Reduction of Antibiotic-induced Biofilm Accumulation of *Pseudomonas aeruginosa* by Quaternized Phytoglycogen

Sarah R. Schooling¹, Karl Klinger², Anton Korenevski², Susan Glasauer¹*

¹Department of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario Canada; ²Department of Mirexus Biotechnologies Incorporated, 574 Hanlon Creek Boulevard, Guelph, Ontario, Canada

*Corresponding author: Susan Glasauer, Department of Mirexus Biotechnologies Incorporated, 574 Hanlon Creek Boulevard, Guelph, Ontario, Canada, Tel: 0015198244120; E-mail: glasauer@uoguelph.ca

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ABSTRACT

Biofilms are oft cited as a factor in the unwanted persistence and recalcitrance of microbial life and a strong research initiative exists to identify, understand and target vulnerabilities. Phytoglycogen is a biodegradable nanoparticulate biomaterial that is purified from crop plants. Importantly, the highly branched glucan structure provides a scaffold on which to construct novel polymers. Functionalized Phytoglycogen (FP) was synthesized using green chemistry principles. Screening of several molecules identified a form of quaternized phytoglycogen which reduced biofilm formation and accretion by *Pseudomonas aeruginosa*. Exposing *P. aeruginosa* to modified phytoglycogen and antibiotic in combination not only substantively reduced biofilms, but also prevented increased biofilm formation, a biological response to sub-optimal antibiotic concentrations. Treatment of pre-grown biofilms with sub MIC antibiotic alone also led to increased proliferation, whereas FP-antibiotic combinations prevented or reduced the extent of this. Swimming, swarming and twitching motility, all critical for biofilm development, were negatively affected by FP. This work supports phytoglycogen as a promising foundational molecule for novel polymers, including those with anti-biofilm function. Critically, in addition to published reports on how sub-optimal antibiotic concentrations promote biofilm formation, we demonstrated a similar effect upon pre-existing biofilms, indicating a further route for the failure of antibiotic therapies.

KEYWORDS: Phytoglycogen; Nanoparticle; Antibiotic; Biofilm; *Pseudomonas aeruginosa*.

INTRODUCTION

Phytoglycogen, a plant-derived form of glycogen, is an emerging and versatile contender in the field of nanotechnology. This ‘nano by nature’ material may be harvested from
sustainable plant crops and extracted as spherical particles with diameter size distributions of 30-100 nm [1]. Each nanoparticle comprises a single molecule of highly-branched glucan, providing a uniform surface physico-chemistry which can be modified by adding functional groups or active pharmaceutical ingredients e.g. antibiotics [2]. Phytoglycogen offers properties such as biodegradability, non-toxicity, near-neutral charge, low immunogenicity [3], high surface hydrophilicity [4, 5] addressing some major limitations of conventional nanomaterials. Phytoglycogen can be purified to low dispersity indices [4], enabling predictable biodistribution and favourable pharmacokinetics. These attributes render phytoglycogen an ideal candidate for exploitation as a nanotechnology, suited for use in a wide range of applications. Modified phytoglycogen and glycogen have been demonstrated as a delivery vehicle for nutraceuticals [6,7] and anti-infectives [8-10], additionally enhancing stability and longevity. Further applications include vaccine adjuvant [11] and the delivery of nucleic acids for use in therapy [12,13].Biofilm development is considered a protective mechanism for the enhanced survival and persistence of microbiota, and is a serious challenge for antibiotic therapies [14]. Because of this, we investigated the impact of antibiotics and functionalized phytoglycogen (FP) on biofilm development and propose that phytoglycogen nanoparticles could serve as a platform to create novel functionalized polymers with activity against biofilm formation and growth.

MATERIALS AND METHODS

Isolation of phytoglycogen
Phytoglycogen nanoparticles were isolated from sweet corn and purified according to Nickels, et al.[4].

Preparation of cationized phytoglycogen: 225 – 450 g of phytoglycogen was dispersed in 0.5 mol.l⁻¹ aqueous NaOH at a concentration of 13% w/w. Then 1.75 molar equivalents (mol reagent/mol anhydroglucose unit) of a 69% aqueous solution of (2, 3-epoxypropyl) trimethylammonium chloride was added to the mixture over the course of 5 h. The mixture was stirred for 24 h at room temperature before adjusting the pH to 7.0 with 6.2 mol.l⁻¹ HCl. The product was precipitated by addition of 2 volumes of ethanol and stored over night at -20°C. The precipitate was collected, washed three times with ethanol, and oven-dried at 80°C to dryness.

Dynamic Light Scattering (DLS) and zeta potential measurements: These were performed on a Malvern Instruments Zetasizer Nano ZSP Instrument, controlled by the Zetasizer 7.11 software. For DLS measurements, a 8 mg.ml⁻¹ solution in deionized water was filtered through a 0.22 µm PES syringe filter into a 4-ml disposable PMMA cuvette. Measurements were run at 25°C, with automatic attenuator and position selection. Results were averaged over three measurements. For zeta-potential measurements, a 0.2% (w/v) solution in 10 m mol.l⁻¹ NaCl was filtered through a 0.22 µm PES syringe filter into a DTS 1070 zeta potential cell. Measurements were run at 25°C, 150 V, with a minimum of 10 zeta runs and automatic attenuator selection. Results were averaged over three measurements.

Elemental analysis: It was performed by the Pregl-Dumas combustion method on a Perkin Elmer 2400 CHNS Analyzer at Wilfrid Laurier University in Waterloo, ON. The degree of substitution by elemental analysis (DSCHN) was calculated from the ratio of nitrogen and carbon content (xN/C) according to the following equation:

\[
DSCH = \frac{M(CAHG) \cdot xN}{C(M(N) - M(C)) \cdot x}
\]

In the equation M(N) is the molar mass of the nitrogen atom (=14), M(CAHG) is the molar mass of all carbons in the anhydroglucose unit (=72), and M(CGTAC) is the molar mass of carbons in the attached group (=72).

1H NMR spectroscopy: 1H NMR spectra were recorded from 5% solutions in D2O on a
Bruker 600 instrument at 600.134 MHz with 16 scans at 348 K. Samples were equilibrated for minimum 10 min at a temperature of 348 K before measurement. The HDO signal at $\delta=4.264$ was used as a chemical shift [16]. Spectra were processed using the Bruker Topspin TopSpin 3.5.b.91 pl 7 software. The degree of substitution by 1H NMR spectroscopy $DS_{NMR}$ was calculated according to the following equation:

$$DS_{NMR} = \frac{(4.33 - 4.60)}{\text{Int} (4.83 - 5.97)}$$

In which Int (4.33–4.60) is the integral of the C$_2$-methine proton of the 3-(trimethylammonio)-2-hydroxyprop-1-yl group (PhG-O-CH$_2$-CHOH-CH$_2$-N(CH$_3$)$_3$), and Int (4.83–5.97) is the integral of all anomeric protons.

**Preparation of sterile native or derivatized phytoglycogen:** Pre-weighed samples of phytoglycogen were aliquoted into glass vials and irradiated to a final dose of 6 kGy using a 60 Co source (McMaster Nuclear Reactor; Hamilton ON). Post-irradiation sterility of samples was confirmed by incubating 20 mg of gamma-irradiated material.ml$^{-1}$ trypticase soy broth at 25°C or 37°C (150 rpm, 48 h). No growth was observed and the materials were deemed sterile. Stock solutions of sterile 100 mg NP or FP.ml$^{-1}$ were made by reconstituting pre-weighed gamma-irradiated samples with sterile milli Q water. Samples were gently agitated and then equilibrated at 4°C (ca. 18 h). Reconstituted samples were stored at 4°C. Phytoglycogen supplementation of solutions sterilized by autoclaving e.g. media, was done by dilution once the autoclaved solution had cooled to a temperature of at least 45°C.

**Microbial strains**

*Pseudomonas aeruginosa* PA01 was maintained as long-term -80°C stock cultures in glycerol (15% vol/vol medium). Stocks were revived by sub-culturing onto trypticase soy agar plates or directly into liquid medium (18 h, 37°C).

**Minimum inhibitory concentration (MIC):** MIC values were determined according to broth micro-dilution methods described in the 2012 CLSI [17] document M07-A9 (bacteria). Mueller-Hinton broth (MHB; Oxoid) was purchased from Fisher Scientific (Canada). Stock solutions of gamma-irradiated NP and FP were reconstituted in sterile MHB. Negative growth controls comprised sterile medium; positive growth controls contained inoculated medium. MIC was recorded as the lowest concentration of an agent which resulted in no growth (optically clear). Where indicated, MIC assessments were additionally performed for the antibiotics tobramycin and ciprofloxacin, in the presence of NP or FP. MIC assays were performed in duplicate within each experiment and were repeated thrice (n=6).

**Motility assays:** Swimming and swarming motility were assessed as previously described using modified M 9 medium (mM 9 medium) [18,19]. Twitching motility was assayed in LB medium supplemented with 1% (wt/vol) bacteriological agar (48 h, 37°C) following Chiang and Burrows [20]. Motility media was supplemented with NP or FP; non-supplemented media was the positive control. Experiments were performed in triplicate, three measurements made on each assessment, and repeated independently three times (n=27). Due to opacity at high concentrations of phytoglycogen, the twitch zone at the agar:plate interface was re-measured after excision of the agar; values were within ± 1 mm.

**Biofilm formation assay:** Overnight culture of *P. aeruginosa* PAO1 in mM 9 medium (37°C,150 rpm, 16-18 h) was diluted 1:4000 in mM 9 or mM 9 supplemented with NP or FP, 3 × 2 ml transferred to sterile glass tubes (18 ×150 mm) and incubated for 20 h (37°C, 150 rpm).Biofilm accumulation was quantitated as described below. Experiments were performed in triplicate replicate and repeated independently a minimum of three times (n=9).

Quantitation of accreted biofilm: Tube or well contents were removed by aspiration.
Loosely attached cells were gently rinsed off with an equivalent volume of sterile 0.9% (wt/vol) NaCl. Adhered biomass was stained with 0.1% (wt/vol) Hucker’s crystal violet (15 min), contents were then aspirated and unbound stain rinsed by excessive washing with water. Samples were air dried. Retained stain was extracted by solubilisation with 33% acetic acid (vol/vol) and transferred to cuvettes or plates and quantitated spectrophotometrically at 570 nm (cuvettes) or 600 nm (plates).

**Impact of cationized phytoglycogen on nascent biofilms:** Biofilm growth and accretion were evaluated as described with the exception that medium transfer was done. Six hours after inoculation sets of tubes were transferred to a biological safety cabinet; tube contents were gently removed by aspiration and immediately replaced with the corresponding pre-warmed solution. The tubes were incubated for a further 14 h, yielding a total incubation time of 20 h. Biofilms formed under non-supplemented mM 9 transfer and no medium transfer were used to determine whether transfer affected final biomass accumulation. Biofilms were quantitated as described previously. Experiments were performed in triplicate replicate and repeated independently a minimum of three times (n=9).

**Dispersion of mature biofilms:** Biofilms were grown as described. At 20 h, sets of tubes were transferred to a biological safety cabinet; culture was removed by aspiration and immediately replaced with the appropriate pre-warmed transfer solution containing 1 mg NP or FP.ml⁻¹. Non-supplemented medium was used as the negative control. Tubes were returned to the incubator, sampled 5, 10, 30 and 60 min post-transfer and biofilm was quantitated as previously described. Experiments were done in triplicate and repeated four times (n=12).

**Evaluation of cationized phytoglycogen to reduce increased biofilm formation due to sub-MIC to bramycin**

Assessments were made in sterile 96 well polystyrene plates. Set up and inoculation followed the MIC protocol except biofilm accretion was assessed at 0, 0.125, 0.25, 0.5, 1, 1.25, and 1.5 X the MIC value (0.4 µg tobramycin.ml⁻¹), in the absence or presence of 1 or 10 mg FP.ml⁻¹. Plates were incubated statically at 37°C. At 20 h, plates were transferred to a biological safety cabinet, well contents were aspirated using a pipette, discarded, the wells rinsed with 0.9% (wt/vol) NaCl, stained and quantitated for biofilm accretion. Experiments were done in quadruplicate replicate and repeated three times (n=12 ± sem).
Combination dosing of biofilms with cationized phytoglycogen and antibiotics

Biofilm growth and treatments were performed in sterile 96 well polystyrene plates. Overnight culture of P. aeruginosa PAO1 in MHB medium (37°C, 150 rpm, 16-18 h) was diluted 1:4000 in MHB, 100 µl transferred to wells and incubated (37°C, 150 rpm). Sterile MHB was the negative growth control. At 20 h, plates were transferred to a biological safety cabinet, well contents were gently aspirated, the wells rinsed with sterile 0.9% (wt/vol) NaCl and the 20 h biofilms were then exposed to CIP or TOB at 0.125, 0.25, 0.5, 1, 2, and 4 X MIC, in MHB with 0, 1 or 10 mg FP.ml⁻¹. Plates were incubated at 37°C for a further 24 h, after which cell contents were aspirated using a pipette, the wells rinsed with 0.9% (wt/vol) NaCl and stained for biofilm. Experiments were done in quadruplicate replicate and repeated three times (n=12 ± sem).

RESULTS AND DISCUSSION

Phytoglycogen was quaternized to have properties dependent upon surface charge, akin to certain metal species [21] or cationic antimicrobials [22,23]. The resulting cationic nanoparticles can be expected to interact with cell surfaces, disrupting function and viability [24]. Cationization was achieved using a synthesis protocol based upon the 12 principles of green chemistry (Table 1), using glycidyl trimethyl ammonium chloride, a short chain Quaternary Ammonium Compound (QAC) of no known antimicrobial efficacy or toxicity to mammalian cells [25, 21]. During Minimum Inhibitory Concentration (MIC) screening a product was identified that caused visual reductions in the blue-green phenazine pyocyanin, a pigment whose production is correlated with virulence factor synthesis and biofilm formation by Pseudomonas aeruginosa PAO1 [26,27]. Physical characterization of this sample indicated that the nanoparticles had an average hydrodynamic diameter of 61.4 nm with associated polydispersity index of 0.19 (dynamic light scattering), a degree of substitution of 0.6 (NMR and elemental analysis) and a zeta potential value of 26.6 mV.

<table>
<thead>
<tr>
<th>Principle</th>
<th>Evaluation</th>
<th>Score</th>
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<tr>
<td>Prevent waste</td>
<td>Small amounts of hydrolysis by-product which is of low toxicity and biodegradable, and NaCl from the catalyst neutralization step.</td>
<td>+ + +</td>
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<tr>
<td>Maximize atom economy</td>
<td>Excellent atom economy- all reagent atoms go into product</td>
<td>+ + +</td>
</tr>
<tr>
<td>Reduce hazardous chemical syntheses</td>
<td>The reagent GTAC is corrosive, toxic and carcinogenic but also non-flammable and degraded into harmless products during the reaction.</td>
<td>±</td>
</tr>
<tr>
<td>Safer chemical/product design</td>
<td>This has not been determined</td>
<td>nd*</td>
</tr>
<tr>
<td>Safer solvents and auxiliaries</td>
<td>Solvent system is water</td>
<td>+ + +</td>
</tr>
<tr>
<td>Increase energy efficiency</td>
<td>Reaction is performed at 40°C</td>
<td>+ + +</td>
</tr>
<tr>
<td>Use of renewable</td>
<td>Phytoglycogen is a renewable resource; GTAC reagent could be</td>
<td>+ +</td>
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resources modified to be renewable
Avoid chemical derivatives No derivatives are used +++
Use catalysts, not stoichiometric reagents Catalytic reaction; no stoichiometric by-products +++
Design chemicals and products to degrade after use Likely biodegradable ++
Real-time analysis to prevent pollution Consumption of reagent is monitored to maximize reaction efficiency and ensure no pollution +++
Minimize potential accidents Low-volatility, non-explosive, ambient pressure ++

+ indicates cationization score
* nd, not determined; low toxicity has been indicated for a similar derivative of phytoglycogen Lu et al. [12]

Table 1: Antimicrobial efficacy or toxicity to mammalian cells.

Cationized phytoglycogen disrupts biofilm formation and growth

*P. aeruginosa*, the model organism for studying biofilms, was used to assess the impact of native (NP) and the Functionalized Phytoglycogen (FP) on biofilm formation. Only supplementation with FP resulted in a concentration-dependent reduction in accreted biofilm, whereas NP had no appreciable impact. This lack of biological response is relevant if NP is to be used as a platform for synthesizing tailored polymers. Treatment of nascent biofilms with FP (Figure 1) proved that it also negatively affected biofilm development, impeding maturation of the nascent biofilms and resulting in near-complete loss of biomass.
Finally, when mature biofilms were exposed to NP or FP, only the latter resulted in average losses of 39%-43% (Figure 2). This was a rapid process, achieved within five minutes and not enhanced by extending treatment up to 60 min. The reduction was less pronounced than for the treatment of nascent biofilms, possibly due to the much shorter exposure time. This is more consistent with a surface cleansing regimen and with the age of the biofilms. Mature biofilms typically have considerable structure. Similar to certain QACs [22], positively-charged nanoparticles, such as FP, may interfere with the interactions between extracellular matrix polymers, affecting the biofilm’s structural integrity.
Figure 2: Short-term exposure of pre-formed *P. aeruginosa* biofilms to cationized phytoglycogen causes a reduction in the associated biomass.

Cationized phytoglycogen reduces induced biofilm formation by sub-MIC tobramycin (TOB)

We hypothesized that the biofilm-disruptive properties of FP could mitigate conditions which promote and/or exacerbate biofilm formation and growth. One such example is the undesirable sub-MIC induction of enhanced biofilm formation by aminoglycosides and other antibiotics [28-30]. As expected, biofilm formation and accumulation increased between 1.3 to 2.1-fold at sub-MICTOB and only declined at TOB concentrations ≥ MIC (Figure 3). Surprisingly, whereas FP suppressed the extent of biofilm formed on glass, when polystyrene (assay wells) was used ca. 2.25-2.5 times more biofilm accumulated relative to non-supplemented medium. Since polystyrene is relatively more hydrophobic than glass, this difference probably reflects interactions between nanoparticles and cells, and responsive changes in cell surface properties. All contribute to cell interactions, affecting initial attachment and adhesion and subsequent biofilm accretion [31]. Yet, in spite of this, when supplemented with FP, biofilm proliferation due to sub-MICTOB was inhibited at ¼-MICTOB and at ½ or ¾-fold MICTOB, biofilm accretion was not only inhibited but also substantively reduced. Normalizing data across the media conditions (0, 1 or 10 mg FP.ml⁻¹) to the absorbance value for biofilm accretion at the same concentration of TOB in 0 mg FP.ml⁻¹ showed that FP caused reduction ratios of 0.82 and 0.69 at ½-MIC and 0.85 and 0.87 at ¾-MIC for 1 and 10 mg FP.ml⁻¹ respectively (equivalent to reductions of 0.62 and 0.31 at ½-MIC and 0.85 and 0.87 at ¾-MIC for 1 and 10 mg FP.ml⁻¹ respectively, when data is normalized to biofilm accretion in non-supplemented medium, no antibiotic). Similar biofilm reduction for non-supplemented medium only occurred once the MICTOB was achieved. That is, FP caused inhibition of the enhanced biofilm formation attributable to sub-MIC values of TOB. Planktonic MIC data found no synergistic effects between TOB and FP since the MICTOB+FP values were 1-2 fold MICTOB.

Figure 3: Cationized phytoglycogen inhibits enhanced biofilm formation attributable to sub-MIC tobramycin.
Combining cationized phytoglycogen and antibiotic enhances biomass reduction of pre-grown biofilms

20 h-old biofilms were treated with ciprofloxacin (CIP) or TOB, both of which are used therapeutically in resolving infections by P. aeruginosa. Planktonic MIC data showed that CIP (MIC 0.125 g.ml\(^{-1}\)), but not TOB, was potentiated by an 8-fold decrease in MIC\(_{CIP}\) in the presence of 1 mg FP.ml\(^{-1}\). Exposing the 20 h-old biofilms to TOB in unsupplemented medium provoked a net increase in biofilm proliferation for the range 0.125 to 2 times the MIC\(_{TOB}\); only at four-fold the MIC\(_{TOB}\) were biofilms reduced by 40%. A similar trend was observed for sub-MIC\(_{CIP}\), with no change at the MIC, and reduction ratios in biomass of 0.64 and 0.73 at two- and four-fold MIC values respectively. The data indicated that both antibiotics possessed concentration ranges which stimulated growth of pre-existing biofilms. Most studies to date have investigated how antibiotics promote initial biofilm formation [28-30] and not how antibiotics may stimulate further proliferation of pre-existing biofilms. This novel finding adds to the incremental knowledge base. That is, under certain conditions, therapies designed to cure may be driving biological responses which exacerbate infections, making these less tractable to treatment. Treatment of biofilms with either TOB or CIP in combination with 1 mg FP.ml\(^{-1}\) did not because a reduction in biofilm proliferation relative to the medium alone control (Figure 4). However, when data were normalized relative to their corresponding medium conditions without added antibiotic s supplementing antibiotic with 1 mg FP.ml\(^{-1}\) was sufficient to prevent antibiotic-induced increase in biofilm accumulation within this medium. In contrast, supplementation with 10 mg FP.ml\(^{-1}\) resulted in reduced accumulation of biofilm at sub-MIC concentrations for both TOB and CIP [32-34]. For example, at the MIC\(_{TOB}\), biofilms were reduced by a factor of 0.9 relative to the corresponding treatment with MIC\(_{TOB}\) in medium alone control; at 0.5 MIC\(_{CIP}\), the biofilm was 0.2 of the corresponding 0.5 MIC in medium alone. That is, a general trend was noted where biofilms treated with antibiotics alone showed elevated biofilm accretion at sub-MIC and required 2-4 times MIC to show similar decreases in biomass. Treatment of biofilms with antibiotics supplemented with 1 mg FP.ml\(^{-1}\) inhibited antibiotic-induced enhanced biofilm accretion, whereas at 10 mg FP.ml\(^{-1}\) there was a net reduction in biofilm biomass.
Cationized phytoglycogen used in combination with antibiotic inhibits biofilm proliferation and enhances reduced biomass of biofilm.

Motility of *P. aeruginosa* is negatively impacted by cationized phytoglycogen

*P. aeruginosa* displays three distinct forms of motility – swimming, twitching, swarming - all of which fulfil roles during infection and are important for migration, attachment and surface colonization, as well as biofilm formation and maturation [35-37]. Concentration-dependent inhibition was observed for all three forms of motility in the presence of FP at concentrations ≥ 0.5 mg/ml. That is, the concentrations employed in assessing how FP could affect biofilm accretion due to stimulation by deleterious agents such as antibiotics would be expected to affect these forms of motility. Supplementation with NP or low concentrations of FP (0.1 mg/ml) resulted in increased motility. The high capacity for water retention by NP [4,5] is likely of consequence to cells on air-exposed agar plates and more targeted approaches, such as the use of flow cells, are required to fully interrogate this. The increased motility promoted by NP, or low concentrations of FP, could signify potential application limits since swarming and twitching motility positively correlate with biofilm formation and growth. However, although NP resulted in enhanced motility (Figure 5a), this did not translate into increased biofilm development. This was an important outcome: stimulation of biofilm by NP would preclude its use in applications such as drug delivery. While motility was impaired at concentrations of ≥ 500 µg FP.ml−1, biofilm biomass was reduced by 50% in the presence of 100 µg FP.ml−1.
Figure 5a: Cationized phytoglycogen reduces all three motility modes of *P. aeruginosa*

Clearly, the biofilm accretion assay indicated greater sensitivity to FP. Collectively, the data indicated that phytoglycogen nanoparticles, isolated from a sustainable source, could be modified using a protocol based on the 12 green principles. The modified nanoparticles inhibited both motility and biofilm formation and development, two critical aspects of the development and progression of infectious disease. These desirable properties could demonstrably act in combination with antibiotics, providing a significant improvement over the action of antibiotics alone. In particular, the modified phytoglycogen could alleviate biofilm proliferation as a response to antibiotic. Phytoglycogen is a promising molecular scaffold for the design and synthesis of novel polymeric constructs which may have future utility as preventatives or co-therapeutics for the treatment and management of biofilms (Figures 5b and 5c).
CONCLUSION

Significance and impact of the study
Biofilms confer increased persistence and recalcitrance of microbial life. Confoundingly, agents who combat these protective structures may promote their growth. Functionalized
Phytoglycogen (FP), a ‘nano by nature’ polymer extractable from sustainable sources and offering benefits over conventional nanoparticles, substantively reduced biofilms and prevented increased biofilm formation in response to sub-optimal antibiotic concentrations. For the first time, we show antibiotic treatment of pre-grown biofilms drives proliferation, whereas FP-antibiotic combinations reduced growth. Swimming, swarming and twitching motility, all associated with biofilm development, were negatively affected by FP. The use of novel polymers to enhance antibiotic performance opens new pathways in antibiotic research.

ACKNOWLEDGEMENTS
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CONFLICT OF INTEREST
KK and AK are employed by Mirexus Biotechnologies Inc.; Mirexus Biotechnologies Inc. also provided financial support and some materials.

SUPPORTING INFORMATION
Supplementary Data Table 1: The cationization process fulfils the 12 principles of green chemistry

REFERENCES


