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Diminishing biofilm resistance to antimicrobial nanomaterials through electrolyte screening of electrostatic interactions

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ABSTRACT

The extracellular polymer substances (EPS) generated by biofilms confers resistance to antimicrobial agents through electrostatic and steric interactions that hinder molecular diffusion. This resistance mechanism is particularly evident for antibacterial nanomaterials, which inherently diffuse more slowly compared to small organic antibacterial agents. The aim of this study was to determine if a biofilm's resistance to antibacterial nanomaterial diffusion could be diminished using electrolytes to screen the EPS's electrostatic interactions. Anionic (+) alpha-tocopherol phosphate (α-TP) liposomes were used as the antimicrobial nanomaterials in the study. They self-assembled into 700 nm sized structures with a zeta potential of −20 mV that were capable of killing oral bacteria (*S. oralis* growth inhibition time of 3.34 ± 0.52 h). In a phosphate (-ve) buffer the -ve α-TP liposomes did not penetrate multispecies oral biofilms, but in a Tris (hydroxymethyl)aminomethane (+ve) buffer they did (depth - 12.4 ± 3.6 μm). The Tris did not modify the surface charge of the α-TP nanomaterials, rather it facilitated the α-TP-biofilm interactions through electrolyte screening (Langmuir modelled surface pressure increase of 2.7 ± 1.8 mN/ m). This data indicated that EPS resistance was mediated through charge repulsion and that this effect could be diminished through the co-administration of cationic electrolytes.

1. Introduction

Bacterial biofilms are structured communities that co-exist within an extracellular matrix [1]. When a biofilm is formed, the bacteria within it become up to 1000 times more resistant to antimicrobial treatment compared to the planktonic organisms [2]. This resistance originates from the creation of subpopulations in the biofilm [3], a higher mutation rate [4], the upregulation of efflux pumps [5], modifications in bacterial lipopolysaccharide (LPS) and a reduction in the diffusion rates of antimicrobial agents in the biofilm matrix, which effectively dilutes the administered agents. These characteristics render it problematic to control biofilm growth once they are established on the surface of materials.

Nanomaterials can physically disrupt biofilms, they can carry antibacterial agents into biofilm communities to control growth [6,7] and, through modification of their surface chemistry, their interactions with the biofilms can be controlled [8,9]. Therefore, it has been suggested that nanomaterials can be designed to penetrate and kill bacteria in biofilm communities [10,11]. However, because each biofilm can show significant variability with respect to the organisms and extracellular components that it contains [12] and nanomaterial diffusion is inherently slower than small organic antimicrobials, designing a nanomaterial that has the surface properties to allow it to efficiently diffuse into a multispecies biofilm after deposition onto a material surface is not a trivial task [13–15]

One approach that could reduce the biofilm resistance to nanomaterial diffusion is to co-administrator a penetration enhancer in order to modify the biofilm interactions with the nanomaterial surfaces. In a similar manner to other biological barriers, *e.g.*, epithelial mucus, bacterial biofilms restrict the diffusion of xenobiotics, within their structured communities, through steric hindrance and electrostatic interactions [16]. The electrostatic interactions in biofilms arise from the outer surface of the bacteria, which are generally negatively charged due to their lipoteichoic acid and lipopolysaccharide components, and

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the extracellular polymer substances (EPS) [17], which can also be negatively charged. Therefore, it seems feasible that cationic penetration enhancers could be useful to screen biofilm electrostatic interactions in attempt to dampen their capability to resist nanomaterial diffusion.

Biofilm electrostatic interactions with antimicrobial nanomaterials could be screened using electrolytes because as electrolyte concentration increases in the biofilm it would be expected that there would be a reduction of the Debye length of the functional groups on the EPS [18]. For example, at an ionic strength of 0.1 mM, the charge effect, *i.e.*, Debye length, should extend by approximately 10 nm, while at 100 mM, it should only extend about 1 nm from the surface of the EPS. This would increase the effective pore size by about 10 nm as the ionic strength is increased from 0.1 to 100 mM, which could have a significant effect on the diffusion of nanomaterials through oral biofilms [19]. Previous work has suggested that electrolyte screening interactions do not influence the diffusion of small nanomaterials encountered during environmental exposure, but there is emerging evidence that it could be significant for larger nanomaterials, *i.e.*, those used to deliver antimicrobial agents as they are typically larger than 10 nm [16,20].

Understanding the screening potential of electrolytes in biofilms could also provide valuable information about the properties of the biofilm EPS. Although it has been stated that the EPS is negatively charged in biofilms it is known that the EPS produced by different species of bacterial varies greatly in composition [21]. These variations generate regions in the EPS that have a different electrostatic charges and different steric interactions due to changes in the component's molecular weight ($0.5–2.0 \times 10^6$ Da) [22]. Studies have confirmed that EPS composition changes influence biofilm interactions with lectins, lipids and the surface of bacteria, but very little work has been performed to understand how the EPS composition influences the access of antimicrobial nanomaterials to the bacteria within the biofilm [23]. One of the reasons is that when fully hydrated, the bulk properties of biofilms can be very similar to those of water, making it difficult to delineate the barrier between the biofilm and the surrounding bulk liquid [24].

The aim of this study was to investigate if the resistance of biofilms to the penetration of antimicrobial nanomaterials could be overcome through the co-administration of electrolytes that screen the biofilms electrostatic interactions with the result of enhancing the nanomaterial's antimicrobial action. The mono alkyl phosphate amphiphile vitamin (+) alpha-tocopherol phosphate (α-TP) was selected as the test antimicrobial agent. Phosphate amphiphiles can form a range of different types of nanomaterials and they are arguably one of the most flexible types of anti-biofilm systems. They can act directly to disrupt bacterial biofilms or they can be loaded with an antimicrobial agent, which they can deliver into biofilms [25,26]. α-TP was specifically selected in this study as it has been shown to form bi-layer islands in aqueous vehicles with a negative surface charge, thus if presented to a biofilm with a negatively charged EPS, electrolyte screening could potentially increase the penetration of these nanomaterials into the biofilm [27]. The naturally occurring α-TP stereoisomer (RRR, + or d) was employed in the study as it has been previously shown to have direct antimicrobial activity, but as it was not easy to extract from natural sources it synthesised from (+) alpha tocopherol (α-T) [18]. An oral multispecies biofilm was used in the study because previous work had suggested that oral biofilms display a net negative charge [19] and thus they would restrict the diffusion of the α-TP into the biofilm by electrostatic repulsion. In addition, it was perceivable that the phosphate nanomaterials and electrolytes could be co-localised for an extended period of time in oral biofilms *in-vivo*, thus the study results may be of practical significance in the field of oral hygiene [28]. In-keeping with the potential practical use of the study data the test agents were always dissolved in a 20% ethanol 80% water vehicle at pH 7.4 as it mimicked an oral healthcare product. The negatively charged phosphate, predicted to have very little effect on the nanomaterial-biofilm interaction, and the positively charged Tris ((hydroxymethyl)aminomethane), predicted to screen the biofilm-nanomaterial interactions through its three ethyl alcohol groups, were used in the study as both these electrolytes are known to be capable of adsorbing at biological interfaces [29]. As the addition of the electrolytes to the biofilm system also had the potential to modify the antimicrobial nanomaterial size, surface polarity and charge these characteristics were assessed using light scattering and fluorescence spectroscopy. Confocal microscopy was used to investigate the multispecies salivary biofilm penetration of the aggregates in the presence of the two different electrolytes [30]. These penetration results were investigated in more detail by studying the effects of the electrolytes on the interactions of the nanomaterials with artificial Gram-positive bacteria membranes, using a Langmuir trough, and the effects of the electrolyte nanomaterial combinations on the bacteria growth inhibition was assessed using a single species of oral bacteria, *Streptococcus oralis,* a primary coloniser in the mouth [31].

2. Experimental section

2.1. Materials

(+) α-T (Type VI, natural extract ≥ 40% purity), phosphorus oxychloride ($POCl_3$) (≥99%), tetrahydrofuran (THF) (anhydrous) (≥99.9%), trimethylamine (≥99%), trifluoroacetic acid (≥99%), Tris hydrochloride (≥99%), cetylpyridinium chloride (CPC) (99.0–102%), brain heart infusion (BHI) broth and glycerol were purchased from Sigma Aldrich, UK. Absolute ethanol, propan-2-ol, hexane fractions (60–80), disodium hydrogen phosphate, monosodium dihydrogen phosphate, blood agar (BA) plates containing blood agar base no. 2 with 5% horse blood, 0.2 μM nylon syringe filters, hydrochloric acid and sodium hydroxide were purchased from Fisher scientific Ltd, UK. Deionised water was used from laboratory supply. Hydroxyapatite discs (5 mm diameter x 2 mm thick) were purchased from Himed inc, USA. Live/ dead ® BacLight™ bacterial viability kit, for microscopy, was purchased from Life Technologies, UK. *S. oralis* NCTC 7864 T was purchased from LGC standards, USA. 1-palmitoyl-2-oleoyl-sn-3-glycerophospho-1-glycerol (POPG) and 1-palmitoyl-2-oleoyl-sn-3-glycerophosphocholin (POPC) were purchased from Avanti polar lipids, USA. Chromatographic paper, 10 mm x 100 m was purchased Whatman, Maidstone, UK. Plastic syringes (1 and 20 mL) were purchased from Terumo, Philippines. Syringe needles were purchased from Macrolance, Ireland. Disposable clear dynamic light scattering cuvettes (macro, PMMA) and disposable folded capillary cells (DTS1070) where purchased from VWR, Germany. Clear sterile polyester adhesive films were purchased from Starlab, UK.

2.2. Methods

2.2.1. (+) α-TP synthesis

(+) α-TP was synthesised as previously described to generate a naturally derived, non-commercially available isoform [18]. In brief, (+) α-T was phosphorylated in the presence of phosphorus oxychloride with triethylamine in anhydrous THF for 3 h at room temperature. The triethylamine hydrochloric acid salt was removed and the solution was hydrolysed in water for 24 h. (+) α-TP was then extracted into hexane, into water at basic pH and then again into hexane at acidic pH to remove the impurities. The product was purified by C18 chromatography (final purity 99%).

2.2.2. (+) α-TP aggregate characterisation

To understand the effects of the electrolytes on the self-assembly of (+) α-TP, fluorescence emission spectra of (+) α-TP (195 μM) dispersions were recorded using a fluorescence spectrometer fitted with a Xenon pulse lamp (Varian Cary Eclipse Fluorescence Spectrometer, Agilent Technologies, UK). A fluorescence cell (Helima fluorescence cell 10 mm, Helima UK Ltd., UK) with a 10 mm path length was used.