Antibiotic penetration and bacterial killing in a *Pseudomonas aeruginosa* biofilm model

Bao Cao1*, Lars Christophersen1, Kim Thomsen1, Majken Sønderholm2, Thomas Bjarnsholt1,2, Peter Østrup Jensen1, Niels Høiby1 and Claus Moser1

1Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark; 2Institute of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark

*Corresponding author. Tel: +45-30950585; E-mail: baocao81@gmail.com

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**Objectives:** Treating biofilm infections successfully is a challenge. We hypothesized that biofilms may be considered as independent compartments with particular pharmacokinetics. We therefore studied the pharmacokinetics and pharmacodynamics of tobramycin in a seaweed alginate-embedded biofilm model.

**Methods:** Seaweed alginate beads containing *Pseudomonas aeruginosa* were cultured in LB medium, sampled at day 1, 3, 5 or 7 and examined for the effect of treatment with tobramycin for 30 min. Treated beads were homogenized and the number of cfu was determined. The antibiotic concentration in the solution of homogenized beads was measured. Finally, beads were examined for live cells by Syto9 staining and for dead cells by propidium iodide staining using a confocal laser scanning microscope.

**Results:** The antibiotic level in each bead was relatively stable (range 30–42 mg/L; MIC = 1.5 mg/L). There were fewer cfu in the tobramycin-treated beads than the non-treated beads (*P* = 0.016) and bacterial killing was reduced as the culture period increased from 1 to 7 days. Throughout the study period, increasing size and more superficial positioning of the microcolonies within the beads were demonstrated by confocal laser scanning microscopy. More dead cells (measured by propidium iodide staining) were observed in the treated group of beads, which supports the results obtained by culture.

**Conclusions:** The present study, simulating the clinical pharmacokinetics of tobramycin, demonstrates fast absorption of tobramycin in an *in vitro* biofilm model. In addition, this model system enables parallel investigation of pharmacokinetics and pharmacodynamics, providing a model for testing new treatment strategies.

**Keywords:** pharmacokinetics/pharmacodynamics, PK/PD, *P. aeruginosa*

**Introduction**

Biofilms are communities of bacteria enclosed in an extracellular matrix consisting primarily of polysaccharides, proteins and extracellular DNA (eDNA).1 Biofilms may be formed on foreign bodies and indwelling devices such as catheters, heart valves and orthopaedic prostheses,2 but may also consist of aggregates of bacteria situated in mucus (e.g. cystic fibrosis mucus) or tissues (e.g. chronic wounds).3–5 Despite increased knowledge, awareness and research over recent decades, biofilm infections are still clinically difficult to manage, let alone treat.

Bacteria growing in biofilms differ significantly from planktonically growing bacteria of the same clone. This has been attributed to findings such as limited nutrition and oxygen, slow growth, increased mutation rates and quorum-sensing activity.6–8 Furthermore, they exhibit properties such as the occurrence of persister cells, matrix chelation and increased production of β-lactamases and liposaccharides, all of which makes them more tolerant to antibiotics and host immune defence mechanisms and more resistant to chemical disinfectants.2 However, unlike the pharmacokinetics (PK) and pharmacodynamics (PD) of free, planktonic microorganisms, there are still unanswered questions regarding these topics in biofilm.

Traditionally the first PK compartment is considered to be blood, from which antibiotics diffuse into the various tissues, which are thus considered the second compartment. When treating infections with planktonically growing microorganisms, the PK/PD parameters of a given antibiotic can be used to predict the optimal dosing for treatment, which is also true for acute infections.9,10 However, biofilms may be defined as ‘a structured consortium of microbial cells surrounded by a self-produced polymer matrix’11 and range in size *in vivo* from 4 to 200 μm in tissues and from 5
to 1200 μm on foreign bodies. Therefore, biofilms consist of material that differs from the surrounding tissue and, at least for the larger biofilms, may also represent a considerable challenge for the distribution of antibiotics. Based on these observations, we suggest considering biofilms as independent compartments with particular PK. Since the established in vitro biofilm models we are aware of do not allow the simultaneous measurement of antibiotics within the matrix and the parallel bacterial killing, we selected our seaweed alginate-embedded biofilm model containing Pseudomonas aeruginosa to test our hypothesis.

Materials and methods

Bacterial strain, medium and antibiotic

WT P. aeruginosa strain PA01 Iglewski was used in this study. Strains were grown in LB broth incubated overnight at 37 °C on a shaking table at 150 rpm. The antibiotic tobramycin sulphate (ApodanNordic, Copenhagen, Denmark) was used. The MIC of tobramycin for our strain was determined to be 1.5 mg/L by Etest (bioMérieux, Marcy-l’Étoile, France).

Preparing the beads

Seaweed alginate beads with and without P. aeruginosa were prepared by a modification of a previously described procedure. In this study we used an alginate concentration of 3% (Protanal LF 10/60, FMC BioPolymer, Drammen, Norway) to increase stabilization of the beads. The alginate was mixed with an overnight culture of P. aeruginosa and the mixture was transferred to a syringe and placed in a syringe pump (Graseby 3100; Ardu Medical Inc., Watford, UK) to increase stabilization of the beads. The alginate was driven through a plastic tube at a flow rate of 40 mL/h into a gelleting bath containing 0.1 M Tris–HCl and 12.5 mM CaCl2. The tip of the plastic tube was fixed exactly 8 cm above the surface of the gelling bath and the beaker containing Tris–HCl was placed on a magnetic stirrer to prevent the beads from clumping. The beads were kept in Tris–HCl for at least 1 h to stabilize. Finally, the beads were washed twice in 0.9% NaCl; 12.5 mM CaCl2 was used as a stabilizer.

The beads were kept in LB broth with CaCl2 at 37 °C on a shaking table at 205 rpm. The antibiotic tobramycin sulphate (ApodanNordic, Copenhagen, Denmark) was used. The MIC of tobramycin for our strain was determined to be 1.5 mg/L by Etest (bioMérieux, Marcy-l’Étoile, France).

Standard curve

To estimate the absorption of tobramycin in the biofilm matrix, non-bacterial beads were exposed to medically relevant peak concentrations of tobramycin (42.9 mg/L). The flask containing the beads was placed on a wobble board at 37 °C for 48 h. Beads were sampled at different timepoints and, after homogenization of the beads, the concentration of tobramycin was measured.

To compare the penetration profile of an aminoglycoside and a glycopeptide, non-bacterial beads were exposed to vancomycin (17.3 mg/L) in a parallel pilot study. The previously described procedure was repeated.

Antibiotic elimination

Elimination of antibiotic from the beads was determined by placing non-bacteria-containing beads in 0.9% NaCl with CaCl2 after treatment and then at specific timepoints measuring the concentration of tobramycin in the beads and surrounding fluid.

Tobramycin measurements

The alginate beads were dissolved using citrate buffer, pH 7, and homogenized mechanically. Subsequently the solution was diluted with 0.9% NaCl. Tobramycin concentrations were measured using an ARCHITECT c4000 analyser (Abbott, IL, USA) according to the manufacturer’s instructions.

Quantitative bacteriology

With the aim of relating the tobramycin concentrations in the beads (mimicking PK) to the effect on P. aeruginosa growing in biofilm (mimicking PD), beads containing P. aeruginosa were sampled at day 1, 3, 5 or 7. For each timepoint, the number of cfu was determined for beads that were treated with tobramycin at a clinical treatment concentration and beads that were not treated. To imitate a clinical treatment the antibiotic exposure time was set to 30 min. With this procedure we simulated the clinical treatment situation since the serum concentrations of tobramycin are known to decrease significantly after 30 min. The beads were subsequently homogenized, serial dilutions made and the suspensions plated onto modified Conradi–Drigalski medium (State Serum Institute, Copenhagen, Denmark), which is selective for Gram-negative bacilli. The number of cfu was determined after overnight incubation at 37 °C. All counts were performed in quadruplicate. Finally, the concentration of tobramycin in the solution of homogenized beads was measured.

Antibiotic effect after clearance

To examine the effect of antibiotic on biofilms after removal from tobramycin suspension, mimicking the antibiotic effect after clearance from serum, beads containing P. aeruginosa were treated with tobramycin, washed twice with 0.9% NaCl and subsequently placed in 0.9% NaCl with CaCl2 on a shaking table. Beads were sampled at 0.5, 1, 2 or 4 h and the previously described procedure was repeated with homogenization of beads, determination of cfu and measurement of tobramycin concentration in both the beads and the surrounding fluid. As a control, untreated beads containing P. aeruginosa were placed in 0.9% NaCl with CaCl2, beads were sampled at the same timepoints and homogenized, and the number of cfu was determined after overnight incubation. All concentration measurements were performed in triplicate and the mean value was used.

Microcolonies of P. aeruginosa in seaweed alginate beads

Treated and non-treated alginate beads sampled on days 1, 3 and 7 were studied using a confocal laser scanning microscope (Zeiss LSM 710, Oberkochen, Germany). Following treatment with tobramycin, the beads were sectioned and stained for 30 min. Viable cells were visualized with Syto9 (green fluorescence), dead cells were stained with propidium iodide (red and yellow fluorescence). We studied microcolonies of bacteria on the surface and the sectioned part of the beads, focusing on representative microcolonies in the central and peripheral parts of the bead. The pictures were subsequently edited using Imaris (Bitplane, Zurich, Switzerland).

A flow chart of the experiments is depicted in Figure S1 (available as Supplementary data at JAC Online).

Statistics

All analyses were performed in Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Student’s t-test for unpaired data was performed for all parametric values. P<0.05 was considered statistically significant.
Results

Antibiotic concentration in alginate

The mean diameter of the alginate beads was 4.5 mm (range 4 – 5 mm). The concentration of tobramycin within the non-bacterial beads reached a higher level than that in the surrounding fluid within 30 min. During the first 12 h the measured concentration increased exponentially and after 16 h the beads were saturated with tobramycin at concentrations ~6-fold higher than in the surrounding fluid (Figure 1). The penetration profile of tobramycin differed markedly from that of vancomycin. In the non-bacterial beads, a higher concentration of vancomycin than that in the surrounding fluid was reached after only 15 min; however, the concentration remained only marginally higher in the beads throughout the 48 h of measurement (data not shown).

In beads containing P. aeruginosa, the concentration of tobramycin was one-third of that in the surrounding fluid after 30 min of treatment (Figure 2b).

Figure 3 shows the concentration of tobramycin in the non-bacterial beads after removal from the antibiotic solution. The antibiotic concentration in the beads decreased as the concentration of tobramycin in the surrounding fluid increased. However, antibiotic washout was considerably slower than absorption, and the beads still contained measurable levels of tobramycin even after 48 h.

Quantitative bacteriology

The concentration of tobramycin in each bead was found to increase slightly throughout our experiment (range 30–42 mg/L). There were fewer cfu/bead in the tobramycin-treated beads compared with the non-treated beads (P< 0.016). More than 99% of the planktonic cells were killed by treatment, but once biofilm formation was established, bacterial killing was reduced to 25% (day 1). Furthermore, both absolute and relative bacterial killing were reduced as the biofilm became older (Figure 2a).

Post-antibiotic effect

After removal from tobramycin, the antibiotic concentration in the beads decreased slowly (Figure 4b). Reduced but continued bacterial killing to ~100 times less than in control beads was observed 4 h after removal of the beads from tobramycin (Figure 4a).

Microcolonies of P. aeruginosa in seaweed alginate beads

Generally, there was a higher density of microcolonies in the periphery of the beads than the central part. Furthermore, the peripheral microcolonies were larger and more vital, as evaluated by means of green fluorescence-tagged bacteria, than the central microcolonies. The size of the peripheral microcolonies increased significantly throughout the study period, from 50 – 60 μm in diameter on day 1, growing to 120 μm on day 3 and reaching 150 μm by day 7, whereas the central microcolonies remained at ~10 μm. We noticed increased spontaneous death in the non-treated beads as the biofilm matured. Although the differences between the treated and non-treated microcolonies were minimal, more red/yellow fluorescence was observed after treatment with tobramycin (Figure 5). A substantial amount of green fluorescence remained even after tobramycin treatment, indicating a substantial number of live cells.
dependent process. However, by means of live–dead staining it
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like aminoglycosides, which work better in aerobic environments,
the biofilm may attenuate the antibacterial activity of antibiotics
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nature of the biofilm.2,18,19 One of the antibiotic classes that has
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Discussion

Biofilms cause persistent infections and several reasons why bio-
films infections are difficult to treat have been reported.14,15 Often
the relevant antibiotic treatment is not sufficient to eradicate
infections completely. The aim of our study was therefore to
develop a model system in which the PK/PD parameters in bio-
films could be further investigated simultaneously.

Consistent with other studies,16,17 we found that biofilms are
significantly more recalcitrant to antibiotics than free, planktonic
cells. Antibiotic tolerance increases with maturation of a biofilm,
and thus bacterial killing was reduced >4-fold in old biofilms
day 7 compared with young biofilms (day 1). The mechanisms
behind this tolerance phenomenon are not thoroughly under-
stood. Although poor penetration of the drug through the layers
of the matrix has been suggested as a possible reason, studies
have shown that the diffusion of antibiotics like fluoroquinolones,
piperacillin, vancomycin and daptomycin is not limited by the
nature of the biofilm.20-22 One of the antibiotic classes that has
been reported to have poor penetration into biofilms is the amino-
glycosides,20,21 since tobramycin binds to the alginate in biofilms,
and this binding of the positively charged aminoglycosides to
negatively charged biofilm matrix polymers retards the penetra-
tion of these drugs.22,23 Accordingly, we found surprisingly good
penetration of tobramycin into the alginate. The drug even
appears to be accumulated in the biofilm matrix. Furthermore,
continued killing of bacteria was observed for at least 4 h
after removal of the beads from the tobramycin, indicating that
the tobramycin was evenly distributed throughout the
beads and there was enough free unbound tobramycin to kill
microorganisms.

Others report that the major mechanism of biofilm resistance
is not retarded antibiotic penetration and binding to alginate, but
rather a low uptake of antibiotics by oxygen-deprived organ-
isms.24 Since mature biofilm creates steep gradients of dissolved
oxygen concentrations that result in internal anoxic conditions
~50 μm from the biofilm surface,25,26 these anoxic zones within
the biofilm may attenuate the antibacterial activity of antibiotics
like aminoglycosides, which work better in aerobic environments,
as transport of the drug across the membrane is an oxygen-
dependent process. However, by means of live–dead staining it
was observed that tobramycin was still able to kill bacteria inside
the beads.

The role of eDNA has been intensively investigated throughout
the last decade. Research has shown that not only does eDNA
have a significant role in stabilizing the structure of biofilms, but
it also promotes tolerance to antimicrobial peptides and amino-
glycosides by chelating cations and thereby restricting diffusion
of cationic antimicrobials.27,28 However, a possible binding of
tobramycin to eDNA was not investigated in the present study.

The panel of pictures taken by confocal laser scanning micros-
copy shows the establishment and development of a biofilm and
its microcolonies over time. Like Coquet et al.,24 we found that the
distribution of microcolonies within the biofilm was highly hetero-
geneous, with a higher density of bacteria in the peripheral regions
and lower density in the central areas. In the pictures of the sec-
tions of beads, the difference between untreated and treated
beads seems slight, but closer observation of the microcolonies
revealed more red/yellow fluorescence in the treated group of
beads, indicating marginally increased killing in the tobramycin
group. However, results of live–dead staining should be inter-
preted with caution, because of inconsistencies associated with the staining method.19 Yet, as a visual supplement we found

Figure 3. Antibiotic washout. Beads were treated with tobramycin
(41.7 mg/L) for 30 min and subsequently placed in 0.9% NaCl with
CaCl₂. Beads were harvested and homogenized at different timepoints.
Antibiotic concentration was measured in beads (filled circles) and in
the surrounding fluid (open squares).

Figure 4. Post-antibiotic effect. (a) Beads containing P. aeruginosa were
treated with tobramycin for 30 min. At 0 h beads were placed in 0.9%
NaCl with CaCl₂. At different timepoints the number of cfu was
determined by culture for treated beads (open squares). As a control
group, untreated beads were placed in 0.9% NaCl with CaCl₂, harvested at
the same timepoints and the number of cfu determined (filled circles). (b)
Beads containing P. aeruginosa were treated with tobramycin and
subsequently at 0 h placed in 0.9% NaCl with CaCl₂. Tobramycin
concentrations in beads (filled circles) and surrounding fluid (open squares)
were determined at various timepoints.
Figure 5. Microcolonies in the alginate beads. Beads containing *P. aeruginosa* were harvested on days 1, 3 and 7. Beads treated with tobramycin for 30 min and untreated beads were studied using a confocal laser scanning microscope. Viable cells were stained green by Syto9 and dead cells were stained red by propidium iodide. Surface and section of beads, ×10. Microcolonies in beads, ×40.
that the results correlated well with the quantitative bacteriology determined by culturing.

With our biofilm model it is possible to investigate the killing of bacteria (PD) in relation to the level of antibiotics (PK). Simultaneous PK/PD investigation of biofilms has been done in only a few biofilm studies, e.g. Hengzhuang et al.\textsuperscript{17,30} The vast majority of PK/PD studies have been done on planktonic cells or flow models. Obviously, all biofilm models are only partial approximations, representing a fraction of the complexity of the in vivo mechanisms. However, some of the advantages of this model are the possibility of measuring and evaluating several parameters, such as simultaneous PK/PD and visualization. The model allows actual measurements of the antibiotic level within the biofilm; as far as we know, this has not been done previously in biofilm models. In other models biofilms are exposed to various concentrations of antibiotics, but the actual levels of antibiotics within the biofilms have not been determined. Furthermore, the treatment of the beads was planned to be as clinically relevant as possible, which is unlike most of the biofilm PK/PD models published. In addition, with this model it is possible to make mathematical calculations of the antibiotic diffusion rate into the biofilm matrix. Finally, the presented biofilm model can also be transferred to animal experiments to test the PD during in vivo PK. In this way, we will be able to explore the tissue as a second compartment, making our biofilm model the third compartment. This would further strengthen the physiology of the biofilm model, since it would not only be a ‘medical’ biofilm consisting of bacterial cells and material, but would also have elements of animal cells, tissue debris and proteins.

However, several modifications can be made to further optimize the model. We are aware that the size of our model is unphysiologically large, although we had deliberately chosen this size to test our hypothesis, but the size of the beads can be reduced to make the model resemble physiological biofilms. The antibiotic concentrations are measured chemically using a method validated for serum or plasma. Since we apply this method to homogenized alginate, the measurements might be biased, but the measured concentrations in the liquid were similar to the calculated concentrations, arguing against this speculation.

Conclusions

We have established a biofilm model that demonstrates fast absorption of tobramycin and a biofilm model in which the PK can be related to the PD simultaneously.

The present study demonstrated changed PK of tobramycin in the alginate biofilm model, indicating that biofilm can be regarded as an independent third compartment and that the behaviour of biofilms differs from that of tissue. Furthermore, the study stresses the fact that the recalcitrance of biofilms is not due to lack of penetration of antibiotics into the biofilm.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


