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Antibacterial, Remineralising and Matrix Metalloproteinase Inhibiting Scandium-doped Phosphate Glasses for Treatment of Dental Caries

Running Title: Anti-cariogenic Scandium-doped Phosphate Glasses

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Abstract

Objectives: Antibiotic resistance is increasingly a growing global threat. This study aimed to investigate the potential use of newly developed scandium-doped phosphate-based glasses (Sc-PBGs) as an antibacterial and anticariogenic agent through controlled release of Sc$^{3+}$ ions.

Methods: Sc-PBGs with various calcium and sodium oxide contents were produced and characterised using thermal and spectroscopic analysis. Degradation behaviour, ion release, antibacterial action against *Streptococcus mutans*, anti-matrix metalloproteinase-2 (MMP-2) activity, remineralisation potential and *in vivo* biocompatibility were also investigated.

Results: The developed glass system showed linear Sc$^{3+}$ ions release over time. The released Sc$^{3+}$ shows statistically significant inhibition of *S. mutans* biofilm (1.2 log$_{10}$ CFU reduction at 6 h) and matrix metalloproteinase-2 (MMP-2) activity, compared with Sc-free glass and positive control. When Sc-PBGs were mounted alongside enamel sections, subjected to acidic challenges, alternating hyper- and hypomineralisation layers consistent with periods of re- and demineralisation were observed demonstrating their potential remineralising action. Furthermore, Sc-PBGs produced a non-toxic response when implanted subcutaneously for 2 weeks in Sprague Dawley rats.

Significance: Since Sc$^{3+}$ ions might act on various enzymes essential to the biological mechanisms underlying caries, Sc-PBGs could be a promising therapeutic agent against cariogenic bacteria.

Key words: Scandium-doped phosphate-based glasses, antibacterial, MMP-2, remineralisation and biocompatibility.
1. Introduction

Antibiotic-resistance is increasingly becoming a key public health risk and threatening decades of advances in the treatment of infections. Extensive and indiscriminate use of antibiotics offers a potent selective force for antibiotic-resistant bacteria. During the past four decades, only a few antibiotics was introduced into the market with an entirely novel mechanism of action e.g. linezolid [1, 2]. With the dearth of new classes of antimicrobial pharmaceuticals on the horizon, the future looks challenging [3].

The scarcity of effective antibacterial agents is a major problem in oral health. Dental caries occurs as a result of complex interactions between acid-producing bacteria (e.g. *Streptococcus mutans*) [4], fermentable carbohydrates and host factors (e.g. teeth and saliva) [5]. Thus far, various agents, e.g. chlorhexidine, cetylpyridinium chloride [6] and triclosan [7], have been developed to inhibit microbial growth or metabolism often by interfering with a single intracellular target. It has been reported however that *S. mutans* developed resistance against antibacterial agents such as cefuroxime, penicillin and tetracycline [8]. Furthermore, the lack of evidence on long-term clinical outcomes and reported side effects of chlorhexidine, for example, limit its use in caries prevention [9]. The potential of new antimicrobial agents for long-term management of caries has been therefore highlighted [10].

Recently, it has been demonstrated that gallium inhibits the growth of various bacteria including *S. mutans* and has the potential to protect the enamel surface under representative oral conditions [11]. Other transition metal ions such as scandium (Sc$^{3+}$) have been shown to inhibit the growth of *Klebsiella pneumoniae* [12], *Escherichia coli* [13] and *Pseudomonas aeruginosa* [14] due to the formation of Sc (III)-enterobactin complex. Because the charge/radius ratio of Sc$^{3+}$ is similar to Fe$^{3+}$, Sc$^{3+}$ could enter the cell in place of Fe$^{3+}$ [15] and exert its toxic effect by displacing Zn$^{2+}$ from enzymes such as RNA polymerase [16]. Sc$^{3+}$ could therefore interfere
with the activity of matrix metalloproteinase-2 (MMP-2), that plays an important role in the control and progression of caries [17, 18].

The use of Sc\(^{3+}\) as an anticariogenic agent could be significantly improved by the development of an effective means of delivery. The use of chemically-durable materials with tailored degradation rates as a source of long-term release of Sc\(^{3+}\) ions could be an option. Phosphate-based glasses (PBGs) have been shown to be an effective system for the delivery of various antibacterial ions e.g. silver [19], copper [20] and gallium [21, 22]. The rate of release of such ions is defined by the overall degradation rate of the glass.

This study aimed to develop novel Sc-doped PBGs and investigate their physical, structural and degradation properties. The role of these glasses as anticariogenic (antibacterial and anti-MMP-2 activity) and remineralising agents were assessed. Finally, the *in vivo* biocompatibility of these glasses was investigated using rat model.

### 2. Materials and Methods

#### 2.1. Glass Preparation

PBGs were produced using sodium dihydrogen phosphate (NaH\(_2\)PO\(_4\)), calcium carbonate (CaCO\(_3\)), phosphorus pentoxide (P\(_2\)O\(_5\)) and scandium oxide (Sc\(_2\)O\(_3\)) (Sigma-Aldrich, Dorset, UK) with a purity of >99% (Table 1). The reagents were weighed, mixed and melted in a quartz crucible (Thermo Fisher Scientific, Loughborough, UK) at 1100 °C for 1 h. The molten glass was then poured into a preheated graphite mould of 5 mm diameter and 10 mm length and left to cool overnight. The resulting glass rods were cut into discs of 5 mm diameter and 2 mm thickness using an Isomet low-speed rotary diamond saw (Buehler Ltd, UK). Powder was prepared by grinding the cooled glass using a Retsch PM100 milling machine (Retsch GmbH, Germany) and used for physical and structural characterization. Discs were used for anticariogenic, remineralising action and biocompatibility studies. Glass extracts, prepared by
soaking the glass disc in 10 mL ultrapure protease free water (Sigma-Aldrich, Dorset, UK) for 72 h at 37 °C, were used to evaluate anti-MMP-2 activity.

2.2. Physical Characterization

2.2.1. Density Measurements

The density measurements were carried out (n=3) using a AccuPyc™ II 1340 Gas Displacement Pycnometry System (Micromeritics Corp, Georgia, USA), which utilizes Archimedes’ Principle using helium gas as the fluid.

2.2.2. Thermal Analysis

Simultaneous thermogravimetric (TG) and differential thermal analysis (DTA) was carried out on a TG-DTA16 instrument (Setaram Labsys™, Lyon, France). Each sample was heated from 40 to 1200 ºC under air at 5 ºC.min⁻¹. The data was baseline-corrected using a blank run.

2.3. Structural Analysis

2.3.1. FTIR Spectroscopy

The FTIR data was collected on a Varian 660-IR FT-IR spectrometer (Varian Inc, California, USA) fitted with a Golden Gate diamond-anvil ATR (attenuated total reflectance) attachment. Spectra were recorded over 4000-400 cm⁻¹ with a resolution of 1 cm⁻¹ and each composed of 16 summed scans.

2.3.2. ³¹P MAS NMR

All ³¹P MAS NMR measurements were acquired at 7.05 T using a Chemagnetics Infinity Plus spectrometer (Bruker, Coventry, UK) operating at a ³¹P Larmor frequency of 121.5 MHz. The data was generated using single pulse experiments utilising a pulse length of 1.5 μs (corresponding to a 30º nutation angle) and a recycle delay of 10 s. Checks on the influence of
The structure of phosphate glasses can be described using the Q\textit{n} terminology, where the \textit{n} represents the number of bridging oxygen’s that a PO\textsubscript{4} tetrahedron has in a P\textsubscript{2}O\textsubscript{5} glass, every tetrahedron can bond at three corners. When it bonds to two bridging oxygen’s, usually in a 3D-network it gives the Q\textsubscript{2} species (it is referred to as metaphosphate glass). Further addition gives Q\textsubscript{1} species (also called pyrophosphate glass, which bonds only to one bridging oxygen). The relative abundances of the Q\textsubscript{1} and Q\textsubscript{2} species [23], determined by fitting Gaussian lineshapes to these resonances. Since Q\textsubscript{1} and Q\textsubscript{2} groups have different O/P ratios, the expected abundances of these species from the nominal
composition of the glass was calculated as follows:  
\[ Q^1 = 2\left(\frac{O}{P} - 3\right) \]
\[ Q^2 = 1 - Q^1 \]
assuming that only Q^1 and Q^2 are present.

### 2.3.3. SEM/EDX

The distribution of Sc^{3+} in the produced glasses was analysed with a Quantax70 EDX chemical microanalysis system (Bruker, Coventry, UK) attached to a Hitachi Tabletop SEM TM3000 (Hitachi High-Technologies Europe GmbH, Daresbury, UK) operating at 15 kV.

### 2.4. Degradation Behaviour

#### 2.4.1. Mass Loss

PBGs discs (n=3) were placed in 25 mL Sterilin Polypropylene Universal Container (Sterilin Ltd, Newport, UK), filled with 10 mL deionised water (Sigma-Aldrich, Dorset, UK) (pH 7.0 ± 0.5) and incubated at 37 °C. At various time points (24, 48, 72 and 120 h), the discs were removed, blot dried, weighed and placed into a fresh solution. The mass loss (M_i) was calculated from \((M_0 - M_i)/A\), where \(M_0\) is the initial weight (mg), \(M_i\) is the weight at time (t) and A is the surface area (mm²). \(M_i\) was plotted against t and the degradation rate (mg.mm\(^{-2}\).h\(^{-1}\)) was determined from the slope of the plot. The solution collected from the mass loss study was used for ion release and pH measurement.

#### 2.4.2. Ion Release

Ion release was conducted by A Spectro Ciros CCD Spectrometer (SPECTRO Analytical Instruments GmbH, Boschstr, Germany) after being calibrated using element standards (Sigma-Aldrich, Dorset, UK) at 0.0–40.0 ppm. Sample analysis was conducted under 1400 W power, 12 L min\(^{-1}\) coolant flow rate, 1 L min\(^{-1}\) auxiliary and nebuliser flow rate with a side-on plasma detection system (SOP) providing minimum detection limits for Sc^{3+}, Ca^{2+} and P^{5+} of 50, 0.5 and 50 ppb respectively. Due to the relatively high Ca^{2+} content of the samples, regular acidic
washout periods with 35% HCl were scheduled every 4-6 sample runs to alleviate possible signal attenuation from residual build up within the nebuliser unit. Data were collected with Smart Analyser of Spectro Smart Studio, Version 2.11.0631.

2.4.3. pH Changes

The pH measurements were recorded using a Hanna Instruments pH 211 Microprocessor pH meter with an attached glass combination pH electrode (BDH, UK) after calibration with colourkey buffer solutions (BDH, UK) pH 4.0 and 7.0; to ensure the accuracy of pH electrode.

2.5. Anticariogenic Potential

2.5.1. Antibacterial Action

For this study, S. mutans NCTC 10449 strain, grown on brain heart infusion agar (BHI, Merck, Sigma Aldrich, Dorset, UK), a solid nutrients-rich medium commonly used for the growth of bacteria, in an anaerobic (N₂: CO₂: H₂, 80:10:10) environmental chamber (Don Whitley MG1000; Don Whitley Scientific, Shipley, UK), was used.

2.5.1.1. Disc Diffusion Assay

This test was carried (n=3) out as a screening to select the glass composition with the highest antibacterial action for the rest of the study. Growth inhibition of S. mutans in the presence of C11, C12 and C13 glasses were analysed using disc diffusion methodology (BSAC Disk Diffusion Method for Antimicrobial Susceptibility Testing, Version 4, 2005) [24]. C20 was used as control. BHI plates were inoculated anaerobically overnight at 37 °C with standardised cultures of S. mutans (~10⁸ cells/mL). The diameter of the inhibition zone (mm) formed around the discs was measured using callipers. Based on the results of this assay, C11 composition was used for the rest of the study; C20 was used as a control.

2.5.1.2. Biofilm Assay
A constant depth film fermenter (CDFF) was used for the Biofilm assay[11] with C20 and hydroxyapatite (HA) discs of similar dimensions (5 mm diameter and 2 mm thickness, 3D Biotek, New Jersey, USA) as controls [25]. HA was used as a control to compare with both Sc-free (C20) and Sc-containing glasses (C11), while C20 glass was used to evaluate the effect of Sc on biofilm growth. The CDFF contained a stainless-steel turntable and held up to 15 polytetrafluoroethylene (PTFE) pans each of which had 5 PTFE plugs that were used to hold substrates and recessed by 200 μm. The PTFE pans were then slotted in so that they were flush with the turntable. A cylindrical glass vessel and two stainless steel end plates enclosed the turntable. The top plate contained an air inlet port attached to two 0.2-μm high-efficiency particulate air filter air vents (Thermo Fisher Scientific, Loughborough, UK) and three medium inlet ports. Prior to use, CDFF was sterilised in a hot air oven (LTE Scientific Ltd., Oldham, UK) at a temperature of 140 °C for 3 h. During operation, the CDFF was incubated at 37 °C, and rotated at a speed of 3 rpm. A 10 mL of S. mutans cultured for 24 h was inoculated into 0.5 L of saliva-type growth medium [STGM, Lab-Lemco powder 1 g/L, yeast extract 2 g/L, protease peptone 5 g/L, type III hog gastric mucin 2.5 g/L, sodium chloride 0.2 g/L, potassium chloride 0.2 g/L, calcium chloride 0.3 g/L] and circulated through the CDFF. The STGM was dripped onto the rotating turntable and distributed over the pans by 2 scraper blades, which also served to maintain the required depth of biofilms on the discs [26]. After 8 h, the fermenter was fed from an 8-litre medium reservoir of STGM, with the waste flowing into an effluent bottle. The STGM was delivered at a rate of 0.5 mL/min mimicking the normal resting-saliva flow rate [27] using a peristaltic pump (Watson and Marlow, Falmouth, UK). At various time intervals (6, 24, 48 and 120 h), the pans were removed aseptically from the CDFF. Discs containing biofilms were placed in 1 mL of PBS and vortexed for 1 min to remove and disperse the attached biofilms into suspension. Serial dilutions of the suspensions were carried out in PBS; 25-μl aliquots of the diluted suspensions were spread onto BHI plates and incubated
anaerobically at 37 °C for 72 h. Log₁₀ values of colony forming units (log₁₀ CFU) were then determined in triplicate and presented.

2.5.2. Anti-Matrix Metalloproteinase-2 (MMP-2) Action

The anti-MMP-2 activity of C20 and C11 extracts were investigated using a purified recombinant human MMP-2 (Anaspec Inc, Fremont, CA, USA) and SensoLyte® 570 Generic MMP Fluorimetric assay kit (Anaspec Inc., Fremont, CA, USA). This kit uses a 5-TAMRA/QXL 570 fluorescence resonance energy transfer peptide (FRET) as a MMP-2 substrate. The MMP-2 activity was evaluated through MMP-2 cleavage of the FRET peptide. 5-TAMRA fluorescence was detected at excitation/emission wavelength of 540/575 nm using a Molecular Devices Flexstation 3 microplate reader with Softmax pro data software. Fluorescence reference standard curve was prepared by measuring relative fluorescence units (RFU) against 5-TAMRA concentrations of 20, 10, 5, 2.5, 1.25 and 0.625 µM, serially diluted in assay buffer containing substrate. The pro-MMP-2 was first activated with 1 mM 4-aminophenylmercuric acetate (APMA) solution and incubated for 1h at 37 °C. Upon activation, 10 ng/mL of pro-MMP-2 with enzyme: substrate ratio of 1:100 was used. MMP-green substrate was then added to the activated MMP-2; this was used as a positive control. For the experimental groups, the extract of C20 or C11 was added to the activated MMP-2 before the addition of MMP green substrate. The green substrate (assay buffer) was used as a negative control. The end-point RFU readings were recorded.

2.6. Remineralising Potential

Enamel blocks (n=15) from the labial surface of sound bovine permanent incisors were successively abraded from the middle third to ~ 0.5 mm depth using a wet 300-grit followed by 1,000-grit carborundum paper on a rotary grinder (Buehler Ecomet 30, Esslingen, Germany). They were then cut to ~6 × 3 mm² sections using a diamond wire saw (WELL Diamond Wire
Saws SA, Le Locle, Switzerland) and painted with nail varnish (MaxFactor Nailfinity; Proctor & Gamble, UK) to leave windows of ~3 × 2 mm² exposed. Then, they were mounted in green stick impression compound (Kerr Impression Compound Sticks Green, Henry Schein Dental UK) and placed in 50 mL polypropylene containers (Sterilin Ltd, Newport, UK). Enamel sections were randomly divided into 4 groups as follows: G1: C20-treated, G2: C11-treated, G3: NaF-treated and G4: water-treated. Samples were subjected to pH cycling (i.e., demineralising medium (acidic challenge) for 6h/day followed by remineralising medium for 18 h/day) for 5 days. After pH cycling, samples were kept in remineralising solution for 2 days. For G1 and G2, the glass disc was mounted alongside the enamel section throughout the experiment. For G3 and G4, the samples were immersed in 228 ppm NaF solution or water respectively for 5 min at each solution change – Figure 1 (a).

The demineralising medium was composed of 2.0 mM CaCl₂, 2.0 mM KH₂PO₄, 0.04 ppm F and 0.075 M acetic acid adjusted to a pH of 4.7 with 1.0 M NaOH. The remineralising medium composed of 1.5 mM CaCl₂, 0.9 mM KH₂PO₄, 150 mM KCl, 0.05 ppm F and 20 mM HEPES adjusted to a pH of 7.0 with 1.0 M NaOH [28].

2.6.1. Surface Roughness (Ra)

Surface roughness (Ra) was measured (n=3) for both exposed and protected area after removal of nail varnish with acetone using non-contact surface profilometry (NCSP; Proscan 2000; Scantron Industrial Products Ltd, Taunton, UK). Samples were scanned in x and y directions with a step size of 0.01 μm. Data were collected with an S5/03 scan head at 0.01 μm resolution, 0.3 mm measuring range, 4 μm spot size, 300 Hz and 1 iteration Kalman filter and analysed using Proscan 2000, version 2.1.1.15.
2.6.2. Integrated Mineral Loss (ΔZ)

Several thin bars were cut from each enamel section, corresponding to either exposed or unexposed areas of the enamel, and samples matched as adjacent pairs. The sections were mounted lengthwise on brass anvils, fixed with nail varnish (MaxFactor Nailfinity; Proctor & Gamble, UK), polished with a diamond-impregnated grinding disc (15 µm particles; Buehler, Lake Bluff, Ill., USA) to 80 µm thickness and mounted on a plastic template along with an aluminium step wedge. Transverse microradiographs (TMR) of the templates were taken using Kodak type 1A high-resolution plates (HTA Enterprises, San Jose, USA) exposed to a Cu Kα X-ray source operating at 10 mA and 30 kV. The exposure time was 25 min and the distance from source to template was 30 cm. The microradiographs were examined microscopically (Leica, Wetzlar, Germany) and images were captured. The integrated mineral loss (ΔZ, vol%·µm), the product of the lesion depth (µm) and the mean mineral loss (vol%) over that depth, was assessed relative to sound enamel, which was assumed to be ~80% v/v mineral [29]. ΔZ was assessed using a computerised image analysis system (TMR2006; Inspektor Research Systems, Amsterdam, The Netherlands) [30].

![Diagrammatic representation showing the steps of remineralisation potential (a) and in vivo biocompatibility testing (b).](image)

*Figure 1: Diagrammatic representation showing the steps of remineralisation potential (a) and in vivo biocompatibility testing (b). Sound enamel was used as a control; enamel window was exposed to pH cycling for 5 days followed by remineralisation for 2 days. G1 & G2 had glass disc alongside the enamel section. For G3 & G4, the enamel section was exposed to NaF or water for 5 min between the medium (demineralisation and remineralisation) exchange.*
2.7. *In vivo* Biocompatibility (Subcutaneous Implantation)

All animal experimentation including surgery and husbandry were conducted on three adult male Sprague Dawley rats (Harlan Ltd. UK) weighing 250–380 g in accordance with the Animal (Scientific) Procedures Act 1986 and Home Office code of Practice and under personal licence PIL 80/10339 and project licence PPL 80/2200. Rats were anaesthetised using 0.25 mL IM Hypnorm (0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone) and 1 mg IP diazepam. A ventral midline incision was made through the skin after disinfection and shaving the fur. A pocket was created between the skin and muscle on either side of the midline incision. C11 and C20 discs were implanted in both pockets. The overlying skin was sutured using with 3/0 Mersilk® sutures (Ethicon, Johnson & Johnson Medical Ltd, UK) and pain relief was administered. Rats were sacrificed by a lethal injection of sodium pentobarbitone after 2 weeks; the glass discs and adjacent tissues were removed. The discs were fixed in 10% neutral buffered formal saline (Genta Medical UK) for a minimum of one week. Following fixation and processing for paraffin embedding, full-face 5 μm histological sections were cut stained with haematoxylin and eosin (H & E) – Figure 1 (b).

2.8. Statistical Analysis

Student’s t-test of log10 CFU, Rₐ and ΔZ was conducted using GraphPad software (San Diego, Calif., USA); continuous variables were expressed as means ± SD and p < 0.05 was considered statistically significant.

3. Results

3.1. Physical Characterization

3.1.1. Density Measurements

There was no significant change in density – Table 1.
<table>
<thead>
<tr>
<th>Glass Code</th>
<th>Composition (mol%)</th>
<th>Density (g.cm$^{-3}$)</th>
<th>Thermal Properties (°C)</th>
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<tr>
<td>Na$_2$O</td>
<td>CaO</td>
<td>P$_2$O$_5$</td>
<td>Sc$_2$O$_3$</td>
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<td>20</td>
<td>45</td>
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<td>C$<em>{11}$Na$</em>{41}$P$<em>{45}$Sc$</em>{3}$ (C11)</td>
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<td>11</td>
<td>3</td>
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<tr>
<td>C$<em>{12}$Na$</em>{40}$P$<em>{45}$Sc$</em>{3}$ (C12)</td>
<td>40</td>
<td>12</td>
<td>3</td>
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<tr>
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<table>
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<tr>
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<th>Width (Hz±50)</th>
<th>Relative Abundance (%±5)</th>
<th>Expected Abundance (%)</th>
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</thead>
<tbody>
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</table>

Table 1: Glass code, composition, density, thermal properties and Q speciation of studied glasses. Tg: glass transition temperature; Tc: crystallization temperature and Tm: melting temperature.

3.1.2. Thermal Analysis

The glass transition (Tg), crystallization (Tc) and melting temperature (Tm) of tested glasses are given in Table 1. The tested glasses showed no significant change in Tg but different crystallization and melting behavior. Two crystallization peaks were detected for C20 in contrast to one peak for Sc-doped glasses. Three melting peaks were detected for C20; one (C13) or two peaks (C11 and C12) were detected for Sc-doped glasses – Figure 2.
Figure 2: Differential thermal analysis for the scandium-doped PBGs in comparison to scandium-free PBGs. Tg: glass transition temperature. Tc: crystallisation temperature. Tm: melting temperature.

### 3.2. Structural Characterization

#### 3.2.1. FTIR Spectroscopy

All glasses showed similar spectra. The band near 1250 cm\(^{-1}\) is assigned to the asymmetric stretching mode of the two non-bridging oxygen atoms bonded to phosphorus atoms in the PO\(_2\) metaphosphate Q\(^2\) units, \(\nu_{as}(PO_2)^-\) [31, 32]. The absorption bands at 1100 and 1000 cm\(^{-1}\) are assigned to the asymmetric and symmetric stretching modes of chain-terminating Q\(^1\) groups, \(\nu_{as}(PO_3)^2^-\) and \(\nu_s(PO_3)^2^-\) respectively [33]. The absorption band near 900 cm\(^{-1}\) is assigned to the asymmetric stretching modes of the P–O–P linkages, \(\nu_{as}(P–O–P)\) [34], and the partially split band centred around 750 cm\(^{-1}\) is assigned to the symmetric stretching modes of these linkages, \(\nu_s(P–O–P)\) [31]. The peak at 540 cm\(^{-1}\) is attributed to O-P-O deformation modes [34] – Figure 3 (a).
3.2.2. $^{31}$P, $^{23}$Na, $^{45}$Sc MAS NMR

The $^{31}$P MAS NMR data from C20 exhibited two resonances at shifts of $\sim$-4 and $\sim$-21 ppm which are ascribed to Q$^1$ and Q$^2$ species, respectively. From Figure 3(b) and Table 1 the studied Sc-free and the Sc-containing glass compositions display similar nominal Q$^1$:Q$^2$ ratios of $\sim$1:3. However, the -4 ppm resonance in the Sc-doped glasses data exhibits a distinct broadening which can be ascribed to the presence of two Q$^1$ resonances at $\sim$-3 ppm and $\sim$-9 ppm, thus suggesting that the incorporated Sc$^{3+}$ ions undergo a preferred ionic association with the Q$^1$ chain-end P species. Table 1 also indicates that there is a divergence between the experimentally measured and theoretically expected Q$^1$:Q$^2$ ratios. As mentioned above, the measured Q$^1$:Q$^2$ ratio is constant for this suite of glass compositions, while the theoretical Q$^1$:Q$^2$ ratio is expected to reduce with a reducing Na$^+$ content. This suggests that the network modification normally induced by an increasing Na$^+$ content is being compensated by the network forming capability of the Sc$^{3+}$ ions. The corresponding $^{23}$Na and $^{45}$Sc MAS NMR data from this suite of glasses are shown in Figures 3 (c) and 3(d). The similarity of these data clearly demonstrates that the Na$^+$ and Sc$^{3+}$ speciation is constant across this glass consistent compositional range, thus corroborating the observed static Q$^1$:Q$^2$ ratios from the $^{31}$P MAS NMR data.

3.2.3. SEM/EDX

SEM-EDX analysis of Sc-doped PBGs showed relatively smooth surfaces with a uniform distribution of Sc$^{3+}$ - Figure 3 (e).
Figure 3: Structural Characterization: (a) FTIR showing similar composition of tested glasses. (b) $^{31}$P NMR spectra showing that $Q^1:Q^2$ of 1:3 for tested glasses with the Sc-doped ones having broader $Q^1$ resonance with two contributions at -3 and -9 ppm than control. (c) $^{23}$Na NMR and (d) $^{45}$Sc NMR spectra are indistinguishable for tested glasses. (e) Top panel shows the SEM images of Sc-doped PBGs; the bottom panel shows the elemental mapping of Sc-doped PBGs with uniform distribution of Sc$^{3+}$ in all samples. The scale bar represents 30µm.

3.3. Degradation Behaviour

3.3.1. Mass Loss

The rate of weight loss of the Sc-doped glasses decreased with increasing Na$_2$O content; all three Sc doped glasses were significantly more stable than the control (Figure 4a). The degradation rates were determined from slopes of the linear plots of mass loss against time. The linear equation of mass loss versus time was presented as \( y = mx + b \), where \( y \) is the mass loss, \( x \) is the slope and \( b \) is the intercept with \( y \)-axis. The calculated degradation rates were $44\pm0.6$, $28\pm0.2$, $23\pm0.2$ and $19\pm0.5 \times 10^{-4}$ mg.mm$^{-2}$ h$^{-1}$ for C20, C11, C12 and C13 respectively.
Figure 4: Degradation Behaviour: (a) Mass loss decreased with increasing sodium oxide content. (b-e) Ion release followed the same trend as the mass loss; the highest Na\(^+\), Ca\(^{2+}\), P\(^{5+}\) release was observed for the highly degrading control glass. The highest Sc\(^{3+}\) release was observed for C11; Sc\(^{3+}\) release was not detected with the control glass. (f) pH remained alkaline during the study period for all tested glasses.

3.3.2. Ion Release

The ion release data are consistent with the measured degradation rates. The highest Na\(^+\), Ca\(^{2+}\) and P\(^{5+}\) release was observed with the highly degrading C20 control – Figure 4 (b-d). No Sc\(^{3+}\) was detected from the C20 glass as expected; Sc\(^{3+}\) release followed the same trend as the degradation rate with C11 releasing the highest amount – Figure 4 (e).
3.3.3. pH Changes

The pH analysis revealed a slight increase in alkalinity with time but a drop in pH was observed at 48 h but the pH remained very close to 7.0 – Figure 4 (f).

3.4. Anticariogenic Potential

3.4.1. Antibacterial Action

3.4.1.1. Disc Diffusion Assay

The results showed that the zones of inhibition (i.e. zones of no visible bacterial growth surrounding the disks) decreased with decreasing Sc\textsuperscript{3+} release – Figure 5 (a); the highest antibacterial action was observed with the highly degrading Sc-doped glass (C11). No zone of inhibition was observed with the control glass (C20) – Figure 5 (a). Accordingly, the C11 was chosen for the rest of the study and C20 was used as a control.

3.4.1.2. Biofilm Assay

At 6 h, C11 displayed a statistically significant (p ≤ 0.004) lower log\textsubscript{10} CFU than C20 and HA controls. At 24 h, the decrease in the log\textsubscript{10} of the mean number of viable cells was not as pronounced as at 6 h, but C11 still showed significantly (p ≤ 0.002) lower log\textsubscript{10} CFU than both controls. However, C11 exhibited no anti-biofilm effects at 48 and 120 h (p>0.05); the greatest activity of C11 was observed at 6 h (1.2 log\textsubscript{10} CFU reduction) – Figure 5 (b).
Figure 5: Anticariogenic potential: (a) Inhibition zone (mm) decreased with decreasing the \( \text{Sc}^{3+} \) release (ie, C11 showed the highest inhibition zone). C20 did not show any inhibition zone. (b) \textit{S. mutans} viable count: C11 displayed a statistically significant reduction in \( \log_{10} \text{CFU} \) than both controls (HA and C20) only at earlier time points (6 and 24 h). (c) Anti-MMP-2 activity: C11 showed a statistically significant reduction in MMP-2 activity than both controls (positive and C20).

3.4.2. Anti-Matrix Metalloproteinase-2 (MMP-2) Action

The fluorescence readings are expressed in relative fluorescence unit (RFU). The fluorescence reading from the substrate control well is the background fluorescence. This background reading was subtracted from the readings of other wells. Low MMP-2 activity value for the negative control indicate small substrate background fluorescence. C11 extract significantly (\( p < 0.01 \)) reduced the MMP-2 activity compared with both positive and C20 controls. Extract of C20 showed no statistically significant (\( p > 0.5 \)) difference in MMP-2 activity compared with positive control – Figure 5 (c).
3.5. Remineralising Potential

3.5.1. Surface Roughness (Ra)

The Ra (µm) values from pH-cycled enamel samples of all groups did not significantly differ statistically from the baseline values – Figure 6 (b).

3.5.2. Integrated Mineral Loss (ΔZ)

TMR analyses showed statistically significant (p<0.05) mineral loss for all tested groups except water-treated when compared with their baselines (sound enamel) – Figure 6 (b). However, the TMR images of pH-cycled enamel in the presence of C11 revealed a characteristic pattern of several alternating hyper- and hypo-mineralisation layers – Figure 6 (c). This pattern was not observed for the rest of groups.
Figure 6: Remineralisation Potential: (a) Surface Roughness (Ra): exposing the bovine enamel to pH cycling did not produce a significant change in Ra value when compared with the baseline (sound enamel) for all tested groups. (b) Mineral Loss (ΔZ): a significant mineral loss was observed for all tested groups after exposure to pH cycling, however, a characteristic pattern of hyper- and hypo-mineralisation was only observed for C11-treated group (c). The white arrows refer to the typical lesion formation where demineralisation was observed by its radiolucency. The red arrow refers the presence of hypermineralised layer as indicated by its radiopacity.

3.6. In vivo Biocompatibility (Subcutaneous Implantation)

The initial in vivo response showed that both implants were surrounded by a thin capsule containing proliferating fibroblasts with minimal and mild inflammatory cell infiltration for C20 and C11, respectively – Figure 7.
Figure 7: Digital image of two glass discs after 2 weeks of subcutaneous implantation in SD rats (a). Histological H&E stained sections showing the biological response of C20 (b) and C11 (c & d) after two weeks of implantation. A thin fibrous capsule, formed around the glass discs and consisting predominantly of fibroblast with mild inflammatory infiltrate, is evident for both glasses. Arrows in b-d refer to the inflammatory infiltrate in the fibrous tissue capsule.

4. Discussion

The degradation and Sc\(^{3+}\) release of the studied glasses was controlled by changing CaO and Na\(_2\)O mole\%. All glasses exhibited a \(T_g\) of \(~335\) °C; consistent with a previous study [35]. The Sc-doped glasses exhibited very similar thermal behaviour (crystallization at 450 °C and melting at 600-700 °C). The presence of two melting peaks for C11 and C12 suggested the presence of two phases. The C20 showed markedly different behaviour with two crystallization and three melting peaks; this might suggest that this glass showed partial phase separation.

The \(^{31}\)P MAS NMR results indicate that all the glasses studied have structures based upon chains of (PO\(_2\))\(^-\) metaphosphate Q\(^2\) units terminated by Q\(^1\) (PO\(_3\))\(^2-\) groups. The relative abundance of Q\(^1\) and Q\(^2\) species does not significantly differ between samples. The \(^{31}\)P MAS NMR of C20 is consistent with a previous study [31] and the measured abundances of Q\(^1\) and
Q\textsuperscript{2} units agree well with the calculated values. However, for C12 and C13, the expected abundance of Q\textsuperscript{1} is significantly lower than the observed. This result suggests that the Sc\textsuperscript{3+} ions do not depolymerise the phosphate network, as expected on the basis of composition alone. The split Q\textsuperscript{1} resonance, observed with Sc-doped glasses, indicates that the presence of Sc\textsuperscript{3+} ions introduces a further degree of disorder into the structure. Since the chemical shifts of \textsuperscript{31}P resonances become more negative with increasing cation potential [36], it is possible that the second Q\textsuperscript{1} resonance at -9 ppm is from PO\textsubscript{3}\textsuperscript{2-} groups that are in the proximity of Sc\textsuperscript{3+} ions. The structural equivalence of the samples suggested by the \textsuperscript{31}P NMR results is supported by the similarity of the FTIR spectra from the four glasses. Except for a slightly stronger peak at 1250 cm\textsuperscript{-1} for the C20 glass, the spectra are indistinguishable. Since this peak is assigned to an asymmetric stretching mode of PO\textsubscript{2} metaphosphate Q\textsuperscript{2} units, this slight decrease in intensity with the addition of Sc\textsuperscript{3+} might hint at some small degree of depolymerisation of the phosphate network. However, it is equally likely that this change is caused by the introduction of more disorder into the network by the presence of Sc\textsuperscript{3+} as suggested by the splitting of the Q\textsuperscript{1} resonance.

The similarity of \textsuperscript{23}Na MAS NMR spectra for tested glasses suggests that Na environments are equivalent for all of them. The broad linewidth suggests that sodium adopts a distorted environment with respect to oxygen. The \textsuperscript{23}Na spectra obtained here are consistent with those of a previous study [37] which suggested that Na adopts a 5-fold coordinated site. Similarly, the \textsuperscript{45}Sc MAS NMR are all indistinguishable, suggesting an equivalence of the Sc\textsuperscript{3+} positions within the glass systems. There are very few \textsuperscript{45}Sc MAS NMR studies of PBGs reported in the literature; however, the spectra presented here are consistent with those reported in a previous \textsuperscript{45}Sc MAS NMR study of Sc\textsubscript{2}O\textsubscript{3}-Al\textsubscript{2}O\textsubscript{3}-NaPO\textsubscript{3} glasses [38], suggesting that the Sc\textsuperscript{3+} ions are octahedrally coordinated with respect to their oxo environments, and each Sc\textsuperscript{3+} position is isolated from all other Sc\textsuperscript{3+} ions occupying the network [38].
The degradation behaviour mirrored ion release. Glass degradation consists of the three synergistic processes: ion exchange, hydration, and finally hydrolysis of phosphate chains [39]. As a result of ion exchange, a gel layer is usually formed on the glass surface (i.e. hydration), and when it leached into the surrounding medium it increases its ionic strength which subsequently decreases the glass degradation rate. The hydrolysis of phosphate chains is pH-dependent (i.e. accelerated in basic solutions) and accompanied by an increase in the pH of the surrounding medium [40]. The degradation study was carried for short time as the ion release is expected to be higher during the first few days, while the surrounding environment becomes saturated with the released ions, prior to getting reduced. Generally, the degradation of the glass is a complex process and usually follows an initial fast degradation, caused by hydration of P-O-P chains, followed by a slow bulk degradation of the glass network and the release of modifying ions. During the fast degradation stage, ions such as sodium is usually exchanged with H$_3$O and the degradation increased linearly with time. During the bulk degradation however, it is no longer linear with time and it is mainly influenced by the interaction between the modifying oxides and the glass network [41].

The anticariogenic potential of Sc-doped PBGs was investigated by studying their antibacterial as well as anti-MMP-2 activity. The results of the disc diffusion assay are promising. However, 75% of bacterial infections involves biofilms [42] and bacteria protected within biofilms are up to 1,000 times more resistant to antibiotics [43] than planktonic cells. Therefore, the impact of most potent Sc-doped PBGs, C11 was investigated on an *in vitro* grown *S. mutans* biofilm using a CDFF that has been reported to match growth conditions in the oral cavity [44]. Based on the charge/radius ratios of the ions [15], Sc$^{3+}$ could have the potential to enter the bacterium in place of Fe$^{3+}$ and exert toxicity by displacing Zn$^{2+}$ from enzymes essential for bacterial division such as RNA polymerase [16]. The ability of Sc$^{3+}$ to displace Zn$^{2+}$ from an enzyme was further investigated by testing the activity of C11 against zinc-dependent MMP-2 which is a major
therapeutic target for caries management. It was also reported that MMP-2 can cleave amelogenin, the major structural protein component of the enamel matrix, into several fragments and therefore, play an important role during tooth development [45]. The results showed that MMP-2 activity was significantly reduced by the presence of C11 extracts.

After establishing the effectiveness of C11 as an antibacterial and anti-MMP agent, its remineralisation potential was investigated. To mimic the in vivo condition, the effect of scandium on bovine enamel was assessed under a well-established pH-cycling conditions [11]. The surface profiling analyses of enamel revealed no statistically significant differences in pH-cycled enamel in the presence of C11 and controls under conditions similar to oral environment. However, an increase in surface roughness was observed for all pH-cycled enamel samples in the presence of controls confirming that surface roughness cannot be directly correlated to any treatment carried in this study. However, the unusual pattern of enamel lesion consisting of several alternating hyper- and hypo mineralisation layers observed with C11-treated samples is consistent with periods of re- and demineralisation. Similar observations have been reported previously for gallium-doped phosphate-based glasses [11]. However, further experiments, utilising chemical analysis, are required to confirm that scandium has a role in inhibiting mineral loss in enamel. The fact that NaF positive control treatment only lasted 5 min in contrast to the C11 sample, which was mounted alongside enamel during the entire experiment, makes it impossible to directly compare both treatments. More importantly, the total number of pH-cycled enamel samples for each condition should be increased in future experiments to validate the observations made in this study. Nonetheless, the results of this study suggest that C11, which inhibited S. mutans growth, could also help to maintain the integrity of the enamel. Demineralisation of enamel is a process by which the mineral ions e.g. calcium and phosphorus are removed from hydroxyapatite crystals. Initially, with minimal mineral loss, the integrity of hydroxyapatite crystals is maintained providing more chance for the lost minerals to be replaced
by remineralisation process. Both demineralisation and remineralisation are dynamic lifelong process. To prevent the loss of hydroxyapatite integrity, maintaining an environment in favour of remineralisation is key [46].

The mild-moderate inflammatory response observed with the glasses tested in vivo would be expected given that they were tested in a dynamic environment and the glasses are degradable in aqueous media. Therefore, this response could be related to the release of breakdown products particularly with the C20 which has a higher degradation rate than C11.

Although Sc (III)-enterobactin complex exerted antibacterial effects is reported [12,13,14,15,16], only limited information on human toxicity of scandium is available. Scandium has shown high affinity to transferrin [47], albumin and globulins [48]. The presence of scandium in nails was suggested as a biomarker associated with low risk of acute myocardial infarction [49]. The average plasma concentration of scandium in a healthy population was found to be 28.6 ng/L [50]. Such levels of scandium are reported to be decreased by physical exercise [51]. The maximum level of scandium ion release in this study was 6 ppm, that is higher than the average plasma concentration reported in a healthy individual [50]. However, the ion release study was carried out in deionised water that has been changed at each time point. In clinical scenario, where the Sc-PBGs could be added to dentifrices, the scandium ion release will be significantly low due to short exposure in oral environment.

5. Conclusion

Novel Sc-doped PBGs developed in this study are structurally equivalent and more disordered than the PBG. Incorporation of scandium oxide produced no significant change in density or glass transition temperature; however, changes in crystallization and melting behaviour were observed. Incorporation of scandium oxide also significantly reduced the degradation rate; the
ion (Ca$^{2+}$, Na$^+$, P$^{5+}$ and Sc$^{3+}$) release data were consistent with the measured degradation rates. During the glass degradation, the pH ranged from 6.6-7.6. Sc-PBGs, C11 in particular, showed a statistically significant inhibition of S. mutans biofilm (1.2 log$_{10}$ CFU reduction at 6 h) and matrix metalloproteinase-2 (MMP-2) activity. When Sc-PBGs were mounted alongside enamel sections, subjected to acidic challenges, alternating hyper- and hypomineralisation layers consistent with periods of re- and demineralisation were observed. When implanted subcutaneously for 2 weeks in Sprague Dawley rats, the C11 glass showed a typical mild inflammatory response. The promising anti-caries potential of this Sc-PBG warrants further animal caries model studies.

Acknowledgements

The authors would like to thank Michael Dixon from Hitachi High-Technologies Europe GmbH (Daresbury, UK) for the SEM analyses. This research was supported by an induction award (University of Liverpool, UK). RS was funded internally by the University of Liverpool, Department of Biochemistry and Cell Biology. EB was funded internally by the University of Liverpool, Department of Human Anatomy and Cell Biology. JVH thanks the EPSRC, the University of Warwick and the Birmingham Science City Program for partial funding of the solid state NMR infrastructure at Warwick. The latter program accessed the Birmingham Science City Advanced Materials Project 1: Creating and Characterising Next Generation Advanced Materials, which derived support from Advantage West Midlands (AWM) and the European Regional Development Fund (ERDF).
References


