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Reduction of *Pseudomonas aeruginosa* biofilm formation through the application of nanoscale vibration

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Short title: *P. aeruginosa* biofilm and nanoscale vibration

1 **Abstract:** Bacterial biofilms pose a significant burden in both healthcare and industrial
2 environments. With the limited effectiveness of current biofilm control strategies, novel or
3 adjunctive methods in biofilm control are being actively pursued. Reported here, is the first
4 evidence of the application of nanovibrational stimulation (“nanokicking”) to reduce the
5 biofilm formation of *Pseudomonas aeruginosa*. Nanoscale vertical displacements
6 (approximately 60 nm) were imposed on *P. aeruginosa* cultures, with a significant reduction
7 in biomass formation observed at frequencies between 200 to 4000 Hz at 24 h. The optimal
8 reduction of biofilm formation was observed at 1 kHz, with changes in the physical
9 morphology of the biofilms. Scanning electron microscope imaging of control and biofilms
10 formed under nanovibrational stimulation gave indication of a reduction in extracellular
11 matrix (ECM). Quantification of the carbohydrate and protein components of the ECM was
12 performed and showed a significant reduction at 24 h at 1 kHz frequency. To model the
13 forces being exerted by nanovibrational stimulation, laser interferometry was performed to
14 measure the amplitudes produced across the Petri dish surfaces. Estimated peak forces on
15 each cell, associated with the nanovibrational stimulation technique, were calculated to be in
16 the order of 10 pN during initial biofilm formation. This represents a potential method of
17 controlling microbial biofilm formation in a number of important settings in industry and
18 medical related processes.

19

20 **Introduction**

21 It is estimated that over 80% of the world's microbial biomass exists in a biofilm state (1).

22 These microbial biofilms represent the preferred mode of growth of bacteria, yeasts,

23 filamentous fungi and protists (2, 3). A microbial biofilm can briefly be described as a

24 consortium of cells enclosed in a self-derived extracellular polymeric substance (EPS),

25 interspersed with water channels, and attached to a surface or each other (4, 5). This enclosed

26 consortium of cells has a greater capacity to resist environmental stresses, and is important as

27 a microbial survival strategy (6).

28 Clinically, the role of biofilms may have been underestimated, but recent guidance aims to

29 correct this (7), as it has been estimated that biofilms account for between 65% and 75% of

30 all infections (8, 9). The transition of planktonic cells to a biofilm community, confers with it

31 a vastly increased tolerance to antibiotics (10) and disinfectants (11). As well as being a

32 survival mechanism for microorganisms, it is possible that organisms growing within a

33 biofilm may be more virulent. In blood stream infections a biofilm phenotype has been

34 associated with a higher mortality rate in contrast to planktonic cells (12), possibly due to

35 dispersal of cells from the biofilm (13). Biofilm studies investigating the capacity of clinical

36 isolates to form biofilms have grouped strains of the same species into low and high biofilm

37 formers, with the latter being shown to have an increased pathogenicity and resistance; this

38 effect has been demonstrated in both bacteria (14, 15) and fungi (16).

39 Within the wider environment, biofilm formation can lead to food spoilage and

40 contamination of food processes resulting in significant financial costs (17). Industrial

41 processes that involve pipelines can suffer significant degradation over time due to microbial

42 influenced corrosion. This is mediated by biofilm formation on the inner surface of the pipe,

43 leading to fouling and corrosion of iron and steel alloys (18, 19).

44 It is well known that eukaryotic cells can respond to mechanical stress and convert these
45 mechanical stimuli into an electrical or biochemical response, a process termed
46 mechanotransduction (20). A recent review has highlighted the multitude of mechanical
47 forces that bacteria can experience when attached to surfaces (21). Yet, our understanding of
48 the response of bacteria to these mechanical forces is less well formed than that of eukaryotic
49 cells. Existing studies have investigated microbial biofilm formation in response to surface
50 acoustic waves induced vibration (22) and acoustic induced vibration (23). Surface acoustic
51 waves induced vibration was effective in reducing the bacterial burden in Foley catheters
52 whereas acoustic vibration was demonstrated to increase biofilm formation of *Pseudomonas*
53 *aeruginosa* in Petri dishes.

54 The application of nanovibrational stimulation by use of the reverse piezo effect to control
55 cell behaviour has been described in a recent review (24). Using this method, precise control
56 of experimental parameters can be achieved which are independent of shear flow, produces
57 negligible heat and minimises variability in the displacements applied across the surface of
58 the Petri dishes (25). Nanovibrational stimulation has previously been applied to endothelial
59 LEII cells (26) and mesenchymal stem cells (24-29). Here, we report the first study of
60 nanovibrational stimulation on the formation of *P. aeruginosa* biofilms.

61 **Materials and methods**

62 **Nanovibrational apparatus**

63 The nanovibrational stimulation apparatus was nominally identical to that used by both
64 Nikukar et al and Curtis et al (25, 26). To perform experiments with 35 mm Petri dishes, six
65 aluminium support discs were cut to 32 mm diameter, 3 mm thickness, polished (to ensure a
66 smooth bonding surface). Six 35 mm tissue culture treated polystyrene Petri dishes (Corning,
67 UK) were bonded onto the six aluminium discs using Loctite 2-part epoxy (Loctite,

68 Hempstead, UK). Each cultureware assembly was subsequently bonded to a piezo transducer
69 (model no. 010–05H Physik Instrumente, Karlsruhe/Palmbach, Germany) by application of a
70 non-solvent glue (Bostik, UK). The transducers provided the required nanoscale amplitudes
71 when driven by a continuous sine wave output from a GWINSTEK AFG-2005 arbitrary
72 function generator (Good Will Instrument Euro B.V., Velhoven, Netherlands). The
73 functionality of the piezo/cultureware assemblies was verified by incrementally driving each
74 one at an audible frequency, i.e. 5 kHz, and listening to the audible output generated. The
75 final set-up of the nanovibrational stimulation apparatus is shown in Figure 1.

76 **Laser interferometry and force estimation**

77 Nanoscale amplitudes were measured by laser interferometry as previously described (25).
78 Measurements were taken at the centre and edge of 35 mm Petri dishes, measurements were
79 performed on 3 separate Petri dishes. An estimation of the maximum force exerted due to
80 nanovibrational stimulation was calculated as previously described (27). The average
81 amplitudes measured at each frequency were used to calculate the maximum force exerted, as
82 there were slightly variations in the amplitudes produced.

83 **Culture conditions, standardisation and experimental conditions**

84 *P. aeruginosa* type strain NCTC 10332 (*P. aeruginosa* 10332) was used for all work in this
85 study. All working stocks of *P. aeruginosa* 10332 were maintained at 4°C on Lysogeny broth
86 agar (Oxoid, Cambridge, UK). *P. aeruginosa* 10332 was propagated in Lysogeny broth (LB
87 [Oxoid, Cambridge, UK]). *P. aeruginosa* 10332 was propagated in LB for 16 h at 37°C with
88 shaking at 250 rpm. The culture was then washed by centrifugation (1,600 x g), resuspended
89 in 1x phosphate buffered saline (PBS) twice then adjusted to an OD_{570nm} corresponding to 1
90 x10⁸ CFU/mL. A working inoculum was prepared in LB broth at 1 x10⁵ CFU/mL. Sterile LB
91 (1 mL) was added to each Petri dish before addition of 1 mL of the *P. aeruginosa* 10332

92 inoculum, giving a final inoculum of 5×10^4 CFU/mL. LB without addition of inoculum was
93 used as a negative growth control. The nanovibrational stimulation apparatus was incubated
94 at 37°C for 24 h in air, with the signal generator connected via crocodile clips to the piezo
95 actuators terminal wires. In all experiments a driving potential of 20 V peak to peak (pk-pk)
96 was used producing an approximately 60 nm displacement. Alteration of frequency was
97 performed by changing the input frequency via the digital control panel of the function
98 generator.

99 **Quantifying biofilm biomass**

100 Filtered crystal violet (CV [Fisher, UK]) was prepared to a 0.1% w/v solution in deionised
101 water (dH₂O). At the experimental end time point the nanovibrational stimulation apparatus
102 was removed from the incubator and the Petri dishes detached from the aluminium support
103 discs. Once detached the supernatants were aspirated and the biofilm was washed twice with
104 1x PBS to remove non-adherent cells. One millilitre of 0.1% w/v CV was added to each Petri
105 dish including the media-only control. Petri dishes were then incubated at room temperature
106 for 15 min. Excess CV stain was removed by washing in dH₂O until subsequent washes did
107 not remove any further excess staining. To quantify the bound CV, 80% v/v ethanol was
108 added, and Petri dishes gently rocked to allow full solubilisation of the bound CV. This
109 procedure was repeated for all experimental conditions, controls and media only control. To a
110 96 multi-well plate (Corning, UK), 100 µl of the solubilised CV was transferred from the
111 Petri dish in triplicate. The 96 multi-well plate was then read at OD_{595nm} using an Infinite
112 F200 Pro plate reader (Tecan Group Ltd, Switzerland).

113 **Enumeration of colony forming units**

114 At end point Petri dishes were removed from the aluminium disc and washed twice with 1x
115 PBS. To disrupt the biofilm, 1 mL of 1x PBS was added to a petri dish and sealed with parafilm.

116 Sonication was performed for 10 min at 15 kHz, with subsequent disrupted and detached
117 biofilm in 1x PBS transferred to a 1.5 mL Eppendorf tube. To ensure full disruption and
118 detachment of the biofilm, the Petri dish was stained with CV, full disruption occurring when
119 the CV staining of the Petri dish was negative. To enumerate the colony forming units (CFU)
120 the Miles and Misra method was performed (30). Serial decimal dilutions were performed in
121 1x PBS and 20 μ l plated on LB agar in triplicate for each dilution. LB agar plates were inverted
122 and incubated at 37°C for 24 h, following which the CFU was calculated by counting the
123 colonies at the easiest to count dilution (~20 – 60 colonies).

124 **Live/dead biofilm imaging**

125 Following test conditions Petri dishes were removed from aluminium discs, as previously
126 described. Supernatant was aspirated, and biofilms washed twice with PBS. A live/dead
127 staining solution was prepared using the LIVE/DEAD BacLight Bacterial Viability Kit for
128 microscopy and quantitative assays (Invitrogen, UK). Briefly, 1 μ l of SYTO9 and propidium
129 iodide (PI) were added per 1 mL of dH₂O. To each Petri dish 1 mL of staining solution was
130 added, Petri dishes were then incubated for 15 min in the dark at room temperature. The
131 staining solution was aspirated, and the biofilm washed twice with dH₂O to halt any residual
132 staining. Biofilms were imaged using an EVOS FL (Life Technologies, UK) all in one
133 fluorescent microscope. Fluorescent images were obtained using the GFP (470/22, 510/42)
134 and Texas Red (585/29, 624/40) lightcubes (Life Technologies).

135 **SEM analysis**

136 Biofilms with (1 kHz applied on inoculation) and without nanovibrational stimulation were
137 grown for 24 h at 37°C as previously detailed. Biofilms were washed twice with 1x PBS and
138 fixed with a 2% w/v para-formaldehyde, 2% v/v glutaraldehyde, 0.15 M sodium cacodylate,
139 and 0.15% Alcian Blue (pH 7.4) solution, and prepared for SEM as described by Erlandsen et

140 al (31), with modification of counter staining process by addition of 1 mL 0.5% w/v uranyl
141 acetate for 1 h, at room temperature in the dark. Following which, progressive dehydration
142 steps were performed with increasing concentrations of ethanol (EtOH), twice for 5 min for
143 each (30% v/v, 50% v/v, 70% v/v, and 90% v/v). Dehydration by absolute and dried absolute
144 EtOH was performed 4x 5 min. Following dehydration steps, substrates were critically dried
145 by addition of hexamethyldisilazane (HDMS) twice and stored in a desiccator overnight.
146 Fixed and dried biofilms were sputter coated with 5 nm of gold using an EMSscope SC500
147 sputter coater (EMS, UK). Examination of samples was performed on a Hitachi S-4100
148 scanning electron microscope under vacuum, operated at 10 kV.

149 **Quantification of ECM components**

150 Biofilms with (1 kHz applied on inoculation) and without nanovibrational stimulation were
151 grown for 24 h following which the supernatant was aspirated and biofilms were washed with
152 1x PBS twice to remove non-adherent cells. Biofilms were harvested in 1 mL 1x PBS and
153 disrupted by scraping with a cell scraper. Biofilms samples were stored at -20°C for a
154 maximum of 1 week prior to processing. Biofilms samples were fully thawed and
155 homogenised by a combination of vortexing and pipetting. Biofilm samples were $0.2\ \mu\text{m}$
156 filtered and the resultant eluent aliquoted into Eppendorf tubes. To measure the protein
157 content of the biofilm samples the Bradford assay was performed (32) with bovine serum
158 albumin as a standard. To measure the carbohydrate content of the biofilm samples, an
159 optimised phenol-sulphuric acid method with glucose standards was performed (33).

160 **Visualisation of potential lateral force production**

161 To visualise any potential production of shear flow, $5\ \mu\text{l}$ of dye composition (30% v/v
162 glycerol, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol), was added
163 concurrently to 2 Petri dishes, with and without nanokicking. A time lapse video was

164 recorded of the dye dispersal, and the experiment repeated in triplicate. Videos were exported
165 and converted to JPG stills using Paxillion Free Image converter software (Softonic,
166 Barcelona, Spain). To quantify the dispersal of the dye, images at 10 s intervals were assessed
167 for the diameter of dispersal in AxioVision V4.8 (Zeiss, Feldbach, Switzerland). Diameter
168 measurements were taken in 4 aspects to average and account for non-uniform dispersal of
169 the dye. Rate of dispersal was calculated at 10 s intervals over a time course of 0 – 100 s.
170 Linear regression curve analysis was performed on the dispersion of a dye over time. Linear
171 function lines were plotted when there was no significant difference between the control and
172 nanokicking replicates.

173 **Statistical analysis**

174 All data were assessed for normality using a Shapiro–Wilk test. For assessing the statistical
175 significance of the alteration of frequency and time of application of nanovibrational
176 stimulation, a one-way ANOVA with Tukey *post hoc* correction was performed. For
177 assessing the statistical significance of observed alteration of biofilm formation kinetic,
178 comparison of CFUs and components of the biofilm matrix a student t-test was performed. In
179 all experiments, statistical significance was achieved when $p < 0.05$. Data were exported from
180 the Infinite F200 Pro plate reader to Microsoft Excel (Microsoft, Redmond, WA, USA).
181 Assessment of normality, statistical analysis and plotting of data was performed in GraphPad
182 Prism 7.0 (GraphPad Software Inc, San Diego, CA, USA).

183 **Results**

184 **Laser interferometry and modelling of maximum force on *P. aeruginosa* cells**

185 Validation of the cultureware assembly was quantified by measurement of the displacements
186 generated by nanovibrational stimulation using a SIOS laser interferometer (Figure 2A).
187 Displacement was observed to increase linearly with increased pk-pk voltage supplied. The
188 theoretical force exerted by the nanovibrational stimulation on a single *P. aeruginosa* cells

189 can be mathematical calculated using Newton's second law, that of force (F) being
190 determined by the mass (m) times acceleration (a) (26, 27). In this case the mass refers to the
191 column of fluid directly above each cell, with the peak acceleration being $A_0(2\pi f)^2$, where f is
192 frequency and A_0 is the vibration amplitude (note that this is half of the total peak to peak
193 displacement). An estimate for this mass is determined by the average surface area of a *P.*
194 *aeruginosa* cell being $1\ \mu\text{m} \times 5\ \mu\text{m}$, with an aqueous column of culture media extending 2
195 mm above (with the density of water used to calculate this mass). Peak values due to
196 acceleration during vibration are calculated as described in the papers by Curtis et al and
197 Nikukar et al (26, 27). Figure 2B shows the modelled peak force exerted per single cell of *P.*
198 *aeruginosa* due to nanovibrational stimulation at frequencies of 100, 200, 400, 500, 1000,
199 2000 and 4000 Hz with a 20 V pk - pk driving potential. Peak forces of 0.4 pN, 1.9 pN, 2.9
200 pN, 11.7 pN, 42.9 pN and 133.5 pN were calculated.

201
202 **Effect of altering the frequency and time of application of nanovibrational stimulation on**
203 ***P. aeruginosa* biofilm formation**

204 Previous literature has demonstrated that vibrating a surface can alter biofilm formation (23).
205 A range of frequencies from 10 Hz to 4 kHz was examined to determine if nanovibrational
206 stimulation alters *P. aeruginosa* biofilm formation. No reduction in biomass at 24 h was
207 observed at frequencies of 10 and 100 Hz ($p > 0.05$) (Figure 3A). A statistically significant
208 reduction in biomass was observed at 24 h, at frequencies of 200 Hz, 400 Hz, 500 Hz, 1 kHz,
209 2 kHz and 4 kHz (52.5%, 52.8%, 54.0%, 64.0%, 41.6% and 38.9% reduction respectively, p
210 < 0.001 one-way ANOVA with Tukey post hoc test).

211
212 It was noted that the reduction in biomass was less at frequencies of 2 and 4 kHz compared to
213 1 kHz, with the reduction at 4 kHz being significantly lower than that of 1 kHz ($p < 0.05$).
214 The greatest reduction of biomass was observed at 1 kHz, while not significantly different to

215 the frequencies of 200 Hz, 400 Hz and 500 Hz, it was consistently lower in all biological
216 replicates. Due to limitations of the equipment (limited number of Petri dishes and set-up
217 time) it was decided to focus on one frequency; therefore, a frequency of 1 kHz was selected
218 for further investigation.

219

220 Biofilm formation has defined temporal stages initiated from the initial reversible attachment,
221 following which irreversible attachment occurs and ultimately biofilm formation and
222 maturation. To determine if the time of application of the nanovibrational stimulation after
223 inoculation influenced biofilm formation, nanovibrational stimulation was applied
224 continuously at 1 kHz from 0, 2, 4 and 6 h after initial inoculation for a total time of 24 h, e.g.
225 0 h equals 24 h stimulation, 2 h equals 2 h no stimulation and 22 h stimulation. When
226 nanovibrational stimulation was applied from 0 h and 2 h, a significant reduction in the
227 measured biomass at 24 h was observed when compared to the control (50.8% and 57.5%)
228 (one-way ANOVA with Tukey post hoc test, $p < 0.001$; Figure 3B). Application of
229 nanovibrational stimulation from 4 h and 6 h after inoculation resulted in no significant
230 reduction in total biofilm formation when compared to control (one-way ANOVA with
231 Tukey post hoc test, $p > 0.05$).

232 **Effect of nanovibrational stimulation on development kinetic of *P. aeruginosa* biofilm**

233 To better understand the observed reduction in biomass at 24 h and the dependence on the
234 time of application of the nanovibrational stimulation, biomass formation kinetics for *P.*
235 *aeruginosa* 10332 was performed. Biomass was assessed using the CV biomass assay at 0, 2,
236 4, 6, 12, and 24 h with and without nanovibrational stimulation at 1 kHz frequency, 30 nm
237 amplitude applied 0 h after inoculation. At 2 h, there was no significant difference between
238 the stimulated *P. aeruginosa* 10332 and control, but at 4 h *P. aeruginosa* subjected to
239 nanovibrational stimulation showed a significantly lower biomass at OD_{595nm} compared with

240 non-stimulated control of 0.3 and 1 (un-paired Student T-test, $p < 0.05$; Figure 4). Without
241 nanovibrational stimulation the exponential formation of the bacterial biofilm continued to
242 12 h and plateaued by 24 h reaching a final average OD_{595nm} of 3.27. With nanovibrational
243 stimulation, exponential biomass formation was not observed through the course of the
244 experiment. At 24 h the biomass was significantly lower at an average OD_{595nm} of 0.94 (un-
245 paired Student T-test, $p < 0.001$).

246 **Nanovibrational stimulation does not reduce the number of cells in the biofilm and** 247 **planktonic phase**

248 Due to the nature of the CV assay, cells and extracellular matrix are both stained by CV, in
249 addition if there is a lower number of cells adhered to the surface, a lower quantity of
250 extracellular matrix may be produced. To investigate if the reduction in biomass was due to a
251 reduction in the number of cells in the biofilm, Miles and Misra counts were performed to
252 enumerate the number of CFUs. In addition, to determine if nanovibrational stimulation could
253 cause dislodgement of cells from the surface, the planktonic CFU was also investigated. No
254 statistically significant reduction in the CFU/cm² was noted ($p > 0.05$) between the control
255 (1.62×10^9 CFU/cm²) and stimulated (1 kHz) biofilm (7.99×10^8 CFU/cm²) (Figure 5) at 24
256 h. There was a mean 10-fold increased recovery of planktonic *P. aeruginosa* when comparing
257 the control (6.94×10^{10} CFU/mL) versus stimulated (6.88×10^9 CFU/mL), however this was
258 not statistically significant ($p = 0.3648$). To determine if there was a difference in the total
259 recovered CFU, the planktonic and sessile CFU recovery were combined. This gave a total
260 recovery of 1.46×10^{10} CFU for the control and 8.44×10^{10} CFU for the stimulated, this
261 difference was not statistically significant ($p = 0.5522$, unpaired Student T-test).

262 **Microscopic examination of the effect of nanovibrational stimulation on *P. aeruginosa*** 263 **biofilm architecture**

264 As no statistically significant reduction in cells numbers was noted, live/dead staining was
265 performed on 24 h biofilms with and without the application of nanovibrational stimulation to

266 visualise the biofilm. Biofilms formed under nanovibrational stimulation at 1 kHz showed a
267 change in structural architecture and density compared to the control (Figure 6A and B). With
268 increased magnification it was shown that there were regions of sparse microbial coverage
269 compared to the controls (Figure 6C). When the number of dead cells was assessed using PI
270 staining, no significant difference ($p > 0.05$) was observed between the control and stimulated
271 biofilms (supplementary table S1). During visual examination of the 1 kHz stimulated
272 biofilms, striated line-like formations were visible; these were interspersed variably across
273 the biofilms, yet they were observed in all technical and biological independent replicates
274 with 1 kHz nanovibrational stimulation. Representative image is shown in Figure 6B and C.
275 When propidium iodide staining was viewed, staining was observed that was concordant with
276 lines observed with the SYTO9 live cell staining (Figure 6B and C PI staining), however this
277 did not appear to be stained cells. Collectively, these observations provide visual evidence of
278 an alteration in the biofilm formation structure due to the 1 kHz nanovibrational stimulation
279 when compared to the control.

280

281 To further visualise the altered biofilm formation of *P. aeruginosa* and the lower biomass due
282 to nanovibrational stimulation, scanning electron microscopy was performed. Control
283 biofilms had confluent growth with microcolonies evident across the Petri dish surface with
284 ECM being visible, (albeit in a dehydrated state due to the ethanol dehydration method used
285 to prepare the samples) (Fig. S1). Comparison of the control biofilms (Figure 7A & B) versus
286 the 1 kHz biofilms (Figure 7C & D) showed a similar pattern of confluence across the Petri
287 dish surface with a higher confluence in the middle and a lower confluence at the edge. In
288 keeping with the fluorescent imaging, regions of lower density of cells was observed in 1 kHz
289 nanovibrational stimulated biofilms compared to the control (Figure 7A & C).

290

291 **Nanovibrational stimulation reduces key matrix components of the ECM of *P. aeruginosa***
292 **biofilms**

293 The reduction in biomass coupled with visual evidence of an altered biofilm structure gives
294 tentative evidence that the ECM produced by *P. aeruginosa* biofilms is reduced due to the
295 nanovibrational stimulation at 1 kHz. To further investigate this hypothesis, quantification of
296 the carbohydrate and protein components of *P. aeruginosa* 10332 biofilms with and without
297 nanovibrational stimulation was performed. The average protein content of the control biofilms
298 was 19.62 $\mu\text{g}/\text{cm}^2$, the average protein content of stimulated biofilms was 6.78 $\mu\text{g}/\text{cm}^2$ equating
299 to a 65.4% reduction which was statistically significant ($p < 0.0001$) (Figure 8). The average
300 carbohydrate content of the control biofilms was 8.42 $\mu\text{g}/\text{cm}^2$, the average carbohydrate content
301 of stimulated biofilms was 2.96 $\mu\text{g}/\text{cm}^2$, equating to a 64.8% reduction which was statistically
302 significant ($p < 0.0001$) (Figure 7).

303 **Discussion**

304 This is the first reported demonstration of a reduction in bacterial biofilm formation due to an
305 induction of a vertical nanoscale vibration approximately 30 nm amplitude, at a frequency of
306 200 Hz – 4 kHz, when applied 0-2 h after inoculation. Biofilm formation can be grouped into
307 a number of key stages: reversible adhesion, irreversible adhesion, proliferation, ECM
308 production and ultimately formation of a mature biofilm (34). The optimal time of application
309 of the nanovibrational stimulus may give an indication that vibration at the nanoscale
310 interferes with the initial attachment of *P. aeruginosa* 10332, as these time periods are known
311 to be within the reversible period of cell attachment to a surface in the current model of
312 biofilm development (34, 35). This would also indicate that the frequencies studied would be
313 ineffective in disrupting pre-formed biofilms. Nanovibrational stimulation, however, cannot
314 completely abrogate the adhesion of *P. aeruginosa* as biofilm growth occurs throughout the
315 24 h growth period, yet the final biomass at 24 h is significantly lower than the controls,

316 suggesting an additional effect to reduction of initial adherence. Initial surface interactions
317 have recently been demonstrated to be mediated by the mechanical activity of type IV pili
318 (TFP) in *P. aeruginosa* on short time scales (36). Persat and colleagues (36) have proposed a
319 molecular model for the surface sensing by TFP, whereby the cell encounters the surface and
320 through attachment and retraction of the TFP, tension is exerted on the TFP, this then
321 activates the Chp system, leading to cyclic adenosine monophosphate (cAMP) synthesis
322 within the first hour of attachment. TFP retraction forces have been measured to be within the
323 pN range (37), this is within the order of the forces generated by the nanovibrational
324 stimulation at a frequency of 1 kHz (approximately 10 pN) (Figure 2). While the forces
325 generated are of the same magnitude it is too early to establish a link between the forces
326 exerted by nanovibrational stimulation and an interaction with the tension forces exerted by
327 TFP.

328 Prior studies using shear flow have demonstrated altered biofilm phenotypes in *P. aeruginosa*
329 PA01 (38). Turbulent flow resulted in the formation of the streamlined patches that in some
330 cases had ripple-like structures perpendicular to the flow. The intersecting and crossing lines
331 observed (Figure 6) were different to the ripple-like formations produced by turbulent flow in
332 the PA01 study. It is of note that no statistically significant reduction in viable cells with the
333 Live/Dead staining was observed between the stimulated and unstimulated biofilms.
334 Exopolysaccharides have previously been shown to play an important role in biofilm
335 formation and structure (39). In this study, carbohydrate and protein content of the *P.*
336 *aeruginosa* 10332 biofilms were significantly reduced due to nanovibrational stimulation at 1
337 kHz. These data give strong indication that the reduction in biomass due to nanovibrational
338 stimulation is as a direct result of a reduction in the carbohydrate and protein content of the
339 biofilm matrix.

340 Acoustic stimulation provided by a speaker has previously been shown to enhance biofilm
341 formation in response to non-uniform micrometre displacements (23). This stimulation
342 method may have promoted accelerated biofilm formation due to clustering of the *P.*
343 *aeruginosa* at early time points (concentric rings) leading to potentially higher levels of
344 quorum sensing molecules. In contrast, our results have shown that uniform nanometre scale
345 displacements result in decreased biofilm formation. This may indicate that biofilm formation
346 can be controlled by the uniformity of the stimulation thereby allowing variable control of
347 biofilm formation dependent on the application *e.g.* it may be beneficial to promote biofilm
348 growth for bio-engineering purposes. A proposed hypothesis of how nanovibrational
349 stimulation reduces biofilm formation is that of initial inhibition/delaying of the adherence of
350 *P. aeruginosa* leading to a reduced quantity of extracellular components of the biofilm being
351 produced *e.g.* delayed attachment leading to a less mature biofilm when compared to the
352 control. It is yet undetermined if molecular mechanisms play a role in this observed
353 mechanism of biofilm reduction.

354 A number of possible confounding factors have also been considered and discounted. It has
355 previously been demonstrated that the rapid expansion and contraction of the piezo ceramic
356 generates negligible heat transfer to the aluminium disc upon which the Petri dish sits (25,
357 28). This means that the effect is unlikely to be due to heating of the culture system (Fig. S2).
358 Shear force mediated effects on adhesion have also been demonstrated in *P. aeruginosa* (40).
359 However, our experimental design minimises the generation of any shear flow by minimising
360 lateral motion of the media, only vertical movement is observed (Fig. S3). The uses of an
361 aluminium disc and Petri dish ensures rigidity and minimises any differential vibration
362 amplitudes, which could also induce shear flow across the growth surface at a frequency of 1
363 kHz and 30 nm amplitude.

364 In conclusion, we have described a novel method of biofilm control using piconewton forces
365 that does not require the use of antibiotics or other chemical agents. This negates the potential
366 for traditional environmental drug resistance mechanisms that have been shown to translate
367 into clinical treatment failures (41, 42). This effect may have a number of potential
368 applications in combating biofilms in the industrial setting and healthcare setting, but further
369 work is required to fully understand the mechanisms by which nanovibrational stimulation
370 causes this effect.

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- 512

513 Figure 1 – Nanovibrational stimulation apparatus. Example of a typical set-up with 35mm
514 diameter Petri dishes attached. The Petri dish is mounted on an aluminium disc, which
515 provides support to the Petri dish allowing uniform displacement across the entire surface
516 area. The Petri dish with aluminium disc is then attached to the piezo then to the large
517 aluminium block underneath, this ensures that the expansion of the piezo results in upwards
518 movement of the Petri dish.

519
520 Figure 2 – Amplitude response to driving potential and frequency with peak force estimation.
521 (A) Petri dish amplitude as a function of piezo driving potential. Interferometry was performed
522 at a range of frequencies measured at the surface of the Petri dish. Measured amplitudes were
523 linearly correlated to driving potential (B) The maximum force exerted due to acceleration as
524 a result of nanovibrational stimulation was calculated using Newton's second law, based on
525 the maximum amplitude measured by interferometry for each frequency, calculated from
526 interferometry data. Data are mean \pm SD, n = 3.

527
528 Figure 3 – *P. aeruginosa* biomass formation is dependent on both frequency and time of
529 application of nanovibrational stimulation. (A) The effect of altering the frequency of the
530 nanovibrational stimulus was performed and the resultant final biomass at 24 h was
531 quantified by CV assay. Frequencies of 200 Hz through to 4 kHz were effective in reducing
532 biomass formation at 24 h. (B) Nanovibrational stimulation at 1 kHz was applied at specified
533 periods after inoculation (0, 2, 4 and 6 h) as indicated on the graph. Resultant biomass at 24 h
534 was quantified by CV assay. A significant reduction in biomass formed was observed when
535 nanovibrational stimulation was applied at 0 and 2 h post inoculation but not at 4 and 6 h post
536 inoculation. Data are mean \pm SD. One-way ANOVA with Tukey post hoc test, *** $p < 0.001$,
537 * $p < 0.05$, n = 3.

538
539 Figure 4 - Biofilm formation kinetic of *P. aeruginosa* 10332 with (dashed) and without
540 nanovibrational stimulation. *P. aeruginosa* 10332 was inoculated in LB broth at 5×10^4
541 CFU/mL and 2 mL seeded to each Petri dish on the nanokicking set-up. Nanokicking set-up
542 was incubated at 37°C for 24 h. A Petri dish was removed at the respective time points,
543 washed with 1x PBS, and crystal violet staining performed. Bound CV was desaturated with
544 80% v/v ethanol and 100 μ l transferred to a 96 well flat bottom microtitre plate (n = 6). Data
545 are mean \pm SD. Unpaired Student T-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 3.

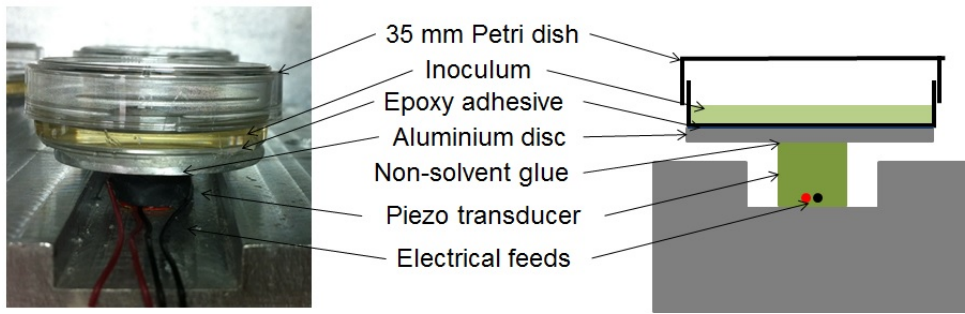
546
547 Figure 5 – Microbial count determination disrupted biofilm and supernatants.
548 Nanovibrational stimulation at a frequency of 1 kHz was applied after inoculation (0 h).
549 Biofilms were disrupted by combination of sonication and cell scraping. Disrupted biofilm
550 was resuspended in 1 mL PBS and Miles and Misra CFU counts performed. Resultant
551 CFU/mL for biofilms were then adjusted to CFU/cm². Circle = Control, Square = stimulated
552 1 kHz. Data are mean \pm SD, n = 3.

553
554 Figure 6 – Nanovibrational stimulation alters *P. aeruginosa* 10332 biofilm architecture.
555 Representative images obtained on EVOS® FL all in one microscope. Syto9 (green - live) and
556 PI (dead - red) images obtained at same fluorescent intensity and combined (merged). (A)
557 Unstimulated controls, scale bar = 400 μ m (B) 1 kHz stimulation non-uniform biofilm
558 formation was observed, scale bar 400 μ m (C) 1 kHz stimulation, unusual biofilm features
559 were observed, Scale Bar = 200 μ m.

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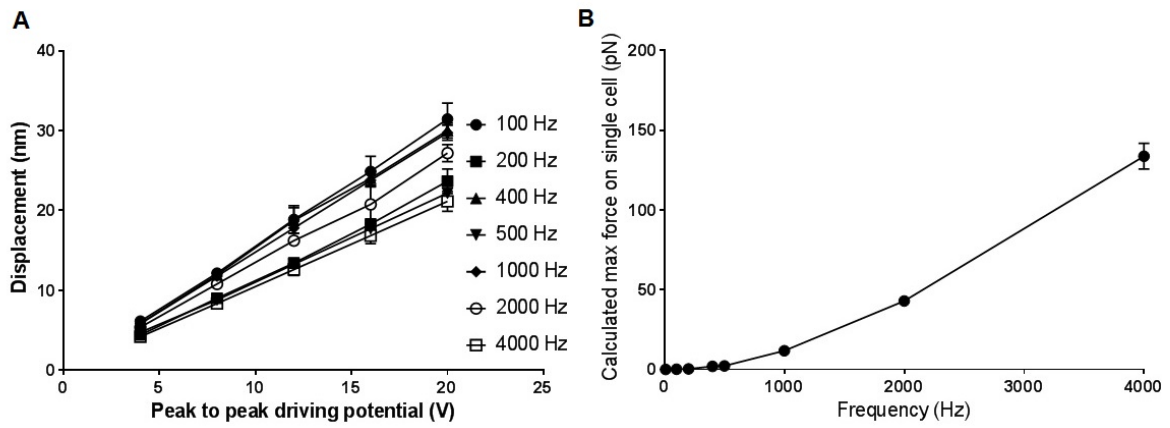
562 Figure 7 – SEM evaluation of control and 1 kHz nanovibrational stimulated *P. aeruginosa*
563 10332 biofilms. Representative SEM images of *P. aeruginosa* 10332. Control biofilm (A)
564 centre of Petri dish and (B) edge of Petri dish. Nanovibrationally stimulated biofilm (1 kHz)
565 (C) centre of Petri dish, (D) edge of Petri dish. Scale Bar = 10 μ m.
566

567 Figure 8 – Nanovibrational stimulation significantly reduces the protein and carbohydrate
568 content of *P. aeruginosa* biofilm ECM. Quantification of protein and carbohydrate content of
569 control and stimulated (1 kHz) *P. aeruginosa* biofilms performed by Bradford assay and
570 phenol-sulfuric acid assay respectively. Data are mean \pm SD, *** $p < 0.0001$, n = 3.
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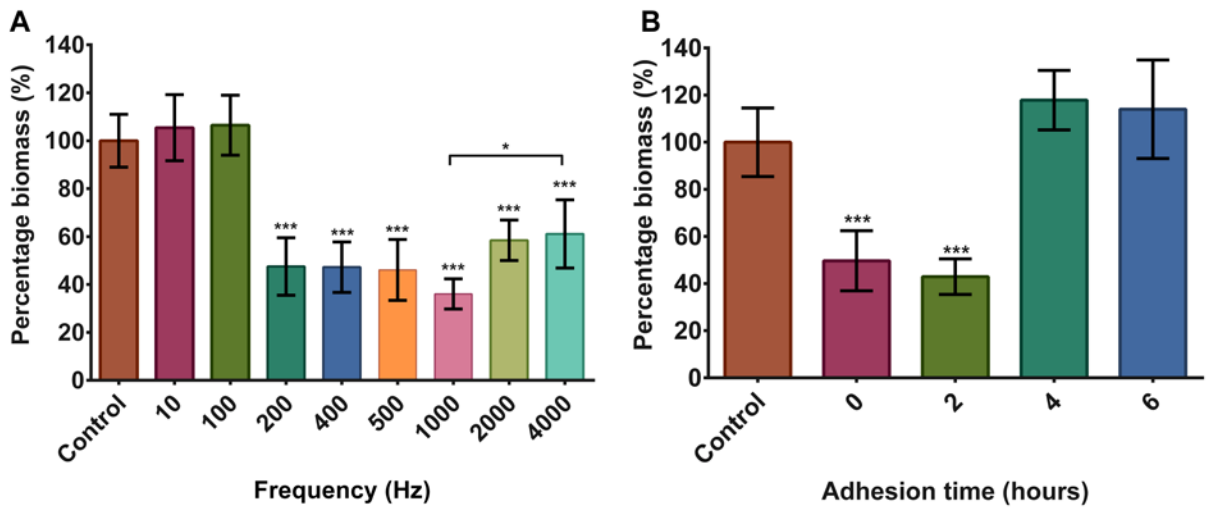
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Figure 1



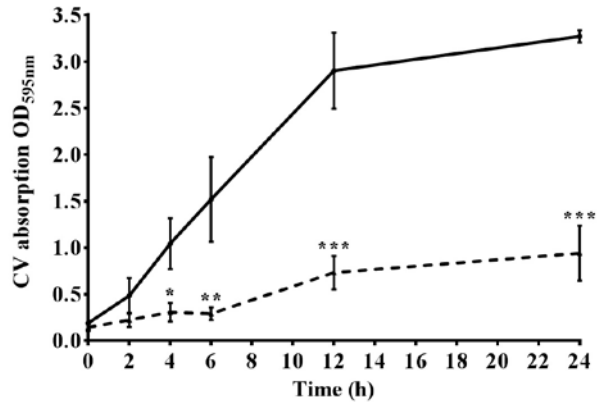
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Figure 2

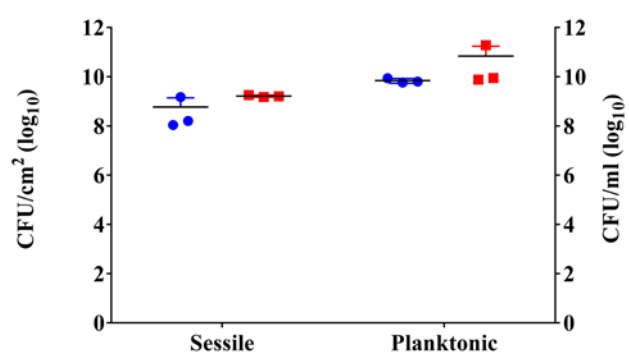


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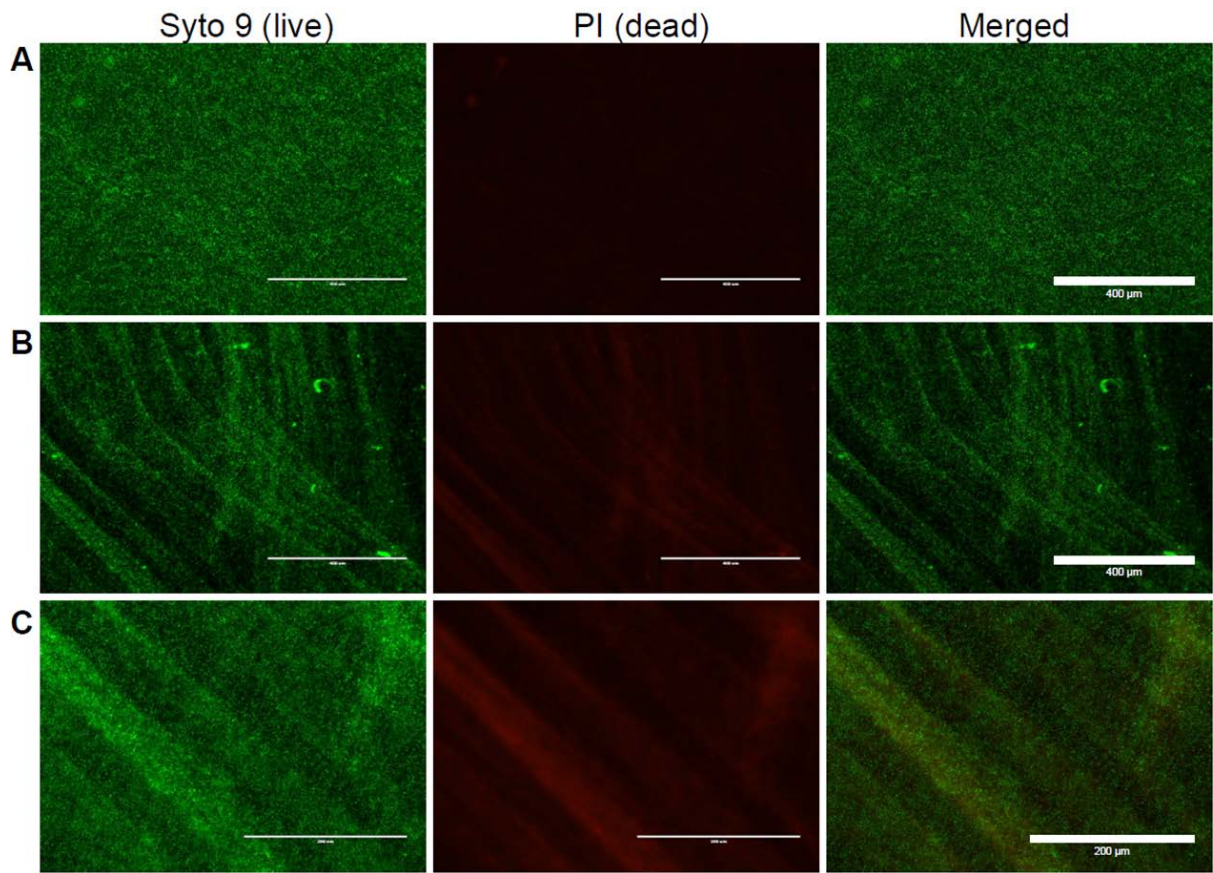
Figure 3



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582 **Figure 4**
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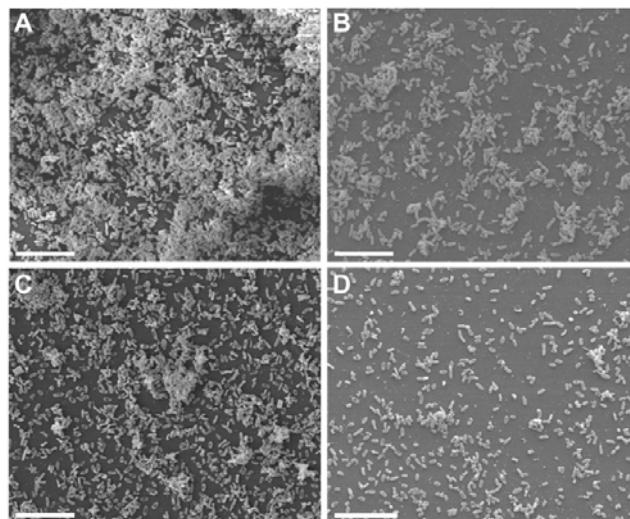


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585 **Figure 5**
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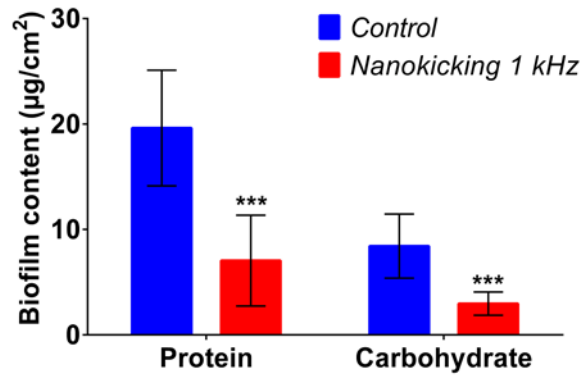
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Figure 6



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Figure 7



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601 **Figure 8**
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