Effects of antibiotic treatment and phagocyte infiltration on development of *Pseudomonas aeruginosa* biofilm—Insights from the application of a novel PF hydrogel model *in vitro* and *in vivo*

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**Background and purpose:** Bacterial biofilm infections are major health issues as the infections are highly tolerant to antibiotics and host immune defenses. Appropriate biofilm models are important to develop and improve to make progress in future biofilm research. Here, we investigated the ability of PF hydrogel material to facilitate the development and study of *Pseudomonas aeruginosa* biofilms *in vitro* and *in vivo*.

**Methods:** Wild-type *P. aeruginosa* PAO1 bacteria were embedded in PF hydrogel situated *in vitro* or *in vivo*, and the following aspects were investigated: 1) biofilm development; 2) host immune response and its effect on the bacteria; and 3) efficacy of antibiotic treatment.

**Results:** Microscopy demonstrated that *P. aeruginosa* developed typical biofilms inside the PF hydrogels *in vitro* and in mouse peritoneal cavities where the PF hydrogels were infiltrated excessively by polymorphonuclear leukocytes (PMNs). The bacteria remained at a level of ~10^6 colony-forming unit (CFU)/hydrogel for 7 days, indicating that the PMNs could not eradicate the biofilm bacteria. β-Lactam or aminoglycoside mono treatment at 64× minimal
inhibitory concentration (MIC) killed all bacteria in day 0 in vitro biofilms, but not in day 1 and older biofilms, even at a concentration of 256× MIC. Combination treatment with the antibiotics at 256× MIC completely killed the bacteria in day 1 in vitro biofilms, and combination treatment in most of the cases showed significantly better bactericidal effects than monotherapies. However, in the case of the established in vivo biofilms, the mono and combination antibiotic treatments did not efficiently kill the bacteria.

**Conclusion:** Our results indicate that the bacteria formed typical biofilms in PF hydrogel in vitro and in vivo and that the biofilm bacteria were tolerant against antibiotics and host immunity. The PF hydrogel biofilm model is simple and easy to fabricate and highly reproducible with various application possibilities. We conclude that the PF hydrogel biofilm model is a new platform that will facilitate progress in future biofilm investigations, as well as studies of the efficacy of new potential medicine against biofilm infections.

**KEYWORDS**

biofilm infection, antibiotic resistance, PF hydrogel, *Pseudomonas aeruginosa*, *in vivo* model

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**Introduction**

*Pseudomonas aeruginosa* is an important opportunistic pathogen responsible for a substantial part of the refractory nosocomial infections (Høiby et al., 2001). In the majority of the problematic *P. aeruginosa* infections, the bacteria are present in the form of biofilms (Tolker-Nielsen, 2014; Hentzer et al., 2003). Bacterial biofilms can be described as aggregated cells embedded in self-produced extracellular polymeric substances. Biofilms are causing persistent infections, e.g., when they are present on implanted material or are present as bacterial aggregates embedded in lung mucus or wound material (Rybtke et al., 2015).

Common clinical biofilm infections include implant or prosthesis-related infections, lung infections in cystic fibrosis patients, and infections in burn patients (Lynch and Robertson, 2008; Høiby et al., 2010a; Song et al., 2013; Wu et al., 2015). The characteristic feature of biofilm infections is an enhanced tolerance to antimicrobials, which makes eradication of the infections almost impossible by conventional antibiotic therapies and host immune defences (Høiby et al., 2010; Wu et al., 2015; Ciofu and Tolker-Nielsen, 2019).

Various biofilm models have been developed and used in biofilm research for at least three decades, including *in vitro* models (static and dynamic systems), *ex vivo* models (tissue and cell cultures), and *in vivo* models (invertebrate or vertebrate animal models) (Lebeaux et al., 2013; Rybtke et al., 2015). However, the lack of chronic features in these biofilm models has long hindered progress in biofilm research. It is essential that the study model exhibits the typical features and symptoms of real chronic infections (Costerton et al., 2003). Clinical outcomes of routine antibiotic susceptibility tests currently correlate poorly with antibiotic treatment outcomes, which may indicate a need for improved assessment platforms. After investigating different materials and substances for their ability to support bacterial biofilm formation, PF hydrogel was selected as an outstanding candidate and used in our biofilm studies. PF hydrogel with cell-compatible features has become a popular synthetic biomaterial used as temporary tissue scaffold and drug delivery vehicle in biomedical sciences (Seliktar, 2012). PF hydrogel is a polymeric material containing plenty of water and can be polymerized together with live cells and/or therapeutic compounds. Importantly, PF hydrogels have been shown to provide cells with a three-dimensional scaffold, thereby enhancing cell survival and cell proliferation (Tibbitt and Anseth, 2009).

Because of the potential advantages of biofilm models built with hydrogel, we designed the present study to investigate systemically the development of bacterial biofilms in an optimized model and to investigate the effects of phagocytes and antibiotics in mono or combination therapy on *P. aeruginosa* biofilm infections.
Materials and methods

Bacterial strains, antibiotics, media, and culture conditions

The bacterial strain used in the present study was *P. aeruginosa* PAO1 (kindly provided by Professor Iglewski, University of Rochester). The antibiotics used in the study were imipenem (β-lactam) (Tienam® imipenem/cilastatin, MSD) and/or gentamicin (aminoglycoside) (Hexamycin®, Sandoz A/S, Denmark). Luria–Bertani (LB) and a growth minimal medium called ABTG (Clark and Maaløe, 1967) media were used for culture of *P. aeruginosa* at 37°C.

Preparation of PF hydrogel biofilm implants

For details on the synthesis of PF hydrogels, please refer to previous publications (Dikovsky et al., 2006; Dikovsky et al., 2008 and Elbert and Hubbell, 2001). The final PF hydrogel product was PEG-DA moieties attached to a cysteine residue on the Eibrinogen backbone, hereafter named as PF hydrogel. The PF hydrogel mixture was made by 0.1% photoinitiator, 2% PEG-DA (w/v) plus PF hydrogel (8 mg/ml), and LB with/without *P. aeruginosa* cells. The mixture of 100 μl was pipetted into the wells of microplate and polymerized under UV light at 365 nm and 5 mW/cm² (VL-6.L 365 nm 1 × 6 W Lamp), and it was demonstrated that UV energy of 5 mW/cm² did not reduce bacterial number or interfere with bacterial viability.

Establishment of biofilm in vitro and in vivo

Twenty-five microliter of ABTG was added to each well containing PF hydrogel (0.1 ml) and cultured at 37°C for 0, 1, 3, and 7 days to enable biofilm formation in vitro. All in vitro samples for bacteriological studies were in triplicates. For the in vivo biofilms, 8- to 9-week-old BALB/cJ mice (Taconic M&B A/S, Denmark or InVivos Pte Ltd., Singapore) were used, and each individual group contained 10 mice. All animal experiments were authorized by the National Animal Ethics Committee (2012-15-2934-0677), Denmark, or ARF SBS/NIE NTU Institutional Animal Care and Use Committee (A-0191 AZ), Singapore. An incision in the left and/or right groin of the mice was made after anaesthesia to expose the peritoneal cavity. The PF hydrogel implants with or without *P. aeruginosa* PAO1 were placed into the right and/or left peritoneal cavity of each mouse. The incisions were closed with a suture and healed without complications.

Evaluation of bacteriology in *P. aeruginosa* biofilms in vitro and in vivo

A moisture swab was taken from the peritoneal cavity before acquiring the implant and spleen from the mouse, and the swab was put into a tube containing 1 ml of sterile saline for quantification of the bacteria in the peritoneal cavity. PF hydrogel implants and spleens were collected from wells (in vitro) or from peritoneal cavities (in vivo) into sterile tubes with 3-ml cold sterile saline. The PF hydrogel implants and spleens were homogenized separately, and all samples were serially diluted and plated on agar plates for determination of bacterial CFU after 24–48 h incubation at 37°C.

Histological evaluation of biofilms in PF hydrogels

The PF hydrogel implants with or without *P. aeruginosa* were embedded in Tissue-Tek and frozen at −20°C to −40°C. Frozen sections (minimum of 50 μm) of the PF hydrogels were made by using a freeze microtome with Cryostat (Leica CM3050S) and were evaluated directly or with live/dead staining (LIVE/DEAD® BacLight™) using a confocal laser scanning microscope (CLSM) (LSM 780, Carl Zeiss) equipped with a laser detector and filter set for monitoring green fluorescence (excitation, 488 nm; emission, 517 nm). Simulated epifluorescence images were generated with an IMARIS software package (Bitplane AG). Additional frozen sections (ca. 8–10 μm) were stained with Methylene Blue and evaluated by light microscopy using an Olympus BX40-DP71 microscope.

Efficacy of antibiotics on *P. aeruginosa* biofilms in vitro and in vivo

The minimal inhibitory concentration (MIC) was 1.25 μg/ml for imipenem/cilastatin (IMP) and 0.5 μg/ml for gentamicin (GM) against planktonic *P. aeruginosa* PAO1. IMP and GM at final concentrations of 64×, 128×, and 256× MIC in ABTG were used in the in vitro study for single or combination treatments. The concentration of IMP and GM used in the in vivo study was 64× MIC for day 0 infections and 128× MIC for longer infections. The treatment was given by intraperitoneal injection, one dose per day for 2 days, and the time points of assessment started at 4 h after the second treatment.

Statistical analyses

The significance of the differences of bacterial CFUs and bactericidal percentages between the groups were analyzed by
using ANOVA (analysis of variance) and Student’s t-test. \( P < 0.05 \) was considered statistically significant. The graphs were generated by using a Microsoft Office Excel program.

Results

Development of \textit{P. aeruginosa} Biofilms in PF hydrogel \textit{in vitro} and \textit{in vivo}

\textit{P. aeruginosa} PAO1 tagged with green fluorescent protein (GFP) was demonstrated to form typical biofilms in PF hydrogels \textit{in vitro} and \textit{in vivo} (Figure 1). For the \textit{in vitro} PF hydrogels, CLSM images visualized clearly that \textit{P. aeruginosa} developed biofilms progressively from single bacteria at day 0, further to microcolonies at day 1, that increased in size at days 3 and 7 (Figure 1A, left panel). The biofilm present at day 3 was histologically evaluated, and distinct biofilm structures and single bacterial cells inside the PF hydrogel were detected (Figure 1A, right panel). For the frozen sections of PF hydrogel implants harvested from mouse peritoneal cavities, light and CLSM microscopy showed single microcolonies at day 1, and biofilm in an extensive area inside the PF hydrogel at day 3, which indicated the PF hydrogel, could be first a shelter for bacteria from host immunity and then a good substance for bacteria to form biofilm in \textit{in vivo} (Figure 1B). It was expected to see extensive green fluorescence emitted from the biofilm bacteria \textit{in vivo} on day 7, consistent with the large number of bacteria detected in the PF hydrogel samples. However, this was not the case, only a few green signals were discovered (Figure 1B). Most bacteria did not emit green light, possibly due to consumption of molecular oxygen by the surrounding large numbers of phagocytes, as oxygen is required for the expression of the fluorophore in GFP (Heim et al., 1994).

Bacterial quantification in \textit{P. aeruginosa} peritoneal biofilm infections in mice

For the \textit{in vivo} PF hydrogels, the bacterial density increased from the inoculum with approximately \( 10^5 \) CFU per hydrogel to approximately \( 10^6 \) CFU per hydrogel within 12 h after inoculation and remained constant at least for 7 days. Moreover, \textit{P. aeruginosa} could be detected in the PF hydrogels on days 14 and 28 after inoculation (Figure 2A), indicating that the PF hydrogel facilitated a long-term bacterial biofilm infection in the mouse peritoneal cavities. The results were highly reproducible regardless the variation of the inoculum between \( 10^2 \) and \( 10^3 \) CFU per hydrogel. Bacteria could be detected in the peritoneal cavities for only 3 days, indicating a transient acute peritonitis. Bacteria were also detected in PF hydrogels that were fabricated without bacteria (sterile hydrogels) and placed in the abdomen together with the bacteria-containing hydrogels. This indicated that the PF hydrogels could be colonized by bacteria from the peritoneal cavity \textit{in vivo}. Bacteria were detected in the spleen for as long as the existence of the peritoneal biofilm infection, which reflects dissemination of \textit{P. aeruginosa} from the peritoneal biofilm into the blood stream (Figure 2A). Nevertheless, the bacteria in the spleens were relatively low in number, approximately \( 10^3 \) CFU per spleen, which might suggest that the animals suffered from only slight bacteraemia, but not sepsis, as the animals exhibited no symptoms and seemed healthy throughout the experiment.

Infiltration of phagocytes in \textit{P. aeruginosa} biofilms

Phagocyte infiltrations were observed in the peritoneal PF hydrogel implants with or without \textit{P. aeruginosa}. The infiltrations differed with respect to the type of phagocytes and the duration of infiltration (Figure 3). The sterile PF hydrogel implants were mainly infiltrated by a thin layer (40 μm) of peritoneal macrophages by day 1, which was significantly reduced by day 3 and completely gone by day 7 (Figure 3A). In contrast, a thick layer (250 μm) of polymorphonuclear leukocytes (PMNs) dominated the infiltration in the PF hydrogels with \textit{P. aeruginosa} in the entire study period (Figure 3B). The PMNs migrated close to the biofilms but could not clear them efficiently as the bacterial CFU continuously was at a level of \( 10^6 \) CFU per PF hydrogel (Figure 2A). On day 14 after intraperitoneal challenge, the hydrogels inoculated with \textit{P. aeruginosa} were found tightly wrapped by the greater omentum in the mouse abdominal cavity, and a large abscess formed (Figure 2B). Strong and durable host immune responses were observed only in the PF hydrogels inoculated with \textit{P. aeruginosa}, in contrast to the sterile PF hydrogels that induced only mild transient inflammatory responses.

Evaluation of antibiotic treatments of biofilms \textit{in vitro} and \textit{in vivo}

Antibiotic treatment at different ages of \textit{P. aeruginosa} biofilms showed that imipenem (IMP) or gentamicin (GM) monotherapy at 64× MIC killed 99.9% of the bacteria merely in day 0 biofilms and eliminated less than 90% of the bacteria in day 1 and older biofilms (Figure 4A), which suggests the importance of early antibiotic treatment. Combination therapy with IMP and GM at 64× MICs improved significantly the bactericidal effect in day 1 and 3 biofilms, indicating that combination therapy worked more efficient than monotherapy. However, it was not possible to eliminate more than 95% of the bacteria in day 1 or older biofilms (Table 1). The killing of the biofilm bacteria was concentration-dependent, i.e., higher doses had better
bactericidal effects in both mono and combination therapies. Nevertheless, the bactericidal differences between higher and lower doses were only significant in the combination treatments, in which the IMP-GM combination at 256× MIC eliminated more than 99.9% of the bacteria in day 1 biofilms (Table 2 and Figure 4A), implicating that both combination therapy and high dosage are key points for elimination of biofilm infections. Extension of antibiotic treatments from 4 to 8 h increased the bactericidal effects of the antibiotics in relatively mature biofilms (Table 3). Hence, in addition to concentration-dependent killing, time-dependent killing was also observed in the biofilm treatments. For the in vivo biofilm infections of P. aeruginosa established in mouse peritoneal cavities, IMP or GM monotherapy killed completely day 0 biofilm bacteria in accordance with the in vitro study. However, mono and combination therapies at 128× MIC (IMP, 160 mg/kg; GM, 64mg/kg) killed less bacteria in day 1 and older biofilms (Table 4 and Figure 4B) compared with the in vitro study (Table 2). The combination therapies killed less than 50% of the bacteria in day 3 and 7 biofilms, exhibiting poor outcomes in vivo; however, the poor killing rates were still significantly better than the killing rates observed for monotherapies (Figure 4B).
Biofilm studies have been established by means of bacteria embedded in agar or seaweed alginate or pre-attached to silicone implants, and these manipulations were shown to protect the bacteria from host immune defences and antibiotics (Christensen et al., 2007; Hengzhuang et al., 2012). However, these models possess several limitations, and the most critical drawback is the high inoculum of $10^7$–$10^8$ CFU required to initiate an in vivo infection, because animals with normal immunity are not susceptible to planktonic *P. aeruginosa* (Moser et al., 1997; Christensen et al., 2007). The accumulation of lipopolysaccharide endotoxin released from the high inoculum of bacteria unavoidably induced severe acute symptoms, such as weight loss, bleeding, dehydration, fever or hypothermia, and even mortality (Rietschel and Brade, 1992; Moser et al., 1997).

Therefore, we appreciate the present biofilm model facilitated by PF hydrogel, which provides a real developing biofilm infection in vivo rather than the prebuilt biofilms transferred directly into animals. This biofilm model also has the advantage of the typical chronic features, such as a persistent large quantity of bacteria in the infected foci, continuous mild bacteraemia for more than 2 weeks, and the hosts in general with a “healthy” appearance including stable body weight with normal body temperature and normal appetite and activity.
Moreover, an important feature of the PF hydrogel is biocompatibility with bacteria (Figure 1) and host phagocytes (Figure 3), which is demonstrated by its use in human medicine for years (Slaughter et al., 2009). Therefore, we pointed out that our PF hydrogel biofilm model is a platform that will facilitate the progress in microbial biofilm studies, especially in vivo.

Biofilm infections are known to be tolerant to immune defenses. Our results confirmed that PMN phagocytes could migrate and distribute all over the bacterial biofilms (Figure 3, right), but it appeared that the PMNs were not able to kill the biofilm bacteria. The reason might be that the aggregated P. aeruginosa in the biofilm inhibited the phagocytosis of PMNs. The biofilm matrix and large size of the aggregates may provide protection against phagocytosis (Moser et al., 2021). Furthermore, Jensen et al. reported that the oxidative burst response induced by biofilm bacteria in PMNs was significantly slower and weaker than that of planktonic bacteria (Jensen et al., 1990). It was demonstrated that rhamnolipids, products of the rhlA gene in P. aeruginosa, efficiently induce PMN lysis (Jensen et al., 2007), and inactivation of the rhlA gene disabled bacterial protection against PMNs (Van Gennip et al., 2009).

Studies evaluating the efficiency of antibiotic mono or combination treatment against biofilm infections are important and valued by the medical community. However, only a few studies have performed such research in models mimicking real biofilm infections. Aminoglycoside in combination with β-lactam antibiotics is often used intravenously in hospitals as the major treatments against P. aeruginosa infection (Ramsey, 1996). Comparison of P. aeruginosa isolates growing as planktonic cells, adherent monolayer, and biofilms showed that the susceptibility to mono treatment with β-lactam, aminoglycoside, and fluoroquinolone was decreasing in the order planktonic cells, adherent monolayer, and biofilm (Aaron et al., 2002). Furthermore, biofilm bacteria, but not adherent bacteria, were significantly more resistant to two- or three-antibiotic combination treatments than the planktonic bacteria (Aaron et al., 2002). Our in vivo results are in accordance with these previous in vitro findings, because day 0 biofilms
similar to adherent cells, could be easily cleared by β-lactam or aminoglycoside mono treatment. Our study further demonstrated that combination therapy at ≥256× MICs was able to eliminate 99.9% of the bacteria in day 1 biofilms (young biofilms), but not in day 3 and day 7 biofilms (mature biofilms), emphasizing that early antibiotic treatment is essential to fully eradicate biofilm infections (Tables 1 and 4, Figure 4B). In general, concentration-dependent killing was demonstrated in our study (Table 2). It was impossible to remove more than 50% of the bacteria in mature in vivo biofilms with combination treatment employing β-lactam and aminoglycoside antibiotic at 128× MICs (Table 4, Figure 4B), which further demonstrated that once the biofilm infection is established, it is impossible to eradicate the infection by antibiotic treatment alone. Smith et al. reported that
combination treatment with β-lactam and aminoglycoside antibiotic produced a longer clinical remission without infection symptoms than β-lactam mono treatment on the management of a pulmonary exacerbation in cystic fibrosis patients (Smith et al., 1999). Herrmann et al. found that combination treatment of colistin and tobramycin showed significantly better bactericidal effects on in vitro P. aeruginosa biofilms compared with the monotherapies (Herrmann et al., 2010). On the other hand, combination antibiotic treatment helps to reduce the risk of antibiotic resistance according to the ESCMID (European Society of Clinical Microbiology and Infectious Diseases) guidelines for the diagnosis and treatment of biofilm infections (Høiby et al., 2015). Therefore, we conclude that combination therapy with high doses of antibiotics potentially can be used to control (but not eradicate) biofilm infections in the clinic.

### Conclusions

In conclusion, we have developed a PF hydrogel-based biofilm model that has many of the features of real biofilm infections, including progressive biofilm development, chronicity, immune response, and antibiotic tolerance. We pointed out that that this biofilm model will be useful for future biofilm research, as well as studies of the efficiency of new potential medicine against biofilm infections.
The study was supported by the Danish Strategic Research Council to MG (CAR) and the National Research Foundation - Technion - National University of Singapore (R-398-000-065-592) to the Regenerative Medicine Initiative in Cardiac Restoration Therapy. We greatly appreciate the financial support for our research from the two foundations. We sincerely thank Dr. Lim Lee Wei, Division of Molecular Genetics and Cell Biology, School of Biological Sciences, NTU, for his kind guidance in relation to our histological work.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by The National Animal Ethics Committee (2012-15-2934-0677), Denmark and ARF SBS/NIE NTU Institutional Animal Care and Use Committee (A-0191 AZ), Singapore.

Author contributions

Study design: HW, ZS, and MG. Major researchers: LS, HW, ZS, JH, HZW, and MP. Figures, tables, and statistical analyses: HW. Manuscript writing and revision: HW, ZS, MG, NH, and TT-N. Project guidance: MG, NH, and TT-N. Partly support of research materials: MR, DS, and TK. Financial support: MG, TK, and NH. All authors contributed to the article and approved the submitted version.

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