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Direct laser writing by two-photon polymerization as a tool for developing microenvironments for evaluation of bacterial growth

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ABSTRACT

Monitoring bacteria growth and motion in environments is fundamental to understand, for instance, how they proliferate and contaminate organism. Therefore, techniques to fabricate microenvironments for *in situ* and *in vivo* studies are interesting for that purpose. In this work we used two-photon polymerization to fabricate microenvironments and, as a proof of principle, we demonstrated the development of the bacteria ATCC 25922 *Escherichia coli* (*E. coli*) into the microstructure surroundings. Two varieties of polymeric microenvironments are presented: (i) a microenvironment doped at specific site with ciprofloxacin, an antibiotic typically used in the treatment of diseases caused by *E. coli* and (ii) micro-fences, which serve as traps for bacteria. These microenvironments, fabricated by two-photon polymerization, may be a potential platform for drug delivery system, by promoting or inhibiting the growth of bacteria in specific biological or synthetic sites.

1. Introduction

Nonlinear optical processes have been extensively used for manufacturing microdevices for different technological areas [1–5], with emphasis to two-photon polymerization (2PP). This technique has several advantages over conventional microfabrication methods, among which we can mention the resolution above the diffraction limit and high spatial selectivity of the two-photon absorption [6,7]. An interesting approach is based on doping the host resin used in the 2PP with compounds of interest (organic dyes and bioactive agents). Such approach enables the fabrication of devices with specific characteristics for different areas of optics [8–10] and biology [11–16]. Furthermore, the manufacture of components using 2PP can be carried out in stages, allowing the fabrication of microdevices with multi-doping components [17]. The application of 2PP has recently been used for fabricating microenvironments and biological devices [18–21], allowing major advances in drug delivery systems and studies of the process of three-dimensional cell migration [15,22–25].

In this paper, we fabricated two types of microenvironments produced using 2PP. The first microenvironment consists of an array of cylinders doped in a specific site with ciprofloxacin, an antibiotic used in the treatment of bacterial infection. In this case, we have evaluated the inhibition of bacterial growth around the doped site of the microenvironment. Such experiment was performed using ATCC 25922 *Escherichia coli* (*E. coli*). The second type of developed microenvironment consists of micro-fences, which is used to trap bacteria. This demonstrates, as a proof of principle, that the microstructures are suitable for monitoring bacterial growth and motion, allowing the evaluation of *E. coli* migration inside the microenvironment.

2. Experimental

Microenvironments were produced *via* direct laser writing by two-photon polymerization (2PP). This is accomplished by using a Ti:sapphire laser oscillator operating at 780 nm with pulse duration of 100 fs. The laser was focused by a microscope objective (0.25-NA or 0.85-NA) in a volume of polymeric resin containing a photoinitiator, which is responsible to trigger the polymerization. The intensity of femtosecond pulses is high enough to induce nonlinear absorption (two-photon absorption) at the focal volume. The quadratic dependence on the intensity, exhibited by the two-photon absorption process, allows spatial confinement of the excitation to the focal volume and, consequently, of the polymerization. The structures were fabricated using laser pulse energy on the order of 0.6 nJ. A sample containing an unpolymerized resin was positioned in the axial z-direction using a motorized stage, and the laser beam was scanned in the x–y-directions with a set of galvanometric mirrors. The laser beam was expanded, after passing the galvanometric mirrors, to fulfill the microscope objective entrance. The experimental setup allows real time visualization of microfabrication using a red LED as an illumination source and a CCD camera for displaying and recording. Further details about the experimental system used for the 2PP microfabrication can be obtained elsewhere [26].

The host resin employed in this work contains two three-acrylate monomers; the first one, tris(2-hydroxyethyl)isocyanurate triacrylate

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(50 wt.%), provides hardness to the structure, while the second monomer, ethoxylated(6) trimethyl-lolpropane triacrylate (50 wt.%), reduces the shrinkage tensions upon polymerization [27]. Lucirin TPO-L [27] is utilized as a photoinitiator. The monomers, together with the photoinitiator, are mixed for 1 h to obtain a homogeneous solution. In order to add the ciprofloxacin into the host resin, initially it is dissolved in ethanol and then added to the solution containing the monomers/photoinitiator. When the antibiotic is added into the resin, the solution is stirred for 30 min. It is then left to rest for 24 h to allow the solvent to evaporate.

Aiming at estimating the ciprofloxacin concentration used in this work, the minimum inhibitory concentration (MIC) of the antibiotic capable of inhibiting bacterial growth was determined. Micro-dilution plates (96 wells) containing serial dilutions of ciprofloxacin (Bayer Pharmaceuticals Inc.) and control (no antibiotics) were prepared with Mueller-Hinton broth made from a powdered stock (Becton Dickinson, Sparks, MD) according to CLSI (formerly NCCLS — National Committee for Clinical Laboratory Standards) guidelines [28]. Briefly, using colonies from freshly streaked plates, an inoculum of 1×10^7 colony-forming units per milliliter (cfu/mL) in the final medium was prepared. Liquid cultures of *E. coli* incubated at 36 ± 1 °C were grown until the mid-exponential growth phase. Using the same culture medium, concentrations from 2.5×10^{-4} to 12.5×10^{-2} μg/mL of ciprofloxacin were prepared to measure *E. coli* inhibition. The growth of *E. coli* was monitored as optical density at 620 nm using a Bio-Rad I Mark Microplate Reader (Bio-Rad Laboratories, Philadelphia, PA). The plates optical densities were read immediately at 620 nm and then incubated at 36 ± 1 °C for 24 h. After incubation, the plates optical densities were read again. The measured absorbance was subtracted from the value obtained before incubation. All experiments were performed in triplicate.

The fabrication of the microenvironment containing the antibiotic is carried out in various stages. First, structures without the antibiotic were produced by placing a drop of the net resin in a glass slide. The resin remains allocated between two spacers and enclosed by a cover slip. Once the fabrication was completed, the sample was immersed into ethanol for approximately 15 min to remove the unpolymerized resin. Secondly, a drop of the resin containing the antibiotic (12.5×10^{-2} μg/mL of ciprofloxacin) was place on a glass slide that contains previously fabricated microstructures. After a careful alignment of the sample position, aiming at fabricating the doped part of the microstructure in a specific site, the second stage of fabrication starts. Again, upon finishing the microstructure fabrication, the sample was rinsed in ethanol to remove unpolymerized resin. This approach does not limit the shape or number of microstructures which may be produced [17].

In general, the dopant content (antibiotic in this case) retained into the bulk of two-photon polymerized microstructures is dependent on their dimensions. Up to the size limit of the microstructure fabricated here (nearly 30 μm), the antibiotic remained into it after sample development.

The fabricated microstructures were characterized by scanning electron and optical microscopies. Bacteria inoculated in microenvironments were monitored for 24 h using optical microscopy.

3. Results and discussions

The result of the MIC experiment is displayed in Fig. 1. Such result shows the absorbance of samples containing distinct ciprofloxacin dilutions at 620 nm as a function of its concentration. The red dot in Fig. 1 represents the absorbance obtained in the absence of the antibiotic. As it can be seen, the absorbance of the solution is near zero for concentrations higher than 7.0×10^{-3} μg/mL, indicating the inhibition of bacterial growth when ciprofloxacin concentration above this value is used.

Fig. 2 shows a scanning electron microscope (SEM) image (tilted view) of the fabricated microenvironment composed of solid cylinders

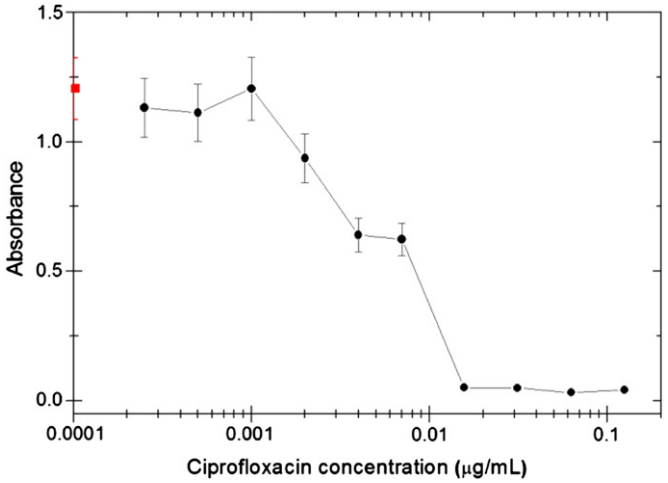

Fig. 1. Absorbance of the ciprofloxacin solutions after 24 h of incubation with *E coli*. The red square indicates the absorbance obtained without antibiotic.

(radius of 15 μm and height of 45 μm). The central cylinder in this microstructure (microenvironment) is doped with 12.5×10^{-2} μg/mL of ciprofloxacin. The SEM micrography reveals that the microenvironment exhibits good integrity and definition, even for the doped cylinder, suggesting that the presence of the antibiotic into the resin formulation does not affect the 2PP process. Furthermore, the microstructures maintain their structural features even after repeated washing procedures, used to leach out the unpolymerized resin before the bacteria were inoculated in the microenvironment. Although some cylinders of the microenvironment present slight differences in height (1 to 3 μm), they do not alter the properties of fabricated microstructures regarding its use as microenvironments.

Bacteria *E. coli* (Mueller-Hinton broth) inoculated in the microenvironment have all the necessary conditions for their development. The monitoring of the bacterial growth in the microenvironment was performed hourly by using an optical microscope. Fig. 3(a) shows an optical microscopy image (top view) of the microenvironment after 1 h of incubation. Clearly, there is a bacteria inhibition halo around the central

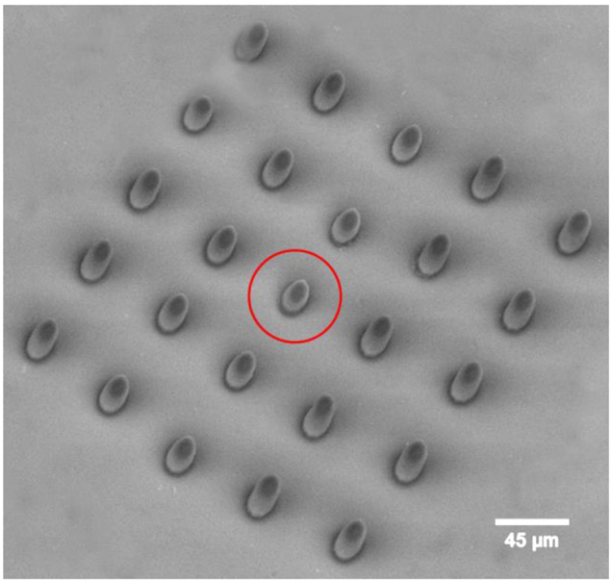

Fig. 2. SEM micrography (tilted view) of the microenvironment composed of solid cylinders fabricated by 2PP. In the center of the microenvironment, the cylinder is doped with ciprofloxacin.