Bacterial cellulose growth on 3D acrylate-based microstructures fabricated by two-photon polymerization

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Abstract
Miniaturized environments have emerged as an excellent alternative to evaluate and understand biological mechanisms. These systems are able to simulate macroenvironments with high reproducibility, achieving many results in a short time of analysis. However, microenvironments require specific architectures that can be reached using laser micromachining techniques, such as two-photon polymerization (TPP). This technique has many advantages, allowing the production of environments without shape limitation and with special features. In this work, aided by the TPP technique, we produce different arrays of microstructures, fabricated using acrylate-based materials, in order to evaluate the growth and development of the Komagataeibacter xylinus bacteria, the micro-organism responsible for producing bacterial cellulose (BC), a natural polymer with several biological applications. BC grown in microenvironments presents similar features to those of biofilm formed in macroenvironments, maintaining their attractive properties. In addition, due to the high optical quality and mechanical resistance of the BC matrices, we use these films as flexible substrates in TPP experiments, obtaining promising results for tissue engineering studies.

1. Introduction
Biomimetic systems with micrometric dimensions can be used in several experiments, achieving fast results and with great accuracy, similar to analyses performed in macroscopic environments. These platforms have emerged as a promising alternative to studying biological mechanisms, aiming, for instance, to understand the movement dynamics of micro-organisms and their growth in controlled environments [1–6]. An interesting micro-organism that can be explored in miniaturized environments is Komagataeibacter xylinus, a species of bacteria responsible for synthesizing bacterial cellulose (BC) [7, 8], a natural renewable polymer. BC presents a uniform structure and morphology. It is also free of lignin and polyoses. It is endowed with unique characteristics, such as high purity and crystallinity, remarkable mechanical properties, good chemical stability, and high water-holding capacity. BC is featured as a completely biocompatible polymer that can be produced in almost any shape due to its high moldability during formation. Therefore, this is an interesting biomaterial that is distinctive in many aspects. Its discovery has shown tremendous potential as an effective biopolymer in various fields, such as tissue engineering [9–12]. In particular, several studies have been carried out in order to apply BC in dressings and biocompatible scaffolds, aiming at, for instance, efficient drug delivery systems [13, 14].

Among the different tools available for biomimetic microenvironment fabrication, the two-photon polymerization (TPP) technique [15–17] has been widely used due to its distinct advantages. In this maskless technique, the local polymerization allows the fast production of three-dimensional (3D) complex platforms, without shape limitation, with high resolution, and in few steps, which makes it a competitive tool in comparison with other simpler micromachining techniques. Several materials can be used in TPP experiments. Usually, these matrices are mixed together with an organic compound known as a
photoinitiator, a molecule responsible for generating free radicals for the polymerization procedure after laser radiation. For biological applications, biodegradable scaffolds are often required [18, 19]. However, acrylate-based materials are also well known to be bio-compatible and non-toxic to several micro-organisms [20–24]. In addition, some studies require a large quantity of 3D scaffolds for different analyses. Therefore, it is possible to use TPP combined with sophisticated replication methodologies, which allow fabrication of several scaffolds with an exceptional reproducibility [24–27]. Beyond that, this technique makes it possible to fabricate structures with specific features, which are developed exclusively for the desired applications [28–34].

In this work, we investigate BC (biofilm) formation in 3D acrylate-based arrays produced via TPP. We compare BC grown on these photopolymered structures with the matrices obtained by conventional means (macroscopic experiments). In addition, we investigate the movement dynamic of Komagataeibacter xylinus bacteria in micrometric labyrinths, evaluating the BC formed after their development. Due to their attractive properties, we also use BC matrices as flexible substrates in TPP experiments, using their porous network to diffuse a liquid through the fabricated microstructures, an interesting feature for tissue engineering applications.

2. Materials and methods

2.1. Acrylate-based samples for photopolymerization experiments

To perform the photopolymerization experiments, we use two acrylate-based samples. The first sample, here called the hybrid sample, is composed of a mixture of equal proportions of the penta-acrylate monomer, known as dipentaerythritol pentaacrylate (SR399—Sartomer), and a silane compound, known as 3-(trimethoxysilyl)propyl methacrylate (A174—Sigma Aldrich). The acrylate monomer is used to provide mechanical resistance to the final structure while the silane material is responsible for increasing the roughness of the structure, allowing better adhesion for the inoculated bacteria. A few drops of an acylphosphine oxide photoinitiator, 2,4,6-trimethylbenzoylphenyl phosphinate (Lucirin TPO-L—Irgacure) are also mixed with the previous compounds (3 wt% in the total sample volume). The second sample is prepared using only acrylate monomers. This sample is composed of two three-acrylate monomers, ethoxylated(6) trimethyl-lolpropanetriacrylate (SR499—Sartomer) and tris(2-hydroxyethyl)isocyanurateretiaacrylate (SR368—Sartomer), also employed in equal proportions and mixed with 3 wt% Irgacure TPO-L. Although photoinitiators are often classified as toxic in biological experiments, the Lucirin TPO-L used in the preparation of these samples is known to be a biocompatible compound with several micro-organisms. In addition, the photoinitiator amount used in our experiments is small enough to be almost entirely consumed during the polymerization procedure.

2.2. Experimental setup used in TPP

3D structures were fabricated using a homemade setup which uses a femtosecond laser (mode-locked Ti:sapphire oscillator—KMLabs) as the excitation source, centered at 790 nm, operating at a repetition rate of 86 MHz and delivering pulses of ∼150 fs. The laser beam is focused on the acrylate-based samples (placed between glass substrates separated by a micrometer spacer) using a 10× microscope objective (NA = 0.25, voxel size ∼6 × 1.5 μm). A pair of galvanometric mirrors is controlled by dedicated software, allowing laser scanning through the sample in two dimensions (x–y plane). A translational stage, which also holds the sample during the photopolymerization experiment, is used to achieve the third dimension on the structure. The laser intensity is controlled using a half-wave plate and a polarizer, positioned in front of the galvanometric mirrors. Due to the difference between the refractive index of the solidified resin and the liquid resin [35], the polymerization process can be monitored in real time aided by an independent imaging system composed of a CCD camera, a telescope and a red LED. At the end of the fabrication procedure, the sample is immersed in ethanol heated at 75 °C for 15 min, in order to remove the unpolymerized resin. Finally, before starting the biological experiments, the fabricated microstructures are sterilized using UV irradiation.

2.3. Komagataeibacter xylinus activation and BC growth

First, the Komagataeibacter xylinus bacteria were activated by transferring the surface of the solid culture plates to a liquid medium [36], where they grew and multiplied. Some BC properties, such as the optical quality and porosity, depend on how the natural polymer is purified (washed) and dried. In figure 1, we can observe three different matrices obtained after distinct washing and drying procedures.

As can be seen in figure 1(a), non-purified BC membranes exhibit a nanofibrous porous network that is highly moldable. Also in figure 1(a), it is possible to observe Komagataeibacter xylinus bacteria inside the formed matrix. When water-soaked membranes were put on support and submitted to drying in an
Figure 1. BC matrices obtained after distinct washing and drying procedures. (a) Non-purified BC membrane dried at room temperature. It is possible to see Komagataeibacter xylinus bacteria inside the formed matrix. (b) BC membranes washed in water and dried in a forced air circulation oven with temperature control at 28 °C for 12 h. (c) BC membranes washed in ethanol and subjected to supercritical drying in a E3000 critical point dryer (using liquid CO₂ as a solvent).

air-circulation oven with the temperature controlled at 28 °C for 12 h (figure 1(b)), the biofilm porosity decreased and the mechanical stability of the natural polymer was improved. Finally, in figure 1(c), the ethanol-soaked membranes were subjected to supercritical drying in an E3000 critical point dryer (Quorum Technologies) using liquid CO₂ as a solvent. The exchange of ethanol in the pores of the membranes with liquid CO₂ was done by a slow change of solvent over 4 h. Supercritical drying was carried out by heating the system to 45 °C and maintaining the pressure at 1200–1300 psi. The system was maintained under these pressure/temperature conditions for 1 h before venting to obtain the BC membrane aerogel [37]. As a result of this procedure, the BC membranes were highly moldable, with appropriate optical quality and with high porosity degree, an interesting feature for several biomedical applications.

To evaluate the bacteria development in photopolymerized microenvironments, the fabricated structures were placed inside Petri dishes with a water slide at the bottom. With the help of a micropipette, a 5 µl drop of the liquid medium containing the bacteria was placed on the surface of the microstructures. Then, each Petri dish was placed in an incubator with air circulation and temperature control at 28 °C for 24 h. After the above procedure, the plate was frozen and lyophilized.

2.4. Characterization of BC grown on the microstructures
To evaluate and characterize the BC grown on the microstructures, we used conventional optical microscopy, scanning electron microscopy (SEM—Zeiss, SIGMA model) and a Fourier-transform infrared (FT-IR) spectrometer (Bruker, Vertex 70 model using an ATR objective).

3. Results and discussion
This section presents the results as a proof of principles regarding BC formation on the photopolymerized microstructures using hybrid and polymeric matrices. Figure 2 shows typical structures fabricated with hybrid materials (left) and acrylate matrices (right).

Komagataeibacter xylinus bacteria were inoculated into the acrylate-based microenvironments for 24 h, receiving all the necessary conditions for their development (as described in section 2.3).

As can be seen in figure 3, the BC film formed in the array composed of hybrid materials presented good adhesion to the structure surface, including inside the cylinders (as shown in figure 3(b)). It is still possible to observe that the thickness of the formed film was limited by the cylinder size.

FT-IR spectroscopy was performed on these grown films in order to evaluate and compare them with macroscopic BC matrices (grown on the usual platforms or experiments). Figure 4 shows these results.

The ATR-FTIR spectrum of the neat BC matrix (black line) shows characteristic bands of BC. Different bands can be identified in the spectrum, which include hydrogen-bonded hydroxyl groups (O–H stretching around 3100 cm⁻¹–3600 cm⁻¹), CH₂ symmetric (around 2890 cm⁻¹) and asymmetric (around 2850 cm⁻¹) stretching, bending motion of absorbed water H–O–H (1650 cm⁻¹ region) and characteristic C–C and C–O–C polysaccharide linkages (multiple bands in the 800–1200 cm⁻¹ region) [38, 39]. When a similar analysis was performed on BC films grown on hybrid microstructures, we observed equivalent bands (figure 4, red line), indicating that the BC microfilms have a similar chemical composition to those macroscopic BC films.
Figure 2. Microstructures fabricated via TPP using acrylate-based materials. (a) Hybrid matrices, which have more roughness on the surface. (b) Polymeric matrices, a typical sample used in TPP experiments.

Figure 3. BC grown on the microstructures fabricated with hybrid matrices. (a) It is possible to observe good adhesion of the formed BC film around the microstructures, including (b) inside the cylinders.

Figure 4. FT-IR spectrum of BC matrices. Black line: macroscopic sample, which was formed according to conventional procedures. Red line: BC formed on the photopolymerized microstructures.
Figure 5. BC grown on polymeric materials. (a) BC film formed after 6 h of bacteria incubation and (b) SEM image of the BC network grown in these structures. (c) BC film formed after 24 h of bacteria incubation and (d) SEM image of the BC network grown in these structures.

Figure 6. Photopolymerized labyrinth used to evaluate the movement dynamics of *Komagataeibacter xylinus* bacteria and BC film formation. The red arrow indicates the small aperture of the structure.

We also evaluate how the BC is synthesized at different incubation times. Figure 5 shows the results when the bacteria are inoculated into the microenvironments for 6 h (figures 5(a) and (b)) and 24 h (figures 5(c) and (d)).

As shown in figure 5(a), even after a short time of incubation, it is already possible to identify a network of nanofibers around the structures (on the bottom). After 24 h of incubation, it is possible to observe a thin film formed on the microstructures, covering the microenvironment. This result is very interesting because it can enable the removal of BC grown in these structures, allowing their application in other experiments.

To investigate the mobility mechanisms of the *Komagataeibacter xylinus* bacteria, we fabricate micrometric labyrinths. In these structures, there is only a small aperture, which we use to inoculate the bacteria. To facilitate our analyses, the labyrinths do not have a ceiling. Figure 6 shows an SEM image of the fabricated labyrinth, and the red arrow indicates the structure aperture.
Again, the bacteria are inoculated in the environment for 24 h and, posteriorly, we evaluate the BC growth on the structures. The results are presented in figure 7.

Bacteria were dispensed only close to the labyrinth aperture. In this experiment, we observed that the biofilm did not grow throughout the entire labyrinth, but preferentially over short distances (∼30–40 µm), using the structure walls to support the adhesion.

As previously mentioned, BC is a natural highly moldable polymer, which can be applied as a flexible substrate in several devices. In addition, the high porosity of the BC membranes is an attractive feature for different applications aimed at the diffusion of liquid media of interest, such as organic dyes or medicines. Here, we also use BC as a substrate for TPP experiments. Figure 8 summarizes some of the results when we fabricated 3D structures using BC as a support material.

To fabricate these structures we used BC matrices as a support material, as presented in figure 1(c), which have a high pore density. During the TPP experiment, the BC film maintained its properties and structural integrity, an important feature for this application. Figures 8(a) and (b) show, respectively, an optical microscopy image and an SEM image of the fabricated structures. To demonstrate a potential application of these structures fabricated on the flexible substrate, we soaked the sample in a solution of rhodamine B dissolved in ethanol (10⁻⁴ M) for 5 min and evaluated the liquid diffusion. After this step, we washed the sample (microstructures and BC substrate) three times in ethanol. As can be seen in figure 8(c), it is possible to observe that the liquid spreads in all directions, including inside the photopolymerized structures. Thus, the results obtained in this experiment can open new possibilities for the use of BC as a flexible substrate, allowing future applications in drug delivery systems and in other tissue engineering fields.
4. Conclusion and final remarks

In this work, we presented 3D acrylate-based structures fabricated via TPP, which were used to evaluate the development of *Komagataeibacter xylinus* bacteria, a micro-organism responsible for BC production. BC formation occurred both in hybrid and polymeric matrices, and their original features were kept, as demonstrated by SEM images and FT-IR spectroscopy. However, we observed better adhesion of these bacteria in the hybrid matrices.

When the bacteria were inoculated in labyrinths, differently from what was expected, BC formation did not occur throughout the structure. We observed the BC growth preferentially over short distances, using the structure walls to support film adhesion.

Using BC as a flexible substrate in TPP experiments, we obtained interesting results. The natural polymer did not change by laser irradiation, maintaining its original features until the end of the photopolymerization procedure. There was a good adhesion of the structures with the natural substrate, even after washing the sample in heated ethanol. Finally, when the structures fabricated on BC substrates were immersed in rhodamine B solution, we were able to verify the liquid diffusion through the structures. This result demonstrated a potential application of these flexible substrates for liquid diffusion mechanisms in miniaturized environments, opening, for instance, a new approach for drug delivery systems, which require fast, controlled and directed diffusion of medicines.

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Conflict of interest

The authors declare no conflicts of interest.

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