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# Apatite formation of bioactive glasses is enhanced by low additions of fluoride but delayed in the presence of serum proteins

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ABSTRACT

Five bioactive glass compositions in the $SiO_2$–$P_2O_5$–CaO–$Na_2O$–$CaF_2$ system (0–32 mol% $CaF_2$) and Bioglass® 45S5 were evaluated for their apatite forming ability in serum-free and serum-containing cell culture media for up to seven days. While $F^-$ ions in low concentrations were found to enhance apatite formation, higher fluoride content caused formation of fluorite and calcite. The presence of serum proteins delayed apatite precipitation for all compositions, while Bioglass® 45S5, despite considerably higher phosphate content (2.6 vs. $\leq$1.1 mol% $P_2O_5$) and high concentrations of $Ca^{2+}$ and $PO_4^{3-}$ in solution, formed only amorphous calcium phosphate.



## 1. Introduction

Bioactive glass (BG) is known to bond to hard and soft tissues [1]. Fluoride-containing glasses are of particular interest owing to their ability to form fluorapatite, which exhibits better chemical stability than fluoride-free apatites [2]. Fluoride has well documented antibacterial properties [3], and in low concentrations fluoride ions increase bone mass and mineral density [4]. Furthermore, fluoride-containing bioactive glasses enhance osteoblast proliferation, differentiation and mineralization [5].

Novel BG compositions are evaluated in vitro for their apatite forming ability in physiologically relevant test solutions such as SBF [6], Tris-buffer solutions [7], Hank's solution [8], and cell culture media [9]. Dissolution and precipitation form an amorphous calcium phosphate surface layer in the early reaction stages [10], which later undergoes crystallization to apatite by $CO_3^{2-}$, $OH^-$, and/or $F^-$ anion incorporation. This surface apatite is able to elicit an interfacial biological response, resulting in bond formation between tissues and the synthetic material, i.e., bioactive fixation [11]. However, pivotal to this bioactivity is controlling the release rates of ionic dissolution products, i.e., $Ca^{2+}$ and $Si^{4+}$ ions [12]. Dissolution kinetics and consequently the rate of apatite formation is directly related to atomic structure [13], which therefore are critical to in vivo performance.

Although in vivo conditions do not parallel simulated in vitro conditions [14], certain proteins induce specific biological effects in simulated model systems [15]. Amino acids [16], proteins [17], and other organic molecules are rapidly adsorbed onto the glass surface and interfere with apatite formation and stability of the precipitated surface layers. However, the majority of dissolution experiments are performed in serum-free (i.e., protein-free) media. The present work investigates the role of serum proteins in the dissolution medium and their influence on ion release and the in vitro apatite forming ability of fluoride-containing bioactive glasses.

## 2. Materials and methods

Five BG compositions in the $SiO_2$–$P_2O_5$–CaO–$Na_2O$–$CaF_2$ system were prepared by conventional melt-quench route as described earlier [18]. Briefly, mixtures of $SiO_2$ (Prince Minerals Ltd., UK), $P_2O_5$, $CaCO_3$, $Na_2CO_3$ and $CaF_2$ (all Sigma-Aldrich, UK) were heated at 1430 °C for 1 h in a platinum–rhodium crucible. The molten glass was water quenched to prevent crystallization, dried, milled and sieved to a particle size $<$38 μm. Glass F0 was the base composition where the ratio of all the oxides was kept constant in order to maintain a fixed network connectivity of 2.13, to which $CaF_2$ was added incrementally (Table 1). Bioglass® 45S5 was

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**Table 1**
Nominal glass compositions (mol%).

| Glass | $SiO_2$ | $P_2O_5$ | CaO | $Na_2O$ | $CaF_2$ | Classification |
|---|---|---|---|---|---|---|
| Bioglass® 45S5 | 46.1 | 2.6 | 26.9 | 24.4 | – | Fluoride-free |
| F0 | 49.47 | 1.07 | 23.08 | 26.38 | – | |
| F4 | 47.12 | 1.02 | 21.98 | 25.13 | 4.75 | Low-fluoride |
| F9 | 44.88 | 0.97 | 20.94 | 23.93 | 9.28 | |
| F17 | 40.68 | 0.88 | 18.98 | 21.69 | 17.76 | High-fluoride |
| F32 | 33.29 | 0.72 | 15.53 | 17.75 | 32.71 | |

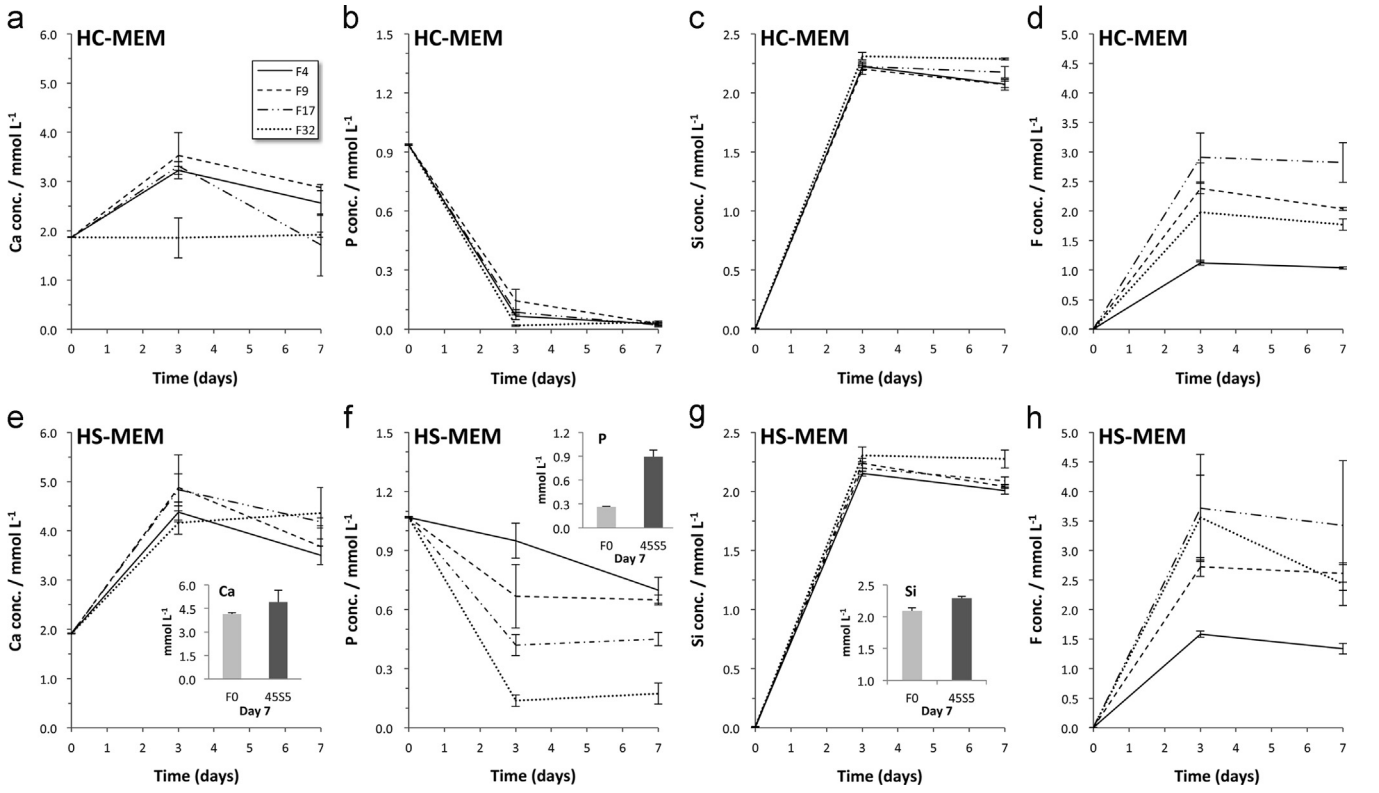


**Fig. 1.** Concentrations of $Ca^{2+}$, P, $Si^{4+}$ and $F^-$ in culture media (a–d) HC-, and (e–h) HS-MEM after immersion of BG. Insets: (d) $Ca^{2+}$ and (e) P concentrations for F0 and 45S5 at day 7 (HS-MEM).

prepared as control. Glass powders were immersed in two dissolution media based on Eagle's Minimal Essential Medium with Earle's Salts (MEM) containing sodium pyruvate as a pH indicator (PAA Laboratories GmbH, Austria). Both media contained 2.2 g $L^{-1}$ $NaHCO_3$ (Sigma-Aldrich, UK), 20 mL $L^{-1}$ HEPES buffer solution (PAA Laboratories GmbH, Austria), and were nominally $Si^{4+}$ and $F^-$ free; HC-MEM (pH = 7.4) was serum-free, while HS-MEM (pH = 7.3) contained 10% of heat-inactivated foetal bovine serum (Sigma-Aldrich, UK). For the dissolution experiments, 75 mg glass powder was dispersed in 50 mL of solution in a polystyrene container. The sealed container was placed in a temperature controlled shaking incubator (IKA® KS4000i control) at 60 rpm and 37 °C for 3 and 7 days. Elemental analysis was performed using a fluoride-selective electrode (Thermo Scientific) for fluoride ions, and by ICP-OES for calcium, phosphorus and silicon ions [9]. The glass powders were analysed by FTIR (20 scans/spectrum; 1 $cm^{-1}$ step size) and XRD (0.03° step size; 4 s step time; standard-short reflection mode using Cu-*K*α radiation).

One-way analysis of variance (ANOVA) with *post hoc* Bonferroni analysis (SPSS Statistics, v.20, IBM Corp.) was used for statistical analysis; $p$ values $< 0.05$ were considered statistically significant. Mean values ± standard deviations are presented.

## 3. Results and discussion

Glass dissolution, by ion exchange and dissolution of the silicate network through a combination of Si–O–Si bond breakage [19] and silicate chain dissolution [20], caused a rapid increase in $Ca^{2+}$, $Si^{4+}$ and $F^-$ concentrations between days 0 and 3. Conversely, P (or $PO_4^{3-}$) depletion closely mirrored apatite formation. On day 3, concentrations of $Ca^{2+}$ and $PO_4^{3-}$ were generally higher in the serum-containing medium, HS-MEM (Fig. 1). Between days 3 and 7, $Ca^{2+}$ and $Si^{4+}$ concentrations decreased slightly, and $F^-$ concentrations remained approximately constant. $PO_4^{3-}$ concentrations decreased in both solutions; however, the decrease was much more pronounced in HC-MEM, coinciding with faster apatite formation.

On day 7, ionic concentrations were also generally higher in the serum-containing HS-MEM medium, with differences in $Ca^{2+}$ and $F^-$ being less pronounced for low-fluoride glasses (F4 and F9).