Supplementary Information (SI) for Biomaterials Science.
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Supporting Information

Gradually-frozen aligned bacterial nanocellulose membranes loaded with gallic acid exhibit enhanced mechanical and dual antithrombotic-antimicrobial properties

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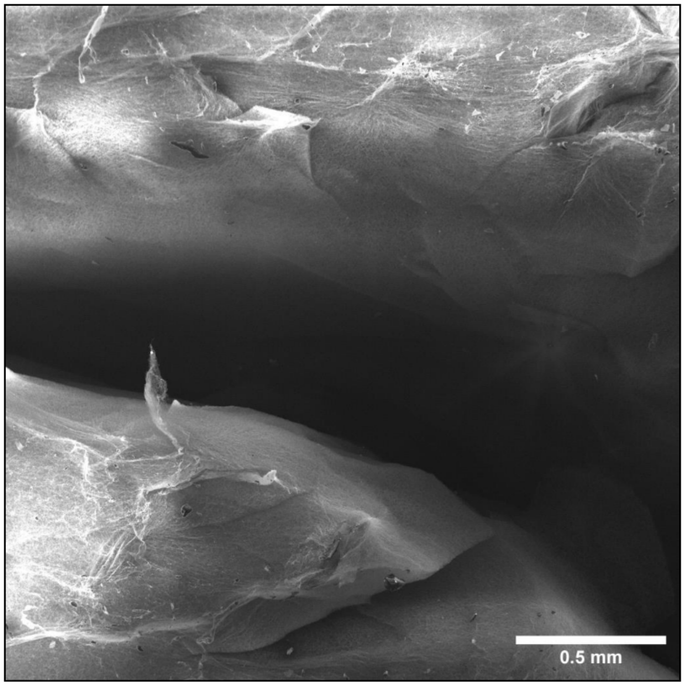
Effects of rapid freezing on the integrity of FD-BNC

To further characterize the effects of rapid freezing on the structural integrity of BNC, SEM imaging was performed (Supporting Figure 1). Multiple fractures in one location of the sample can be seen, hypothesized to be directly related to the stresses which occur during rapid freezing of the samples in liquid nitrogen.



Supporting Figure 1. The surface of freeze-dried BNC (FD-BNC), presenting with multiple macro/micro-fractures formed during the freezing process.

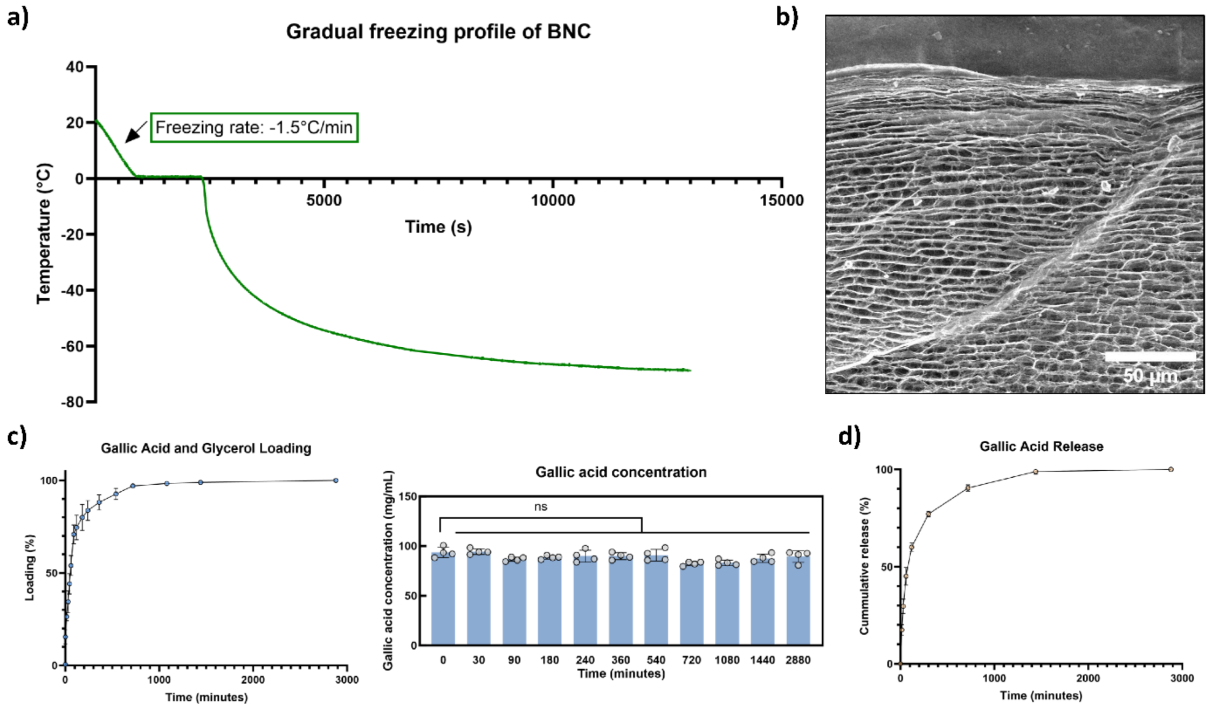
Gradual Freezing Validation

To validate the freezing rate of BNC while gradual freezing (GF-BNC), the temperature of the BNC inside the isopropanol container was recorded using a thermistor (Supporting Figure 2a) at about a 1 Hz sampling rate. The slope of the best-fit line on the temperature versus time data was recorded as the freezing rate of the material. The gradual freezing process directly contributes to the aligned nature of GF-BNC, which aids in mechanical strength and fluid transfer properties (Supporting Figure 2b).

Gallic acid/glycerol loading and release profile

Gallic acid and glycerol loading in GF-BNC were quantified using mass changes over time (Supporting Figure 2c). Over a period of 48 hours, the mass of 1 cm × 1 cm GF-BNC samples submerged in the GG solution was monitored. The resulting mass change, displayed as a percentage of final mass at each time point, demonstrates rapid absorption in the first 12 hours and stabilization of the material as the loading period ends after 48 hours. To ensure that bulk loading of GG was occurring, UV-vis quantification of the loading solution was performed throughout the loading period. This revealed no significant change in the loading solution over the 48-hour loading period, indicating that bulk loading of GG into GF-BNC was achieved.

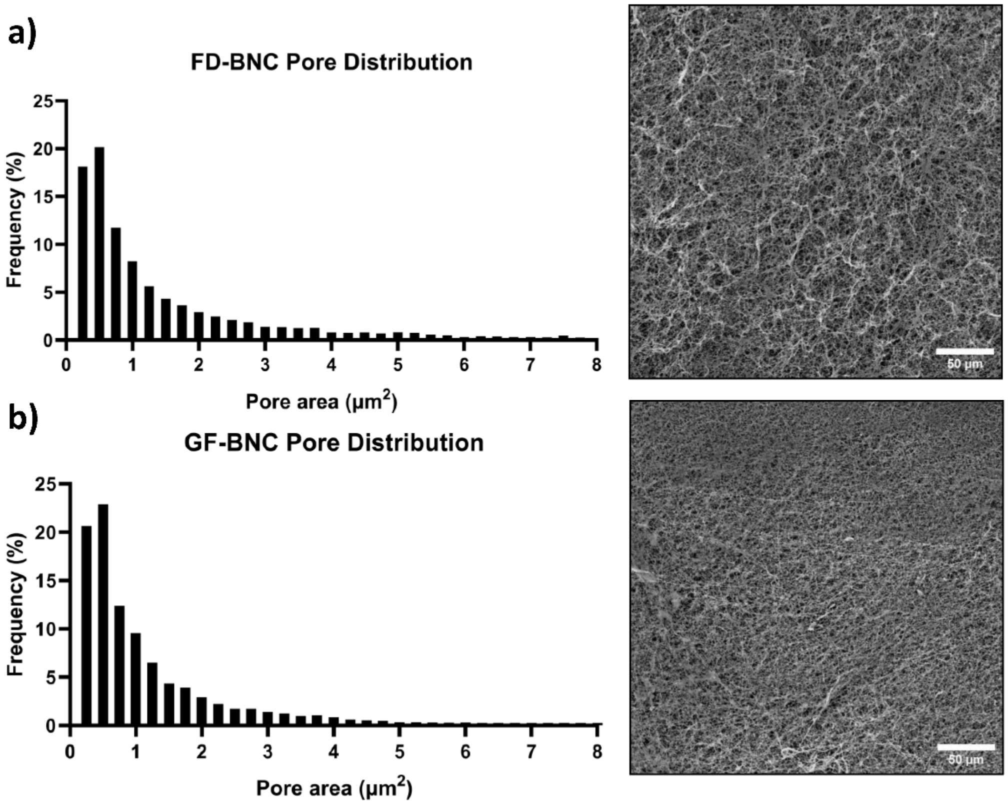
Gallic acid release was determined by submerging GF-GG-BNC in phosphate buffer saline (PBS) at 37°C and performing UV-vis quantification of gallic acid at set time intervals (Supporting Figure 2d). Cumulative GG release was detected using 1 cm × 1 cm GF-GG-BNC samples submerged in 800 µL of PBS, which was refreshed at each time point and 200 µL samples from the previous wells were used to quantify gallic acid release using absorbance measurements from a plate reader (SpectraMax M2, Molecular Devices) from 200 nm - 400 nm. A calibration curve was prepared to relate the concentration of the released gallic acid peaks of the spectra were compared to known concentrations of GG in PBS to the absorbance measurements, with a linear calibration curve (R2 > 0.97) observed in the concentration ranges of 5 mg/mL to 100 mg/mL for gallic acid. Cumulative gallic acid release of all repeats was calculated and compared as a percentage of the release after 48 hours.



Supporting Figure 2. a) Temperature profile of BNC during gradual freezing. **b)** Gradually-frozen BNC (GF-BNC) presents with directional alignment in its cross-section **c)** Gallic acid and glycerol loading in GF-BNC were quantified by monitoring the change in mass over a 48-hour period. To ensure bulk loading, gallic acid concentration in the GG loading solution was quantified throughout the data collection period. No significant change in gallic acid concentration over time was detected. **d)** Gallic acid release in PBS was monitored using UV-vis spectroscopy.

Pore size distribution of FD-BNC and GF-BNC

To better understand the effects of rapid and controlled-rate freezing on material morphology, pore size distribution was determined from SEM images using image thresholding and particle size identification in ImageJ (Supporting Figure 3a, b).

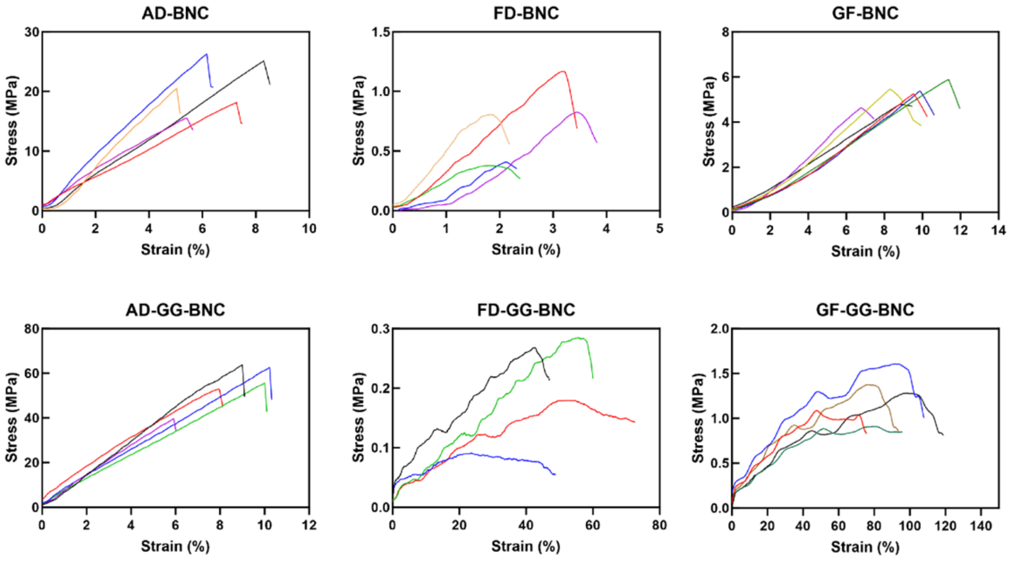


Supporting Figure 3. Representative SEM images and pore size distribution for a) FD-BNC and b) GF-BNC samples.

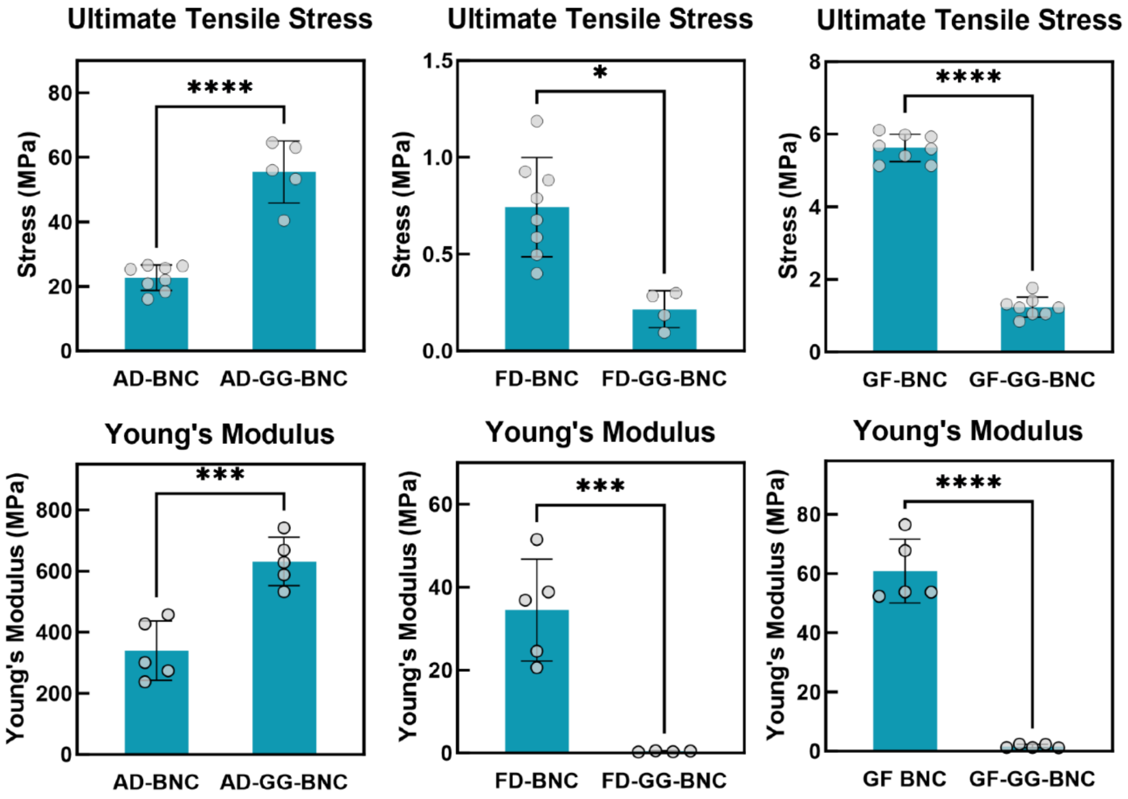
Uniaxial tensile testing

All samples were subjected to repeated uniaxial tensile testing to characterize their mechanical properties. The stress-strain curves (Supporting Figure 4) provide information on the strain at rupture as explored in the main report, but also ultimate tensile strength (UTS) and Young's modulus and the repeatability of each (Figure S5). When subjected to tensile stress, freeze-dried BNC (FD-BNC) demonstrated both poor stress retention and poor repeatability. Under identical conditions, samples of FD-BNC showed highly varied mechanical properties, which was also seen during testing of the glycerol/gallic acid (GG) loaded FD-BNC (FD-GG-BNC) samples. As hypothesized in the main report, this is expected to be a result of freezing-related fractures in FD-BNC from the drying process. Air-dried BNC (AD-BNC) exhibited a UTS greater than both AD-BNC and gradually-frozen BNC (GF-BNC). The thin, compact AD-BNC membranes hold a high density of BNC fibers, allowing for greater resistance to stress.

One of the key features expected in GG loading is the plasticizing effect of glycerol on BNC materials. As discussed in the main report, plasticizers are known to greatly increase the elasticity of a material while also decreasing its mechanical strength. As seen in Supporting Figure 5, a decrease in mechanical strength was evident for both FD-GG-BNC and GF-GG-BNC, while AD-GG-BNC increased in strength when compared to AD-BNC. This is indicative of inadequate GG incorporation for AD-GG-BNC. The drastic changes expected to be brought about by the plasticizer can be similarly seen when examining Young's modulus. A significant decrease in Young's modulus directly indicates both an increase in elasticity and decrease in strength for FD-GG-BNC and GF-GG-BNC compared to their dried counterparts, while AD-GG-BNC showed an increase in Young's modulus compared to AD-BNC.



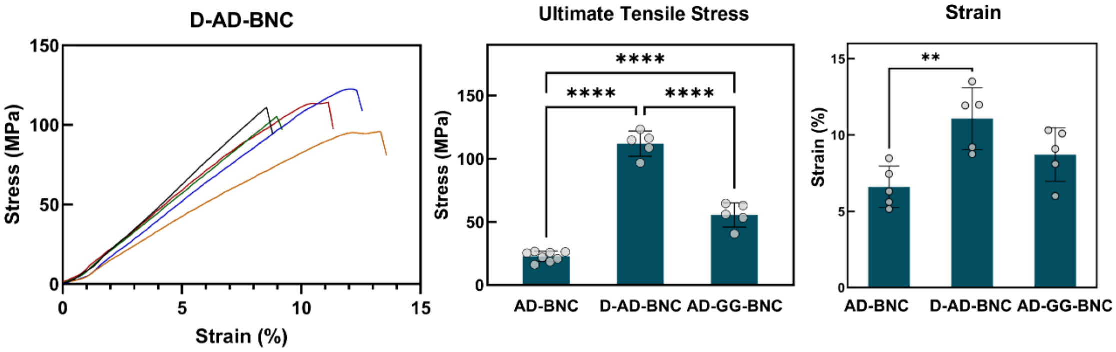
Supporting Figure 4. Stress-strain curves of dried and gallic acid/glycerol (GG)-loaded BNC samples.



Supporting Figure 5. Ultimate tensile strength (UTS) and Young's modulus comparison between the dried BNC materials and their gallic acid/glycerol (GG)-loaded counterparts. All graphs are reported as mean ± standard deviation. $****P \leq 0.0001$, $***P \leq 0.001$, $*P \leq 0.05$.

Double air-dried BNC investigation

Glycerol is a known plasticizer for polymeric biomaterials. The expected effect of a plasticizer, as detailed in the report, is that the strain at rupture of a sample will significantly increase, while the UTS should decrease. With this effect not being seen for AD-GG-BNC, it is hypothesized that GG loading in the material is insufficient. During the rinsing process, if GG is rinsed from the surface and structure of AD-BNC with water and the material is allowed to dry a second time, the material is expected to become increasingly compact. This would amplify the effect of the internal hydrogen bonding resulting from evaporation and decrease the already limited empty space within the material. To validate the hypothesis that the change in mechanical properties of AD-GG-BNC can be attributed to a second wetting and drying of the material, not GG loading, double air-dried BNC (D-AD-BNC) was subjected to tensile testing (Supporting Figure 6). By re-hydrating AD-BNC and allowing it to air-dry a second time, a significant increase in UTS could be seen when compared to AD-BNC. Moreover, the strain at rupture of D-AD-BNC was also significantly improved from AD-BNC, indicating further that the change in mechanical properties of AD-GG-BNC when compared to AD-BNC can be attributed to the rehydration and drying of the membranes.



Supporting Figure 6. Ultimate tensile strength (UTS) and strain at rupture for air-dried BNC (AD-BNC), double air-dried BNC (D-AD-BNC), and gallic acid/glycerol air-dried BNC (AD-GG-BNC). All graphs are reported as mean ± standard deviation. ****$P \leq 0.0001$, **$P \leq 0.01$.