

In vitro evaluation of Pseudomonas aeruginosa chronic lung infection models: Are agar and calcium-alginate beads interchangeable?

Bruna Gaelzer Silva Torres, Rana Awad, Sandrine Marchand, William Couet,

Frédéric Tewes

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8	Torres, Bruna Gaelzer Silva ¹ ; Awad, Rana ¹ ; Marchand, Sandrine ^{1,2} ; Couet, William ^{1,2} ; Tewes,
9	Frederic ¹ .
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11	
12	¹ - Inserm U1070, Université de Poitiers, CHU de Poitiers, 1 rue Georges Bonnet, 86073 POITIERS
13	Cedex 9, France.
14 15	² - Laboratoire de Toxicologie-Pharmacocinétique, CHU of Poitiers, 2 rue de la Milétrie, 86000 Poitiers, France
16	
17 18	Corresponding authors at: INSERM, U1070, UFR de Médecine Pharmacie, Université de Poitiers, 1 rue Georges Bonnet, TSA 51106, 86073 Poitiers Cedex 9, France
19	E-mail address: ftewes@univ-poitiers.fr

20 Abstract

21 Animal models of chronic lung infection with *P. aeruginosa* (*PA*) are useful tools to improve antibiotic 22 (ATB) treatment. Two main models based on the pulmonary instillation of PA embedded in agar or 23 calcium-alginate beads are currently used. However, these two polymers used to prepare the beads 24 have different properties; for example, agar is a neutral polysaccharide while alginate is anionic. We 25 hypothesized that the effect of an ATB on PA entrapped in agar or calcium-alginate beads depends on 26 its physicochemical properties, including charge, and concentration. To test this hypothesis, PAs were 27 entrapped in agar or calcium-alginate beads dispersed in a growth medium containing either 28 tobramycin (TOB), selected as a cationic ATB, or ciprofloxacin (CIP) selected as a neutral zwitterionic 29 ATB. In vitro, time-kill curves evaluating the efficacy of ATBs over time were performed by measuring 30 the light emitted by a bioluminescent PA for 42 hours in the presence of ATB concentrations ranging 31 from 0 to 100 times the MIC. In the presence of CIP, time-kill curves obtained with PA trapped in agar 32 or calcium-alginate beads were comparable, whatever the CIP concentration used. In the presence of 33 TOB, a clear difference was observed between the kill curves obtained with PA embedded in agar or 34 calcium-alginate beads. While PA trapped within agar displayed the same susceptibility than the 35 planktonic one, it was unresponsive to TOB for concentrations up to 1-fold MIC when trapped in 36 calcium-alginate. At 10-fold the TOB's MIC, the luminescence emitted by PA01 in the agar beads was 37 reduced by 95% after 40 h, whereas it returned to the same initial value for PA01 trapped in alginate-38 based beads. The reduction in TOB efficiency was even greater when alginate-based beads were 39 dispersed in a mucus-simulating medium. These results show that the agar and alginate beads models 40 can be interchangeable only for uncharged ATB, such as CIP, but not for cationic ATB, like TOB. In vitro 41 experiments performed in this study could be a quick way to evaluate the effect of each model on a 42 given ATB before performing animal experiments.

43 **1. Introduction**

Animal models of chronic lung infection with Pseudomonas aeruginosa (PA) are useful tools to 44 improve antibiotic (ATB) treatment of these infections in human. The first murine model of chronic 45 bronchopulmonary infection with PA was developed by pulmonary instillation of PA embedded in agar 46 47 beads [1]. The aim of embedding PA within agar beads is to physically restrain the bacteria in the 48 airways by creating an interface between the bacteria and the host that protects them from each 49 other. This leads to persistent stimulation of host defenses and to inflammation that mimics the 50 pathophysiology of chronic lung infection observed in human [2]. A second model was then developed 51 using calcium-alginate beads to replace the agar beads. These alginate-based beads better simulate 52 in vivo conditions in which mucoid PA are embedded within self-produced alginate found in pulmonary 53 biofilms in cystic fibrosis (CF) patients [3-5].

54 Both models are still used today to evaluate the efficacy of ATB to treat chronic lung infections [2, 4, 55 6-8]. However, these two polymers used to form the beads have different properties; for example, 56 agar is a neutral polysaccharide while alginate is anionic. Thus, they could interact differently with 57 ATBs, especially in relation with their charge, and thereby might alter their diffusion, binding, and as 58 a result their efficacy. Therefore, using TOB and CIP as representatives of cationic and non-ionized 59 ATBs, respectively, the aim of this study was to determine whether these models have an impact on 60 the efficiency of some ATBs or whether they can be considered interchangeable. Indeed, we 61 hypothesized that the effect of an ATB on bacteria entrapped in agar or calcium-alginate beads could depend on its physicochemical properties, such as ATB charge and concentration. To test this 62 63 hypothesis, in vitro time-kill curves-like, measuring ATB efficacy over the time have been performed 64 by recording the light emitted by a bioluminescent PA for 42 hours for different TOB and CIP concentrations. 65

66 2. Methods

67 2.1. Materials

A *P. aeruginosa PAO1* strain, hereafter referred as *PAO1*, which was made bioluminescent by chromosomal integration of the luxCDABE operon, was kindly provided by Professor Patrick Plesiat (Centre National de Référence de la résistance aux antibiotiques, Centre Hospitalier Universitaire de Besançon, France). Tobramycin (TOB) and ciprofloxacin (CIP), were purchased from Sigma Aldrich (France).

73 2.2. Luminescence kinetics using planktonic *P. aeruginosa*

74 PAO1 was grown overnight in cation-adjusted Mueller-Hinton Broth (MHB; Sigma-Aldrich, France) in 75 an orbital shaker at 37°C. The optical density of a suspension measured at 600 nm (OD₆₀₀) was adjusted 76 to 0.03 (\approx 1-2 x 10⁷ cells/mL)[9] in MHB (after subtracting MHB absorbance value) and the suspension 77 was incubated at 37°C. Once the exponential phase of growth was reached (approximately 3 hours, evaluated by measuring the OD_{600} every 15 min), 100 μ L of the bacterial suspension, having a 78 79 concentration adjusted to obtain an initial luminescence of 10 000 RLU, were dispersed in the wells of 80 a low-binding (to avoid biofilm formation), white, flat-bottom, 96-well microplate (Greiner®, ref: 81 655904, France). Then, 100 μL of MHB containing the 2 times concentrated ATB was added. ATBs 82 concentrations used, expressed in number of times the minimum inhibitory concentration (MIC; 0.025 83 mg/L for CIP and 0.5 mg/L for TOB) were 100, 50, 25, 10, 5, 2, 1, 0.5, 0.25, and 0. The plates were sealed with a clear, gas permeable, moisture barrier membrane (4titude®, ref: 4ti-0516/96) and 84 85 luminescence was recorded every 30 minutes for 42 hours at 37°C using an Infinite M200 Pro 86 microplate reader (Tecan, France). The use of luminescence enables each well to be used as its own 87 control over time, thus limiting the number of wells required in the study and minimizing well-to-well variations by normalization. Thus, luminescence values recorded at different times were normalized 88 89 by the initial luminescence recorded for each well so that all kinetics curves start at 1 on the y-axis. 90 Plates were shaken before each measurement (n= 12-16).

91 2.3. Luminescence kinetics using biofilms adsorbed on an abiotic surface

92 For biofilms formation, the OD₆₀₀ of an overnight suspension was adjusted to 0.03 in MHB and 200 μ L 93 were dispensed in the wells of high-binding (to facilitate bacteria adherence), white, flat-bottom 96-94 well microplate (Greiner[®], ref: 655074, France). Plates were sealed using an air-permeable membrane 95 and incubated in an orbital shaker at 37°C. After 24 h, the supernatants were removed, and the wells 96 were rinsed 4 times with 150 µL of PBS pH 7.4. The washing procedure is critical, with the main 97 emphasis on preserving biofilm integrity. Then, 200 µL of MHB containing the 1 time concentrated 98 ATB were added. The plates were sealed with a clear, gas permeable, moisture barrier membrane, 99 and then the luminescence was recorded every 30 minutes for 42 hours at 37°C using the microplate 100 reader (Tecan, France) (n= 12-16). In the presence of planktonic bacteria, the value of the initial 101 luminescence was set around 10 000 RLU in each well by adjusting the bacteria concentration. For 102 biofilms, the initial number of bacteria could not be changed and the initial luminescence values used 103 to normalize the data ranged from 2000 to 5000 RLU.

104 2.4. Luminescence kinetics using *P. aeruginosa* entrapped in polysaccharides beads

105 2.4.1. Agar beads preparation

106 Agar beads loaded with PAO1 were prepared using an adapted version of the method described by 107 Growcott et al. [2]. Fresh PAO1 suspension prepared in MHB was cultured to exponential growth 108 phase and then adjusted to an OD₆₀₀ of 0.3. Two mL of this suspension was washed with PBS pH 7.4 109 and bacteria were concentrated in 1 mL of PBS. Then, this suspension was dispersed in 9 mL of 2% m/v molten agar solution (agar; VWR, France), which was then emulsified into 100 mL of warmed 110 111 (48°C) paraffin oil (Sigma Aldrich, France) containing 0.01% v/v of sorbitan monooleate (SPAN[®]60, 112 Sigma Aldrich, France) using a mechanical stirrers model RZR-2021 (Heidolph, France) at 1300 rpm. 113 Then, under moderate mixing (300 rpm), the emulsion was cooled for 1 hour by pouring pieces of ice 114 around the beaker to produce agar beads, which were centrifuged at 5,000 g for 10 min, and then 115 washed 4 times with PBS pH 7.4. Sterile beads were prepared under the same conditions by using 1 116 mL of PBS instead of 1mL of bacterial suspension and their size distribution was determined by laser

117 light diffraction (Microtrac[®] X100 particle size analyzer). Evaluations of the size of some *PAO1*-loaded

118 beads were performed by microscopic observation.

119 2.4.2. Alginate beads preparation

Alginate (Alginic acid sodium salt from brown algae; Sigma Aldrich, France) beads were produced using the same emulsification process than for agar beads preparation. However, after the emulsification step, the gelation of the droplets containing the alginate at a concentration of 2% m/v was induced by adding dropwise and under stirring 20 mL of 0.1M TRIS-HCl buffer pH 7 containing 0.1M of CaCl₂ to the emulsion. After 2 hours of moderate stirring (300 rpm), beads were centrifuged at 5,000 g for 10 min and then washed 4 times with a 0.9 % m/v NaCl solution. Sterile beads were prepared by the same

126 method to determine their size distribution.

127 2.4.3. Time-luminescence kinetics with polymer beads

128 *PAO1* loaded into freshly prepared beads were dispersed in MHB and bioluminescence was adjusted 129 to obtain the same luminescence intensity than with the experiments done using planktonic bacteria 130 (\approx 10000 RLU). Then 100 µL of beads suspension was dispensed in a low-binding (to avoid biofilm 131 formation), white, flat-bottom, 96-well microplates (Greiner[®], ref: 655904, France), followed by 100 132 µL of 2 times concentrated ATB solution in MHB. The plates were sealed with a clear, gas permeable, 133 moisture barrier membrane and luminescence was recorded every 30 minutes for 42 hours at 37°C 134 using the microplate reader (Tecan, France) (n= 12-16).

135 In traditional *in-vivo* models of *PA* chronic lung infection, polymer beads are instilled into the animal 136 lungs, and after 3-4 days when the bacterial burden is stable, they are exposed to ATB [4]. Under these 137 conditions, biofilms can be created inside the beads before being in contact with ATB [10]. Thus, to 138 evaluate the effect of biofilm formation within the beads, *PAO1*-loaded agar beads were incubated 139 under stirring for 48 hours at 37°C in MHB, and then washed 4 times with PBS pH 7.4. Afterward, the 140 luminescence of the bead suspension was adjusted to 10000 RLU in MHB and luminescence kinetics 141 were recorded in the presence of various TOB concentrations as described above. 142 Most of the PA chronic lung infections are associated with the presence of abundant mucus which can 143 be an additional barrier to ATB efficacy [11, 12]. To test the effect of the components of the mucus on 144 the calcium-alginate beads model, additional luminescence kinetics were performed by dispersing the 145 beads in an artificial sputum medium (ASM) instead of the MHB, in the presence of different TOB 146 concentrations. ASM was prepared following the protocol of Sriramulu et al. [13]. It contains 5 g/L of 147 mucin from porcine stomach (Sigma-Aldrich, France), 4 g/L of low molecular-weight salmon sperm 148 DNA (Sigma-Aldrich), 5.9 mg/L of the iron-chelator diethylene triamine pentaacetic acid (Sigma-149 Aldrich), 5 g/L of NaCl (Sigma-Aldrich), 2.2 g/L of KCl (Sigma-Aldrich), 5% (v/v) of egg yolk emulsion 150 (Sigma-Aldrich) and 5 g/L of casein amino acids (Sigma-Aldrich).

151 2.5. Antibiotic diffusion measurements

152 Effective diffusivities of CIP and TOB within the two gels were determined by assaying the amount of 153 ATB that crossed a layer of gel as a function of time. In these experiments, 2% m/v agar or calcium-154 alginate gel layers were prepared in membrane-free Thincerts[®] 24-well plate inserts (Greiner bio-one; 155 France). The thickness of the gel layers was adjusted to 0.45 cm by trimming off the excess of gel. 156 Inserts were placed in a 24-well plate and equilibrated for 1 hour at 37°C with 1.2 mL and 0.25 mL of 157 TRIS-HCl buffer pH 7 in the acceptor and donor compartment, respectively. Then, TRIS-HCl buffer in 158 the acceptor compartment was removed and replaced by a TRIS solution containing the ATB at a 159 concentration of 10 or 100 times the MIC. At different times (10, 20, 30, 40, and 60 min for CIP and 160 30, 60, 90, 120, and 150 min for TOB) 500 μ L of the acceptor compartment was removed and replaced 161 by the same volume of warm, ATB free, TRIS buffer pH 7 (n= 4 per ATB concentration). ATBs 162 concentrations were determined using the analytical methods described on 2.7. The cumulative amount of ATB per centimeter square (ng/cm²) versus time was plotted to check linearity and calculate 163 164 the slope (ng/sec/cm²). Apparent permeability (cm/sec) was calculated by dividing the slope by the 165 donor compartment initial ATB concentration (ng/cm³). Effective diffusivity (cm²/sec) was calculated by dividing the apparent permeability by the gel layer thickness (cm). 166

167 2.6. TOB binding to alginate

168 Calcium-alginate gels were formed by adding 200 µL of alginate solution at 2% m/v in Eppendorf tubes 169 and then, after centrifugation, 600 µL of 0.1M TRIS-HCl buffer pH 7 containing 0.1M of CaCl₂. After 4 170 hours of gelation, 550 μ L of liquid was removed and 500 μ L of TOB solution in TRIS-HCl was added. 171 After incubation for 7 days at 37 °C, the free TOB concentration in the liquid phase in equilibrium with 172 the gel was determined by the LC-MSMS method described below. A control without alginate gel was 173 treated in the same conditions to evaluate the TOB stability and plastic tube binding. No significant 174 change in TOB concentration was observed in the control over the 7 days. The data for three 175 experiments on binding of TOB to calcium-alginate gel were analyzed as described by Nichols et al. 176 [14] using a linear adsorption isotherm equation, B = RC, where B is the amount of TOB bound in a 177 unit volume (in $\mu g/mL$), C is the free TOB concentration ($\mu g/mL$), and R is a constant of proportionality.

- 178 2.7. Antibiotic assays
- 179 2.7.1.CIP assay

180 CIP was assayed using an Infinite M200 Pro microplate reader (Tecan, France) as a fluorimeter. Stock 181 solutions of CIP in acidic water with 0.1 mM HCl were prepared at 1 mg/mL. Standard solutions having 182 CIP concentrations of 100, 50, 25, 10, 5, and 1 ng/mL as well as QC solutions having CIP concentrations 183 of 75, 15, and 2.5 ng/mL were prepared in PBS pH 7.4 or TRIS-HCl pH 7. Then, 250 μL of these solutions 184 and samples were added in the wells of a low-binding, white, 96-well microplate (Greiner®, ref: 185 655904, France) and the fluorescence intensity at 430 nm was measured after excitation at 278 nm. 186 Coefficients of determination of linear regressions of calibration curves were higher than 0.99 and QC and standards biases were lower than 15%. 187

188 2.7.2. TOB assay

TOB was assayed using an LC-MSMS method previously described [15]. Briefly, a Waters Alliance 2695 separation module coupled with a Waters Micromass Quattro micro API tandem mass spectrometer was used. Chromatographic separation was done with an X bridge C18 column (5.0 μm, 150 by 2.1 mm); Waters, St-Quentin en Yvelines, France) and a mobile phase composed of 0.1% (vol/vol) formic acid in water and 0.1% formic acid in acetonitrile (75:25, vol/vol) flowing at 0.2 ml.min⁻¹.

194	Quantification was performed in the positive-ion mode with multiple-reaction monitoring of m/z
195	transitions 468.2 \rightarrow 163.1 for TOB and 448.2 \rightarrow 160.2 for sisomycin, the internal standard (1 µg/mL).
196	TOB concentrations of the standard solutions were 1000, 750, 500, 100, 25, 5, 2.5, and 1 ng/mL and
197	750, 100, and 2.5 ng/mL for the QC solutions. Coefficients of determination of linear regressions of
198	calibration curves were higher than 0.99 and QC and standards biases were lower than 15%.
199	2.8. Data analysis
200	All data analyses were performed using the R software version. 3.4 and the package "ggplot2 v. 3.10"
201	was used to plot smoothed mean luminescence values versus time using a generalized additive model
202	(GAM) as smoothing method. The shaded area around the mean value curve is the 95% confidence
203	level interval for the mean values predicted from the GAM smoothing model.

204 3. Results

Bioluminescence kinetics of planktonic *PA* and adsorbed biofilms of *PA* in the presence of CIP or TOB. In order to obtain references for comparing the data obtained with PAs trapped in polymer beads, luminescence kinetics were first recorded for planktonic PAs and PAs adsorbed on plastic surfaces as biofilms in the presence of CIP (Figure 1) or TOB (Figure 2).





210 Figure 1: Time-luminescence curve obtained with planktonic PAO1 (black dashed lines), biofilms of 211 PAO1 (black solid lines), PAO1 trapped in agar beads (red solid lines), or PAO1 trapped in calcium-212 alginate (blue two-dash lines) beads exposed to various CIP concentrations in MHB expressed in the 213 number of times the MIC (Top of each graph). The green horizontal curve (Control -) is the signal obtained in the absence of bacteria. For each well, luminescence intensity was normalized to the 214 value measured at time zero. The gray band around the lines is the 95% confidence level interval of 215 216 the mean values predicted from the GAM smoothing model (n = 6-16). The mean values between 217 different conditions are significantly different in the absence of overlapping of these gray-shaded 218 areas.

In the absence of ATB, luminescence produced by planktonic *PAO1* or biofilms of *PAO1* increased similarly for 5 hours (Figure 1 and 2). For biofilms, the luminescence was then stable for the time of the experiment, while it decreased slowly for the planktonic bacteria. The addition of CIP at a low

concentration, e.g. 0.25 times the MIC, decreased the bacteria growth rate in both growing mode and
a plateau was reached only after 15 hours. At this CIP concentration, biofilms had higher normalized
luminescence at the plateau compared to values got without ATB. For CIP concentrations comprised
between 2 to 100 times the MIC, biofilms formed on the bottom of 96-well plates were less susceptible
to CIP than the planktonic bacteria (Figure 1). A higher luminescence decrease rate and a greater
extent of the luminescence decrease were obtained with the planktonic bacteria compare to biofilms.



228

Figure 2: Luminescence kinetics obtained with planktonic PAO1 (black dashed lines), biofilms of 229 230 PAO1 (black solid lines), PAO1 trapped in agar beads (red solid lines), or PAO1 trapped in calcium-231 alginate (blue two-dash lines) beads exposed to various TOB concentrations in MHB expressed in the number of times the MIC (Top of each graph). The green horizontal curve (Control -) is the signal 232 233 obtained in the absence of bacteria. For each well, luminescence intensity was normalized to the value measured at time zero. The gray band around the lines is the 95% confidence level interval of 234 235 the mean values predicted from the GAM smoothing model (n = 6-16). The mean values between 236 different conditions are significantly different in the absence of overlapping of these gray-shaded 237 areas.

The overall time-luminescence profiles obtained with CIP or TOB against planktonic or biofilm of *PAO1* were similar and planktonic bacteria were generally more susceptible to both ATBs than biofilms (Figure 1 and 2). However, for ATB concentrations below 25 times the MIC, biofilms were less sensitive to TOB than CIP. For example, no effects were observed with biofilms exposed to TOB at concentrations up to 0.5 times the MIC (Figure 2), whereas CIP showed an effect from 0.25 times the MIC (Figure 1). In addition, for biofilms exposed to ATB concentrations ranging from 1 to 5 times the MIC, greater luminescence rebounds were observed with TOB than with CIP.

245 Bioluminescence kinetics of PA entrapped in agar or calcium-alginate beads. Both types of beads 246 were prepared using a similar emulsification method and comparable monodisperse size distributions 247 were obtained (supplementary material 1). Their mean diameters of $68 \pm 23 \,\mu\text{m}$ and $81 \pm 27 \,\mu\text{m}$, for 248 calcium-alginate and agar, respectively, were not significantly different (p = 0.58, two-tailed t-test). 249 Luminescence kinetics acquired with PAO1 trapped in agar or calcium-alginate beads were nearly 250 similar for all tested CIP concentrations (Figure 1). The only difference observed was an initial increase 251 in luminescence over the five first hours with the alginate beads, while the luminescence decreased 252 immediately with the agar ones for CIP concentrations between 0.5 and 10 times the MIC. Thus, no effect of the polymer forming the beads was observed in the presence of CIP. For CIP concentrations 253 254 between 0.5 and 10 times the MIC, the luminescence profiles obtained with PAO1 entrapped in both 255 types of beads were close to those obtained with biofilms adsorbed on the bottom of the 96-well 256 plates. However, for higher CIP concentrations, the profiles were similar to those obtained with 257 planktonic bacteria.

Although no effect of the type of beads used was observed on the CIP effectiveness, for certain TOB concentrations, the luminescence profiles were changed depending on the type of beads. For TOB concentrations lower than 0.5 times the MIC, and higher than 25 times the MIC, no effect of the polymer was observed and the same profiles were obtained with agar or calcium-alginate beads (Figure 2 - Supplementary materials 4). For these TOB concentrations ranges, the kinetics were similar to those obtained with planktonic bacteria. However, between these TOB concentrations, *PAs* entrapped in alginate-based beads were less susceptible to TOB than those entrapped in agar beads.
For this TOB concentration range, *PAs* embedded in the alginate beads were also less susceptible to
TOB than the bacteria present in the biofilms adsorbed on the bottom of the 96-well plates and the
planktonic bacteria.



268 Figure 3: Luminescence kinetics obtained with PAO1 freshly trapped in agar beads (solid lines) or for 269 270 48 hours (dashed lines) before being dispersed in MHB containing different concentrations of TOB expressed in the number of times the MIC (Top of each graph). The blue horizontal curve (Control -271 272) is the signal obtained in the absence of bacteria. For each well, luminescence intensity was 273 normalized to the value measured at time zero. The gray band around the lines is the 95% 274 confidence level interval of the mean values predicted from the GAM smoothing model (n=12-16). 275 The mean values between different conditions are significantly different in the absence of 276 overlapping of these gray-shaded areas.

PAs freshly entrapped in calcium-alginate beads were less susceptible to TOB than those freshly entrapped in agar beads (Figure 2). However, in *in vivo* experiments, beads are instilled into the animals' lungs and are exposed to ATB only after 2-3 days when the bacterial burden is stable. During this period, *PAO1* can form biofilms in the beads that can influence the effect of ATB. To evaluate this, 281 luminescence kinetics using agar beads that did not appear to interfere with the TOB effect, were 282 incubated for 48 hours in MHB prior to the addition of TOB. Luminescence kinetics measured from 283 fresh or 48-hours-old agar beads were almost similar (Figure 3). *PAs* in 48-hours-old agar beads were 284 slightly less susceptible to TOB than *PAs* in the fresh beads only for TOB concentration around the 285 MIC. Additionally, *PAs* trapped into fresh or 48-hours-old agar beads were around 10 times more 286 susceptible to TOB than *PAs* freshly trapped into calcium-alginate beads.

Effective diffusivity of TOB and CIP in agar and calcium-alginate gels at 2% m/v. To determine whether the decrease in the efficacy of TOB on *PAs* trapped in calcium-alginate beads compared to *PAs* trapped in agar beads could be due to a decrease in TOB diffusion, the effective diffusivity of the two ATBs were evaluated in both gels by measuring their transport rate through a layer of these gels (Figure 4).



Figure 4: A) Scheme showing the test used to determine the ATBs effective diffusivities. B) CIP and TOB effective diffusivity values measured at 37°C in 2% (m/v) agar and 2% (m/v) calcium-alginate gels. * The diffusivity percentage of the two ATBs in the gels relative to their diffusivity in pure water was calculated using the diffusivity of the CIP in pure water (6.9 x 10^{-6} cm²/s) and the diffusivity of TOB in pure water (5.6 x 10^{-6} cm²/s) at 37°C obtained from Stewart [16].

The CIP effective diffusivity values at 37°C were similar in both gels, with values of $1.5 \pm 0.2 \times 10^{-6} \text{ cm}^2/\text{s}$ and $1.4 \pm 0.4 \times 10^{-6} \text{ cm}^2/\text{s}$ in 2% (m/v) agar and 2% (m/v) calcium-alginate gel, respectively (Figure 4). TOB effective diffusivity in agar gel ($1.6 \pm 0.7 \times 10^{-6} \text{ cm}^2/\text{s}$) was similar to that of CIP in this gel. In the calcium-alginate gel, the TOB effective diffusivity was reduced to $0.8 \pm 0.5 \times 10^{-6} \text{ cm}^2/\text{s}$. TOB interaction with the calcium-alginate gel was further investigated by measuring its binding at equilibrium with this gel. The concentration dependence of the TOB binding to 2% (m/v) calcium alginate gel was linear within the concentration range tested (MIC at 100 times MIC - Figure 5A), which supposes the absence
 of saturation. Nichols *et al.* [14] also found a linear binding of TOB to sodium alginate in this range of
 TOB concentrations. The constant of proportionality between the amount of TOB bound to alginate
 and the free TOB concentration was around 3.7.





Figure 5: Measurement of TOB binding to alginate. A) Log-log plot of the concentration dependence of TOB binding to calcium-alginate gel. B) Linear TOB adsorption isotherm described by B = RC, where B is the amount of TOB adsorbed in a unit volume (μ g/mL), C is the free TOB concentration (μ g/mL), and R is the constant of proportionality.

When adsorption reaches equilibrium instantaneously, solute effective diffusivity (*De*) in a gel can be related to its diffusivity in water by the following equation: De = D(1 / (R + 1)) [14]. Thus, using the diffusivity of TOB in the water at 37°C calculated from the Wilke-Chang correlation at 5.6 x 10⁻⁶ cm²/s [16], the effective diffusivity of TOB in the calcium-alginate gel was 1.2 x 10⁻⁶ cm²/s.

317 Bioluminescence kinetics of PA entrapped calcium alginate beads dispersed in MHB or artificial sputum medium. The effect of mucus components on the efficacy of TOB was accessed by measuring 318 319 the luminescence kinetics of PA entrapped in calcium-alginate beads dispersed in artificial sputum 320 medium (ASM) in the presence of different TOB concentrations (Figure 6). For TOB concentrations of 321 up to 2 times the MIC, no difference was observed in the kinetics obtained with calcium-alginate beads dispersed in MHB or ASM. However, for TOB concentrations greater than 2 times the MIC and up to 322 323 50 times the MIC, the ASM components further decreased the efficacy of the TOB compared to the 324 results obtained with alginate-based beads dispersed in MHB. Higher luminescence values were

325 obtained in ASM and rebounds in the luminescence was observed after 40 hours for TOB



326 concentrations up to 50 times the MIC.

327 Figure 6: Luminescence kinetics of PAO1 obtained for different concentrations of TOB (expressed as 328 329 a number of times the MIC against planktonic PAO1). PAO1 were entrapped into calcium-alginate 330 beads dispersed either in MH broth (dashed lines) or in artificial sputum medium (ASM) (solid lines). 331 The blue horizontal curve (Control -) is the signal obtained in the absence of bacteria. For each well, 332 luminescence intensity was normalized to the value measured at time zero. The gray band around 333 the lines is the 95% confidence level interval of the mean values predicted from the GAM smoothing 334 model (n=12-16). The mean values between different conditions are significantly different in the 335 absence of overlapping of these gray-shaded areas.

336 4. Discussion

Pseudomonas aeruginosa (PA) is responsible for pulmonary chronic infections in patients with chronic respiratory diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). These PA infections are usually associated with the presence of biofilms in the lungs that are impossible to eradicate [5, 17, 18]. This problem is worsening due to the occurrence of multidrugresistance (MDR) PA worldwide [19]. In this context, new methodologies for treating these pulmonary 342 biofilms are being developed [20]. For example, new therapeutic approaches aim to improve the 343 penetration of ATBs into biofilm/mucus gels by ATB pegylation [12], or by loading them into 344 nanoparticles able to penetrate these gels [21, 22]. Some approaches aim at maintaining high ATB concentration in the lung [23], optimizing ATBs dosage with PK-PD studies [4], or combining several 345 346 ATBs together or with anti-quorum sensing or biofilm dispersants [24]. To evaluate these strategies, 347 good in vitro and in vivo models simulating pulmonary biofilms conditions in human are central. The 348 two most common models used to create chronic pulmonary PA infections in rodents are pulmonary 349 instillation of PA confined in agar beads or calcium-alginate beads. To determine whether these two 350 beads models can be considered interchangeable, PA-loaded agar or calcium-alginate beads were 351 challenged with two clinically relevant ATBs, the TOB and the CIP, doing time-kill-like experiments. In 352 order to provide a high throughput-screening test for easy assessment of the ATB efficacy, a 353 bioluminescent PAO1 was used.

354 Use of bioluminescent bacteria seems an interesting tool to quickly evaluate the kinetics effect of 355 an antibiotic on bacteria grown either planktonically, as biofilms or immobilized within polymer 356 beads. Time-kill curves are traditionally made by following the variation of concentration of bacteria 357 able to form a colony versus time. This method is time-consuming and allows only a limited number of time points. For biofilms, it is even more complicated as you have to extract the bacteria from the 358 359 biofilm matrix [12, 24]. Monitoring the luminescence produced by transformed bacteria allows live 360 measurements and the acquisition of many points for precise kinetics. [25]. Still, bioluminescence is a 361 more complex parameter than a simple bacteria count as its value depends on the concentration of 362 living bacteria but also their metabolic state [26, 27]. Yet, bioluminescence permits the observation of 363 living bacteria that are metabolically active but unable to multiply to create a colony, such as bacteria found in biofilms [26, 28]. One of the weaknesses of the use of bioluminescence could be the difficulty 364 365 to access the presence of persisters and ATB tolerant bacteria described in biofilms [29] that have low 366 or almost no metabolic activity and thus should produce much less light than the active bacteria. 367 Anyhow, persisters generally do not produce a visible colony in 20-24h and thus are normally not detected by traditional time-kill experiments. Another limitation of the use of bioluminescence is the needs of O_2 for the reaction that produce light. Studies have shown that O_2 diffusion can be limited in alginate gels and biofilms [30-32]. Its penetration is generally limited to 50 to 90 µm down from the surface of biofilms [30, 33]. Thus oxygen diffusion can be a restriction for using bioluminescence with thick biofilms, as those formed on abiotic surfaces, or with large alginate beads [33], but for beads having diameters lower than 100 µm, oxygen diffusion limitation should not be responsible for a decrease in light production.

375 For adherent biofilms and planktonic cells, ATBs concentrations higher than the MIC induced a fast 376 decrease of the luminescence. This decrease was faster and more intense for planktonic PAO1 than 377 for biofilms. Biofilm cells are less susceptible to antimicrobials than their planktonic counterparts [20, 378 29, 34]. Thus, the results obtained here are excepted if the bioluminescence correlates with the 379 bacterial survival and metabolic state. The kinetics obtained with planktonic PAO1, adherent biofilms, 380 and with PAs entrapped in both types of beads displayed a concentration- and time-dependent change 381 in luminescence with both ATBs. Time-kill curves with similar behaviors were established with 382 planktonic PAO1 treated with these two ATBs using the traditional bacterial counting method [35, 36]. 383 Except for TOB in calcium-alginate beads, ATBs concentrations around the MIC induced an initial 384 decrease in luminescence that was followed by a rebound. In traditional time-kill experiments 385 performed with these ATBs at these concentrations, a "regrowth" of the bacterial population is often 386 observed [35, 36]. This observation is generally attributed either to the adaptation of bacteria to the 387 ATB (mutation) or to the selection of a subpopulation of bacteria that are already resistant but has a 388 slower growth rate than the majority of the bacteria in normal condition. This luminescence rebound 389 could be attributed to the growth of this kind of bacteria. For biofilms and for CIP concentrations 390 between 0.25 and 0.5 times the MIC, a higher normalized luminescence value was observed at the plateau compared to the value obtained without ATB. This increase could be due to a stimulation of 391 the bacterial metabolism by CIP, as observed by other studies that showed that sub-MIC CIP 392 393 concentrations stimulate PA biofilm formation and virulence [37, 38].

394 Agar and calcium-alginate beads are interchangeable for evaluating uncharged ATB such as the 395 fluoroquinolone CIP, but not for cationic aminoglycosides such as TOB. Luminescence kinetics 396 obtained in the presence of a large range of CIP concentrations (0 to 100 times the MIC) were virtually 397 the same for *PAO1* trapped in calcium-alginate or agar beads. The size distributions of the two types 398 of beads did not differ significantly, so their size should hardly influence the kinetic profiles. Therefore, 399 both bead models seem interchangeable with CIP. For TOB however, a net difference was observed 400 between kinetics obtained from PAs entrapped in agar and calcium-alginate beads. In alginate beads, 401 PAs were up to 10 times less susceptible to TOB than in agar beads. In these experiments, beads were 402 produced and immediately exposed to ATB. Initially, in these conditions, PAs entrapped are planktonic 403 bacteria. Thus, at the beginning of the kinetics, the differences observed between the two types of 404 beads cannot be attributed to phenomena such as the presence of low metabolically active bacteria 405 found in biofilms. In addition, the effect of TOB on PAs entrapped freshly or for 2 days in agar beads 406 prior to addition of TOB was almost similar for all tested TOB concentrations. This suggests that PAO1 407 biofilm formed in agar beads had little effect on the TOB susceptibility compare to the effect of 2% 408 m/v calcium-alginate. Thus, when using non-mucoid bacteria such as PAO1 that do not produce 409 alginate, the agar and calcium-alginate beads models are not interchangeable when testing cationic 410 ATB such as TOB.

411 The calcium-alginate beads model was developed based on the finding that many PA found in CF 412 patients are present as mucoid biofilms containing a large proportion of alginate in their EPS matrix. 413 Numerous studies evaluating the effect of ATBs on these biofilms have shown that their efficacies are 414 reduced compared to those obtained with free planktonic bacteria. Initially, it was thought that the 415 ATBs tolerance of bacteria in these biofilms was due to the limitation of ATBs transport in biofilms due 416 to the presence of the alginate-containing EPS matrix. [14, 39, 40]. So, the penetration of various ATBs 417 within biofilms of different PAs, or within calcium-alginate gels made from commercial seaweed 418 alginate or alginate from clinically relevant mucoid variants of PA was measured using various 419 methods [14, 39, 41-43]. With the exception of some positively charged ATBs, such as

420 aminoglycosides, which interact with negatively charged alginate [14, 39, 44-46] or calcium-alginate 421 [39], most ATBs, including CIP, readily penetrated biofilms and alginate gels at sufficiently high 422 velocities to exclude a limiting phenomenon that would contribute to antimicrobial tolerance [14, 39, 423 44]. Even though TOB penetration in alginate-based biofilms is delayed [42], some studies considered 424 to have a minor effect on the ATB tolerance [47]. In our study, the effective diffusivity of CIP at 37°C calculated from transport experiments through both gels was around 1.5 x 10⁻⁶ cm²/s, which is 5 times 425 426 lower than its value in water [16]. Even with this reduction, luminescence kinetics obtained in the 427 presence of CIP with both types of beads were highly similar to those obtained with planktonic bacteria. TOB effective diffusivity in agar $(1.6 \pm 0.7 \times 10^{-6} \text{ cm}^2/\text{s})$ was close to that of the CIP in this gel 428 429 and was reduced by 3.5 fold in relation to its diffusivity in water. This effective diffusivity was 2 times lower than the value found by Gordon et al. [39] in a similar 2% m/v agar gels (3.0 x 10⁻⁶ cm²/s). TOB 430 effective diffusivity in 2% (m/v) calcium-alginate gel ($0.8 \pm 0.5 \times 10^{-6} \text{ cm}^2/\text{s}$) was 2 times lower than in 431 432 2% (m/v) agar gel and 7 times lower than in water [16]. This value was in the same range than the 433 value found by Gordon et al. [39] in 2% (m/v) alginate containing calcium lactate as a gelling agent 434 $(0.65 \pm x \ 10^{-6} \text{ cm}^2/\text{s})$. TOB diffusivity in the calcium-alginate gel was also assessed from its binding at equilibrium to this gel as described by Nichols et al. [14]. It was evaluated at 1.2 x 10⁻⁶ cm²/s, which is 435 436 in the same range of values as calculated from the transport experiment across a calcium-alginate gel 437 layer. According to Stewart, P. S. [47] and Nichols et al. [14], the time required for a molecule to 438 achieve 90% of the dispersing medium concentration at the center of a spherical polymer bead is estimated by $t_{90} = 0.37 \times R^2/De$, where R is the radius of the bead and De is the effective diffusivity 439 of the molecule in the polymer. Therefore, with a diffusivity of $0.8 \pm 0.5 \times 10^{-6} \text{ cm}^2/\text{s}$ and beads having 440 a maximal diameter of 100 μ m, 12 seconds should be needed for TOB to obtain 90% of the dispersing 441 442 medium concentration at the center of beads. Thus, only a decrease in the diffusivity of TOB in alginate 443 by a factor of 1000 to 10,000 could explain by itself the difference in efficacy against PAO1 entrapped in calcium-alginate compared with those trapped in agar. 444

The reduction of TOB diffusivity seems not a major mechanism of TOB resistance of *PA* in calciumalginate beads. However, several studies proposed that the delay in TOB penetration give to the bacteria the time to adapt to the ATB and adopt a more antimicrobial-tolerant state before killing concentrations of the ATB can be achieved [48]. In agreement with this suggestion, hydrolysis of alginate by alginate lyase enhanced the activity of TOB in biofilms by dissolving the biofilm matrix [49]. Also, PA exposures to sub-lethal concentrations of TOB results in phenotypic alterations such as increased EPS productions or activation of ATB efflux pump [50].

452 Alginate beads dispersed in artificial sputum media could be a good model to simulate pulmonary 453 biofilms. In cystic fibrosis (CF) patients, the conductive zone serves as a bacterial reservoir where the 454 bacteria are organized in mucoid biofilms within the mucus, protected against ATBs and host defenses 455 [5]. These two gels that are biofilm and mucus are formed of negatively charged polymers that could 456 behave as barrier able to reversibly bind cationic ATBs and slow down their penetration [12, 51]. This 457 reduction in ATBs penetration rate could give the bacteria enough time to adapt to the ATBs [40, 48]. 458 In vivo PA biofilm patches found in the pulmonary mucus of chronically infected CF patients have sizes 459 ranged from 5 to 200 μ m in diameter [5, 18, 52]. With a high frequency, *PAs* found in these infections 460 are able to create mucoid biofilms having a high concentration in alginate [34]. Thus, alginate beads 461 having the size of the biofilm clusters found in the CF patients lungs dispersed in mucus or mucus-462 simulating medium could be a good model for evaluating anti-biofilm strategies. In the present study, 463 we have shown that the dispersion of the alginate beads in ASM further reduced the efficacy of TOB 464 compared to the same beads dispersed in MHB. This result is in agreement with Müller et al. [45] 465 study showing a significant reduction of TOB efficacy against PA biofilms grown on 96-well plates in 466 the presence of mucus compared with those grown in the absence of mucus. Also, several studies 467 have shown that the presence of mucus altered the PA biofilms behaviors [53] and promote the expression of several genes responsible for ATB resistance [54, 55]. 468

In human, most planktonic bacteria are killed by the ATB and the residual bacteria are eradicated bythe immune system. For biofilms, however, due to the presence of the EPS matrix, immune cells

471 cannot reach the bacteria and the residual cells that are not killed by the ATB might be able to restore 472 the biofilm. Also, bacteria in biofilms are less susceptible to ATBs than their planktonic counterparts. 473 Therefore, ATB concentrations required to eradicate bacterial infections with biofilms are higher than 474 the clinical targets usually used to treat infections caused by planktonic cells. These concentrations 475 are difficultly achievable via systemic administration and pulmonary biofilms eradication seems only 476 possible by ATB inhalation due to the possibility to achieve high ATB concentrations in biofilms while 477 avoiding side effects and toxicity. The mucus barrier is often neglected in *in vitro* tests employed to 478 evaluate the efficacy of inhaled ATBs. This model could be highly relevant in testing the efficacy of 479 inhaled ATB, whose development to treat pulmonary lung infections is increasing [21, 56].

In CF patients, activated polymorphonuclear leukocytes (PMNs) are accumulating around the bacterial aggregates [5]. PMNs impact on *PAs* behavior and release a large amount of extracellular DNA that could affect cationic ATB efficacy [57, 58]. Thus, a model using small beads of calcium-alginate dispersed in medium simulating mucus could be supplemented with immune cell lines that could aggregate around the beads, which would make it possible to study the effect of the three barriers (biofilm, mucus, and agglomerated immune cells) that an ATB must pass to reach the bacteria.

486 **5.** Conclusion

These results show that the agar and alginate beads models can be interchangeable only for uncharged ATB, such as CIP, but not for all ATB. In vitro experiments performed in this study could be a quick way to evaluate the influence of each model on a given ATB before performing animal experiments.

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