

# 1 **Chitosan films for microfluidic studies of single bacteria and perspectives for antibiotics** 2 **susceptibility testing**

3 Julie Tréguier<sup>a\*#</sup>, Loïc Bugnicourt<sup>b\*</sup>, Guillaume Gay<sup>c</sup>, Mamoudou Diallo<sup>b</sup>, Salim Islam<sup>a#</sup>,  
4 Alexandre Toro<sup>d</sup>, Laurent David<sup>b</sup>, Olivier Théodoly<sup>e</sup>, Guillaume Sudre<sup>b</sup>, Tâm Mignot<sup>a</sup>

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6 Affiliations:

7 <sup>a</sup> Laboratoire de Chimie Bactérienne, Institut de Microbiologie de la méditerranée, CNRS-Aix  
8 Marseille University (UMR7283), 31 Chemin Joseph Aiguier, 13009 Marseille, France.

9 <sup>b</sup> Ingénierie des Matériaux Polymères, Université Claude Bernard Lyon 1, Université de  
10 Lyon, CNRS UMR 5223, 15, bd Latarjet 69622 Villeurbanne Cedex France.

11 <sup>c</sup> Morphogénie Logiciels SAS, 12 rue Camoin Jeune, 13004 Marseille, France

12 <sup>d</sup> Laboratoire de Biologie, Centre Hospitalier de Martigues, 3 bvd des Rayettes, 13117  
13 Martigues, France

14 <sup>e</sup> Laboratoire Adhésion et Inflammation. INSERM U1067 CNRS UMR 7333. 163, avenue de  
15 Luminy, 13288 Marseille cedex 09, France

16

17

18 \* Co-first authors

19

20 # Current address :

21 Julie Tréguier : Institut de Génomique Fonctionnelle, 141, rue de la Cardonille, 34094  
22 Montpellier cedex 5, France

23 Salim Islam : Centre INRS–Institut Armand-Frappier, 531, boulevard des Prairies, Laval  
24 (Québec) H7V 1B7, Canada

25

26 correspondence to:

27 Tâm Mignot: [tmignot@imm.cnrs.fr](mailto:tmignot@imm.cnrs.fr)

28 Guillaume Sudre: [guillaume.sudre@univ-lyon1.fr](mailto:guillaume.sudre@univ-lyon1.fr)

29 Olivier Théodoly: [olivier.theodoly@inserm.fr](mailto:olivier.theodoly@inserm.fr)

30

## 31 **Abstract**

32 Single cell microfluidics is powerful to study bacteria and determine their susceptibility to  
33 antibiotics treatment. Glass treatment by adhesive molecules is a potential solution to  
34 immobilize bacterial cells and perform microscopy but traditional cationic polymers such as  
35 poly-lysine deeply affect bacterial physiology. In this work, we chemically characterized a class  
36 of chitosan polymers for their biocompatibility when adsorbed to glass. Chitosan chains of  
37 known length and composition allowed growth of *Escherichia coli* cells without any deleterious  
38 effects on cell physiology. Combined with a machine-learning approach, this method could  
39 measure the antibiotics susceptibility of a diversity of clinical strains in less than 1 hour and  
40 with higher accuracy than current methods. Last, chitosan polymers also supported growth of  
41 *Klebsiella pneumoniae*, another bacterial pathogen of clinical significance. The low cost of  
42 chitosan slides and their simple implementation makes them highly versatile for research as  
43 well as clinical use.

44

## 45 **Introduction**

46

47 In recent years, microfluidics coupled with live-cell imaging have revolutionized  
48 bacteriology, testing directly the impact of rapid and controlled environmental transitions on  
49 cell physiology. With the advent of super-resolution microscopy, the bacterial cell can now be  
50 further explored at unprecedented resolution, tracking cellular processes one molecule at a time

51 (1). The impact of these methods is not limited to basic research because single cell approaches  
52 are unquestionably powerful at determining antimicrobial susceptibility (AST, Antimicrobial  
53 Susceptibility Testing) in record time (2, 3).

54

55 However, a major technical bottleneck with the implementation of single cell  
56 approaches for super-resolution or AST, is the immobilization of bacterial cells. Agar surfaces  
57 have been widely used and support the growth of a wide range of bacterial species. However,  
58 this method has several limits:

59 (i) Agar surfaces are not compatible with high-end microscopy (HEM) methods  
60 that require cells to adhere to glass, for example Total Internal Reflection  
61 Fluorescence Microscopy (TIRFM) and all single molecule microscopy  
62 techniques (PALM, STORM, STED).

63 (ii) Because the adhesion of bacterial cells to Agar surfaces is generally weak, these  
64 surfaces cannot be manipulated in aqueous environments and the experimental  
65 conditions are generally set by diffusion through the agar substrate. However,  
66 this approach does not allow rapid changes of the medium or injection of  
67 chemicals and thus the kinetics and precise dose-dependent effects are poorly  
68 controlled (3, 4).

69 Alternative methods have remedied these issues by growing bacteria immediately in contact  
70 with a glass surface. Because most bacteria do not directly adhere to glass, immobilization  
71 procedures are required, which include direct physical immobilization of the bacteria in micro  
72 channels or glass functionalization by adhesive polymers. The use of micro-channels is  
73 certainly compatible with HEM and it allows fast AST with high accuracy (2, 5). However, this  
74 method requires expert handling, complex nanolithography to produce the channels and  
75 extensive development to be used for the study of a given bacterial species. Alternatively,  
76 bacterial adhesion on glass can be obtained by functionalizing a glass slide with adhesive  
77 polymers/molecules. This approach can also be difficult because the polymer must be fully  
78 biocompatible and the functionalization procedure and surface chemistry can be complex.  
79 Indeed, although this approach has been widely used for eukaryotic cells, the choice for  
80 polymers biocompatible with bacteria is limited. Cationic polymers such as poly-lysine bind  
81 glass surfaces effectively and promote adhesion of a wide range of bacterial species. However,  
82 poly-lysine also generates cell envelope stress and has been shown to dissipate/diminish the  
83 membrane potential in several Gram negative of Gram positive species (e.g. *Escherichia coli*  
84 or *Bacillus subtilis* (6–8)). For clinical microbiology applications, this issue is particularly  
85 sensitive because changes in the membrane potential can directly affect antimicrobial  
86 susceptibility (9) and thus produce false negative or even worst, false positive results in AST.  
87 Thus, there is a need in developing new functional polymers with neutral effects on bacterial  
88 physiology for single cell AST studies.

89

90 This work originated from the observation that chitosan polymers can support bacterial  
91 adhesion and motility on surfaces (in the case of *Myxococcus xanthus* and *Bacillus subtilis* (1)).  
92 Herein, we investigated if chitosan-treated glass slides could also support bacterial growth when  
93 inserted in commercial microfluidic chambers. Using a specific chitosan polymer (with a high  
94 degree of acetylation) and a new controlled functionalization procedure, we showed that  
95 Chitosan-Coated Slides (CCS) can support the growth of *E. coli* during multiple generations  
96 without any effects on bacterial fitness. Using clinical *E. coli* strains obtained from intestinal  
97 and urinary tract infections ((I/U)TI) of known antibiotics susceptibilities, we showed that CCS  
98 allowed fast direct determination of AST. Last, CCS can be derived to promote growth of other  
99 so-called ESKAPE pathogens such as *Klebsellia pneumoniae*, which also raise significant  
100 problems for antibiotics treatment (10). We conclude that chitosan-based functionalization

101 procedures are promising for their application in bacterial single cell studies for basic research  
102 but also potentially, in clinical contexts.

103

## 104 **Results**

105

### 106 **Functionalizing glass slides with chitosan polymers**

107 Chitosan is a linear polysaccharide composed of randomly distributed  $\beta$ -(1 $\rightarrow$ 4)-linked D-  
108 glucosamine and N-acetyl-D-glucosamine units (Figure S1A). Its physicochemical properties  
109 are highly dependent on its macromolecular parameters (i.e. average molar mass  $M_w$ , and  
110 degree of acetylation DA). A control of these parameters is needed to ensure robustness when  
111 studying the physicochemical and biological behavior of chitosan polymers. Indeed, growth  
112 and motility were not always reproducible when glass slides were coated with raw commercial  
113 chitosan and this variability could be due to the poor chemical characterization of commercial  
114 stocks, which contain chains of variable DA, molar mass and statistical distribution of the acetyl  
115 groups.

116 Consistent with this, size exclusion chromatography (SEC-MALLS/RI) analysis performed on  
117 chitosan from a commercial source (Sigma-Aldrich, See Methods), revealed an important  
118 dispersity in polymer chains length (Polydispersity Index  $\bar{D} = 2.65$ ). It is essential to control  
119 the dispersity of chitosan chains because slight variations in molar mass and DA of chitosan  
120 polymers can be associated to a wide range of biological responses: cell adhesion, wound  
121 healing and even bacterial stasis and lysis (11, 12). As a general trend, it is mandatory to  
122 determine and control each molecular parameter in order to understand their impact on bacterial  
123 physiology and to ensure reproducibility of our experiments.

124 To this aim, we first generated a large library of chitosan polymers with various DA and molar  
125 masses (13) (Chito-library). Different molar masses ( $M_w$ , 180 kg/mol and high  $M_w$ , 460 kg/mol)  
126 were obtained by selecting chitosan from different source (shrimp or squid). To control the  
127 acetylation levels, the polymer chains were re-acetylated *in vitro* to produce DAs of 1%, 10%,  
128 15%, 25%, 35%, 45%, and 55% (14). Each polymer was characterized by SEC to control its  
129 molar mass and by  $^1\text{H}$  NMR to measure its DA (Figure S1A, see Methods).

130

131 Flat and homogeneous layers of polymer in the nanometer range (i.e. 15 nm < thickness < 150  
132 nm) were obtained by Spin-coating of chitosan solutions with controlled concentration and pH  
133 (Figure 1A). Substrates were chosen to be either silicon wafers for physicochemical  
134 characterization, or boro-silicate glass coverslips for bacterial single cell studies (Figure S1B,  
135 no incidence of the substrate was noticed on the physicochemical characteristics of chitosan  
136 layers). The thickness, uniformity, wettability and morphology of chitosan ultrathin films  
137 prepared from the chito-library were systematically examined by ellipsometry, tensiometry,  
138 optical microscopy, profilometry or atomic force microscopy (Figure 1B). Whatever the  
139 formulations studied, thickness and wettability of chitosan layers were highly reproducible (e.g.  
140  $23.3 \pm 1.3$  nm, and  $37.8 \pm 1.2^\circ$ , for chitosan formulation of DA 55%,  $[\text{c}] = 0.67\%$ ,  $M_w = 180$   
141 kg/mol, with  $n = 10$ ), with a Root Mean Square (RMS) roughness lower than 1 nm. The detailed  
142 information on the physicochemical properties of the chitosan thin films will be described in a  
143 specialized dedicated publication.

144

145 We thus successfully generated homogeneous CCS of known polymer molar mass, DA and  
146 thickness. By varying the chitosan macromolecular parameters and chitosan solution  
147 characteristics, more than 50 different chitosan coatings were thus prepared to be screened for  
148 their ability to support bacterial proliferation.

149

150

## 151 **Specific chitosan polymers promote adhesion and normal growth of *E. coli* cells**

152 We next tested the ability of the various types of CCS to support the adhesion and ultimately  
153 growth of the main laboratory *E. coli* K12 strain. To perform this screening, we divided our  
154 CCS library in nine representative subclasses, based on source, DA and additional treatments  
155 (Table 1). Each CCS type was then mounted at the bottom of a microfluidic cassette and tested  
156 for *E. coli* adhesion and growth (see Methods).

157 We found that while LB-grown *E. coli* cells did not adhere to uncoated glass slides, they  
158 adhered to all CCS types, showing that chitosan can indeed promote adhesion of *E. coli*.  
159 However, while *E. coli* cells did generally proliferate on these surfaces, growth was frequently  
160 abnormal, evidenced by cell filamentation and morphological aberrations (Figure S2A).  
161 Nevertheless, one type of CCS obtained with chitosan polymers of DA 55%,  $M_w$  of 156 kDa  
162 and thickness of 32 nm supported normal growth (C5, Figure 2A, Table 1). To further  
163 characterize this chitosan class, we tested 156 kDa polymers of varying DAs and found that  
164 DAs  $\geq 50\%$  were required for biocompatibility (Table 1). In addition, formulation was important  
165 because acid rinsing negatively impacted the biocompatibility of the procedure (Table 1).

166 We next characterized the ability of C5 to promote adhesion and growth in detail. *E. coli* K12  
167 formed mono-layered micro-colonies and could be monitored for up to 6 generations after  
168 which the cells started growing above the focal plane defined by the glass slide. Expansion of  
169 the *E. coli* micro-colony in 3D could occur because tight adhesion of the monolayer forced the  
170 daughter cells to grow away from the immediate surface, which has been shown to act as driving  
171 force for bacterial colony and biofilm development (15). To test this possibility, we analyzed  
172 *E. coli* cell adhesion to C5 by Reflection Interference Contrast Microscopy (RICM), a technique  
173 that allows imaging of intimate cell contacts with glass surfaces (16). RICM revealed that each  
174 cell remained in close contact with the glass surface by adhering along their axis. Surface escape  
175 was due to steric constraints and vertical growth of bacteria adhered via their cell poles (Figure  
176 2B, Movie S2). In contrast, when we performed RICM on a CCS type that created abnormal  
177 cell torsions, it was apparent that the dividing cells only adhered via the cell poles, explaining  
178 cell detachment and the emergence of torsions (Figure S2B). Consistent with the RICM results,  
179 *E. coli* cells remained attached to C5 event when subjected to shear stress of up to  $12 \text{ dyn/cm}^2$   
180 (which is comparable to shear stress generated in aorta (17), see Methods).

181 We next tested whether C5 created detectable stress on K12 *E. coli* growth. *E. coli* K12 cells  
182 grew exponentially with a generation time similar to the generation of *E. coli* grown under  
183 agitation in liquid culture at room temperature (Figure 2C, C5 experiments were conducted at  
184  $25^\circ\text{C}$ . C5 also supported growth of *E. coli* at  $37^\circ\text{C}$  but all described experiments were performed  
185 at  $25^\circ\text{C}$  to avoid the use of a thermo-controller system). Cell morphology, measured by the  
186 aspect ratio (length/width) remained stable over time, showing that it was not affected on C5  
187 (Figure 2D). Last, to test whether C5 generates long term cellular defects, we allowed *E. coli*  
188 cells to develop on C5 until they reached stationary phase and became quiescent for 3 days.  
189 These cells resumed growth normally after fresh medium was injected, showing that long term  
190 exposure to C5 does not affect cell viability (Figure S2C). We conclude that C5 is a well-  
191 adapted chitosan to grow *E. coli* K12 cells on glass surface in microfluidics chambers.

192

193

## 194 **CCS allow fast Antibiotics Susceptibility Testing (AST)**

195 Beyond their obvious use in research applications, CCS could provide a fast and reliable tool  
196 for AST. For this, CCS should be significantly faster and at least as reliable as currently used  
197 methods. To test this, we incubated *E. coli* K12 on C5 and injected Ampicillin, which rapidly  
198 resulted in the typical cell elongation and formation of a bulge in the septal zone that precludes  
199 cell lysis (Figure 3A, Movie S3). The approximate time-to-death was  $\sim 120 \text{ min}$  (Td), consistent

200 with the kinetics described in other single cell experiments (18). On C5, Ampicillin generates  
201 the same cellular defects as in other studies and could thus be used for AST.

202

203 We next tested whether antibiotic susceptibility may be determined in less time than the  
204 measured Td (as detected by irreversible cell lysis). Indeed, although *E. coli* cells lyse after  
205 2hrs, the action of ampicillin is first characterized by abnormal cell elongation (Figure 3A).  
206 Thus, early detection of abnormal cell morphologies would provide a fast method to assess the  
207 action of Ampicillin. To do this reliably and computationally, we designed a machine-learning  
208 based morphometric method that discriminates abnormal cell morphologies from WT cell  
209 morphologies and detects the effect of antibiotics at different treatment times (Figure S3A-C  
210 and Methods). Briefly, following segmentation and determination of cell contours, this method  
211 allows the direct counting of cells with normal morphologies and thus the determination of  
212 growth curves. This approach could readily determine growth curves of an *E. coli* strain isolated  
213 from a urinary tract infection and treated with increasing doses of Ertapenem, a relatively large-  
214 spectrum Carbapenem standardly used at the hospital (UTI227, Figure 3B). Lethal Ertapenem  
215 effects could be detected as early as 50 minutes after addition of the antibiotic with 95%  
216 confidence (Figure 3B-3C). Thus, combined with our computational detection method, CCS,  
217 and here specifically C5, is a promising tool for fast AST.

218

#### 219 **CCS can be used to measure the Minimal Inhibitory Concentration of clinical *E. coli*** 220 **isolates**

221 To test the potential clinical application of CCS more broadly, we next obtained a collection of  
222 15 clinical isolates derived from Urinary Tract (UTIs, 14 isolates) and Intestinal Tract  
223 Infections (ITI, 1 isolate) and tested their ability to grow on C5. We determined that 70% of the  
224 clinical strains adhered and grew normally on C5 but that this number could be improved to  
225 85%, if the thickness of C5 were increased to 66 nm (Table 1), showing that thickness is another  
226 important parameter to increase the application spectrum C5 to most *E. coli* clinical strains.

227 In current clinical practice, the antibiotic susceptibility of a given bacterial strain is determined  
228 by its so-called Minimum Inhibitory Concentrations (MIC), which corresponds to the lowest  
229 antibiotic concentration that prevents growth. To test if MICs determined on C5 can be directly  
230 compared to MICs determined by standard methods, we further selected two clinical strains of  
231 known MICs (as determined by Vitek2, Biomérieux) for Mecillinam (UTI704 MIC=2) and  
232 Ertapenem (UTI227 MIC $\leq$ 0.5) and measured their MICs on C5, extracting growth rates with  
233 our computational methods (Figure 3D). In both cases, the results showed remarkable  
234 consistency with the Vitek method and in fact, the CCS method was more sensitive allowing to  
235 determine that the UTI227 Ertapenem MIC is between 0.01 and 0.05 mg/ml (Figure 3D, Table  
236 2). To further test the validity of the method, we tested the consistency of the measurements  
237 over various range of Ertapenem concentrations for UTI227 and showed that its Mecillinam  
238 MIC on C5 also matches the Vitek-determined MIC (Table 2). Further MIC measurements on  
239 additional clinical strains UTI687 and UTI698 on Ofloxacin and Mecillinam, respectively also  
240 showed good consistency with Vitek measures (Table 2). In conclusion, CCS appears a  
241 promising tool to measure MICs rapidly and accurately in hospitals.

242

243

#### 244 **CCS can be used to measure the Minimal Bactericidal Concentration of antibiotics**

245 The MIC does not measure microbial death *per se* and thus it cannot distinguish bactericidal  
246 from bacteriostatic effects. This can be problematic for treatment, especially since it was  
247 discovered that antibiotic treatment can induce bacterial persistence, a seemingly dormant state  
248 that could be associated with chronic infections (19). Using the Minimum Bactericidal  
249 Concentration (MBC), the lowest antibiotic concentration resulting in bacterial death, would in

250 general be more appropriate but it is a highly time-consuming procedure because it requires re-  
251 growing the bacteria after antibiotic treatment. However, as we show above, the CCS  
252 technology allows direct observation of *E. coli* cell lysis in the presence of Ampicillin and is  
253 therefore a potential tool to determine the MBC of an antibiotic directly and rapidly (Figure  
254 3A). In addition, the microfluidic environment of CCS allows detection of cell death, for  
255 example using dyes such as Propidium Iodide (PI) that only bind the bacterial DNA if the  
256 bacterial membrane is irreversibly altered. The use of PI can improve detection sensitivity,  
257 especially if the detection method is automated. Indeed, addition of PI on *E. coli* treated-  
258 Ertapenem allowed cell death detection on C5, suggesting that this method could be used to  
259 determine MBCs in clinical contexts (Figure 3E, Movie S4).

260  
261

### 262 **CCS can be adapted to promote surface growth of *Klebsiella pneumoniae***

263 Next, we were interested in testing whether C5 could be useful to study other clinically-relevant  
264 pathogens. In particular, and along with UTI *E. coli* strains, *K. pneumoniae* is a member of the  
265 so-called ESKAPE pathogens, characterized by the high resistance of clinical strains to  
266 antimicrobial compounds and thus a growing concern in hospital environments (10). Indeed, *K.*  
267 *pneumoniae* could readily grow on C5 (but at 66 nm thickness), with normal morphology and  
268 generation time (~40 min, Figure 4A-B, Movie S5). Thus, C5 is a versatile substratum for  
269 bacterial adhesion, and could be used in hospitals for AST of ESKAPE pathogens.

270 We next wondered if additional “*Klebsiella*-compatible” chitosans could be identified. As  
271 discussed above, most tested chitosan polymers are not compatible with *E. coli* K12 and it could  
272 be interesting to identify “species-specific” polymers for AST. To do this, we further screened  
273 the Chito-library and successfully identified one additional CCS type, C11 (DA of 35 %, Mw  
274 of 557 kDa and thickness of 101 nm) that also supported *Klebsiella* adhesion and growth  
275 without detectable effect on bacterial fitness (Figure 4B, Figure S4). Importantly, C11 did not  
276 support growth of *E. coli*. In total, the results suggest that CCS is adaptable to the study of  
277 multiple bacterial species and that depending on their chemical structure chitosan substrates  
278 can either be derived to support adhesion and growth of multiple bacterial species or more  
279 specifically, to grow a given bacterial species or even perhaps strain.

280

### 281 **Discussion**

282 In this work, we report a new glass functionalization procedure that supports bacterial adhesion  
283 and growth without any detectable physiological stress, contrarily to currently used  
284 polycationic polymers such as Poly-lysine. This technique allows studies of bacteria at the  
285 single cell level in simple microfluidic devices without the need of complex lithography or  
286 alternative physical immobilization techniques. Because the chemistry of the chitosan polymers  
287 and glass functionalization procedures are well established, the method is robust and highly  
288 reproducible. Moreover, CCS are long-lived and their integrity is not altered after storage of up  
289 to 6 months. Thus, CCS are highly versatile and provide a viable alternative to other and often  
290 more technically challenging microfluidic single cell approaches.

291

292 Although we characterized one CCS type in details and showed its potential for studies of *E.*  
293 *coli* and *K. pneumoniae* for basic and clinical purposes, we also show here that CCS can be  
294 derived for the studies of multiple strains and species, in particular ESKAPE pathogens. We  
295 also performed preliminary tests of the ability of C5 in promoting growth of a wide range of  
296 Gram negative and Gram positive bacteria. In our hands, C5 supported growth of *Vibrio*  
297 *cholera*, *Myxococcus xanthus*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa* (C5 also  
298 supported *Pseudomonas* twitching motility) but cell adhesion for these species was arguably  
299 not optimal. Nevertheless, CCS could be optimized for these species by testing different C5

300 thicknesses or alternatively, by isolating other CCS-types as we performed for *Klebsiella*  
301 *pneumoniae*.

302 The variable effects of chitosan between species and even within species is not too surprising  
303 because the biological properties of chitosan can vary widely based on composition and  
304 formulation. For example, chitosan polymers of large size (> 550 kDa) and high degree of  
305 acetylation (>50%) are known to exert bacteriostatic effects on some bacteria (11). In addition,  
306 adhesion likely depends on the surface properties of the bacteria. In *E. coli*, phenotypic and  
307 genotypic diversity is very wide (20) and thus it is possible that some isolates fail to adhere  
308 (albeit a minority) because they have different surface properties (for example if they carry  
309 particular LPS O-antigens). An interesting avenue for future developments will be to test  
310 whether composite CCS made from several chitosan polymers increase the array of species and  
311 strains that may be grown on a single type of slides.

312  
313 The applications of CCS in the field of bacterial cell biology are evident as such technology  
314 supports studies of any cellular processes, cell division, but also perhaps for studies of more  
315 complex population structures such as micro-colonies, biofilms and communities. Using a  
316 collection of *E. coli* strains, we typically observe that the bacteria first proliferate in two  
317 dimensions which we have shown by RICM occurs due to tight adhesion. The bacteria  
318 eventually proliferate away from the surface when space becomes a limiting factor for  
319 proliferation (Figure 2B). However, we also observed that some *E. coli* strains colonize the  
320 entire surface in 2D and thus form a single layer biofilm (Figure S5, Movie S6). The formation  
321 of *E. coli* micro-colonies on a surface has been shown to depend both on adhesion strength and  
322 preferential adhesion of the polar regions (which we also observe here, Figure 2B, (21)). Thus,  
323 it is likely that expanded micro-colonies are obtained depending on adhesion strength.  
324 Screening conditions that support biofilm formation for a particular strain could be achieved by  
325 defining a compatible adhesion range, either by modulating the ionic strength of the medium  
326 and/or changing the chitosan thickness, molar mass and DA.

327  
328 The search for rapid phenotypic assays to determine antibiotics susceptibility is now a global  
329 priority to save on the use of large spectrum antibiotics and limit the spread of multiple  
330 antibiotics resistance in hospitals (22). In current clinical practice, AST is generally performed  
331 using semi-automated methods that measure growth in bulk cultures in liquid (i.e. VITEK, (23))  
332 or solid media. These methods only yield MICs estimate and the more accurate methods (i.e.  
333 antibiotic gradients or E-tests (24)) are time-consuming and costly. Moreover, all of these  
334 phenotypic antibiotic susceptibility testing require from 18 to 24 hours to provide an estimate  
335 of antibiotic susceptibility. Single cell microscopy approaches are powerful alternatives  
336 because they measure MIC as well as MCBs directly, more precisely and sometimes in less  
337 than 30 min, for example in microchannel chips (2, 25). This technology however suffers from  
338 important drawbacks linked to sophisticated manipulation and high species-specific use,  
339 making its generalization in clinical practice difficult. Also, this method precludes  
340 morphometry analysis because the bacteria are maintained in channels that directly constrain  
341 their shape. Direct morphometry analysis for rapid AST has shown promising results on  
342 bacteria embedded in agarose (3). However, in this case, the antibiotics were added indirectly  
343 by diffusion through the agarose making it difficult to control the exact concentrations and  
344 potentially slowing their action. In this context, CCS could provide an interesting alternative as  
345 we have shown that it can be applied reliably for two major ESKAPE pathogens and it combines  
346 the advantages of both above approaches, allowing direct antibiotic injection and morphometric  
347 analyses. The CCS method is more sensitive and ~10-20 times faster than traditional plate  
348 assays (here 50 min). A machine-learning based computational approach appears promising to  
349 measure MICs in automated fashion. The method still needs testing at higher throughput, but

350 the results establish a proof of principle that its application for MIC determination is feasible.  
351 In addition, one system that could exploit it directly, the so-called Accelerate Pheno System  
352 (APS, Accelerate Diagnostics) is currently being implemented in hospitals (26, 27). Similar to  
353 CCS, APS relies on surface immobilization of bacteria on glass slides in microfluidic channels.  
354 However, in absence of more adapted coating, the APS uses poly-lysine and Indium Tin Oxide  
355 (ITO) facilitated gel electro-filtration to immobilize bacteria (US Patent N°7341841B2). It is  
356 therefore likely that this procedure perturbs the bacterial cell envelope, affecting the proton-  
357 motive force (6–8) and thus downstream, the accurate measurement of MICs. Indeed, lowering  
358 the proton motive force can artificially result in increased antibiotic resistance for some classes  
359 of antibiotics and thus generate false results (9). In the future, adapting CCS to support growth  
360 of most clinical pathogens could make this technology an interesting tool for AST.

361

## 362 **Material and Methods**

363

### 364 **Materials**

365 Chitosans with low degrees of acetylation (DA) and different molar masses ( $M_w$ ) were  
366 purchased from Mahtani Co.ltd: a medium molar mass CS (CS<sub>156</sub>: DA 1.0%;  $M_w$  = 156.1  
367 kg/mol,  $D$  = 1.78, batch 243)) and a high molar mass CS (CS<sub>557</sub>: DA 2.4%;  $M_w$  = 557.2 kg/mol,  
368  $D$  = 1.39, , batch 114). They were reacylated to DA ranging from 1 to 80% using a procedure  
369 previously described.(21) Acetic acid (AcOH), hydrogen peroxide (40% w/w), sulfuric acid  
370 (96% w/w), hydrochloric acid (HCl, 37%), propan-1, 2-diol and ammonium hydroxide were  
371 purchased from Sigma Aldrich. Sterile and non-pyrogenic water was purchased from Otec®.  
372 Silicon wafers (doped-P bore, orientation (100)) were purchased from Siltronix® and glass  
373 coverslips (75 x 25 x 0.17 mm<sup>3</sup> #1.5H D263 Schott glass) from Ibidi.

374

### 375 **Chitosan preparation**

376 CS was subjected to filtrations in order to remove insolubles and impurities before any use. CS  
377 was first solubilized in an AcOH aqueous solution, followed by successive filtrations through  
378 cellulose membrane (Millipore®) with pore sizes ranging from 3 µm to 0.22 µm. CS was then  
379 precipitated with ammonium hydroxide and washed by centrifugation with deionized water  
380 until a neutral pH was obtained. The purified CS was finally lyophilized and stored at room  
381 temperature.

382 In order to investigate the effect of DA on the film properties, CS with various DA were  
383 prepared by chemical modification using acetic anhydride, for both CS of different molar  
384 masses (14). CS was first dissolved in an AcOH aqueous solution (1% w/w) overnight. A  
385 mixture of acetic anhydride and 1,2-propanediol was then added dropwise in the CS solution  
386 for at least 12 h under mechanical stirring. The amount of acetic anhydride added was calculated  
387 according to the DA aimed. The final solution was finally washed and lyophilized in the same  
388 manner as after the filtration step. The DA of the different CS prepared was determined by <sup>1</sup>H  
389 NMR (Bruker Advance III, 400 MHz). For CS<sub>156</sub>, the DA obtained are: 9.0 %, 14.5 %, 25.6 %,  
390 35.3 %, 41.9 % and 52.2 %. DAs close to those obtained for CS<sub>156</sub> were obtained for CS<sub>557</sub>: 8.0  
391 %, 12.2 %, 21.5 %, 34.0 %, 45.3 % and 52.6 %.

392

### 393 **Film preparation**

394 Silicon substrates and glass coverslips were cleaned from organic pollution using a piranha bath  
395 (H<sub>2</sub>SO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub>, 7/3 v/v) heated at 150 °C for 15 min, and then rinsed with deionized water  
396 (resistivity of 18 MΩ.cm). They were then subject to ultra-sonication in deionized water for 15  
397 min and dried under a flux of clean air. The substrates (glass or silicon) were then placed into  
398 a plasma cleaner (Harrick Plasma®) for 15 min in order to generate the silanol groups at the  
399 surface for a better adsorption of CS polymer chains.



400 In the meantime, CS was solubilized overnight in a solution of deionized water (Otec<sup>®</sup>) with  
401 AcOH, under magnetic stirring and at room temperature. The amount of acid added was  
402 calculated in stoichiometry compared to amine groups available along the CS polymer chain.  
403 CS solutions with different concentrations ranging from 0.3% to 1% for CS<sub>557</sub> and  
404 concentrations ranging from 0.5% to 2% for CS<sub>156</sub> were investigated in this study.  
405 The films were finally formed onto silicon substrate by spin-coating at 2000 rpm until the  
406 solvent evaporates completely (5 min). After spin-coating, films were stored 24 h at room  
407 temperature before being characterized (unless mentioned otherwise). Some of the films were  
408 finally rinsed in AcOH aqueous solution (pH 4) for 5 min so that only the adsorbed chains of  
409 chitosan remain on the sample; the samples were then immersed in a water bath and finally  
410 dried under a flux of clean air (thickness < 3 nm in all cases).

411

#### 412 ***Surface topography***

413 *AFM.* The surface morphologies were carried out by atomic force microscopy (AFM) (CSI  
414 Nano-observer). AFM probes with spring rate close to 40 N/m were purchased from Bruker.  
415 The AFM images were processed using Gwyddion software.

416

#### 417 ***Thickness measurement***

418 The film thickness was measured on the silicon wafers using spectroscopic ellipsometry. On  
419 glass coverslips, the measurements were carried out using profilometry on scratched films. The  
420 consistency of the results obtained by ellipsometry or using profilometer profiles independently  
421 of the substrate used for a given chitosan solution permitted to use the measurement by  
422 ellipsometry as reference.

423 The ellipsometer (SOPRA GES-5E) was set at an incident angle of 70°, very close to the silicon  
424 Brewster angle. At least three measurements were done on each film at different positions in  
425 order to verify the film homogeneity. Data were then processed using WINELLI (Sopra-SA)  
426 software. A Cauchy model was used to fit experimental data ( $\cos A$ ,  $\tan \Psi$ ), in the spectral range  
427 of 2.0-4.5 eV, depending on fits and regression qualities, to evaluate the thickness. The UV  
428 parameters  $A$  and  $B$  were respectively set to 1.53 and 0.002.

429 A mechanical profilometer (Veeco Instruments) equipped with a cantilever of 2.5  $\mu\text{m}$  in  
430 diameter was used to measure film thickness on glass coverslips. For this purpose, the samples  
431 were previously scratched by tweezer to locally remove CS film. Data analysis was performed  
432 with VISION V4.10 software from Veeco Instruments.

433

#### 434 ***Wetting measurements***

435 Contact angles were measured using a tensiometer (Easydrop, Kruss) kit out with a camera  
436 connected to a computer equipped with a drop shape analysis software. To put down the liquids  
437 drop on the surface, a Hamilton syringe of 1 mL and a needle of 0.5 mm of diameter were used.  
438 “Static” measurements correspond to the angle determined 10 seconds after water drop  
439 deposition.

440

#### 441 ***Strains, Cell Cultures and Media preparation***

442 Strains used were either lab strains (K12) or clinical strains obtained from the Laboratoire de  
443 Biologie - Centre Hospitalier Martigues.

444 *E. coli* and *K. Pneumoniae* cells were grown in ion-adjusted Luria-Bertani (LB) medium until  
445 exponential phase ( $\text{OD} = 0.5 \pm 0.1$ ) and diluted in LB to an OD around 0.01. The LB media  
446 was prepared using 10 g/L bacto-casitone (BD, 225930), 5 g/L NaCl (Biosciences, RC-093), 5  
447 g/L bacto Yeast extract (BD, 212750) and osmosed water supplemented with 0.46  $\mu\text{g/L}$   $\text{MgCl}_2$ ,  
448 3.21  $\mu\text{g/L}$   $\text{CaCl}_2$ , 5.02  $\mu\text{g/L}$   $\text{ZnCl}_2$  and 6.15  $\mu\text{g/L}$  KCl. The cell suspension was then directly

449 added to microfluidic channels. Loaded microfluidic chambers were centrifuged 3 min at 1000  
450 rcf (Eppendorf centrifuge 5430R) to maximize cell adhesion.

451

#### 452 ***Preparation of chitosan slides and microfluidic chambers***

453 Microfluidic channels were prepared from commercially-available six channel systems (sticky-  
454 Slide VI 0.4, IBIDI) that were directly applied to the surface of chitosan-coated slides. The  
455 dried chitosan was rehydrated by addition de-ionised MilliQ water for at least 5 min.

456 After centrifugation, the microfluidic channels were connected to a syringe and a pump  
457 (Aladdin syringe Pump WPI). Remaining non-adherent cells were thus removed trough a rinse  
458 step: 1.5 ml rinse with a 1.5ml/min flow followed by 1.5 ml with 5ml/min flow. The work flow  
459 was set at 3ml/h. Adhesion strength was assessed by increasing the flow in the channel. The  
460 shear stress was calculated by the following formula, given by IBIDI:  $\tau = \eta \cdot 176.1 \cdot \Phi$  were  $\tau$  is  
461 the shear stress (dyn/cm<sup>2</sup>),  $\eta$  the dynamical viscosity (dyn.s/cm<sup>2</sup>) and  $\Phi$  the flow rate (ml/min).  
462 In absence of data about LB dynamical viscosity, we hypothesize that it is close to cell culture  
463 medium which is around 0.0072 dyn.s/cm<sup>2</sup>. On C5, adhered cells resisted shear forces above  
464 12.3 dyn/cm<sup>2</sup>, indicating that they were firmly adhered.

465

#### 466 ***Dyes, Antibiotics treatment and MIC determination***

467 Propidium iodide (PI) is used as a DNA stain that cannot cross the membrane of live cells,  
468 making it useful to differentiate healthy cells from dead cells. *E. coli* cells were immobilized to  
469 C5 chitosan on microfluidic chamber in presence or absence of antibiotics. Immediately before  
470 acquisition, the channel was rinsed with LB supplemented with 3mg/L Ertapenem (Sigma  
471 Aldrich) and 50 $\mu$ L/ml PI (Sigma Aldrich, P4170).

472 For MIC determination different channels were prepared simultaneously with the same cell  
473 suspension. Antibiotics Ertapenem, Ampicillin (Sigma Aldrich), Mecillinam (Sigma Aldrich)  
474 and Oxofloxacin (Sigma Aldrich) were prepared at different concentrations (one channel  
475 contained only LB as a control) and added to each channel just before image acquisition (every  
476 3 min for standard acquisition). The MIC was defined for the lowest antibiotic concentration  
477 that induced cell death/stasis.

478

#### 479 ***Microscope acquisition and Image manipulation***

480 Images were acquired with a Nikon phase contrast microscope (TE2000) equipped with a  
481 motorized stage, a Nikon perfect focus system and 100X objective lens. For technical  
482 convenience, experiments were performed at 25°C, a condition that supported both *E. coli* and  
483 *K. pneumoniae* growth. Standard Image analysis were performed under MicrobeJ a Fiji-Plugin  
484 developed for the analysis of bacteria (28).

485

#### 486 ***Reflection Interference Contrast Microscopy (RICM)***

487 RICM was performed with a Zeiss Observer inverted microscope (Carl Zeiss, Jena, Germany)  
488 equipped with a Zeiss Neofluar 63/1.25 antilex objective, a crossed-polarizers cube, and a  
489 C7780 camera (Hamamatsu, Tokyo, Japan) with an adjustable field and aperture stops. The  
490 source was an X-cite 120Q lamp (Exfo, Mississauga, Canada) coupled to a narrow bandpass  
491 filter ( $\lambda=546\text{nm}\pm 12\text{ nm}$ ).

492

#### 493 ***Image segmentation***

494 Image segmentation procedures were developed in Python. In order to provide a streamlined  
495 analysis procedure, we used the parameter-free threshold setting algorithm “iso\_data” from the  
496 scikit-image python package (29) to extract the contours of the bacterial cells.

497 For each contour, we then perform a singular value decomposition from the numpy library (30)  
498 to retrieve aligned and centered contours for each bacteria. We use defect analysis (provided

499 by the opencv library) to detect the septum and split the contours. If the defects attributed to the  
500 septum are distant of less than 0.5  $\mu\text{m}$  and their center is less than 0.3  $\mu\text{m}$  from the cell center,  
501 the contour is considered to be composed of two cells, and is therefore split.

502 From the detected and split contours, we then extract relevant morphometric data:

- 503 • the contour area,
- 504 • the contour length or perimeter,
- 505 • the longer of the min area rectangle,
- 506 • the width of the min area rectangle,
- 507 • the circularity defined as  $4\pi A/\ell^2$ . It is equal to 1 if, the contour is perfectly circular, lower  
508 than 1 otherwise,
- 509 • the inverse of the aspect ratio of the enclosing rectangle, (width/length), always lower than  
510 1,
- 511 • the ratio of the minimal rectangle area to the cell area, (which should be close to one for a  
512 wild-type rod-shaped cell).

513

### 514 **Image annotation and training**

515 In order to constitute a training-set to apply supervised machine learning, we developed a web-  
516 based dashboard based on plotly-dash toolset (<http://plot.ly/dash>). The annotation tool allows  
517 classifying the detected contours in 5 categories: normal, divided, abnormal, dead and invalid.  
518 We annotated 7 assays corresponding to 8300 contours.

519

### 520 **Outlier detection**

521 From the annotated contours, those marked as "normal" were used to train a single class scalable  
522 vector machine classifier provided by the scikit-learn library (31) More precisely we fit a  
523 OneClassSVM object over 75% of the annotated data and use the remaining 25% over the above  
524 defined morphometric data. The trained classifier is then used on all the detected data to remove  
525 invalid contours from the count on each image.

526

### 527 **Sensitivity criterion**

528 For each assay, the growth rate  $G$  is computed by performing a linear regression of the logarithm  
529 of the number of detected bacteria versus time.

530

$$531 \quad N(t) = N_0 2^{t/\delta_t} \Leftrightarrow \log_2 N(t) = \log_2 N_0 + t/\delta_t$$

532 The reported error is the 95% confidence interval. We use the `scipy.stats.theilslopes`  
533 method (32) to perform the linear regression. A given growth assay is considered to survive if  
534 the growth rate of  $0.2 \text{ h}^{-1}$ . This corresponds to a doubling time  $\delta = \ln(2)/G$  lower than 200  
535 min. This cut off was chosen as it is longer than the microscopy acquisition span (Figure S3D).

536

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629

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634

### 635 **Disclosure of competing interests**

636 The chitosan polymer characterization, coating procedure and applications is being patented in  
637 “A film of chitosan and a device comprising the same deposited on a substrate and uses thereof”  
638 Priority European patent application: EP18305666.2 (30/05/2018)

639

### 640 **Figure Legends**

641

#### 642 **Figure 1. Functionalization of glass slides with Chitosan polymers**

643 **(a)** Protocol for glass surface modification and characterization of chitosan layers (See Methods  
644 for details).

645 **(b)** Chemical characterization of Chitosan thin films. Thin films are characterized by wetting  
646 measurements, morphology imaging (AFM) and their thickness was determined by  
647 ellipsometry (See Methods for details).

648

649 **Associated Supplemental Figure S1.**

650 (a) Procedure for chitosan purification and preparation (See Methods for details).

651 (b) Photographs of a silicon wafer before and after surface modification. In this example the  
652 wafer was covered with a chitosan solution of concentration 0.67%, DA 25% and  $M_w = 180$   
653 kg/mol. The layer shows high homogeneity.

654

655 **Figure 2. Growth of *E. coli* on selected C5 Chitosan slides.**

656 (a) Growth of *E. coli* K12 on C5. Shown are snapshots separated by 54 min after growth  
657 initiation (left panel). See associated Supplemental movie S1 for the full time-lapse. Scale bar  
658 = 2  $\mu\text{m}$ .

659 (b) Adhesion of *E. coli* on C5 as measured by Reflection Interference Contrast Microscopy  
660 (RICM). *E. coli* is shown by Nomarski (DIC, left) revealing the three-dimensional organization  
661 of the *E. coli* micro-colony and by RICM to reveal the adhesion sites (observed as dark areas,  
662 right). Note that the cells remain tightly adhered to the Chitosan surface even at the latest time  
663 points when the micro-colony clearly expands above the focal plane. White arrows point to  
664 areas where the cells remain adhered by the cell pole only, allowing them to grow away from  
665 the Chitosan surface. See associated Supplemental movie S2 for the full time-lapse. Scale bar  
666 = 2  $\mu\text{m}$ .

667 (c) Growth of *E. coli* K12 on C5. Shown is an exponential fit of the number of cells as a function  
668 of time.

669 (d) Morphology of *E. coli* on C5 over time. The aspect ratio are determined from phase contrast  
670 images of adhered cells and correspond to the ratio between the lengths of the long axis and the  
671 short axis of the cell.

672

673 **Associated Supplemental Figure S2.**

674 (a) Morphological aberrations on Chitosan. Shown is *E. coli* K12 growth on C8 (Table 1). Note  
675 that the cells show abnormal twisted filaments that gradually detach over time. Scale bar = 2  
676  $\mu\text{m}$ .

677 (b) RICM of *E. coli* on torsion-inducing chitosan. On C8, RICM reveals that *E. coli* K12 cells  
678 are mostly attached by the cell poles, explaining their twisted shape due to the combined growth  
679 and local adhesion points. Scale bar = 4  $\mu\text{m}$ .

680 (c) C5 shows no long-term toxicity. Shown are *E. coli* K12 cells that resume growth after being  
681 left for three days in exhausted medium and addition of fresh medium. Cell growth resumes  
682 normally. Scale bar = 8  $\mu\text{m}$ .

683

684 **Figure 3. C5-CCS can be used for fast AST of *E. coli* clinical strains**

685 (a) Ampicillin treatment is effective on C5. Note the characteristic Ampicillin-induced  
686 morphological transitions, cell elongation and the formation of a septal lytic “bubble” that  
687 precludes cell death. See associated supplemental movie S3 for the full-time lapse.

688 (b) Trained detection of Ertapenem effects on growth of *E. coli* clinical strains. Measured  
689 growth curves for strain UTI227 with varying concentrations of ETP. Fitted growth curves  
690 computed from the number of detected cells across time are color-coded with respect to ETP  
691 concentration. For each curve, the plot symbol is circular if the cells survive, and diamond  
692 shaped if the cell population stalls or shrinks due to cell death.

693 (c) Estimation of the minimal diagnostic time. We performed an estimation of the growth rate  
694 for varying time spans for all assays and determined for each time span the fraction of assays  
695 for which the response could be ascertained with a 95% confidence interval.

696 (d) Comparison of the MICs as measured on CCS with MICs obtained at the hospital. The  
697 MIC is determined for growth rates  $\leq 0$  obtained at given antibiotics concentrations. Note that

698 the hospital (Vitek) and CCS-determined MICs for Mecillinam are similar for UTI704, but that  
699 the CCS method measures MICs as low 0.05 for UTI227 in the presence of Ertapenem.  
700 (e) Detection of cell death by Propidium-Iodide (PI) staining. PI only stains the bacterial DNA  
701 of permeable dead cells, which fast and sensitive quantification of MBCs. See associated  
702 supplemental movie S4 for a typical time lapse.

703

704

705

706 **Associated Supplemental Figure S3.**

707 (a) Cell contour detection of a dividing bacterium after imaging by 100X Phase Contrast  
708 microscopy.

709 (b) Quantification of the extracted contour. The orange outline reveals the convex Hull of the  
710 detected contour. The red and gray dots are convexity defects used to detect the septum. Both  
711 red dots are automatically assigned to the septum. Further quantifications were based on the  
712 ratio of the area corresponding to the bacterial contour to the area of the minimal rectangle  
713 encompassing this contour (rectangular fill).

714 (c) Detection training of abnormal (and thus antibiotic sensitive) cells. Shown is an outlier  
715 detection obtained with the Scikit-learn one class scalable vector machine from the annotated  
716 dataset. For clarity, we only display the data corresponding to two dimensions, circularity and  
717 rectangular fill (see Methods for other parameters). Blue symbols: classified as normal; red  
718 symbols: classified as abnormal. Overall, there is a 10% rate of false negatives (normal contours  
719 labeled as abnormal) in both the training and test sets. This performance induces a noise in the  
720 growth curves and globally results in a 10% uncertainty in the computed growth rate. This  
721 uncertainty (which could be reduced with enhanced segmentation procedures) does not  
722 significantly affect the accuracy of MIC measurements.

723

724 **Figure 4. *Klebsiella pneumoniae* grows on CCS**

725 (a) Growth of *Klebsiella pneumoniae* on C5. Shown are snapshots separated by 30 min after  
726 growth initiation (left panel). See associated Supplemental movie S5 for the full time-lapse.

727 (b) Growth of *Klebsiella pneumoniae* on C5 and C11. Number of cells as function of time and  
728 corresponding exponential fits are shown.

729

730 **Associated Supplemental Figure S4:**

731 Growth of *Klebsiella pneumoniae* on C11. Shown are snapshots separated by 30 min after  
732 growth initiation (left panel). Scale bar = 2  $\mu$ m.

733

734 **Associated Supplemental Figure S5: Single layer colonization of C5 by *E. coli* clinical  
735 strains.** Shown is the monolayer development of UTI698. Time points are separated by 54 min.  
736 Scale bar = 2  $\mu$ m. See also associated movie S6

737

738

739 **Tables**

740

741 **Table 1. chitosan types and Adhesion and proliferation of *E. coli* K12**

742

743 **Table 2. MIC determination in clinical strains**

744

745 **Supplemental movies:**

746 Movie S1: Growth of *E. coli* K12 on C5

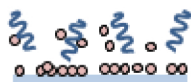
747 Movie S2: RICM of *E. coli* K12 on C5

- 748 Movie S3: *E. coli* K12 in the presence of Ampicillin
- 749 Movie S4: *E. coli* UTI 227 in the presence of Ertapenem and PI
- 750 Movie S5: *K. Pneumoniae* growth on C5
- 751 Movie S6: *E. coli* monolayers on C5



**a***Purified chitosan**Solubilization in water*

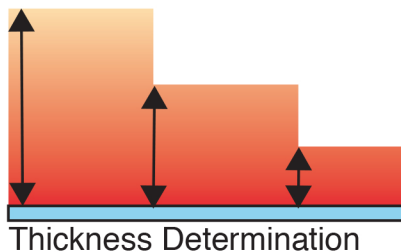
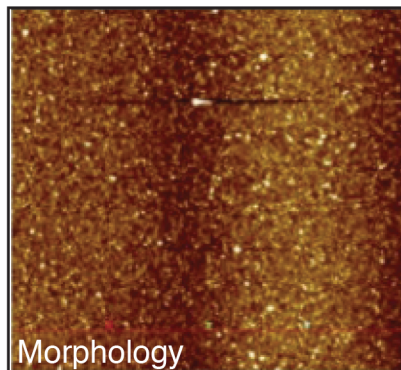
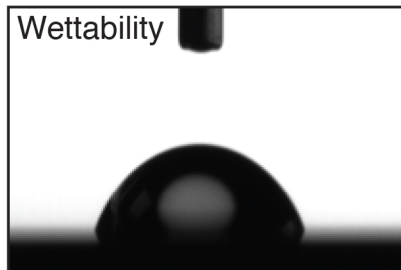
Water adjusted to pH 4

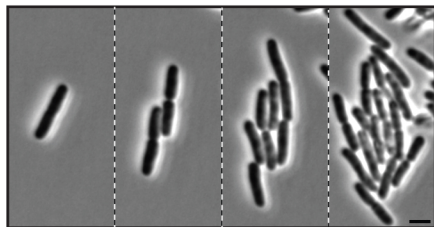
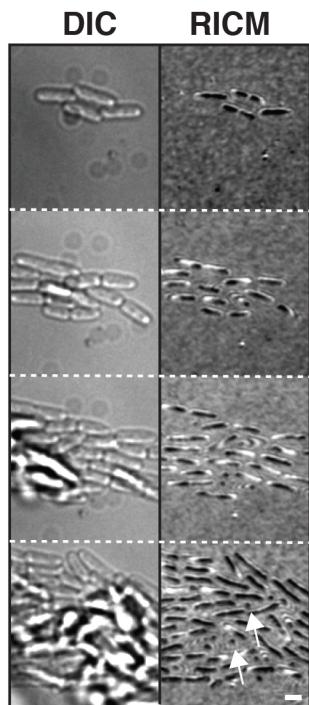
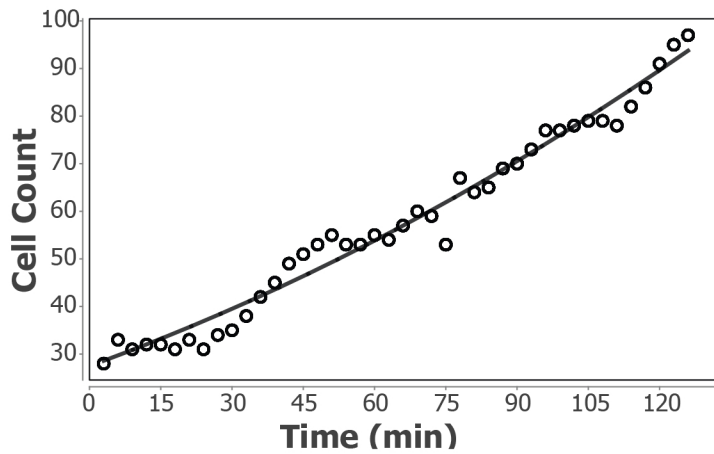
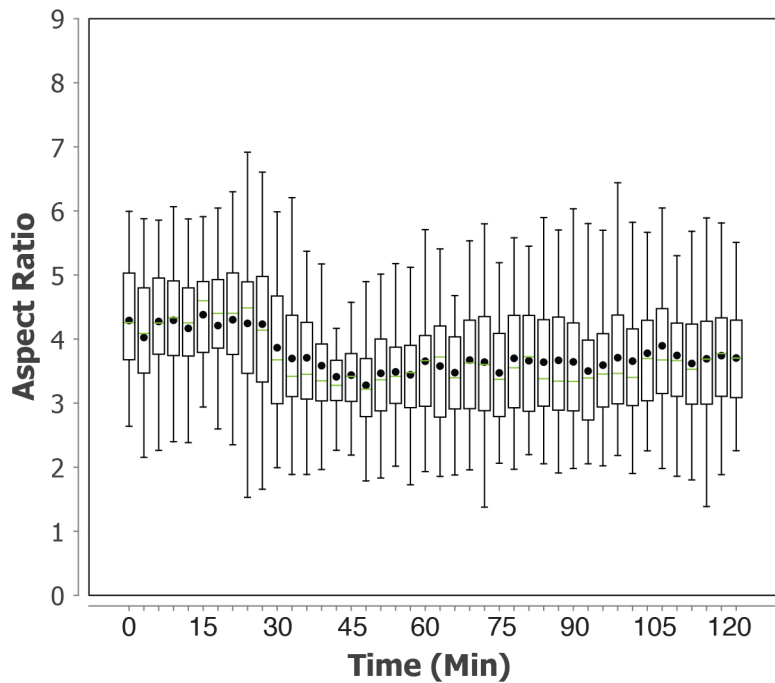
*Glass coverslip**Cleaning and activation*

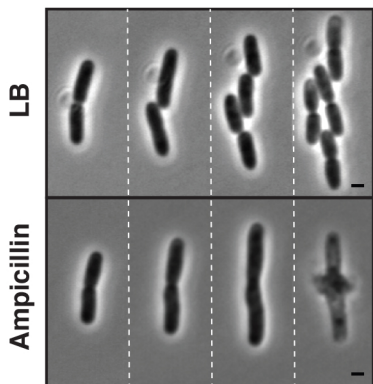
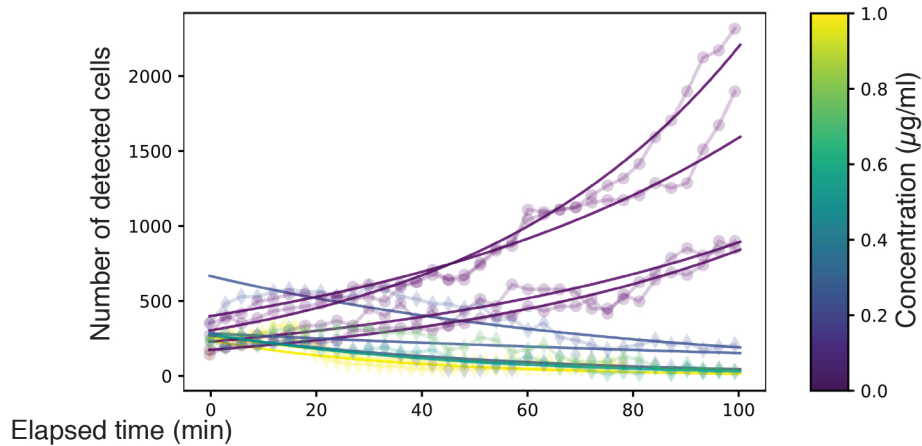
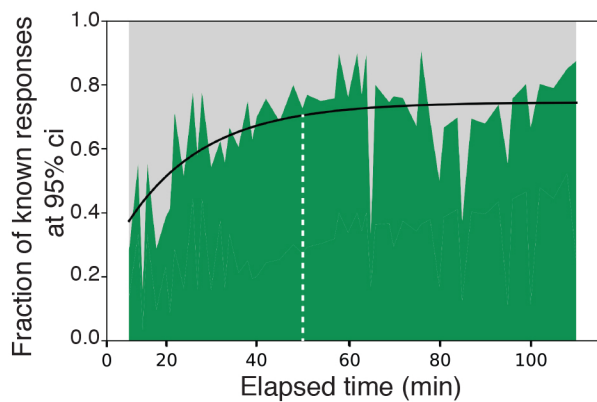
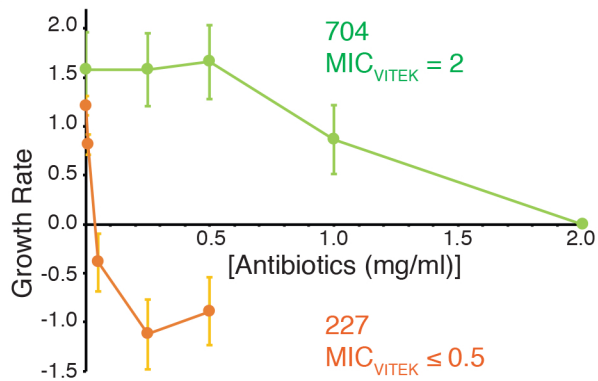
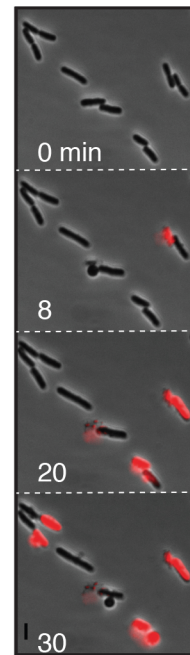
Piranha bath and plasma treatment

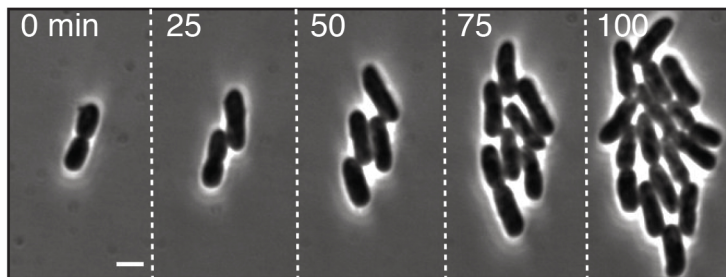
*Spin-coating of chitosan solution**Physicochemical characterization***b**

Wettability



**a****b****c****d**

**a****b****c****d****e**

**a****b**