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Fluoride incorporation in high phosphate containing bioactive glasses and *in vitro* osteogenic, angiogenic and antibacterial effects

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ABSTRACT

Objectives. To manufacture and assess bioactivity of low fluoride/high phosphate (low F^-/high P_2O_5) bioglasses (BGs). Then the effects of BG-conditioned medium on osteoblast-like cell behavior and BG particles on bactericidal activity were investigated.

Methods. BGs (0–7% F^- content, constant 6.33% P_2O_5 in mol%) were designed and produced. BG particles was immersed in Tris Buffer solution or α-MEM to determine apatite formation and ion (Ca, P, Si and F) release. Osteoblast-like cells MC3T3-E1 were treated with BG-conditioned medium and assessed for cytotoxicity, pre-osteogenic and pro-angiogenic responses. Antibacterial ability was explored by incubating sub-gingival bacteria with BG particulates.

Results. Rapid apatite formation was observed in F^- containing BGs after only 2–8 h immersion in Tris buffer solution. In the F^- free group, apatite was not detectable until 72 h. Peak Ca, P and F release into Tris buffer was at 2 h immersion, and then the levels decreased. In α-MEM, apatite formation in all the BGs was undetectable until 72 h immersion. Alkaline phosphatase activity, cell number, collagen formation, bone-like mineral nodules and osteogenic gene expression of MC3T3-E1 cells were significantly promoted in low F^- BG (P6.33F1) conditioned medium. MC3T3-E1 VEGF gene expression was increased, and protein production was dose-dependently promoted with F^- BG-conditioned medium. After incubation with BG particulates, the growth of sub-gingival bacteria, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, was significantly inhibited; the antibacterial activity being dependent on the F^- content of the BGs.

Significance. These results show that low F^-/high P_2O_5 BGs significantly accelerated apatite formation and promoted both pre-osteogenic and pro-angiogenic responses of MC3T3-E1 osteoblast-like cells and inhibited the growth of periodontal pathogens *in vitro*. These BGs may prove useful as bone graft substitutes.

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1. Introduction

Globally, the need for bone defect repair arises due to trauma, tumor, osteoporosis and other causes of skeletal tissue loss. In dentistry, periodontitis and peri-implantitis are common diseases associated with bone loss requiring treatment. BG grafts, when exposed to body fluids, form a bone like apatite layer on their surface (a process termed 'bioactivity'), which is capable of forming a strong bond with the living bone. For this reason they are widely utilized in dental and orthopedic applications [1].

Phosphate plays a vital role in BG bioactivity by forming a $CaO–P_2O_5$ rich bilayer: the new surface for apatite formation [2,3]. ^{31}P and ^{29}Si magic-angle-spinning nuclear magnetic resonance (MAS-NMR) spectroscopy study demonstrates that phosphate is present largely as an orthophosphate phase in BGs results in an increase of the apatite deposition rate, which potentially promotes BG bioactivity [4,5]. In the design of BGs, network connectivity (NC) is considered an important factor as it represents a measure of the number of bridging oxygen atoms per network forming element and an indicator of BG solubility, reactivity and ultimately bioactivity [6].

In fluoride containing BGs, fluoride complexes with calcium and sodium rather than forming Si–F bonds in BG structure, this results in a decrease in the compactness of the BG network [7–9]. Brauer et al. studied fluoride containing BGs by ^{19}F MAS-NMR and demonstrated the formation of fluorapatite (FAp, $Ca_{10}(PO_4)_6F_2$) [8], which is more acid resistant compared with hydroxyapatite (HA), has better stability and slow of degradation kinetics [10]. Numerous *in vitro* and animal studies have demonstrated that fluoride can regulate bone-forming cell activities and bone resorption [11–17], such as, affecting the RANKL/OPG system directly or indirectly [18], regulating BMP/Smads signaling pathway [12] or inhibiting NFATc1 gene expression to decrease osteoclastic activity [13]. Based on the characteristics of fluoride itself and the potential of forming FAp, local delivery of fluoride could reduce demineralisation rate as well as enhancing re-mineralization to increase mineral density and clinically impact on the treatment strategies for osteoporosis [13,19]. However, high levels of systemic fluoride are known to cause skeletal and dental fluorosis characterized by debilitating changes in the skeleton, and marked mottling and discoloration in the teeth [19,20]. Nonetheless, the addition of fluoride into BGs and subsequent local delivery at beneficial concentrations would make such BGs more suitable than existing compounds for dental and orthopedic problems.

Grafting can fail because of insufficient vascularization deep within the body of the graft, thus angiogenesis and associated patent vascular network is crucial for optimal bone formation [21] and subsequent bone:graft contact, 'osseointegration' [22]. Vascular endothelial growth factor (VEGF), released by osteoblastic and other cells, can promote differentiation of local mesenchymal stem cells into endothelial cells and subsequently activate the transmembrane VEFGR2 receptors in endothelial cells, which in turn activates several pathways responsible for angiogenesis [23–26]. This response would be expected to encourage bone formation secondary to increased vascularization throughout the graft substitute.

Another cause of graft failure is bacterial infections which hinder the repair of bone defects [27]. In particular, some oral pathogens associated with periodontal disease have also been associated with dental implant and defect repair failure [28]. Fluoride is widely incorporated into dental restorative materials, to encourage FAp formation, to reduce demineralisation and enhance re-mineralization, it also has anti-microbial properties [29]. Fluoride inhibits the dental plaque acid production that can result in demineralization [30,31]. It acts directly as an enzyme inhibitor to interfere bacterial metabolism [32] and forms metal-fluoride complexes, most commonly AlF^{4-}, which interact with F-ATPase and nitrogenase enzymes resulting in inhibition of bacterial activity [33]. If fluoride released from BGs is available to influence local cell behavior, then fluoride would be a useful constituent of BGs to reduce graft failure due to inappropriate vascularization and infection.

We have created a series of BGs with high, constant phosphate content but with a varying and low fluoride addition, with a fixed BG NC. To determine whether such BGs maintained the characteristics that would make them suitable for potential use *in vivo*, the BGs bioactivity both in Tris buffer solution and cell culture medium were examined. Then, we further assessed the potential of BGs as modulators of biological behavior of osteoblast-like cells and bactericidal activity of the BG particles *in vitro*.

2. Materials and methods

2.1. BG synthesis

BGs in the system $SiO_2–P_2O_5–CaO–Na_2O–CaF_2$ (Table 1) were prepared by the melt-quench route. Briefly, mixtures of analytical grade SiO_2 (Prince Minerals Ltd., Stoke-on-Trent, UK), P_2O_5, Na_2CO_3, $CaCO_3$ and CaF_2 (Sigma-Aldrich Company Ltd., Gillingham, UK) were weighed in the appropriate amounts to give a batch size of 200 g. The batch was mixed thoroughly and placed in a platinum/rhodium crucible, and heated at 1360 °C for 60 min in an electrically heated furnace (Lenton EHF 17/3, Hope Valley, UK). After melting, the BGs were quenched rapidly into deionized water and the resulting frit was washed with ethanol then dried in a drying cabinet at 37 °C overnight. 100 g of each BG was ground in a Gyro mill (Glen Creston, London, UK) for two sets of 7 min and sieved by a mesh analytical sieve (Endecotts Ltd., London, UK) with a size of 38 μm to obtain fine powder. The amorphous structure of the BGs was tested

Table 1 – Bioglass compositions. Compositions in mol% with increasing CaF_2 content and constant P_2O_5. NC fixed at 2.08.

Glass	SiO_2	Na_2O	CaO	P_2O_5	CaF_2
P6.33F0	38.14	29.62	25.91	6.33	0
P6.33F1	37.59	29.38	25.70	6.33	1.00
P6.33F3	36.57	28.85	25.25	6.33	3.00
P6.33F5	35.55	28.33	24.79	6.33	5.00
P6.33F7	34.53	27.81	24.33	6.33	7.00

P6.33F0 named ICSW9 in previous publications [3–5].