tensor in a local neighborhood. The parameter σ (expressed in pixel units) is a critical parameter that determines the scale of the analysis. It should have a value roughly close to the structure of interest (e.g. thickness of the cellulose filament). The smallest local window available was 1 px, which was the set value used for this analysis. From the plugin, the “Color Analysis”, “Distribution”, and “Dominant Direction” modes were used to analyze images. *OrientationJ* was also used to obtain maximum frequencies from the angle distribution to assess the orientation coherency in several imaging techniques (equation (1)).

$$\text{Orientation coherency} = 2 \langle \cos^2 \alpha \rangle - 1 \quad (1)$$

where α represents the angle between an individual fiber and the average fiber orientation. The coherency ranges from 0 to 1, where 0 represents an isotropic image without any preferential orientation and 1 represents a perfectly aligned distribution image²².

Polarization-resolved second-harmonic generation microscopy (PSHG). Semi-wet A-BNC and BNC films were stained for 5 min with 5 μ L with an aqueous solution of 0.25 mg/mL *Brightener 28* (seen as bright blue, exc 365 nm) in Milli-Q water. Then, they were observed under a custom-made multiphoton microscope system, which works both as a two-photon excitation fluorescence microscope (TPEF) and as a polarization-resolved second-harmonic generation microscope (PSHG)^{40,62} (SLN Research Facility, Dr. Pablo Loza-Alvarez, Institute of Photonic Sciences, Castelldefels, Spain). The illumination source is a Ti Sapphire oscillator that generates a linearly polarized laser of a wavelength of 810 nm in a frequency of 76 MHz and a 180 fs pulse. The laser then gets reflected in Galvo mirrors and the rotation of such linearly polarized light is performed by the rotation of a $\lambda/2$ plate, to illuminate the sample by different angles of excitation, from 0° to 180°. The linear polarization of the incident beam was evaluated from 0 to 180 degrees in steps of 10 degrees using a $\lambda/2$ plate, taking frames at each step. After reflection in a dichroic mirror, the beam excites the sample through an inverted microscope objective (28.25X Olympus). Immersion oil was added. By reflection, TPEF signal is obtained backward after reflecting Nikon fluorescence cubes and passing through IR filter. SHG signal is captured by a microscope objective (NA=1.1), and obtained after reflecting a dichroic mirror that rejects backscattered laser light and modifies the ellipticity of the polarization states and passing through narrowband and IR filters. Brightfield, TPEF, and PSHG image stacks were obtained from 0° to 180°, as well as Z stacks. Further analysis was performed pixel by pixel using the stack options “Z project” and “plot Z-axis profile” from the heat map filtered image stacks in ImageJ. The *OrientationJ* ImageJ plugin was used to assess the orientation coherency of the fibers in the images (equation (1)).

Interferometry. A-BNC and BNC films were dried on top of a glass coverslip. 3D images of 450 x 350 x 1.50 μ m³ (A-BNC) and 85 x 100 x 1.50 μ m³ (BNC) were obtained with a high-resolution dual-core confocal and interferometry profilometer (Leica DCM 3D optical profilometer), using a “retouching surface” operator.

Scanning Electron Microscopy (SEM). A-BNC and BNC films were fixed flat on top of aluminum SEM holders with adhesive carbon tape. Images were obtained using a high-performance SEM (QUANTA FEI 200 FEG-ESEM) without metallization at EHTs of 5-10 kV and 50 Pa (low vacuum conditions).

Small Angle X-ray Scattering (SAXS). The scattering measurements were performed at NCD-SWEET beamline at ALBA Synchrotron light facility, in Cerdanyola del Vallès (Barcelona). A monochromatic X-ray beam of 8 keV ($\lambda = 0.154$ nm) was set using a Si (1 1 1) channel cut monochromator. An array of Be lenses was employed to collimate the X-ray beam, obtaining a beam size at the sample position of 50 ×

150 μm^2 ($V \times H$). The scattering patterns were recorded using a Pilatus3 S 1M detector (Dectris®), which consists of a pixel array of 1043×981 ($V \times H$) with a pixel size of $172 \times 172 \mu\text{m}^2$. The scattering vector q (defined as $q = 4\pi\sin(\theta)/\lambda$, being θ is the scattering angle and λ the X-rays wavelength) was calibrated using Silver Behenate as reference, obtaining a sample to detector distance of 6700 mm. Dry A-BNC and BNC samples were fixed flat on 0.50 cm $\varnothing$ metal rings, where the samples were held by the edges. Samples were scanned perpendicularly to the beam direction, with an acquisition time of 30 s. Azimuthal integration of the obtained signal was done using PyFAI, limited by 270° of azimuthal range due to the intrinsic detector gaps limitations but enough to evaluate the 180° of the 2-fold fiber symmetry. The azimuthal distribution was normalized against the maximum value. The integrated scattering pattern generated a distribution of the diffraction intensity along the Debye-Scherrer ring, $I(\Phi)$ (arb. u.), vs azimuthal angle, Φ ($^\circ$). A fitting of the distributions was performed using the OriginLab (OriginLab Corporation) non-linear function Lorentz equation (equation (2)) from where the orientation parameters FWHM, OI and S (equation (3-5)) could be obtained to quantify the alignment of the BNC nanofibers.^{26, 31}

FWHM stands for full width of the half-maximum intensity of the azimuthal profiles from the selected diffraction and it can be obtained directly from the fitting. FWHM ranges from 0° to 180° and low values are correlated with higher orientation of the sample (34). OI (also as f_c) is defined as an orientation index and it is complementary to FWHM. The OI ranges from 0 to 1, with 0 describing a random arrangement and 1 describing a perfect nanofibrils orientation. S is the Herman's order parameter, obtained with MatLab using equation (4-6). Similar to OI, a value of $S = 0$ means the nanofibers are randomly oriented, while $S = 1$ indicates a full nanofiber alignment.

$$y = y_0 + \frac{2\{\text{rm A}\}}{\pi} \cdot \frac{\omega}{4(x - x_c)^2 + \omega^2}$$

2

$$OI = \frac{180^\circ - fwhm}{180^\circ}$$

3

$$S = \frac{3\cos^2\Phi_{c,z} - 1}{2}$$

4

$$\cos^2\Phi_{c,z} = 1 - 2\cos^2\Phi_{200,z}$$

5

$$\cos^2\Phi_{200,z} = \frac{\sum_0^\pi I(\Phi) \sin\Phi \cos^2\Phi}{\sum_0^\pi I(\Phi) \sin\Phi}$$

6

Thermal conductivity. The thermal conductivity, k , of a material is a measure of its ability to conduct heat.

Thermal conductivity measurements of A-BNC and BNC were performed using the 3ω -method and a novel contactless frequency-domain approach designed by L. A. Perez,⁵² et al., and labeled after Anisotropic Thermoreflectance Thermometry (ATT). The later methodology is particularly suitable to study thermally anisotropic materials (e.g. aligned BNC), since it delivers the angular distribution of the thermal conductivity perpendicular to the surface of the sample. Measurements were performed with the described system on A-BNC films dried on silicon wafers at room temperature, to which a gold coating was added.

The 3ω -method is based on electrically heating a thin planar resistor using an AC harmonic current I_0 at a frequency ω , and subsequently measuring the resultant voltage drop at the first (V_ω) and third ($V_{3\omega}$) harmonics. By defining the normalized temperature coefficient of resistance as equation (7) with R_0 the resistance of the resistor at the temperature T_0 , the amplitude of the AC component of the temperature oscillations induced can be determined as equation (8).

$$\beta \equiv (1/R_0)[\partial R/\partial T] \quad (7)$$

$$\Delta T_{AC} = 2|V_{3\omega}| \beta |V_\omega| \quad (8)$$

By solving the 2D heat equation for the geometry of a linear heat source supported on a semi-infinite medium, the thermal conductivity κ of such medium can be obtained as equation (9).

$$\kappa = -P_0 2\pi l I (\partial \Delta T_{AC} / \partial \ln 2\omega) \quad (9)$$

With P_0 the total dissipated power at the resistor and l the length of the resistor. Since the AC current frequency determines the thermal penetration depth according to $1/q = \sqrt{\alpha/i2\omega}$, for thick films supported on a semi-infinite medium (i.e. a substrate) the ΔT_{AC} vs $\ln 2\omega$ curve shows low and mid-frequency regimes that primarily correspond to the substrate and the supported film, respectively. From the slope $(\partial \Delta T_{AC} / \partial \ln 2\omega)$ of both regions it is straightforward to determine their thermal conductivity.

Statistics were performed on 7 different resistors thermally evaporated on BNC films yielded to obtain the thermal conductivity.

Transmission electron microscopy (TEM). A-BNC and BNC films were dried on top of a copper TEM grid. Images were obtained using a high angular range TEM (JEOL 1210 TEM), operating at 120 kV with an

ORius 831 SC 600 Gatan camera. Selected Area Electron Diffraction (SAED) was used to obtain the diffraction pattern from the A-BNC and BNC films, to assess its crystallinity.

Evaluation of BNC and A-BNC as Cell Carriers: in vitro experiment. A human dermal fibroblast (hDF) cell line (1BR.3.G, ECACC 90020507) was used to evaluate the performance of BNC and A-BNC as adherent cell carriers, and how the structuration of A-BNC influences the cell growth. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 2 mM GlutaMax (Gibco), and 10% fetal bovine serum (FBS; Gibco) at 37°C in 10% CO₂. The subculture routine for 1BR.3.G consisted on split sub-confluent cultures (70-80%) 1:3 to 1:6 i.e. seeding at 2-4x10,000 cells/cm² using 0.05% trypsin/EDTA; 10% CO₂; 37°C. hDF were cultured on dry sterile samples of A-BNC and BNC films. In brief, cellulose samples were autoclaved (121°C, 20 min) and dried on autoclaved glass microscope slides at room temperature in a laboratory hood for 6 hours, and finally irradiated with UV light for 30 min to ensure sterility. Slides were placed in Ø 8.5 cm Petri dishes and preconditioned with cell media, before seeding 1.5M cells per dish. Cell media (20 mL) was changed every two days. After 4 and 7 days of culture, images were obtained with an inverted microscope (Nikon Eclipse Ts2). From optical microscope images, the orientation coherency was measured with the *OrientationJ* plugin for ImageJ (equation (1)).

Statistical analysis. Quantitative data are expressed as means ± standard deviation. Statistical analyzes were performed with Graph Pad Prism 8 software using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was accepted when P-values were ≤0.05 and summarized as * = P≤0.05, ** = P≤0.01, *** = P≤0.001, **** = P≤0.0001 for the calculated P-values.

Declarations

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Credit

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Figures

Figure 1

Methodologies to align BNC fibers. **A-B** Top-down and bottom-up reported methodologies to align bacterial cellulose fibers, alongside with the outcomes. **C** Strategy presented in this work: benefitting from the natural BNC biosynthesis.

Figure 2

Methodology to obtain aligned BNC (A-BNC). **A,B** The bacteria are anchored on an agar surface at the bottom of the culture tube filled with liquid culture media, from where bacterial nanocellulose is synthesized through several enzymes. BNC fibrils are linearly extruded from the bacteria through pores. CS (cellulose synthase); PGM (phosphoglucomutase); UGP (UDPGlc pyrophosphorylase); GK (glucokinase)³⁵. The dissolved oxygen (DO) gradient formed in the culture tube is one of the driving forces of this method: *K. xylinus* is an aerobic bacteria and requires oxygen to survive. **C,D** Optical images of the macro-structure of aligned fibers (A-BNC) within the tube.

Figure 3

Transparency and chemistry of A-BNC. **A** Pictures of A-BNC and BNC, respectively **B-C** Pictures of A-BNC and BNC on top of a micron-mesh, indicating a hazier BNC than A-BNC. **D** Dry A-BNC show iridescence effects under the incidence of white light. **E** FTIR indicates that A-BNC and BNC have similar chemical composition. However, the A-BNC spectra shows more intense transmittance peaks in the OH and CH region.

Figure 4

Morphology and structure analysis. **A** Picture of the as produced bacterial nanocellulose films, containing BNC and A-BNC. The top (**B, C, D**) and bottom (**E, F, G**) rows contain the images obtained by TEM, SEM and AFM of BNC and A-BNC, respectively. **B, E** TEM images and Selected Area Electron Diffraction (SAED) patterns of BNC and A-BNC samples. **C, F** SEM micrographs of BNC and A-BNC samples (Insets: color analysis from the *OrientationJ* plugin for ImageJ). **D, G** AFM images of BNC and A-BNC samples (Insets: isotropy distribution computed using MountainView8).

Figure 5

Nanoscale density study with SAXS. **A,B** SAXS scattering signal of BNC and A-BNC. **C** integration of the spectra as an intensity vs azimuthal degree plot from -90° to 90° . A-BNC showed an intensity peak centered around 0° , whereas BNC gave no peak at all. **D** Table with the orientation quantitative parameters orientation index (OI), full width at the half-maximum intensity (FWHM), and Herman's order parameter (S-parameter) values²⁶ for BNC and A-BNC.

Figure 6

Polarization-resolved second harmonic generation (PSHG) microscopy. Stacks of 18 images (frame/10° light polarization) were analysed with ImageJ using a heat map and Z projections. **A** Maximum and minimum SHG intensity projections of 150 x 150 μm^2 for BNC and A-BNC, along with the intensity colour map. **B** Plot of the SHG intensity for each angle polarization, along with their alignment coherency, obtained with the *OrientationJ* plugin for ImageJ.

Figure 7

Behaviour of A-BNC and BNC under polarized light. Visualization under a polarized light microscope at 0° and 45° (incident polarized light beam vs sample orientation) of **A,D** BNC samples, **B,E** A-BNC samples and **C,F** N-shaped A-BNC (Insets of whole films in A-C).

Figure 8

Hydrophilicity and cell substrates evaluation of A-BNC and BNC. **A** Pictures of the process of wetting of a A-BNC film with a colored water drop. **B,C** Measurement of the apparent contact angle (ACA) of A-BNC and BNC films, respectively. **D,E** Optical images of confluent cell cultures at day 7 on A-BNC and BNC, respectively. **F** Orientation coherency plot of A-BNC, BNC and the slide (control), computed with the *OrientationJ* plugin of ImageJ. *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 9

Thermal conductivity study of A-BNC. **A** 3ω measurements of A-BNC at the parallel and perpendicular directions were evaluated, indicating a maximum 5-fold increase of the thermal conductivity in the parallel direction of A-BNC fibers. **B** Thermal conductivity of A-BCN measured by anisotropic thermoreflectance thermometry, showing similar results as shown in **A**. **C** Comparison of the obtained measurements to the standard collagen⁴⁹, PLA⁵⁶ or PE⁵⁴ used in implants. **D** Table of the reported thermal conductivity of polymeric materials such as PE⁵⁴, PLA⁵⁶, collagen⁵⁵, vegetal cellulose^{57,58}, paper⁵⁹ and silk⁴⁹.

Supplementary Files

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