Effect of silicon level on rate, quality and progression of bone healing within silicate-substituted porous hydroxyapatite scaffolds

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Received 1 February 2006; accepted 15 May 2006

Abstract

The osseous response to silicon (Si) level (0, 0.2, 0.4, 0.8 and 1.5 wt% Si) within 5 batches of matched porosity silicate-substituted hydroxyapatite (SA) scaffold was assessed by implantation of 4.6 mm diameter cylinders in the femoral intercondylar notch of New Zealand White rabbits for periods of 1, 3, 6 and 12 weeks. Histological evaluation and histomorphometric quantification of bone ingrowth and mineral apposition rate (MAR) demonstrated the benefits to early (≤1 week) bone ingrowth and repair through incorporation of Si, at all levels, in porous hydroxyapatite (HA) lattices as compared to stoichiometric (0 wt% Si) HA. The group containing 0.8 wt% Si supported significantly more bone ingrowth than all other groups at 3 and 6 weeks (\(P<0.05\)), initially through its elevated MAR between weeks 1 and 2, which was significantly higher than that of all other Si-containing groups (\(P<0.05\)). The level of silicate substitution also influenced the morphology and stability of the repair, with elevated levels of bone resorption and apposition apparent within other Si-containing groups at timepoints >3 weeks as compared to the 0 and 0.8 wt% Si groups. At 12 weeks, the net amount of bone ingrowth continued to rise in the 0, 0.8 and 1.5 wt% groups, apparently as a result of adaptive remodelling throughout the scaffold. Ingrowth levels remained highest in the 0.8 wt% Si group, was characterised by a dense trabecular morphology in the superficial region graduating to a more open network in the deep zone. These results highlight the sensitivity of healing response to Si level and suggest that an optimal response is obtained when SA is substituted with 0.8 wt% Si through its effect on the activity of both bone forming and bone resorbing cells.

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Keywords: Bioactivity; Bone graft; Bone ingrowth; Calcium phosphate; Hydroxyapatite; Silicon

1. Introduction

Bone mineral has a similar crystallographic structure to hydroxyapatite (HA) \[1\], a hydrated calcium phosphate (chemical formula \(\text{Ca}_{10}^{}(\text{PO}_4)^{\text{6}}(\text{OH})_2\); Ca:P ratio 5:3 (1.67)). Unlike HA, bone mineral exhibits calcium, phosphate and hydroxyl deficiency (reported Ca:P ratios 1.37–1.87 [2,3]), internal crystal disorder, and contains various cationic and anionic substituents, principally carbonate (up to 8 wt%), making it more like an A–B-type carbonate-substituted apatite [4,5]. Potential trace level substituents include sodium (up to 0.8 wt%) and magnesium (up to 0.5 wt%), while ultra-trace level substituents include potassium, strontium, zinc, fluorine, chlorine and silicon (Si) [2,4,6,7]. All these factors produce an apatite that is insoluble enough for stability, yet sufficiently reactive to allow the sub-microscopic (5–100 nm) crystallites to be constantly re-absorbed and reformed in vivo, providing a ready source of inorganic nutrients of metabolic significance [8] such as Si [7,9], levels of which can vary with age and sex [10].

Many commercially available synthetic bone graft substitutes comprise calcium salts, glasses or glass ceramics, often incorporating phosphate ions. In the
1970s, Carlisle [11] reported that Si deficiency resulted in abnormal bone formation, confirmed by Schwarz [12] who also identified it as a cross-linking agent in connective tissue [13] and its importance to vascular health [14], while Hench reported the direct bonding of bone and muscle to a range of bioactive, Si-containing glass ceramics [15]. Increasing evidence supports the hypothesis that the presence of Si contributes to enhanced bioactivity of some bioactive glasses and glass ceramics in vitro [16–18], with a significant up-regulation of osteoblast proliferation and gene expression (including BMP-2) when exposed to the ionic dissolution products of bioactive glasses [16,17]. Moreover, orthosilicic acid present in physiological concentrations (5–20 μM) stimulated collagen type I synthesis and enhanced osteoblast differentiation in cell lines, while supra-physiological concentrations (50 μM) effected a smaller increase in collagen synthesis [19]. Work on pseudo-wollastonite (psW, α-CaSiO₃) demonstrated the synergistic effect co-treatment with Si and calcium has in stimulating bone cell activity compared to treatment with either element in isolation [20].

These studies demonstrate the benefits of delivering controlled levels of Si to a wound site to facilitate bone repair. A method for substituting silicate ions into the HA lattice has been developed [21] with the hypothesis that site-specific substitution of silicate ions (SiO₄^4−) for phosphate ions (PO₄^3−) at ultra-trace levels will enhance the bioactivity of HA through either its effect on surface chemistry (surface availability of Si and its influence on surface charge) or controlled local bioavailable Si release. Lapine (0 and 1.2 wt%) [22] (0 and 0.8 wt% Si) [23] and ovine defect models (0, 0.8 and 1.5 wt% Si) [24], demonstrated that the rate and quality of bone apposition was enhanced with Si substitution. Moreover, transmission electron microscopy (TEM) studies of the bone/implant interface between dense HA granules substituted with 0, 0.8 and 1.5 wt% Si implanted for 6 and 12 weeks demonstrated localised dissolution of all apatites, at a rate and extent linked to the level of substitution, suggesting a synergistic mechanism by which increasing the substitution level could influence local serum concentrations of free Si in vivo [25], although subsequent studies failed to find a significant difference in performance between the 0.8 and 1.5 wt% Si-substituted dense granules [24]. In vitro studies of silicate-substituted apatite surfaces [26–28] demonstrated changes in surface charge and surface silicate ion species with the 0.8 wt% material showing higher bioactivity [26,29]. Protein adsorption, osteoblast-like cell attachment and subsequent cell response (such as alkaline phosphatase activity and collagen I synthesis) may also be linked to these parameters [29,30].

Given reports in the literature of Si content in bone reaching levels as high as 0.5 wt% in active calcification sites within the chick [11] and varying in the femoral shaft (40 ppm) and epiphysis (460 ppm) of rhesus monkeys [31], the aim of this study was to investigate the influence of varying levels of silicate content (0.2–1.5 wt% Si) on the rate, quality and volume of bone apposition within porous silicate-substituted hydroxyapatite (SA) scaffolds, to determine whether there was an optimal substitution level beyond which additional Si substitution was either superfluous or detrimental to bone biology.

2. Materials and methods

Four grades of SA powder (Si contents 0.2, 0.4, 0.8 and 1.5 wt%) were synthesised using the aqueous precipitation route [21] by maintaining parity between the number of moles of orthophosphoric acid (H₃PO₄) required for synthesis of stoichiometric hydroxyapatite (HA) with the number of moles of [H₃PO₄ + silicon acetate (Si(CH₃COO)₄)] in the SA precipitations, while keeping the number of moles of calcium hydroxide Ca(OH)₂ constant, so preserving the Ca/(Si + P) ratio as 1.67 (Table 1).

Porous HA and SA scaffolds with no significant variation in porosity level (as-sintered total porosities 72.9 ± 1.4 and 73.4 ± 4.3%; strut porosities 22.2 ± 2.3 and 23.9 ± 4.9%) were produced using a novel slip foaming technique [32]. Strut porosity is the fraction of porosity within the scaffold struts (pores <10 μm in diameter) while macroporosity describes the bulk of the pore fraction [33]. Once foamed, the slips were cast in rectangular plaster of Paris moulds (6 × 8 × 2 mm) and allowed to air dry before sintering at either 1250 °C (HA) or 1300 °C (SA) for 2 h; Fig. 1 shows typical porosity features. For implantation cylindrical specimens (4.62 ± 0.09 mm diameter; 6.51 ± 0.54 mm length) were machined from the sintered plaques.

2.1. Chemical analysis

Samples were obtained by crushing sintered scaffolds to a <50 μm particle size. Si levels were determined by X-ray fluorescence (XRF) (Ceram Research, Stoke on Trent, UK). Phase purity was assessed using X-ray diffractometry (XRD), on a Siemens D-5000 X-ray diffractometer in flat plate geometry with monochromatic Cu-Kα radiation and the X-ray generator operated at 40 kV and 40 mA. Data were acquired from 25 to

Table 1

<table>
<thead>
<tr>
<th>Wt% Si</th>
<th>No. of moles</th>
<th>Predicted molar ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca(OH)₂</td>
<td>H₃PO₄</td>
</tr>
<tr>
<td>HA</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>SA02</td>
<td>0.2</td>
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</tr>
<tr>
<td>SA04</td>
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<td>0.5</td>
</tr>
<tr>
<td>SA08</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>SA15</td>
<td>1.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Note: HA stands for hydroxyapatite, SA for silicate-substituted hydroxyapatite.
before both the joint capsule and skin incisions were closed with interrupted Vicryl sutures. Anaesthesia was reversed by Temgesic (0.25 ml/kg i.v.) and animals were isolated in a recovery room overnight. Animals were subsequently allowed full use of their knees and all were fully mobile within 24 h of surgery. Implants were retrieved for histological and histomorphometric analysis at 1, 3, 6 and 12 weeks. The appearance of surrounding tissue, the extent of healing at the site of implantation, the mobility of the joint and any abnormalities were noted. After sacrifice, operated femora were completely removed and all soft tissue stripped from the bone. The sample size for each bone graft substitute (BGS) material was \( n = 4 \) per implant retrieval time point.

### 2.2. Biological assessment

#### 2.2.1. Implantation procedure

UK Home Office regulations for the care and use of laboratory animals were observed throughout this study (Animals (Scientific Procedures) Act, 1986). Before implantation into mature female New Zealand White rabbits, all specimens were dry heat sterilised at 170°C for 90 min. Following general anaesthesia, operations were performed bilaterally; a single cylindrical defect (4.5 mm external diameter) mounted in a compressed air powered drill. During drilling, the defect site was bathed in sterile saline to minimise local heating and to remove debris. On completion of drilling, a single cylinder was press-fitted into place and the site further washed with sterile saline.

#### 2.2.2. Histology and histomorphometry

Specimens were trimmed and placed immediately in 70% ETOH for at least 4 days. Fixed tissues were dehydrated and embedded in Technovit resin. The resin blocks were sectioned in the sagittal plane and processed through to semi-thin (5–10 μm) sections (Exakt technique) [35]. At least three sections were obtained from each implant; one left unstained for fluorescence microscopy, the others stained with either toluidine blue or Goldner’s trichrome. Histomorphometry, using point counting [36], determined the total volumes occupied by bone ingrowth, implant and porosity for each specimen, allowing calculation of both the absolute (bone fraction within the whole defect site) and normalised (bone fraction in the space not occupied by scaffold within the defect site) percentages of bone ingrowth [37]. The mineral apposition rate (MAR) for animals in the 3, 6 and 12 weeks survival groups was determined through administration of fluorochrome labels 7 days apart in the 2 weeks preceding sacrifice (i.e. at weeks 1+2, 5+6 or 10+11) [38]. As a control the MAR was determined for non-operated trabecular bone >5 mm from the defect site. To aid histological and histomorphometric examination, regional analysis zones (peripheral = defect edges, superficial = towards the articular surface, deep = towards the medullary canal) were defined within the implants and the progression of events such as angiogenesis and new bone formation monitored within these zones.

#### 2.2.3. Statistical analysis

Variations in responses between treatments were assessed using a one-way analysis of variance, where differences between treatment groups were evaluated using Tukey HSD post-hoc testing. All tests were run using KaleidaGraph statistical software (v 4.0, Synergy Software, USA) at a significance level of \( \alpha = 0.05 \).

### 3. Results

#### 3.1. Scaffold chemistry

Elemental analysis confirmed Si levels within the scaffolds, with a mean deviation of 0.03 wt% from the design levels (Table 2). The Ca/P ratio was greater in substituted materials than that in stoichiometric HA (1.67), although the actual Ca/P and Ca/(P+Si) levels were slightly elevated with respect to the theoretical values, particularly in the lower wt% Si additions (Table 1). However, agreement between the ICDD standard pattern for HA and the reflection peaks obtained from X-ray diffraction of sintered specimens was consistent for all PHA and PSA scaffolds (Fig. 2a). No other phases were detected with levels >0.5 wt%. The peaks were narrow indicating a high level of crystallinity for all compositions; no clear trend was observed in peak intensity with increasing Si levels.

All FTIR spectra (Fig. 2b) exhibited the characteristic OH\(^-\) band at 3570 cm\(^{-1}\) and PO\(_4\)\(^{3-}\) bands between 960 and...
were also present, as were weak bands in the region associated with the $\text{CO}_2^-$ v3 vibration mode (1550–1410 cm$^{-1}$), and broad bands in the 2000–2200 cm$^{-1}$ region, possibly due to surface adsorbed $\text{HPO}_4^{2-}$ groups. However, the relative intensity of the $\text{OH}^-$ band was slightly reduced in PSA08 and PSA15, while the relative ratios of the band heights associated with $\text{PO}_4^{3-}$ vibration modes varied with increasing silicate level (most notably for bands at 960 and 630 cm$^{-1}$) and three additional bands were observed at 950, 890, 840 and one at 500 cm$^{-1}$, their relative intensity also increased with silicate addition.

3.2. Histology

3.2.1. One week in vivo

Clear differences were observed between samples. Broadly, cellular infiltration and organisation, neovascularisation and tissue (new bone) penetration was more advanced within the peripheral porosity of all PSA scaffolds, with cellular infiltration notably more organised within the superficial porosity of PSA08 and PSA15, while capillary penetration was more advanced throughout deep and peripheral zones of all PSA scaffolds (Fig. 3a), extending throughout central and central-superficial zones of PSA08 and PSA15. The presence of isolated regions of new bone tissue within central zones was more consistently observed within PSA scaffolds whereas new bone tended to be restricted to very peripheral regions in PHA. Furthermore, there were distinct variations in new bone morphology between the PHA and various PSA grades. Within PSA08 (Fig. 3c) and PSA15 the bone was dense in appearance (numerous, thick trabeculae) compared to the ‘finer’ ingrowth observed in PSA02 and PSA04 (Fig. 3b) (numerous, thin trabeculae). The more sporadic ingrowth within the periphery of PHA implants showed a sparser network of thick trabeculae. The bone within all scaffolds predominately comprised highly cellular, disorganised woven bone, with bone surfaces populated by highly active cuboidal osteoblastic cells, consistent with mesenchymal-type bone formation.

3.2.2. Three weeks in vivo

By 3 weeks vascular networks were established within the deep and peripheral zones of PHA, PSA02 and PSA04, while capillaries had penetrated throughout the porosity of PSA08 and PSA15.

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<p>| XRF data and molar ratios for the four grades of silicate-substituted hydroxyapatite (SA) in comparison to stoichiometric hydroxyapatite (HA) |</p>
<table>
<thead>
<tr>
<th>CaO</th>
<th>P$_2$O$_5$</th>
<th>SiO$_2$</th>
<th>Ca/P</th>
<th>Ca$_4$(P$_2$ + Si)</th>
<th>Wt% Si</th>
<th>Wt% SiO$_4^{2-}$</th>
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<tr>
<td>HA</td>
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<td></td>
<td>1.68</td>
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<tr>
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<tr>
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<td>1.76</td>
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<td>55.4</td>
<td>38.1</td>
<td>3.09</td>
<td>1.84</td>
<td>1.68</td>
<td>1.47</td>
</tr>
</tbody>
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Fig. 2. (a) XRD patterns and (b) FT-IR spectra of the stoichiometric and silicate-substituted porous hydroxyapatite scaffolds.
Bony integration was well advanced within all samples, with complete penetration of bone throughout deep and central zones, however, the bone ingrowth morphology varied widely (Fig. 4). Again a dense trabecular network of ingrowth was observed in PSA08 and PSA15 (Fig. 4d & e), with some pores in PSA08 in 2D section appearing to be almost completely filled with new bone. In contrast, ingrowth within PSA04 (Fig. 4c) was generally sparser while that in PSA02 (Fig. 4b) was moderately dense but sporadic in distribution within the porosity. Ingrowth within the PHA was generally well distributed throughout the porosity with a moderately dense/intermediate trabecular morphology (Fig. 4a).

Bone surfaces of PSA04 also had a high degree of osteoclastic/phagocytic activity, with a high proportion of scalloped bone surfaces and pitted PSA strut surfaces (Fig. 4f). Moreover, much of the ingrowth within PSA04 appeared to be woven in nature. Some evidence of bone remodelling and cell mediated scaffold resorption were also evident in PSA15, PSA08 and PSA02 and deposition of more organised lamellar bone was apparent within PSA15 and particularly PSA08. Fluorochrome labelling provided further evidence, demonstrating decreasing levels of 1 week labelled woven bone with increasing Si content from 0 to 0.8 wt% (Fig. 5a–d) and evidence of direct apposition of bone on strut surfaces (Fig. 5b) and development of pore bridging trabeculae (Fig. 5c). Similar levels of woven bone were observed in the peripheral zones of PSA08 and PSA15 with some labelled lamellar bone within the porosity of PSA08 at 1 week (Fig. 5d). At 2 weeks woven bone apposition still dominated in the central zones, with increasing lamellar apposition in both PSA08 and PSA15.

3.2.3. Six weeks in vivo

By 6 weeks the vascular network within all scaffolds continued to mature and was established throughout the porosity of all PSA scaffolds. There was no apparent difference in the depth of penetration of bone ingrowth into the scaffolds from 3 and 6 weeks. However, trabeculae were thicker throughout PSA08 and PSA15 while within the central porosity of PHA and throughout PSA04 and PSA02 the bone morphology was less dense (Fig. 6). Bone surfaces within all scaffolds were occupied by active plump osteoblasts or possessed a scalloped topography indicating that differing phases of an active remodelling cycle were in progress. This remodelling was particularly marked in PSA02 and PSA15 (Fig. 6c) where bone surfaces were occupied by both active osteoblast seams and resorption pits, with the degree of cellular activity being particularly marked in PSA15. In contrast scalloped bone surfaces dominated in PSA04 (Fig. 6a) and scaffold struts appeared pitted, particularly in the superficial zones. The level of cellular activity was reduced in PSA08, which had a more organised, mature appearance (Fig. 6b).
3.2.4. Twelve weeks in vivo

At 12 weeks bone had penetrated throughout the deep, central and peripheral–superficial zones of all scaffolds. The distribution of bone within PSA02 and PSA04 was sporadic while that within the other scaffolds varied with location (Fig. 7). Moreover the bone had a highly ‘wasted’ morphology in PSA02 where bone surfaces were predominantly scalloped (Fig. 7a), whereas in PSA04 trabeculae were thicker and bone surfaces were occupied by both active osteoblasts and osteoclasts (Fig. 7b). Within PHA, there was a clear pattern of denser bone in the subchondral region that became more open towards the deep zone. Moreover, bone within the peripheral regions tended to be denser than that in central regions where there were active areas of remodelling. Within PSA15, there was some bone remodelling in deep regions (Fig. 7d) and active bone remodelling in central and superficial zones with a reduction in bone in these areas. Within PSA08 a dense trabecular morphology prevailed in the superficial regions with a gradual reduction in density throughout the central-deep zones (Fig. 7c) while bone surfaces were predominantly quiescent, with sporadic instances of apposition and/or resorption.

3.3. Bone volume

The volume of bone in-growth (both absolute and normalised) within PSA08 was consistently greater than within the other samples throughout the study (Fig. 8). The level of absolute bone volume (ABV) within PSA08 was significantly greater than within PHA and PSA15 at 1 week (Fig. 8a), than all specimens at weeks 3 and 6 (Fig. 8b & c).
and than PSA02 and PSA04 at week 12 (Fig. 8d). Except in week 1 data, the ABV for PHA and PSA15 were consistently higher than those for PSA02 and PSA04. However, this variation was not statistically significant, save for PHA vs. PSA02 at 3 weeks (Fig. 8b), PHA and PSA15 vs. PSA04 at 6 weeks (Fig. 8c) and PHA vs PSA02

Fig. 5. Fluorochrome labelling of new bone at 1 and 2 weeks post op. in: (a) PHA, (b) PSA02 and (c, d) PSA08. Inset = same field of view in bright field white light. Bar = 100μm.

Fig. 6. Bone morphology at 6 weeks within macroporosity of: (a) PSA04, (b) PSA08 and (c) PSA15. Bar = 100μm.
and PSA04 at 12 weeks (Fig. 8d). There was no statistical variation in the level of ABV between PSA02 and PSA04 throughout the study, nor between PHA and PSA15. This pattern of statistical significance was maintained when normalised for pore volume.

Fig. 9 shows the normalised bone volume (NBV) data from implants retrieved at 1–12 weeks, grouped for scaffold chemistry. There was a significant increase in bone volume from 1 to 3 weeks for all groups, however there was no further significant variation from 3 to 12 weeks for PSA02 and PSA04. In contrast, while there was no statistical variation in bone volume from weeks 3 to 6 for PHA, PSA08 or PSA15, there was a significant increase from weeks 3 and 6 to week 12 for all three.

3.4. Mineral apposition rate

MARs calculated over the 1–2 week period within implants retrieved at 3 weeks demonstrated elevated rates of apposition compared to internal controls within all scaffolds (Fig. 10a, small asterisks within box plots). Furthermore, the MAR within PSA08 was significantly higher than within all other silicate scaffolds, while the MAR within PHA was significantly higher than that within PSA15 (Fig. 10a, large asterisks). There was no significant difference between the MAR of control bone and bone within PSA08 during the 4–5 week period from implants retrieved at 6 weeks. The MAR within all other scaffolds was significantly higher than control bone, particularly within PSA02 and PSA15 (Fig. 10b, small asterisks). The MAR within PSA08 was significantly lower than within all other scaffolds, whereas the MARs within PSA02 and PSA15 were significantly higher than within all other scaffolds (Fig. 10b, large asterisks).

As expected the MAR of bone within scaffolds during the 10–11 week period was generally lower than at earlier time points, however within all silicate-substituted scaffolds the MARs were significantly elevated compared to control bone (Fig. 10c, small asterisks). The MARs of PSA02 and PSA15 were higher than all other scaffolds (significant for PHA and PSA08, Fig. 10c, large asterisks), while the MAR of PSA08 was significantly lower than that of all other PSA scaffolds, similar to the trend in the 4–5 week period (Fig. 10c, large asterisks). Interestingly, the MAR of bone within PHA was also significantly lower than that within PSA scaffolds other than PSA08 (Fig. 10c, large asterisks).

4. Discussion

4.1. Substitution of silicon within the apatitic lattice

XRF, XRD and FT-IR analyses, confirmed the phase purity and Si level within each scaffold. The significant increase in Ca/P ratio from stoichiometric HA (1.67) in all PSA materials would normally result in a significant loss (>0.5 wt% of any additional phase) in phase purity due to decomposition during sintering, with additional peaks
expected in XRD patterns symptomatic of CaO or tetracalcium phosphate. Such peaks were not seen (Fig. 2a), suggesting that the Si present within the materials was incorporated in the crystallographic structure in such a way as to stabilise the apatitic phase and supporting Gibson’s proposal that the Si was incorporated as tetrahedral silicate ions substituted into the HA lattice on phosphate sites,\[21\], with charge balancing maintained through loss of hydroxyl ions, as per the following equation:

\[
\text{Ca}^{2+} (\text{PO}_4^{3-})_{6-x}(\text{SiO}_4^{4-})_x(\text{OH})_{2-x}
\]

Consistent with this mechanism, a slight loss in relative intensity was seen in the characteristic OH\(^-\) band in FT-IR spectra of PSA08 and PSA15 (Fig. 2b), indicating a loss of occupancy at this site. Moreover, variation in relative intensities of the bands associated with the various phosphate stretches, in addition to the presence of additional bands attributable to Si–O stretching vibrations (840, 890, 950 cm\(^{-1}\)) or Si–O bending vibrations (500 cm\(^{-1}\)) in silicate tetrahedra \[39\] could indicate the loss of

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**Fig. 8.** Variation in absolute volume of new bone within the various PHA and PSA scaffolds at: (a) 1, (b) 3, (c) 6 and (d) 12 weeks (data: box limits = upper/lower quartiles, error bars = max/min, line = median, cross = mean, outliers = o). [*P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001].

**Fig. 9.** Variation in normalised volume of new bone within the various PHA and PSA scaffolds with time in vivo (data: mean, error bars = SD). [*P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001].
phosphate groups and/or symmetry at the site caused by substitution of silicate species in all PSA spectra (Fig. 2b).

4.2. Biological sensitivity to silicate substitution and substitution level

Evidence of Si’s influence on bone mineralisation [11,31,40,41], metabolism [19,42–44], collagen synthesis [19,45] and crosslinking [46] is compelling. In this study, both the presence and level of Si had a profound effect on bone apposition. At the very early time point (<1 week) Si appeared to accelerate the apposition of a loose trabecular network of immature woven bone within the periphery of all PSA scaffolds. The degree of early bone formation and extent of scaffold penetration appears to correlate well with the rate of angiogenesis, where capillary formation and penetration was far superior in all grades of PSA at 1 week than in PHA. A link between dietary Si, atherosclerosis, arterial and connective tissue health has been proposed [14], suggesting a manifold role for Si in bone healing through direct physiological action on vascularisation, bone metabolism and bone quality (matrix organisation and mineralisation). Whether this is a surface charge alteration affecting protein adsorption, cell attachment and differentiation, or a direct interaction between cell metabolism and bioavailable free Si is unclear. Protein conformation is sensitive to surface chemistry [47]; when calcium phosphates and specific proteins are combined under the correct conditions, calcium phosphate dissolution [48,49], nucleation [50] or ageing [51] can be modulated as can adsorbed protein species [49], conformation [52] and stability [53] demonstrating a reciprocal relationship between protein adsorption and calcium phosphate chemistry. More specifically, the quality and quantity of proteins adsorbed in vitro is sensitive to silicate substitution levels, which has a downstream effect on osteoblast-like cell attachment and metabolism [29]. In this study, cellular organisation and neo-vascularisation at 1 week were particularly advanced close to the pore surfaces within the PSA scaffolds. A close relationship between newly formed capillaries and HA surfaces was observed in a study of angiogenesis following HA implantation where immunohistochemical localisation also demonstrated VEGF association with the HA surface [54]. This would suggest that incorporation of silicate ions into the lattice supports and may even promote this association, perhaps

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Fig. 10. Variation in the mineral apposition rate of new bone laid down within the various PHA and PSA scaffolds between weeks: (a) 1–2, (b) 4–5 and (c) 10–11 (data: box limits = upper/lower quartiles, error bars = max/min, line = median, outliers = o). [*P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001. Small asterisks within the box plots denote level of significance against matched controls].
by influencing surface charge and wettability, highlighting the importance of surface chemistry on the interaction between proteins and cells with bioceramic surfaces at these early time points. However, as healing progressed (<3 weeks) it was clear that the osseous response was also sensitive to the level of Si addition. Increasing the amount of Si appeared to influence the organisational level of new bone within the scaffolds and increasing Si to ≥0.8 wt% promoted apposition of lamellar bone at earlier time points. Moreover, throughout the study the amount of new bone was always greatest within PSA08, reflecting faster apposition at earlier time points and retention of an interpenetrating trabecular network within the porosity of this treatment group at later time points. Bioavailable Si is critical in the development and structural integrity of connective tissue [13, 45, 55] and has been implicated in the hydroxylation of proline intracellularly during procollagen synthesis, in forming crosslinks at hydroxylsine/lysine sites [46] and in stabilising the glycosaminoglycan network [13]. Moreover, Si-modulated up-regulation of collagen synthesis exhibits dose dependency where supra-physiological concentrations of Si (1.4 μg/ml) were less effective than treatments at concentrations of 0.28 or 0.56 μg/ml [19], levels more similar to the physiological concentration of Si in human serum. Increasing the level of Si substitution within the HA lattice raises its dissolution rate in vivo, suggesting a synergistic mechanism by which the amount and rate of free Si may be released from the surface of implanted materials with greater levels of substitution [25]. It is therefore unsurprising that a dose dependant effect similar to the biochemical studies [19] is observed here. There was an optimal level of Si, which stimulated rapid bone matrix synthesis with apparently enhanced organisation; higher levels that increased the free Si concentration offered no advantage.

At 3 weeks the less organised, sporadic morphology and irregular pitted bone surfaces in PSA02 and PSA04 reflected an interruption/imbalance in the cycles of bone apposition, which was erratic (chiefly PSA02) and bone resorption, which was extensive (chiefly PSA04), continuing through to week 12. This could not be directly attributed to a variation in osteoblastic activity or bone metabolism, but anecdotal evidence suggested a relative up-regulation in what appeared to be osteoclastic activity. This was in contrast to deceased osteoclast surface and number on ovariectomised rats treated with Silanol compared to controls [42], and decreased osteoclast activity in vitro when incubated in the presence of sodium-aluminium-silicate [56]. The osteoclast response to carbonate-substituted and stoichiometric HAs demonstrated that osteoclast attachment and activity were sensitive to surface energy [57] and it is well known that pH plays an important role in modulating osteoclastic behaviour. Measurements of the point of zero charge (PZC) [28] and zeta potential [27] have shown that surface charge on silicate-substituted apatites was more electronegative than stoichiometric HA with its near neutral PZC of 7.3 ± 0.1 [58], and may be responsible for raising osteoclastic activity in line with or in excess of the rise in osteoblastic activity associated with exposure to the lower levels of Si examined in this study. This resulted in a minimal variation in bone volume from 3 to 12 weeks (Fig. 9) and an alteration in the trabecular structure from one with numerous pore-spanning thin trabeculae at 1 week to one dominated by thin regions of bone apposition on pore surfaces interspersed by a few thick trabeculae from 3 to 12 weeks in both PSA02 and PSA04. Osteoclastic activity was also seen in PSA08, with evidence of bone remodelling and cell-mediated scaffold resorption. However, the structural integrity of the trabecular network within PSA08 at 3 weeks was maintained up to 12 weeks post-operatively. Furthermore, although a variation in bone morphology was noted with time, the reduction in bone density within the scaffold was not as significant as that observed in regions of PHA and PSA15. In PHA the reduction in ingrowth density within deep and central regions may be attributable to mechanical remodelling according to Wolff’s law and represents a process of incorporation/adaptation of the PHA scaffold by local bone tissue [33, 59]. This suggests that the presence of optimal Si levels promotes the retention of dense bone morphology within the osseous environment, despite local fluctuations in biomechanical demand. This hypothesis is supported by a decrease in the humeri strength of broiler chickens with one wing immobilised on a diet supplemented with sodium fluoride compared to no variation in sodium silicate supplemented birds [60]. Moreover, similar retention of bone density was reported for bone apposition around bioglass and HA/TCP coated implants [61], where the HA/TCP and control groups suffered a significant decline in bone volume and trabecular thickness with time compared to the bioglass-coated implants. This disparity in remodelling behaviour suggests that optimal levels of bioavailable Si may stimulate bone development under both healing and steady-state conditions.

Cellular activity appeared to be elevated within PSA15 scaffolds at 12 weeks, which had a significantly higher MAR compared with local bone controls (Fig. 10). MARs did not differ significantly at this time point between stoichiometric PHA or PSA08 and control bone, particularly interesting given the elevated levels of new bone observed within PSA08 compared to all other treatment groups, and given the lower MARs at both 6 and 12 weeks relative to all other silicate-substituted scaffolds. Si was shown to influence the immune response as a modulator of arginine mediated function [62] where inadequate dietary Si was found to impair splenic T-lymphocyte proliferation in response to an immune challenge. The expression of interferon gamma by T cells is known to inhibit osteoclast development and activation [63, 64], thus from this study, it appears that the key to obtaining an optimal response to silicate-substituted apatites may be in ensuring a Si level which influences bone metabolism through a balanced action on the mechanisms controlling both osteoclastic and osteoblastic cellular activity.
5. Conclusions

This investigation demonstrated the benefits to early (<1 week) bone ingrowth and repair through incorporation of Si, at all levels, in porous HA lattices compared to stoichiometric (0 wt% Si) HA in matched (equivalent) porous scaffolds when implanted into the distal femoral condyle of New Zealand white rabbits. The rate and stability or equilibrium position of repair at later timepoints was sensitive to Si level, where 0.8 wt% Si initially promoted fast bone apposition at 1–2 weeks and subsequently supported a greater volume of bone ingrowth throughout the study, supporting the hypothesis that there is an optimum level of Si, here 0.8 wt%, required to achieve the most favourable biological response to a calcium-phosphate bone graft substitute. This appears to be through the substituted silicate's influence on both the very early events at the scaffold interface via variation in surface physio-chemistry and subsequently on the metabolism of both bone forming and bone resorbing cells, presumably by providing trace levels of bioavailable Si, which affects equilibrium levels of bone ingrowth within the scaffold. The authors further suggest that the presence of an optimal level of Si in the scaffold may in part overcome the effects of either mechanical stress shielding or poor host bone metabolism in defect repair.

Acknowledgements

The authors would like to acknowledge the support and technical assistance of Dr Suhur Saeed, Tom MacInnes, Charlie Campion and Katherina Guth. This work was supported in part by EPSRC Grant No. AF/99/0077 and by ApaTech Ltd., UK.

References


