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Role of gallium and silver from phosphate-based glasses on in vitro dual species oral biofilm models of *Porphyromonas gingivalis* and *Streptococcus gordonii*

Sabeel P. Valappil *, Marc Coombes, Lucy Wright, Gareth J. Owens, Richard J.M. Lynch, Christopher K. Hope, Susan M. Higham

Department of Health Services Research and School of Dentistry, University of Liverpool, Research Wing, Daulby Street, Liverpool L69 3GN, UK

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ABSTRACT

Phosphate-based glasses (PBGs) are excellent controlled delivery agents for antibacterial ions such as silver and gallium. The aim of this study was to assess the potential utility of novel PBGs combining both gallium and silver for use in periodontal therapy. To this end, an in vitro biofilm model with the putative periodontal pathogen, *Porphyromonas gingivalis*, and an initial colonizer, *Streptococcus gordonii*, was established. The effect of increasing calcium content in gallium–silver-doped PBG on the susceptibility of *P. gingivalis* was examined. A decrease in degradation rates (30.34, 25.19, 21.40 $\mu g\ mm^{-2}\ h^{-1}$) with increasing PBG calciumcontent (10, 11, 12 mol.% respectively) was observed, correlating well with gallium and silver ion release and antimicrobial activity against planktonic *P. gingivalis* (approximately 5.4 $\log_{10}$ colony-forming units (CFU) reduction after 24 h by the C10 glass compared with controls) and *S. gordonii* (total growth inhibition after 32 h by C10, C11 and C12 glasses compared with controls). The most potent PBG (C10) was evaluated for its ability to inhibit the biofilm growth of *P. gingivalis* in a newly established constant-depth film fermentor model. The simultaneous release of silver and gallium from the glass reduced *P. gingivalis* biofilm growth with a maximum effect (1.92 $\log_{10}$ CFU reduction) after 168 h. Given the emergence of antibiotic-resistant bacteria and dearth of new antibiotics in development, the glasses, especially C10, would offer effective alternatives to antibiotics or may complement current therapies through controlled, localized delivery of gallium and silver ions at infected sites in the oral cavity.

1. Introduction

Periodontal diseases are a group of inflammatory diseases of the gingiva and the supporting structures of the periodontium. Plaque-related chronic periodontitis accounts for up to 60% of tooth loss in the UK and the annual cost of NHS periodontal therapy in 2001/2002 was £174 million [1]. This is of serious concern, as recent studies provide increasing evidence that periodontitis may be a risk factor for severe systemic conditions such as arteriosclerosis, myocardial infarction and stroke; increase the risk for preterm, low birth weight babies; and pose threats to those with chronic diseases, such as diabetes, respiratory diseases and osteoporosis [2,3]. Conventional periodontal therapy involves scaling or root planning, but in more severe cases antimicrobial agents such as doxycycline, metronidazole, minocycline or combinational antimicrobial chemotherapy are used as adjuncts. However, bacteria growing in a biofilm have been reported to be 1000 times more resistant to antimicrobial treatments than their planktonic counterparts [4] and are responsible for >80% of microbial infections in humans [5]. The putative periodontal pathogen *Porphyromonas gingivalis* is detected in dental plaque samples within 6 h following professional tooth cleaning [6,7], and numbers of *P. gingivalis* increase at sites of periodontal disease [8]. It has also been reported that a preformed streptococcal substratum is required for its incorporation into a biofilm [9]. The early appearance of *P. gingivalis* in the development of dental plaque biofilms was substantiated by the findings from Periasamy and Kolenbrander [10], who reported that *P. gingivalis* has the ability to interact with a variety of different stage colonizers and that it exhibits widespread mutualism with initial (*Streptococcus gordonii* and *Actinomyces oris*), early (*Veillonella* sp.), middle (*Fusobacterium nucleatum*) and late colonizers (*Aggregatibacter actinomycetemcomitans*). Further, *P. gingivalis* displayed specificity with initially colonizing streptococci as it forms biofilms with *S. gordonii* [10] but not with *Streptococcus mutans* [9] or *S. cristatus* [11]. We hypothesized that the ability of *P. gingivalis* to co-aggregate with *S. gordonii* [10] would allow us to establish in vitro dual-species biofilm models which could be used to evaluate the antimicrobial action of novel silver- and gallium-doped phosphate-based glasses (PBGs).

* Corresponding author. Tel.: +44 151 706 5299; fax: +44 151 706 5809.
E-mail address: S.Valappil@liv.ac.uk (S.P. Valappil).

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The high occurrence of antibiotic resistance among bacteria, coupled with the potential role of periodontitis as a risk factor for severe systemic conditions, indicates the increasing demand for alternative strategies to combat these infections. From this perspective, silver, (Ag^+) or gallium (Ga^{3+}) ions, which have a broad spectrum of bactericidal activity with controlled release from PBGs, have attracted considerable interest in recent years [12–16]. The antibacterial mechanism of silver was proposed to be due to the inhibition of DNA replication and proteins becoming inactivated after contact with silver ions [17]. It was also reported that silver ions have the potential to destabilize the intercellular adhesion forces within bacterial biofilms [18]. Ga^{3+} is reported to be capable of interacting with: iron-dependent enzymes, such as ribonucleotide reductase, which inhibit DNA synthesis [19]; superoxide dismutase and catalase, which protect against oxidant stress [20]; and enzymes involved in oxidative phosphorylation, such as cytochromes [21]. Moreover, the capability of Ga^{3+} to interfere with iron-dependent enzymes suggests that Ga^{3+} might act concurrently on multiple targets [21]. Therefore mutation of a single intracellular target might not yield high-level Ga^{3+} resistance in targeted bacteria. The aims of this study were (i) to produce a range of gallium- and silver-doped PBGs; (ii) to establish an in vitro biofilm model of the putative periodontal pathogen *P. gingivalis*; and (iii) to probe the glass degradation and ion release, and relate these to the results from biofilm growth studies. The findings from this study may lead to the use of the PBGs to deliver silver and gallium ions to combat the putative periodontal pathogen *P. gingivalis*.

2. Materials and methods

2.1. Bacterial strain and growth

P. gingivalis ATCC 33277 was maintained on fastidious anaerobic agar (FAA; Bioconnections, UK), supplemented with 5% horse blood (TCS Biosciences, UK) and *S. gordonii* DL1 (Challis strain) on brain heart infusion agar (BHI). The bacteria were grown in an anaerobic (N_2:CO_2:H_2, 80:10:10) chamber (Don Whitley MG1000) at 37 °C.

2.2. Preparation of antibacterial PBGs

PBGs for this study were produced using NaH_2PO_4 (BDH, ⩾98%), P_2O_5 (Sigma, ⩾97%), $CaCO_3$ (BDH, ⩾98.5%), Ga_2O_3 (Sigma, 99.99%) and Ag_2SO_4 (Sigma, 99.99%). The required amount of each reagent was weighed and added to a quartz crucible (Fisher Scientific, UK). The crucible was then placed in a preheated furnace at 1100 °C for 1 h, after which the molten glass was poured into graphite moulds, which had been preheated to 350 °C. The glass samples were allowed to cool to room temperature, and the resulting glass rods were cut into discs (diameter, 5 mm; thickness, 2 mm) using an Isomet low-speed rotary diamond saw (Buchler Ltd, UK). Gallium–silver-doped glasses of general composition $(CaO)_x(Na_2O)_{47-x}(P_2O_5)_{45}(Ga_2O_3)_3(Ag_2O)_5$(where $x = 10$, 11 and 12, abbreviated herein after to C10, C11 and C12, respectively) were prepared along with a sample without gallium and silver (CNP), of composition $(CaO)_{20}(Na_2O)_{35}(P_2O_5)_{45}$.

2.2.1. Degradation study of antibacterial PBGs

C10, C11, C12 and CNP glass rods (diameter, 5 mm; thickness, 2 mm) were placed in plastic containers, which were filled with 50 ml of deionized water (pH 7 ± 0.5) and placed in an incubator at 37 °C. At various time points (2, 4, 6, 8, 24, 32 and 48 h) the three discs were taken out of their respective containers and excess moisture was removed by blotting the samples dry with tissue before they were weighed. All the discs were placed into a fresh solution of ultrapure water and placed back into the 37 °C incubator. To obtain the rate of weight loss, the initial weight (M_0) of each sample was measured, as was the weight at time t (M_t), to give a weight loss per unit area; thus, weight loss = $(M_0 - M_t)/A$, where A is the surface area (mm^2). The measurements were carried out in triplicate, and the data plotted as weight loss per unit area against time. The slope of this graph (determined by fitting a straight line of the form $y = mx$ through the origin) gave a dissolution rate value which was converted to $\mu g\ mm^{-2}\ h^{-1}$.

2.2.2. Ion release study of antibacterial PBGs

Parallel to the degradation analyses, ion release studies were conducted and the medium was analysed using inductively coupled plasma atomic emission spectrometry (ICP-AES) to determine the elements present in the samples. An ICP-AES spectrometer (Spectro Ciros CCD, UK) was used and the instrument was calibrated for the predicted concentration in the range 0.1–1000 ppb by mixing single element standards obtained from Sigma and diluted in ultrapure water. The data were plotted as ion release in ppm against time (h).

2.3. Disc diffusion assay

C10, C11 and C12 glasses were investigated for their ability to inhibit planktonic growth of *S. gordonii* and *P. gingivalis* using disc diffusion methodology (BSAC Disk Diffusion Method for Antimicrobial Susceptibility Testing, Version 4, 2005). BHI plates were inoculated with a standardized culture of *S. gordonii* and FAA plates were inoculated with a standardized culture of *P. gingivalis*. C10, C11, C12 discs of 5 mm diameter and 2 mm thickness were then placed on the inoculated plates. CNP samples were used as negative controls. All the BHI plates were incubated overnight in air at 37 °C while FAA plates were incubated for 72 h in ananaerobic chamber. The diameters of any zones that had formed around the discs were measured in triplicate using callipers. The zones of inhibition were presented in mm as the test values minus that of the control, CNP.

2.4. Liquid broth assay

The ability of the C10, C11 and C12 glasses to inhibit planktonic growth of *S. gordonii* and *P. gingivalis* was also assessed in a modified tryptic soy broth (TSB; Becton, Dickinson and Company, UK) with a controlled iron content (iron = 74 $\mu g\ dl^{-1}$) by the addition of a chelating agent, 2,2′-diphyridyl (Sigma Aldrich UK), at a final concentration of 0.5 mM [22]. The chelating agent was added to the medium to ensure any free iron in the medium, which could interfere with the antimicrobial effect of gallium [23], was chelated [22]. Diluting the TSB medium to 40% of the manufacturer's recommendations and supplementing it with hemin (5 $\mu g\ ml^{-1}$) and menadione (1 $\mu g\ ml^{-1}$) has been reported to support good growth of *P. gingivalis* [24].

A 25 ml volume of modified TSB was poured into sterile containers and inoculated with a standardized culture of *S. gordonii* or *P. gingivalis* (optical density of 0.05 at OD_{600}). A single glass disc (5 mm diameter and 2 mm thickness) of C10, C11, C12 or CNP was added to each container. The CNP disc was used as a control and all cultures were incubated at 37 °C in an anaerobic chamber. At predetermined time points, samples were taken and serially diluted in phosphate-buffered saline (PBS; Oxoid) and 25 µl of the suspension and each dilution was spread onto FAA plates. The plates were then incubated anaerobically at 37 °C for 48 or 72 h. For each type of disc, viable counts (the numbers of colony-forming units (CFU)) were determined in triplicate.

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