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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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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 micro-environments, 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.

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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 with 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...
(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 according to CLSI (formerly NCCLS—National Committee for Clinical Laboratory Standards) guidelines [28]. Briefly, using colonies from freshly streaked plates, an inoculum of $1 \times 10^7$ colony-forming units per milliliter (cfu/mL) in the final medium was prepared. Liquid cultures of E. coli incubated at $36 \pm 1 ^\circ C$ were grown until the mid-exponential growth phase. Using the same culture medium, concentrations from $2.5 \times 10^{-4}$ to $12.5 \times 10^{-2} \mu g/mL$ 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 $36 \pm 1 ^\circ C$ 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 ($12.5 \times 10^{-2} \mu g/mL$ of ciprofloxacin) was placed 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 $7.0 \times 10^{-3} \mu g/mL$, 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 fabricated by 2PP. In the center of the microenvironment, the cylinder is doped with ciprofloxacin.
cylinder due to the presence of the antibiotic ciprofloxacin in this cylinder. The darker appearance of the antibiotic-doped cylinder compared to the non-doped ones is caused by the light absorption of ciprofloxacin, which presents a yellowish color in solution.

In Fig. 3(b) it is displayed a magnification of the region where inhibition of bacterial growth was observed. In order to evaluate the range of antibiotic action in the microenvironment, the image was analyzed using the scheme illustrated in Fig. 3(c): the inhibition zone was divided into concentric rings with an inner radius \( r_i \), starting from the edge of the doped microstructure (yellow in Fig. 3(c)), and thickness \( \Delta r_i \). Then, the number of bacteria in each ring was counted with the aid of an image software. Subsequently, the density of bacterial per unit of area was obtained. In this approach \( r_i \) varies from 0 to 25 \( \mu m \), and \( \Delta r_i \) was kept constant at 2.5 \( \mu m \). Fig. 4 shows the bacteria density as a function of \( r_i \).

As seen in Fig. 4, the density of bacteria grows monotonically with \( r_i \), saturating when \( r_i \) reaches approximately 12 \( \mu m \) in about 0.7 bacteria/\( \mu m^2 \), that corresponds to the bacteria density observed in the areas not affected by the antibiotics. Such value (0.7 bacteria/\( \mu m^2 \)) also corresponds to the bacteria density around the undoped cylinders (in average). The results presented in Fig. 4 show that the inhibition zone has a maximum range of approximately 10 \( \mu m \), being more effective as one gets closer to the microstructure impregnated with ciprofloxacin, as expected. Therefore, such kind of microenvironment could be used to carry out a quantitative analysis of antibiotics action in drug delivery systems. It is worth mentioning that previous studies have demonstrated that the resin and photoinitiator employed did not present toxicity to cells [22].

Microenvironments fabricated by 2PP can be a useful approach to study the dynamics of bacterial migration. We have fabricated microenvironments composed by a matrix of micro-fences, as the one displayed by the SEM micrography (tilted view) presented in Fig. 5. Such SEM image reveals that the microstructures exhibit good integrity and definition. These microenvironments are called as microtraps and were used to study the motion and entrapment of E. coli. Each of the microtraps showed in Fig. 5 is 35 \( \mu m \) long, presents an aperture of 7 \( \mu m \) and was designed without a ceiling to facilitate the real time visualization of the migration and trapping of bacteria.

As in the previous experiment, E. coli was incubated in the microenvironment, providing all the conditions necessary for their development. By optical microscopy we monitored bacteria in the microenvironment for 1 h, recording the results at time intervals of 5 min. Such images were analyzed and the number of bacteria in each microtrap was accounted as a function of time. Fig. 6(a) shows the average number of bacteria trapped per microtrap as function of time. Such results correspond to an evaluation of 5 microtraps.

Fig. 6(a) clearly shows that the number of bacteria trapped in the micro-fences increases as function of time. The error bars in this figure represent the standard deviation of the average number of bacteria trapped. The higher error bars observed for the data at 60 min are probably related to the fact that, for longer times, some of the entrapped
bacteria leave the microtrap through the top. Such fact causes a higher dispersion in the number of entrapped bacteria that is reflected in the standard deviation. The inset in Fig. 6(a) displays images of bacteria trapping for 5 and 60 min. In Fig. 6(b), as an illustration, it is shown images of bacteria in regions outside the microtraps, for incubation times of 5 (top image) and 60 min (bottom image). The average number of bacteria determined, in this case, is about 35. Such value remains approximately constant during the time of the experiment (60 min).

It is interesting to note that the number of bacteria outside the microtraps is higher than inside it for times smaller than 40 min. For longer times, the average number of entrapped bacteria approaches the one found in regions outside the microtraps. This result reveals, as a proof of principle, that specially designed microenvironments can be used to study bacterial migration and increase its density in a determined area. Regarding the fact that some bacteria left the designed microtraps along the time through the uncovered top, it could have been easily avoided by designing covered environments.

4. Conclusion

Microenvironments were fabricated using 2PP to evaluate the development of E. coli. Initially the inhibition of bacterial growth was observed around the site of the environment doped with the antibiotic ciprofloxacin. By analyzing the images obtained from this microenvironment it was possible not only to determine the maximum range of inhibition (12 μm), but also to perform a quantitative study on the bacterial density in the inhibition halo. The microtraps were also employed to study bacteria (E. coli) trapping and migration. The presented approach is a promising technique to study bacterial growth and migration, as well as to fabricate scaffolds using biocompatible polymers, opening a new possibility to study advanced drug delivery systems.

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