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LETTER

The response of *Pseudomonas aeruginosa* biofilm to the presence of a glass polyalkenoate cement formulated from a silver containing glass

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Biofilms are microbial derived sessile communities characterized by cells that are irreversibly attached to a substratum, or to each other, and embedded in a matrix of extracellular polymeric substances that they have produced [1]. Such microorganisms exhibit an altered phenotype with respect to growth rate and gene transcription [2]. Biofilms forming on implanted medical devices are problematic as the extra cellular matrix exported by the microorganisms along with the changes in their physiology result in the requirement to remove the device to effect a cure [3, 4]. *Pseudomonas aeruginosa* is a human opportunistic pathogen that colonizes biotic and abiotic surfaces and has emerged as a primary source of nosocomial infections [5], especially in cystic fibrosis sufferers and immuno-compromised patients [6]. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect if the tissue defences are compromised in some manner, such as in severe burns sufferers [7]. In the United States, *P. aeruginosa* ranked first among all nosocomial pathogens related to pneumonia in intensive care units reported to the National Nosocomial Infection Surveillance System [8]. *Pseudomonas* sp. in general and *P. aeruginosa*, specifically, has resistance to antibiotics including aminoglycosides and quinolones, and this is steadily increasing [9]. The mechanisms of biofilm formation are poorly understood and effective prevention and therapeutic strategies still need to be developed for device-associated infections. Treatment with antibiotics can slow down biofilm progression by eliminating planktonic cells and interfering with biofilm metabolism [10], but complete removal is rare. Other eradication methods that have been employed include prevention of initial attachment of bacterial cells by constructing materials into which antimicrobial agents have been incorporated [10] and minimizing biofilm formation by the disruption of quorum-signalling molecules, allowing for improved inactivation and removal [11]. Glass polyalkenoate cements (GPCs), formed by the reaction between an ion-leachable glass and an aqueous solution of polyacrylic acid (PAA) [12], are both antibacterial and cariostatic [13]; properties related to their ability to release beneficial amounts of therapeutic ions [14, 15]. Studies have shown that inhibition of bacterial growth correlates with zinc (Zn^{2+}) and silver (Ag^{+}) ion release from novel GPCs [16, 17]. Zn^{2+} has been shown to inhibit multiple activities in the bacterial cell including glycolysis, transmembrane protein translocation and acid tolerance [18]. It also influences pH, which rises throughout glycolysis via the action of Zn^{2+} on the bacterial cell wall and therefore leaves an excess of OH^{-} that results in a favourable bioactive response [19]. The minimum Zn^{2+} concentration required for *P. aeruginosa* inhibition is 6.02×10^{-4} μg/mL [20] and 8 μg/mL for biofilm inhibition [21]. Ag^{+} is also a known antibacterial agent [22, 23]. To have antimicrobial efficacy against *P. aeruginosa* bacteria, Ag^{+} must be released in biocidal concentrations of 1.102×10^{-6} μg/mL [24] and 5 μg/mL for biofilm inhibition [25]. Ag^{+} avidly binds to negatively charged components in proteins and nucleic acids, thereby causing structural changes in bacterial cell walls, membranes and nucleic acids that affect viability [26].

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It is accepted that novel Zn–Ag–GPCs are antibacterial against a clinical isolate of *P. aeruginosa* [16, 17], but the objective of the work reported herein is to determine whether such a material can inhibit the proliferation of a biofilm forming from *P. aeruginosa*.

A glass (56.04 SiO_2, 32.76 ZnO, 0.33 Ag_2O and 10.87 Na_2O mol%) was synthesized by mixing appropriate amounts of analytical grade reagents in a ball mill (1 h), then firing the mixture in a mullite crucible (1480 °C, 1 h), prior to shock quenching into water. The resulting frit was ground down and all further work was undertaken on sub 25 µm particles. The GPC was prepared by mixing 0.5 g glass with 0.2 g PAA (Mw, 210,000, Ciba Specialty Polymers, Bradford, UK) and 0.25 mL distilled water on a clean glass plate with a dental spatula in ambient laboratory conditions. Cement constructs (Fig. 1) were produced by placing the cement into moulds (4 mmØ, 6 mm ht) and maturing in an oven (37 °C, 24 h). Ion release from the GPC was already reported [16, 17], but is included here (Fig. 2) for completeness. In agreement with the literature [27], the majority of Zn^{2+} (1.5 ppm) and Ag^{+} (0.2 ppm) release occurred in the first 24 h.

The bacterial strain used in this study was *P. aeruginosa* (PAO1), a clinical wound isolate [28], obtained from the Iglewiski laboratory (University of Rochester, NY, USA). Bacterial cultures were grown overnight in Lysogeny Broth (LB broth, Difco, Oxford, UK) supplemented with 0.5% of 1 M Glucose (Glu) solution (Sigma-Aldrich, Dublin, Ireland) added in a Sanyo MIR-162 Incubator (San Diego, USA) at 30 °C. The turbidity of the culture was then measured (at 595 nm) using a spectrophotometer (6300 Series Spectroscopy, Jenway, Staffs, UK). The culture was then diluted using fresh LB/Glu Broth to achieve an absorbance value of 0.05. The biofilm assay was then carried out using a 96-well microplate (Costar, Corning Incorporated, NY, USA). The procedure was adapted from Merritt et al. [29]. The blank was 200 µL of fresh LB/Glu Broth. Two controls were used; 200 µL of the diluted overnight culture of *P. aeruginosa* was pipetted in 16 wells each. These were checked after 12 and after 24 h. To check the effect of the GPC on biofilm, 200 µL of the diluted overnight culture was incubated (in 16 wells) for 12 h to form biofilm; the GPC was then added aseptically and biofilm levels were checked after another 12 h of incubation (24 h in total). Figure 3 offers a schematic diagram of the contents of each well.

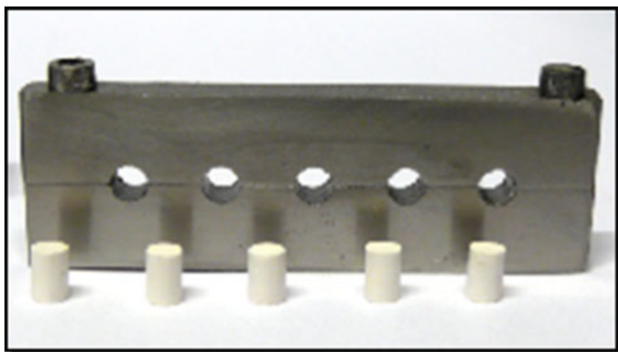

Fig. 1 Samples of GPC alongside the mould

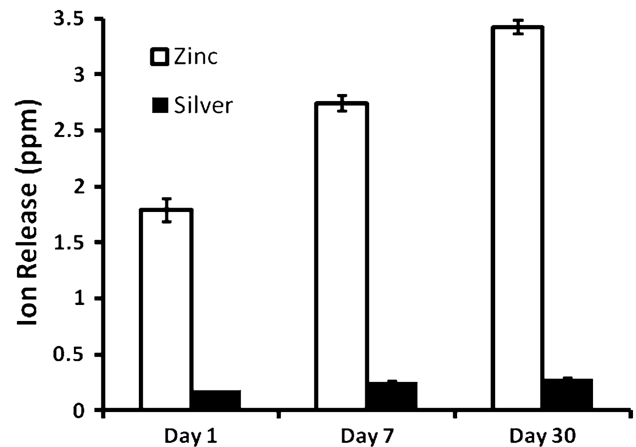

Fig. 2 Cumulative ion release profiles for Zinc and Silver from the GPC

The microplate was incubated at 30 °C (24 h), after which it was overturned, its contents emptied and the wells rinsed out three times with sterilized distilled water. 250 µL of 0.1% crystal violet stain (Sigma-Aldrich, Dublin, Ireland) was pipetted into each of the wells used (residence time, 5 min). The contents were then removed and rinsed out three times with sterilized distilled water. 276 µL of a 20:80% acetone:ethanol mix (Sigma-Aldrich, Dublin, Ireland) was pipetted into the used wells and left for a further 5 minutes to solubilize the attached biofilm. The absorbance of the wells was then tested at 595 nm on a Microplate Reader (BioTek, Mason Technology, Dublin, Ireland) to determine if there was any difference in the absorbance between the contents of the wells containing, and those free of, GPC at the different time intervals. Figure 4 shows that the absorbance values of the wells containing biofilm after 12 h was 0.571 (SD 0.206) and after 24 h 0.924 (SD 0.219). The wells containing the biofilm and the GPC (that was added after 12 h growth) had an absorbance of 0.302 (SD 0.076). Therefore a significant statistical difference ($P < 0.001$) was observed at 12 h between wells containing biofilm and those containing biofilm and GPC (*T* test; Microsoft Excel, California, USA). The experiment was repeated with the biofilm left to grow for 24 h before the addition of the GPC (Fig. 4), which shows that the absorbance values of the wells containing the biofilm and the GPC (added after 24 h growth) had an absorbance of 2.02 (SD 0.411).

Fig. 3 Contents of the 96-well microplate used to analyze the biofilm and biofilm removal

Broth LB\Glu	Blank	Bacterial Biofilm After 12hr	Bacterial Biofilm After 12hr	Blank	Bacterial Biofilm After 24hr	Bacterial Biofilm After 24hr	Blank	Bacterial Biofilm After 12hr & Cement	Bacterial Biofilm After 12hr & Cement	Blank	Blank
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		
X		X	X		X	X		X	X		

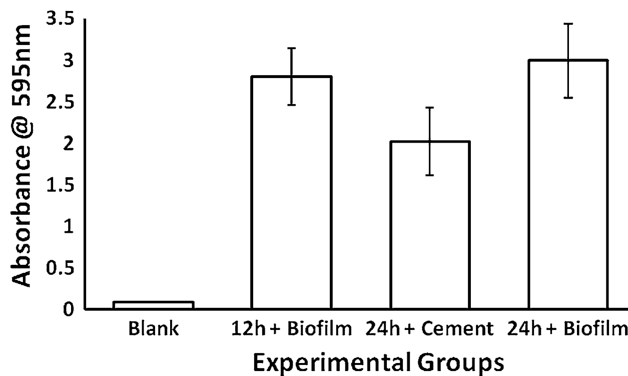

Fig. 4 Absorbance levels of the growth of biofilm in the well plates containing biofilm alone and those containing the biofilm and GPC

The literature reports a similar study using polymethylmethacrylate (PMMA) cement (VersaBond; Smith & Nephew, Memphis, TN, USA), where high absorbance rates, similar to those in the wells containing solely biofilm in this study, indicated that PMMA was unable to eliminate biofilm [28]. Therefore the reduction in absorbance in the wells that contained the novel Zn–Ag–GPC in this study indicates that the cement partially eliminated the biofilm present and prevented the formation of any more. Biofilm formation on medical devices has severe health consequences as it provides a sanctuary for bacteria, which are tolerant to both host defence mechanisms and antibiotic therapies. This study has shown that a novel Zn–Ag–GPC can inhibit such biofilm formation and partially reduce the levels of biofilm in vitro indicating that it may be possible to produce coatings or cements from this novel Zn–Ag glass that retard or partially inhibit biofilm formation from *P. aeruginosa*.

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