Acta Biomaterialia 9 (2013) 5771–5779

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Surface properties and ion release from fluoride-containing bioactive glasses promote osteoblast differentiation and mineralization in vitro

E. Gentleman [a,b,d,*], M.M. Stevens [a,b,c], R.G. Hill [e], D.S. Brauer [e,f,*]

[a] Department of Materials, Imperial College London, London SW7 2AZ, UK
[b] Institute of Biomedical Engineering, Imperial College London, London SW7 2AZ, UK
[c] Department of Bioengineering, Imperial College London, London SW7 2AZ, UK
[d] Craniofacial Development & Stem Cell Biology, King's College London, London SE1 9RT, UK
[e] Department of Materials, Queen Mary University of London, London E1 4NS, UK
[f] Otto Schott-Institut, Friedrich-Schiller-Universität, Jena, Germany

ARTICLE INFO

Article history:
Received 6 June 2012
Received in revised form 8 October 2012
Accepted 30 October 2012
Available online 2 November 2012

Keywords:
Bioactive glass
Fluoride
Osteoblast
Bone regeneration

ABSTRACT

Bioactive glasses (BG) are suitable for bone regeneration applications as they bond with bone and can be tailored to release therapeutic ions. Fluoride, which is widely recognized to prevent dental caries, is efficacious in promoting bone formation and preventing osteoporosis-related fractures when administered at appropriate doses. To take advantage of these properties, we created BG incorporating increasing levels of fluoride whilst holding their silicate structure constant, and tested their effects on human osteoblasts in vitro. Our results demonstrate that, whilst cell proliferation was highest on low-fluoride-containing BG, markers for differentiation and mineralization were highest on BG with the highest fluoride contents, a likely effect of a combination of surface effects and ion release. Furthermore, osteoblasts exposed to the dissolution products of fluoride-containing BG or early doses of sodium fluoride showed increased alkaline phosphatase activity, a marker for bone mineralization, suggesting that fluoride can direct osteoblast differentiation. Taken together, these results suggest that BG that can release therapeutic levels of fluoride may find use in a range of bone regeneration applications.

1. Introduction

Bioactive glasses (BG) have the ability to bond directly with living tissue via the formation of a hydroxycarbonate apatite-like layer on their surface, and are therefore utilized in a variety of dental and orthopaedic applications [1,2]. However, this is not their only mechanism of action; BG can also be designed to release ions that stimulate specific cell behaviour. Indeed, we have recently reported on BG that release strontium ions, the active component of the osteoporosis drug strontium ranelate, which act to both promote osteoblast activity and inhibit osteoclast activity [3].

Fluoride is widely recognized for its ability to prevent dental caries as it inhibits dentine and enamel demineralization [4]; however, it also affects the axial skeleton. That is, clinical examinations of osteoporotic patients have demonstrated that fluoride treatment stimulates bone formation [5]. Nevertheless, in placebo-controlled, double-blind clinical trials, sodium fluoride (NaF) was found not to be efficacious in preventing osteoporosis-related fractures [6,7]. In subsequent detailed analyses of these trials, fluoride's lack of efficacy was attributed to its substitution into bone apatite, creating structurally and mechanically inferior bone, as mineral crystal size and crystallinity increased [8]. Consequently, fluoride's use as an osteoporosis treatment was largely dismissed. Moreover, this seemed judicious, as osteomalacia was reported in patients treated with NaF [9], and skeletal fluorosis, which is endemic in areas with high levels of fluoride in the ground water, is known to cause a range of skeletal abnormalities [10].

As clinical trials with fluoride have failed to demonstrate clear anti-fracture efficacy, other osteoporosis drugs have come into favour. Anti-catabolic agents such as bisphosphonates, which block osteoclast-mediated bone resorption via inhibition of the mevalonate pathway [11], are widely prescribed. However, anti-catabolic agents only prevent further bone resorption and do not promote bone formation. Furthermore, long-term bisphosphonate use has been associated with osteonecrosis of the jaw [12] and atypical, low-energy femur fractures [13], which has created reluctance among some clinicians to prescribe them. Anabolic agents such as recombinant parathyroid hormone (PTH), which stimulate osteoblast-mediated bone matrix formation [14], are also often

* Corresponding authors. Addresses: Craniofacial Development and Stem Cell Biology, CFD19, Floor 27, Tower Wing, Guy's Hospital, Dental Institute, King's College London, London SE1 9RT, UK. Tel.: +44 20 7188 7388 (E. Gentleman), Otto-Schott-Institut, Friedrich-Schiller-Universität, Fraunhoferstr. 6, 07743 Jena, Germany. Tel.: +49 3641 948510 (D.S. Brauer).

E-mail addresses: eileen.gentleman@kcl.ac.uk (E. Gentleman), delia.brauer@uni-jena.de (D.S. Brauer).

1742-7061/$ - see front matter © 2012 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2012.10.043

administered. However, PTH is expensive and requires daily subcutaneous injections. As a result, alternative treatments, such as inexpensive NaF, have recently been re-examined [15].

In vitro studies point towards a small dosing range for NaF that will successfully promote osteoblast activity [16], and recent meta-analyses have shown that fluoride is efficacious in preventing osteoporosis-related fractures when administered at doses lower than those used in the widely reported clinical trials [17]. Furthermore, in vitro studies have demonstrated that fluoride has an anabolic effect on bone, enhancing osteoblast proliferation [18,19] and alkaline phosphatase activity [19–21]. The effect, however, seems to be strongest on osteoblast precursors as opposed to mature cells [22,23], suggesting that fluoride may promote osteoblast differentiation or maturation. Moreover, cells isolated from animals administered fluoride in vivo showed higher proliferative potential than those isolated and subsequently treated in vitro [24]. Taken together, these data suggest the importance of the timing of the administration of fluoride, and support a role for short-term treatment of less mature cells as a route for gaining the greatest benefit. Therefore, therapies such as BG that have the potential to locally administer therapeutic doses of fluoride over a predictable time frame may be useful for stimulating new bone formation.

One important factor in designing BG is network connectivity (NC), a measure of the number of bridging oxygen atoms per network forming element and an indicator of BG solubility, reactivity and bioactivity [25]. Here, by holding the ratio of network former to network modifier constant whilst adding CaF_2, we created a BG series with increasing amounts of fluoride, but with constant NC (assuming fluoride only associates with calcium, i.e. Si–F bonds are not formed [26]). This allowed us to examine the effects of fluoride in BG on human osteoblast cells in vitro without complications caused by changes in the silicate network structure. We also separated the effects of ions released from BG from the effects elicited by cells' complex interactions with the apatite-forming BG surface and examined how both factors influenced human osteoblast activity.

2. Materials and methods

2.1. Preparation of BG discs

BG in the system SiO_2–P_2O_5–CaO–Na_2O were prepared by a melt–quench route. CaF_2 was added whilst the NC and the ratio of all other components were kept constant (Table 1). Mixtures of analytical grade SiO_2 (Prince Minerals Ltd., Stoke-on-Trent, UK), P_2O_5, $CaCO_3$, Na_2CO_3 and CaF_2 (all Sigma–Aldrich, Gillingham, UK) were melted in a platinum–rhodium crucible for 1 h at 1430 °C. A batch size of 100 g was used. After melting, BG were rapidly quenched in deionized water (dH_2O) to prevent crystallization. BG rods were produced by casting the melt into pre-heated graphite moulds (10 mm diameter) and subsequently annealing at the glass transition temperature, which varied between 514 and 439 °C [27]. Rods were sectioned into 1 mm thick discs on a low-speed diamond saw (IsoMet®, Buehler GmbH, Düsseldorf, Germany) using isopropanol as a coolant. Prior to cell culture experiments, BG discs were sterilized under ultraviolet light for 2 h on each side and pre-conditioned in 1 ml of culture medium (RPMI 1640 with 1× penicillin/streptomycin solution; Invitrogen, Paisley, UK) for 4 days. Cell culture medium was exchanged daily and stored at −20 °C.

Table 1
BG compositions (in mol%).

CaF_2	SiO_2	P_2O_5	CaO	Na_2O
0	49.47	1.07	23.08	26.38
1.00	48.98	1.06	22.85	26.12
4.75	47.12	1.02	21.98	25.13
9.28	44.88	0.97	20.94	23.93
13.62	42.73	0.92	19.94	22.79
17.76	40.68	0.88	18.98	21.69

2.2. Elemental analysis of ions released from BG

Culture medium samples were diluted by a factor of 10 in dH_2O, and concentrations of calcium and silicon were measured on an inductively coupled plasma–optical emission spectrometer (ICP-OES; iCAP 6000, Thermo Scientific, Waltham, MA, USA). Fluoride concentration was measured using a fluoride-selective electrode (Orion 9609BNWP with Orion pH/ISE meter 710; Thermo Scientific). Calibration was performed using standard solutions prepared using either dH_2O or Tris buffer for ICP-OES and fluoride measurements, respectively.

2.3. Cell culture with BG discs

The human osteosarcoma cell line, Saos-2, was obtained from the European Collection of Cell Cultures (Salisbury, UK) and cultured under standard conditions (37 °C, 5% CO_2/95% air, 100% humidity) in RPMI 1640 with 10 vol.% foetal bovine serum (FBS) and 2 mM L-glutamine (all from Invitrogen). One BG disc per well was seeded at 30,000 cells cm^{-2}. The surrounding tissue culture plastic was likewise seeded at 30,000 cells cm^{-2} to assess both the effects of culture in contact with the BG and that resulting from exposure to their dissolution products, as previously described [28]. The culture medium was exchanged three times per week and stored at −20 °C. Discs were moved to fresh wells prior to assays to allow separate analyses of cells cultured in contact with discs from those only exposed to their dissolution products.

For experiments with NaF, 30,000 cells cm^{-2} were seeded in 96-well plates and cultured for up to 28 days in medium as above, supplemented with 100 μM NaF (Sigma) for the indicated time period. Otherwise cells received standard culture medium, which was exchanged three times per week.

2.4. X-ray diffraction (XRD) of BG surfaces

After 7, 14 or 28 days of culture, cells were removed and BG discs were rinsed in dH_2O and air dried. Surfaces were then examined by XRD (X'Pert PRO MPD, PANalytical, Cambridge, UK; 40 kV/40 mA, Cu K_α, collected at room temperature) and analysed using X'Pert HighScore Plus software (v2.0, PANalytical, The Netherlands) and the International Centre for Diffraction Data database. Diffraction patterns were compared to reference patterns of hydroxycarbonate apatite (JCPDS 19-272) and fluorapatite (JCPDS 31-267).

2.5. Scanning electron microscopy (SEM) to visualize cells and BG apatite formation

After 7, 14 and 28 days in culture, some BG discs were rinsed with phosphate-buffered saline (PBS) and fixed for 40 min in 2.5% (w/v) glutaraldehyde in sodium cacodylate buffer (pH 7.3) at 4 °C. The discs were then rinsed with PBS, dehydrated in a graded ethanol series, incubated with hexamethylsilasane (Sigma), sputter coated with gold and viewed on an FEI Inspect F scanning electron microscope (FEI, Eindhoven, Netherlands) using the secondary electron mode at 10 kV. Other BG discs used for cell culture analyses and XRD were also analysed by scanning electron microscopy (SEM) to visualize apatite formation. Discs were sputter coating with gold/palladium and viewed in an scanning electron microscope as above.