Laser Interferometry Analysis of Ciprofloxacin Diffusion through *Pseudomonas aeruginosa* Biofilm

**Arabski M**, Wąsik S and Drulis-Kawa Z

1Department of Microbiology, Institute of Biology, Jan Kochanowski University in Kielce, Świętokrzyska 15, 25-406, Kielce, Poland
2Department of Molecular Physics, Institute of Physics, Jan Kochanowski University, Świętokrzyska 15, 25-406, Kielce, Poland
3Department of Pathogen Biology and Immunology, University of Wrocław, Przybylszewskego 63/77, 51-148 Wrocław, Poland

**Abstract**

In this study the novel application of laser interferometry method in quantitative analysis of drugs distributions in bacterial biofilms is presented. Ciprofloxacin diffusion through *P. aeruginosa* PAO1 biofilm was used as a model system. It was determined that 0.759 µmol (25.32%) of ciprofloxacin was transported through biofilm after 2400 s from initial amount of 3 µmol. Additionally, laser interferometry method was used to calculate the amount (mol) of drug accumulated in the biofilm formed on nucleopore membrane. We observed that the amount of ciprofloxacin into biofilm is 0.366 µmol (12.2%) after 2400 s. These results were in accordance with measurements obtained by standard cultivation methods.

In conclusion, laser interferometry technique might be useful tool in real time calculation of drug concentration in bacterial biofilm as well as its transport through that structure. From clinical point of view, this important information might be used in modeling of antibiotics distribution in correlation with their biological effects on bacterial biofilms.

**Keywords:** Biofilm; *Pseudomonas*; Diffusion; Laser interferometry

**Introduction**

Bacterial biofilm is a surface attached form of unicellular microorganisms that come together to form a community and encased in an exopolysaccharide (EPS) [1]. Biofilm formation is a one of the major causes of failure in antimicrobial therapies, like antibiotic treatment in persistent bacterial infections. The EPS shield bacteria from opsonization and phagocytosis [2,3]. Moreover, bacteria in biofilms are less susceptible to antibiotics than their planktonic counterparts [2,4]. This effect might be explained by limitation of antibiotic penetration into biofilm [2]. The surface layers of biofilm are exposed to a lethal dose of antibiotic and these diffusion barriers protect against transport of the antibiotic dipper into the structure [2]. The resistance of biofilm forming bacterial cells against antibiotics might be conditioned by physiological limitations of microorganisms that exist in a recalcitrant phenotypic state in an altered chemical microenvironment within the biofilm [2].

Nowadays, the research methodology of antibiotic penetration analysis into bacterial biofilms is based on bioassay analysis [2], analysis of antibiotic penetration rates through the biofilm formed on cell culture inserts [5] or analysis of IR bands of the biofilm in regions of spectrum associated with nucleic acids in the presence of antibiotics (ATR/FT-IR) [6].

In the present work, we propose a new method in quantitative analysis of antibiotic diffusion through bacterial biofilm; laser interferometry. In our previous studies, this technique was successfully used in the measurements of *Proteus* lipopolisaccharides (LPS) interactions with colistin [7], chitosan [8], saponin [9], antibiotics and liposomes diffusion [10,11]. In this study, the application of laser interferometry technique is presented in quantitative analysis of ciprofloxacin distributions in *Pseudomonas aeruginosa* biofilm. The aminoglycosides (tobramycin and gentamycin) as well as fluoroquinolones like ciprofloxacin are the most commonly used antibiotics in the treatment of *P. aeruginosa* infections. Our previous studies indicated that ciprofloxacin was more efficient in inhibition/eradication of biofilm structure in comparison to gentamycin [12]. Therefore, laser interferometry method was utilized in presented paper to evaluate the role of ciprofloxacin distribution in biofilm structure in bacteria eradication process.

**Material and Methods**

**Culture conditions, membrane and chemicals**

*P. aeruginosa* PAO1 was cultivated under aerobic conditions in Luria-Bertani medium at 37°C. Polymeric nuclear track membrane (nucleopore) with pores diameter 0.9 µm was purchased from Joint Institute for Nuclear Research in Dubna, Russia. Ciprofloxacin was purchased from Krka (Novo Mesto, Slovenia).

**CV staining of *P. aeruginosa* biofilm**

Biofilm of *P. aeruginosa* PAO1 was formed at 37°C in LB (Luria-Bertani) medium for 96 h in stationary conditions in (i) NUNC MaxiSorp microtitre plates for analysis of antibacterial effect of ciprofloxacin on *P. aeruginosa* biofilm or (ii) on sterile nucleopore membrane with pores diameter of 0.9 µm, as element of membrane system from laser interferometry equipment. The microtitre plates or nucleopore membranes with formed mature biofilm were washed 3 times by 300 µl or 2 ml of 0.9% NaCl solution, respectively and were stained by crystal violet (0.4%) for 15 min. After staining the probes were washed in the same conditions as above and absorbance was measured with Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland) at 531 nm (microtitre plates). The %
Antibacterial effect of ciprofloxacin on P. aeruginosa biofilm

The P. aeruginosa PAO1 biofilm was formed in NUNC MaxiSorp microtitre U-bottom plates for 72 h at 37°C in LB medium. Ciprofloxacin was derived from stock solution (10 mg/ml) and added to the P. aeruginosa PAO1 biofilm (after 72 h incubation at 37°C in LB medium and (washed 3 times by sterile medium) to give a final concentration in the range of 0.75-12 µmol/ml in the wells. The plates were incubated at 37°C for 2400 s (time of laser interferometry analysis). After incubation the wells were washed 3 times by 300 µl or 2 ml 0.9% NaCl and stained by crystal violet (0.4%) for 15 min. After the staining the probes were washed in the same conditions as above and absorbance was measured with Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland) at 531 nm.

Data analysis

The data were analysed using the Statistica (StatSoft, Tulsa, OK, USA) software package. All the values in this study are expressed as the mean ± SD from three independent experiments. The differences were compared by the ANOVA test.

Results

Analysis of PAO1 biofilm formation by CV staining

Figure 1 shows the results of P. aeruginosa PAO1 biofilm formation for 24-96 h at 37°C in LB medium under stationary conditions in microtitre plates. The spectrophotometric analysis (CV staining) as well as imageJ software analysis (Figure 2) of plates and nucleopore membranes, respectively, shown that the biofilm is most efficiently formed after 72 h. Moreover, imageJ analysis was used for determination of % of membrane covering by biofilm (Figure 3). We concluded that the 126 value of grey level is a cut-off point below which 32291 pixels from total analyzed 36180 (membrane with biofilm after 72 h image)
Laser interferometry analysis of ciprofloxacin distribution in PAO1 biofilm

Figure 4 shows experimental set-up of laser interferometry equipment. It consists of a Mach-Zehnder interferometer with a He-Ne laser, a measuring system with membrane (place of biofilm formation), a TV-CCD camera and a computer with a system for the acquisition and processing of interference images. The laser light is spatially filtered and is transformed by the beam expander into a parallel beam ca. 80 mm wide and then split into two beams. The first beam goes through the investigated membrane system with formed bacterial biofilm parallel to the biofilm surface, while the second goes directly through the compensation plate to the light detection system. As a consequence of the superimposition of these beams, respective interference images are generated. The images (interferograms) depend on the refraction coefficient of the solute, which in turn depends on the substance concentration. The concentration of antibiotic transported through biofilm formed on membrane or into biofilm is calculated on the basis of changes of refraction coefficient in function of time.

Figure 5 shows the amount of ciprofloxacin (at the initial concentration of 3µmol/ml) transported through biofilm P. aeruginosa PAO1 formed on nucleopore membrane for 72 h. In the control experiments the amount of drug transported through bare membrane increased rapidly for initial times (till 1440 s) and then the “plateau effect” was observed when the system reached the steady state. The kinetics of antibiotic diffusion through membrane covered by biofilm had a steady character for 2400 s. At the end, the amount of transported ciprofloxacin was at the level of 25.32 % (0.0723 µmol) of total amount of drug diffusing across bare membrane (0.285µµmol). It indicated that 0.759 µmol of ciprofloxacin was transported through biofilm after 2400 s.

Additionally, laser interferometry method was used to calculate the

![Diagram](image_url)
amount (mol) of ciprofloxacin accumulated in the *P. aeruginosa* biofilm structure during the diffusion process. Figure 6 shows that the amount of antibiotic decreased from $6.00 \times 10^{-8}$ to $3.66 \times 10^{-8}$ mol after 2400 s. It indicates that the amount of ciprofloxacin into biofilm is 0.366 µmol after 2400 s (12.2 % of initial amount of antibiotic; 3 µmol).

**Effect of ciprofloxacin on PAO1 biofilm**

Figure 7 shows the effects of ciprofloxacin treatment at different concentrations (0.75-12 µmol/ml) for 2400 s at 37°C on *P. aeruginosa* PAO1 biofilm. The concentrations higher than 0.75 µmol/ml reduced the mass of biofilm statistically significant (p<0.001).

**Discussion**

*P. aeruginosa* causes chronic lung infection. Persistent microbial infection by *P. aeruginosa* is the major cause of morbidity and mortality in patients with cystic fibrosis (CF). *P. aeruginosa* strains isolated from CF patients with advanced stages of disease are distinctive because about 85% have mucoid colony morphology (overproduction of the EPS alginate and O-acetylated linear polymer of D-mannurionate and L-gulurionate). It suggests that mucoid *P. aeruginosa* cells have a distinct survival advantage in the CF lung environment [15]. So, analysis of drug distributions in mucoid structure formed by *P. aeruginosa* strains is crucial for effective clinical practice directed to bacteria eradication. In this study the laser interferometry method was applied to determine the antibiotic (ciprofloxacin) diffusion efficacy through *P. aeruginosa* biofilm structure.

Ciprofloxacin is an antibacterial agent of the 4-quinolone group derived by systematic modification of the nalidixic acid. The antibacterial properties of ciprofloxacin are associated with (i) inhibition of the intracellular enzymes (DNA-gyrase, topoisomerase IV) and (ii) the efficient transport of antibiotic molecules through bacterial envelopes and cytoplasmic membranes [16,17]. The effective transport of ciprofloxacin through biofilm is strongly associated with its biophysical properties. This drug formed four different microspecies molecules (neutral, zwitterion, positively and negatively charged) depending on the pH of the solution and this diversity promotes efficient diffusion through bacterial biofilm [17].

Using ciprofloxacin as model antibiotic, we showed that 25.32 % of 3 µmol antibiotic solution was transported through PAO1 biofilm (Figure 5) and the amount of ciprofloxacin accumulated in the biofilm was 12.2% of initial drug amount after 2400 s of treatment (Figure 6). Ciprofloxacin in this initial amount (3 µmol) was sufficient to PAO1 biofilm eradication (Figure 7).

In conclusion, laser interferometry analysis of drug distribution though bacterial biofilm might be useful in modeling of antibiotics diffusion in correlation with their biological effects. The practical application of laser interferometry might be associated with antibacterial efficacy tests especially used for biofilm eradication. Two elements can be determined using interferometry: (i) quantitative analysis of drug diffusion through biofilm, and (ii) drugs distribution in biofilm structure.

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References

1. Mah TF, O’Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9: 34-39.

2. Anderl JN, Franklin MJ, Stewart PS (2000) Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother 44: 1818-1824.

3. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318-1322.

4. Evans DJ, Allison DG, Brown MR, Gilbert P (1991) Susceptibility of Pseudomonas aeruginosa and Escherichia coli biofilms towards ciprofloxacin: effect of specific growth rate. J Antimicrob Chemoth 27: 177-184.

5. Shigeta M, Tanaka G, Komatsuawara H, Sugai M, Suginaka H, et al. (1997) Permeation of antimicrobial agents through Pseudomonas aeruginosa biofilms: a simple method. Chemotherapy 43: 340-345.

6. Suci PA, Mittelman MW, Yu FP, Geesey GG (1994) Investigation of ciprofloxacin penetration into Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemoth 38: 2125-2133.

7. Arabski M, Wąsik S, Dworecki K, Kaca W (2007) Laser interferometric determination of ampicillin and colistin transfer through cellulose biomembrane in the presence of Proteus vulgaris O25 lipopolysaccharide. J Memb Sci 299: 269-275.

8. Arabski M, Davydova VN, Wąsik S, Reunov AV, Lapshina LA, et al. (2009) Binding and biological properties of lipopolysaccharide from Proteus vulgaris O25 (48/57) with chitosan. Carbohydrate Polymers 76: 481-487.

9. Arabski M, Wąsik S, Dworecki K, Kaca W (2009) Laser interferometric and cultivation methods for measurement of colistin/ampicillin and saponin interactions with smooth and rough of Proteus mirabilis lipopolysaccharides and cells. J Microbiol Methods 77: 179-183.

10. Arabski M, Wąsik S, Piskulak P, Goźdz N, Slezak A, Kaca W, et al. (2011) Analysis of antibiotic diffusion from agarose gel by spectrophotometry and laser interferometry methods. Polim Med 41: 25-32.

11. Arabski M, Wąsik S, Grzeskiewicz H, Druś-Kawa Z, Gubernator J, et al. (2012) Laser interferometric determination of liposome diffusion through artificial membranes: Interferometry-Research and Applications in Science and Technology. INTECH.

12. Gula G, Waszczuk K, Olszak T, Majewska J, Gotszalk T, et al. (2012) Piezoelectric tuning fork based mass measurement method as a novel tool for determination of antibiotic activity on bacterial biofilm. Sensors & Actuators: B. Chemical 175: 34-39.

13. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671-675.

14. Arabski M, Konieczna I, Sołowiej D, Rogoń A, Kolesińska B, et al. (2010) Are anti-Helicobacter pylori urease antibodies involved in atherosclerotic diseases? Clin Biochem 43: 115-123.

15. Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, et al. (2004) Alginate production affects Pseudomonas aeruginosa biofilm development and architecture, but is not essential for biofilm formation. J Med Microbiol 53: 679-690.

16. Weigel LM, Steward CD, Tenover FC (1998) gyrA mutations associated with fluoroquinolone resistance in eight species of Enterobacteriaceae. Antimicrob Agents Chemother 42: 2661-2667.

17. Hernández-Borrel J, Montero MT (2003) Does ciprofloxacin interact with neutral bilayers? An aspect related to its antimicrobial activity. Int J Pharm 252: 149-157.