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Microarchitecture and Radiological Flow Pattern of Cocoon-like Nanocellulose Hydrogels

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This work investigates fluid mechanics and structural properties inside bacterial cellulose hydrogels in from of macroscopic anisotropic ellipsoid body cocoon-like 3D scaffolds. The hydrogels were evaluated by injections of iopamidol, a radiopaque medium used in medical radiological procedures. Results showed that hydromechanical behavior of BC hydrogels may be used to characterize its internal structure. It is suggested that internal barriers and flow distribution anisotropy may be used to develop novel therapeutic applications based on the tissue engineered multifunctional possibilities of the biomaterial.

Keywords: *Radiological analysis, Flow patterns, Bacterial nanocellulose, Tissue engineering, 3D Hydrogels.*

1. Introduction

The development of advanced bacterial nanocellulose (BNC) hydrogels should rely on their internal structure and fluid mechanics properties[1-3]. For drug delivering systems and tissue engineering constructs the ability to withhold a great amount of water is of no additional value if structural barriers cannot be used to sustain therapeutic effects, to segregate antagonist biochemical properties such as redox pairs, and to control degradation, tissue uptake and drug release rates[4].

Pseudo-homogeneous and non-uniform, non-isotropic hydrogels have potentially important applications in novel tissue engineering 3D constructs[2,5-12]. Hydrogels are been discussed and postulated as strong candidates for the development of smart materials, mostly because they may mimic actual living systems, including cells, tissues and organs[13-21]. Their capacity to hold water in a cross-linked structure is among the most frequently cited desired property[4,22-24]. Nevertheless, fluid mechanics of hydrogels are not fully exploited. Literature reports on BNC hydrogels are limited to membranes and small (< 1 cm^3) bodies. We have recently produced biosynthetic hydrogels based on networked and entangled fibers of bacterial cellulose. They may be grown until macroscopic bodies (≥100 cm^3) that resembles silk cocoons[13]. The main difference between cocoon-like hydrogels and membranes is that the first are composed of a fibrous membrane with high fiber density that completely involves a less dense fibrous network, while membranes exhibit two distinct sides: an air-exposed upper surface, with high fiber density and a lower immersed surface that exhibits a higher porosity. Those cocoons can be created by *Gluconacetobacter hansenii* (formerly known as *Acetobacter xylinum*) in well defined hydrodynamic culture media. Macroscopic bodies that can vary from a few to more than one hundred cubic centimeters may have very complex internal structures that are challenging to characterize[25]. Usual characterization techniques assess static properties of biomaterials without considering the aqueous environment capacity to direct and retain exogenous biomolecules and other biological or mineral compounds and their mutual interactions. In this work we consider the hydromechanical characterization of bacterial cellulose hydrogels tailored to present particular 3D constructs suitable for several tissue engineering applications. The flexibility of contrast injection technique is used to follow fluid patterns and to analyze internal structure, permeability, construct resistance, elasticity and swallow properties based on radiological videos and images (see Table 1 for terminology adopted in this work).

Radiopaque contrast agents are used in computer tomography (CT) scans and other radiologic (X-ray) imaging to characterize blood vessel flows, organs, and other non-bony tissues[26]. We found that they may be also useful to characterize hydrogel constructs based on bacterial cellulose for applications in tissue and organ engineering.

Here, we report the use of a nonionic contrast agent (Iopamidol solution) to characterize qualitative the internal structure of nanofibrous hydrogels, by means of X-Ray imaging of the injected solution flow.

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Table 1. Glossary of hydrogel characteristics and hydromechanical flow-relatated definitions.

Parameter	Definition
BNC	Bacterial nanocellulose
Cocoon	A macroscopic hydrogel ellipsoid body formed by interlaced and entangled bacterial cellulose fibers, produced by particular agitated and defined culture medium
Granular cocoon	A cocoon with internal discrete granules of cellulose fibers inside the hydrogel
Percolation	The process of natural or forced (field-driven) internal fluid diffusion, advection or trickling
Perfusion	The process of forcing a fluid through the hydrogel body skin to its external environment (air, liquid, tissue or organ)
Permeability	Capacity of the hydrogel body to allow gases or liquids to pass through due to pores or openings in its internal structure
Pseudo-homogeneous cocoon	A gelatinous dispersed fibrous phase of bacterial cellulose
Skin permeability	Permeability of the exterior membrane of BNC hydrogel cocoons

2. Materials and Methods

The strain of *G. hansenii* ATCC 23769 from the Collection of Tropical Culture (CCT, André Tosello Foundation, Campinas-SP, Brazil) was used to synthesize bacterial cellulose structures. The culture medium was prepared by dissolving 25.0 g mannitol, 5.0 g yeast extract and 3.0 g bactopeptone in 1 liter of water. pH was adjusted to 6.6 with NaOH.

The macroscopic 3D hydrogels were produced according to the method previously described[13]. Briefly, an aliquot of 5% of a growing culture that had been stirred vigorously to release cells from the cellulosic mass was inoculated in 100 mL conical flasks containing 50 mL of sterile medium culture (sterilized at 121°C for 20 min). Experiments were conducted on a rotary shaker (Nova Ética Mod 430/RDBP, Vargem Grande Paulista, SP, Brazil) operating at rotational frequency of 3.3 Hz, for 14 days at 30°C. All experiments were carried out in triplicate. Large ($\geq$ 100 cm^3) BNC hydrogel bodies were harvested from the rotary shaker flasks and treated with 0.1M NaOH at 90°C for 20 min in order to remove impurities and cells that remained adhered to cellulose, and thoroughly washed in water until neutral pH. Hydrogel cocoons were finally washed with distilled water. After synthesis, purification and washing, samples were conditioned in a 20% ethanol solution for storage at 4°C. Before submitted to radiological analysis, hydrogel constructs were submitted again to autoclave treatment at 121°C for 20 min.

Bacterial cellulose cocoons were injected with a 10 mL hypodermic, Luer Lok tip, clarified polypropylene barrel syringe (Becton Dickinson Indústria Cirúrgica Ltda, Curitiba-PR, Brazil), filled with Iopamiron® 370, a nonionic contrast agent (iopamidol 755 mg·mL-1, Schering) delivered through stainless steel needles (Becton Dickinson, BD Medical Systems). Visualization was performed under a CIGNUS/VMI Meditech S.A. Medical Technology angiographic system (Cardio Centro Diagnóstico, SociCor - Instituto do Coração SOCIMED, Tubarão-SC, Brazil). X-ray source was adjusted to 40 kV and 400 mA. Iopamidol, $C_{17}H_{22}I_3N_3O_8$, a nonionic, water-soluble contrast agent with molecular weight of 777 g·mol-1, which is used in myelography, arthrography, nephroangiography, arteriography, and other radiological procedures (PubChem Project, National Center for Biotechnology Information, 2009) was withdrawn from a 50 mL vial and manually delivered under laminar flow conditions using a clinical syringe. Reynolds number was estimated at 256 ± 26 for cocoon analysis with some intermittent injections. Injections were carried out through a needle carefully positioned to show advection and contrast agent spreading. An angiography stainless steel needle of 0.64 mm of internal diameter and 40 mm length was used in BNC cocoons.

Previously developed cocoons were used for characterization and internal granular BNC cocoons hydrogels of 1 $g{\cdot}cm^3$ density were both injected with 1 mL of nonionic contrast for 8 seconds at 125 µL·s-1. A regular bevel (sharp angular tip at the end of the cannula) and regular wall thickness needle was used, thus allowing piercing the cocoon external skinny membrane with low penetration force and minimal drag, following manufacturer's guidelines.

The micromorphology and microstructure of the 3D hydrogels were evaluated by Scanning Electron Microscopy (SEM, Philips, XL-30). For the observations, samples with diameters of 5 mm were liofilized and placed over an aluminum support and sputtered with gold.

3. Results and Discussion

Figure 1 shows a photograph of a 3D hydrogel (cocoon) of approximately 60 g produced in a conical flask with 50 mL of culture medium. Excess and perfused fluid may be seen around the hydrogel body. density of 0.99 $g{\cdot}cm^{-3}$(0.01 Its water embedded saturated volume is approximately 120 cm^3. The dimensions of the cocoons depend on the initial volume of the medium and flask geometry as reported previously[13]. BNC cocoons exhibit a gelatinous structure in their inner part, completely enclosed by a nanofibrous denser membrane. This membrane is formed under specific hydrodynamic conditions, where the cocoon-like body is continuously surrounded by a living bacterial layer, which forms a progressive shell membrane that covers the gelatinous inner part. In large bodies, this external membrane is a dense, interconnected, cellulose nanofiber network that resembles a skin.

In tissue engineering completely homogeneous and isotropic materials may not be as useful as structured scaffolds

where cells are phenotypically differentiated according to their local microenvironment. The cocoons described here are hierarchically structured from their internal core up to their external membrane. The internal structure of the developed cocoons can be visualized against intense lightning, as shown in Figure 2.

Three regions can be identified: i) a transparent, uniform mass that surrounds the body, delimited by a skinny membrane; ii) an intermediary, diffuse network of entangled fibers and granules (Shown by arrows in Figure 2a) and iii) discrete, denser fiber agglomerates forming different geometries (Figures 2b and c). These fiber agglomerates form cellulose bundles with diameters ranging between hundreds of micrometers and few millimeters, as displayed in details in Figure 2d. These micro and millimetric scaled features are responsible for the flow pattern of fluids inside the cocoons, acting as structured barriers that define the flow geometry and regime, and flux direction.

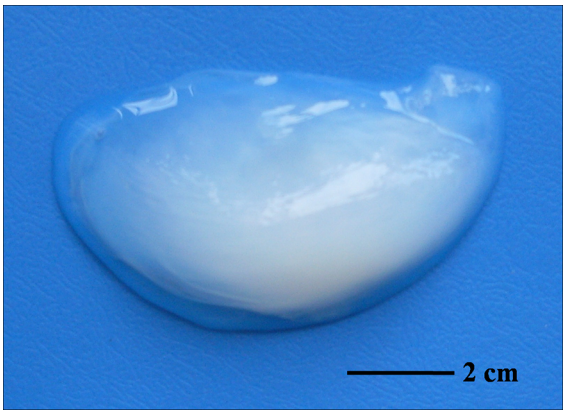

Figure 1. A pseudo-homogeneous bacterial cellulose cocoon laying on a flat surface just after removal from a 20% ethanol solution.

Figure 3 shows SEM micrographs of a liofilized cocoon. The voids observed in Figure 3a were probably formed as a result of fibril islands coalescence, resulting in free spaces between these islands. The external surface of the 3D cocoon exhibits a dense random assembly of interconnect ultrafine fibers network structure, composed of fibers with average diameter ranging from 50 to 100 nm and relative fiber density of 0.91 (Figure 3b). Examination of the fractured section (Figure 3c) revealed an internal micro architecture that consists of a hierarchically organized arrangement constructed of fibrous cell units, which form a honeycomb-like morphology.

Iopamidol internal flow distribution and recirculation patterns in bacterial cellulose hydrogels were examined in ellipsoid (cocoon-like) samples as shown in Figure 4. Strong dependence on internal and external skinny membrane texture and fiber density was observed.

Recirculating flow was noticed when the contrast agent was tangentially injected in the pseudo-homogeneous cocoon however, surprisingly, with spiral patterns formed in the plane perpendicular to the tangential injection path. Figure 4a shows needle positioning and radiological flow image after 4 seconds of injection. This is most likely due

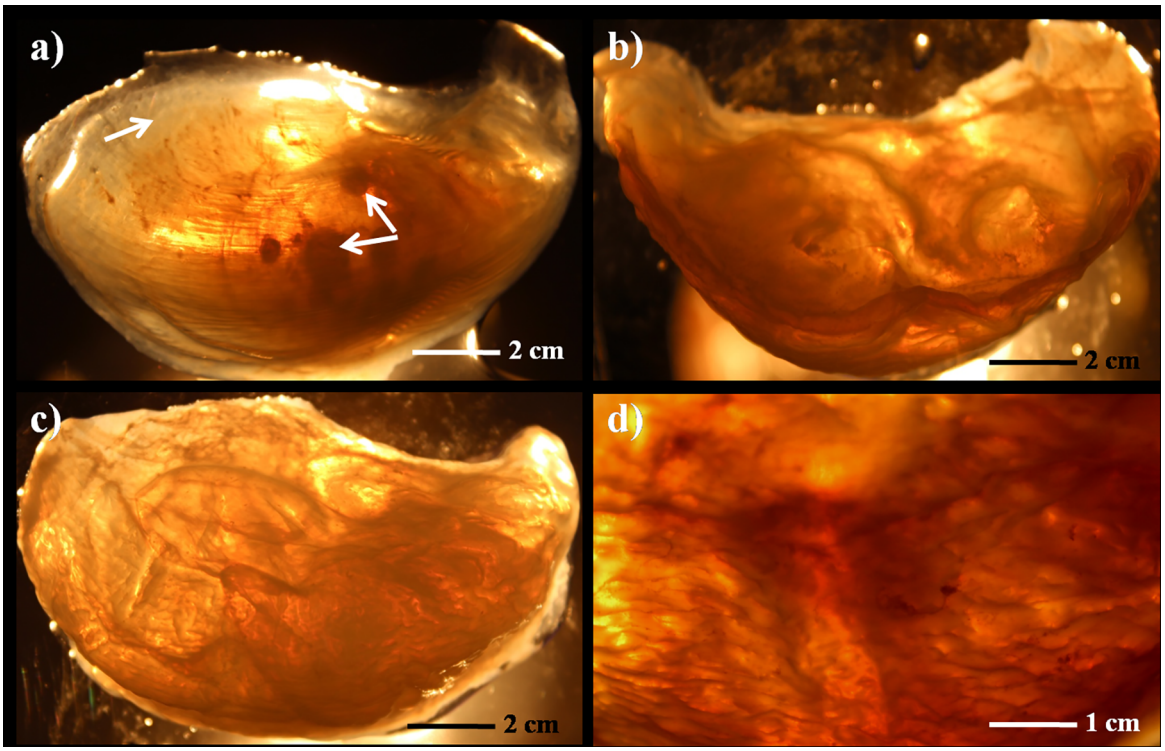

Figure 2. Optical images of a bacterial cellulose cocoon showing the internal structure of cellulose fibers inside the hydrogel: a) Granular cocoon; b)-d) Pseudo-homogeneous cocoon.

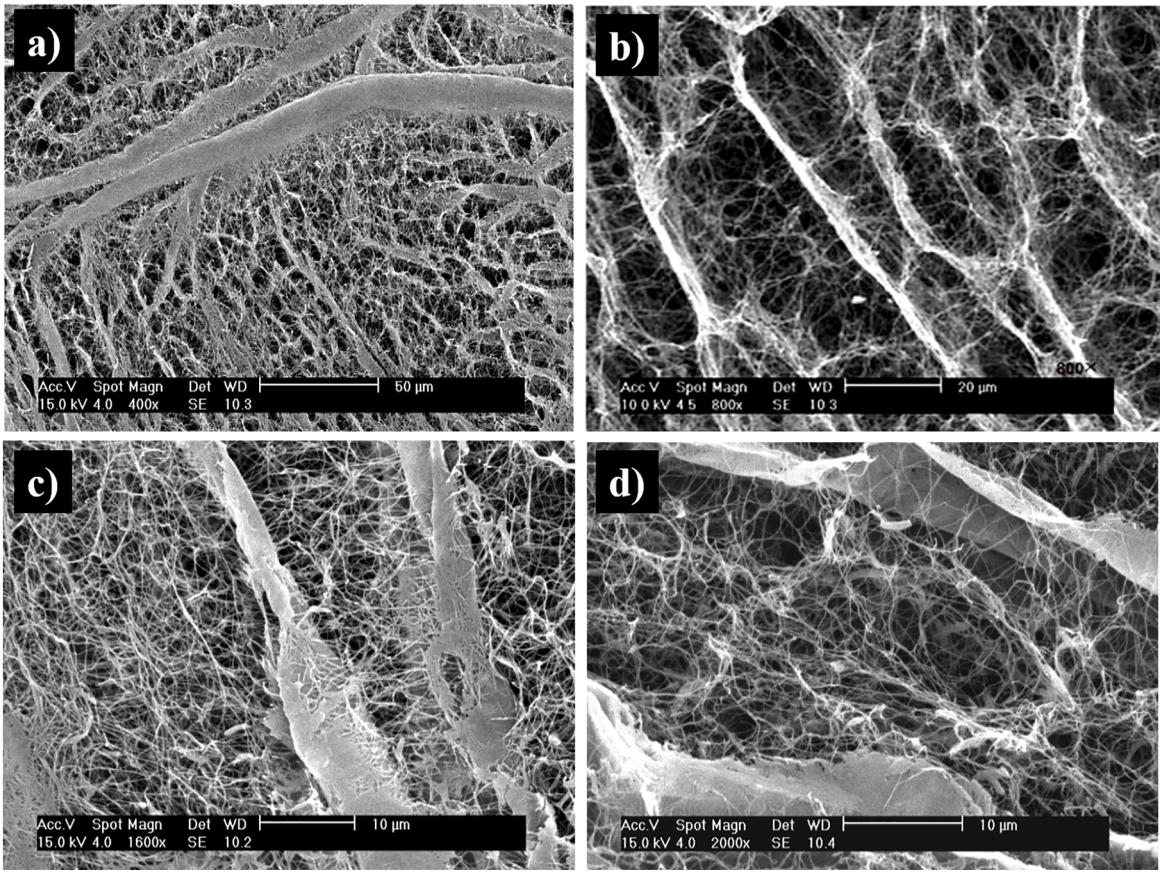

Figure 3. Scanning electron microscopic (SEM) image showing several layers of internal membranes, naturally developed during BNC synthesis under agitated culture.

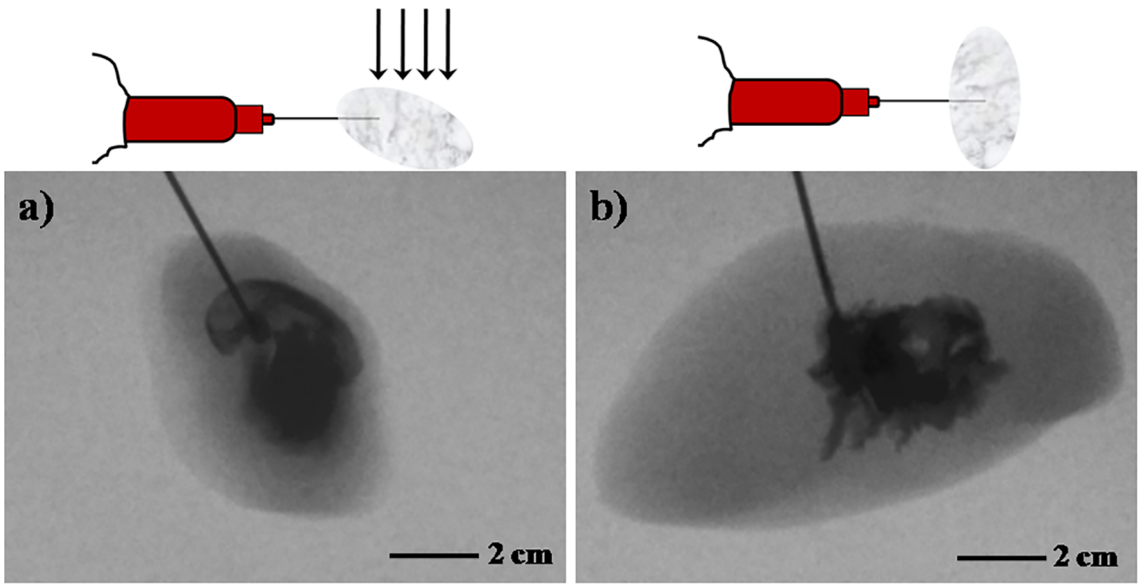

Figure 4. Contrast agent (iopamidol solution) injection scheme and radiological top view images of bacterial cellulose hydrogels under laminar injection flow: a) Pseudo-homogeneous BNC cocoon and the developed spiral-like fluid recirculation; b) Granular BNC cocoon and the percolation ramification pattern, around internal fiber granules. Down arrows represent incident X-ray bean direction.

to the preferential channels formed around fibril aggregates and pseudo-membrane structures internally formed during the bacterial cellulose synthesis[13]. When contrast agent is injected in the more granular cocoon (Figure 4b), a cabbage flower type of pattern emerges, revealing internal aggregates, vacuoles and dense fibril islands. Besides the external skinny membrane that is typical of those cocoons and other BNC bodies, a subcutaneous dense external involucrum is also seen by visual inspection and revealed by the contrast. It is easily seen that this external layer concentrates most of the BNC mass of macroscopic hydrogel bodies.

The outer membrane exhibits enough resistance to hold the geometrical rearrangement, also allowed by the natural elasticity of the external membrane (skin). Only under relatively high pressure and very saturated conditions, we have observed contrast (iopamidol) solution perfusion. Further studies are needed to evaluate diffusion, permeation, percolation and perfusion of macromolecular compounds, in particular of transcription factors, hormones, large proteins, and nucleic acids in the macroscopic bodies of BNC hydrogels. Three-dimensional macroscopic bodies synthesized by static and agitated cultures of *G. hansenni* under appropriate conditions allow for a variety of applications in the biomedical field, particularly in drug delivery/reaction systems and tissue engineering implants.

4. Conclusions

We have demonstrated that iopamidol, a nonionic contrast agent, can be used to characterize bacterial cellulose hydrogel macroscopic bodies. Those 3D constructs are potential candidates for smart tissue engineering scaffolds, particular due to their compartmentalization, that can be somewhat controlled by adjusting hydrodymechanical forces and culture media parameters. The quick spread of iopamidol, a 777 g·mol-1 molecule, inside bacterial cellulose hydrogel constructs indicates that similarly large molecular weight hydrophilic agents may be able to easily diffuse (permeate) in their internal gel structure. On the other hand, the surrounding dense fibrous membrane of BNC cocoons do not allow easy path to their exterior (perfusion). Further studies are needed to evaluate diffusion, permeation, percolation and perfusion of macromolecular compounds in the macroscopic BNC hydrogels reported here. It is suggested, though, that they constitute a valuable platforms for drug tests, tissue development and multifunctional scaffolds.

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