Bone tissue reactions to biomimetic ion-substituted apatite surfaces on titanium implants

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The aim of this study was to evaluate the bone tissue response to strontium- and silicon-substituted apatite (Sr-HA and Si-HA) modified titanium (Ti) implants. Sr-HA, Si-HA and HA were grown on thermally oxidized Ti implants by a biomimetic process. Oxidized implants were used as controls. Surface properties, i.e. chemical composition, surface thickness, morphology/pore characteristics, crystal structure and roughness, were characterized with various analytical techniques. The implants were inserted in rat tibiae and block biopsies were prepared for histology, histomorphometry and scanning electron microscopy analysis. Histologically, new bone formed on all implant surfaces. The bone was deposited directly onto the Sr-HA and Si-HA implants without any intervening soft tissue. The statistical analysis showed significant higher amount of bone–implant contact (BIC) for the Si-doped HA modification \( (P = 0.030) \), whereas significant higher bone area (BA) for the Sr-doped HA modification \( (P = 0.034) \), when compared with the non-doped HA modification. The differences were most pronounced at the early time point. The healing time had a significant impact for both BA and BIC \( (P < 0.001) \). The present results show that biomimetically prepared Si-HA and Sr-HA on Ti implants provided bioactivity and promoted early bone formation.

Keywords: bioactivity; biomimetic; hydroxyapatite; osseointegration; implant; in vivo

1. INTRODUCTION

Pure titanium (Ti) and Ti alloys are well-established, standard materials in oral and orthopaedic implants, because of their favourable combination of mechanical strength, chemical stability and biocompatibility [1]. In order to improve bioactivity and enhance osseointegration, several methods for surface modification or coating, including plasma-spraying, grit blasting, acid etching, anodization or calcium phosphate coatings have been extensively studied [2–4].

Several techniques have been used to coat Ti implants with HA, the most common of which is the plasma-spray technique [4]. However, plasma-sprayed HA coatings are approximately 30–50 \( \mu \)m in thickness and are prone to delaminate from the metal substrate in certain situations owing to their poor bonding strength, which creates a weak interface that may eventually lead to implant failure [8–11].

The biomimetic deposition of calcium phosphate onto surfaces of implant materials is a technique originally developed by Kokubo et al. [12]. This method allows HA and other calcium phosphate surfaces to be deposited on substrates in a simulated body fluid (SBF) under physiological conditions of temperature and pH, [13] on complex geometrical shapes.

In the biomimetic method, a layer of usually rough and porous, calcium-deficient apatite grows on the surface of implants. It is well known that the tissue and cell response could be considerably influenced by the
composition and topography of the implant surfaces [14]. Biomimetically produced apatite surfaces may, therefore, be useful in facilitating early bone ingrowth into porous surfaces without the potential for coating debris, macrophage infiltration, fibrous tissue encapsulation and eventual coating failure, which may occur with the plasma-sprayed HA coating [15]. Furthermore, these surfaces can be made osteoconductive and osteoinductive by the incorporation of osteogenic agents, such as BMP-2 [16–18].

Strontium (Sr) and silicon (Si) are elements that can substitute calcium and phosphate in the inorganic phase of bone. For this reason, Sr and Si are naturally occurring trace elements that accumulate in the bone [19,20]. Sr has been proved to increase bone strength and increase the bone mineralization rate and enhance the osteoblast proliferation, differentiation and collagen production [23,24].

Ion-substituted apatite, deposited biomimetically on Ti implants, has been suggested as a potential way to improve surface bioactivity and enhance implant osseointegration. In a previous study by Xia et al. [25], Sr ions were chemically bonded and successfully incorporated into the structure of apatite.

Bra˚nemark et al. [26] have shown that, in humans, osseointegration is achieved three to four months after implantation and that remodelling takes place over a 1 year period under functional conditions. The rat is an interesting experimental model because the bone formation around the implant is achieved in 28 days [27,28]. Bone turnover and resorption in the rat, which is several times faster and higher than in humans [29]. Most investigations have focused on long-term osseointegration, reporting that, in the rat, four weeks after implantation, the bone–implant interface is characterized by a mature bone directly in contact with the implant [27–29].

The aim of this study was, therefore, to investigate the effects of these new ion-substituted apatite biomimetic surface modifications for implant osseointegration at the early stage of implantation in an animal model.

2. MATERIAL AND METHODS

2.1. Implant preparation

Eighty threaded implants (2 mm in diameter, 2.3 mm in length) were manufactured by machining a commercially pure (grade II) Ti rod and dividing it into four groups. Three of the groups were biomimetically surface modified with Sr-substituted apatite (Sr-HA), Si-substituted apatite (Si-HA) and HA surfaces, respectively, whereas the remaining unmodified group served as Ti (control) implants.

The ion-substituted HA surfaces consist of a double layer on the Ti surface, which is made up of HA, Sr-HA or Si-HA as the top layer (formed by biomimetic surface modification process) and crystalline Ti dioxide (TiO2) as the inner layer (formed by heat and alkaline treatment of Ti implants).

The biomimetic process has been described in detail in a previous study [25]. Briefly, phosphate-buffered solution (PBS) was used as the soaking medium. For the HA surfaces, the ion composition of the PBS was Na+ (145 mM), K+ (4.3 mM), Mg2+ (0.49 mM), Ca2+ (0.91 mM), Cl− (143 mM), H2PO4− (1.6 mM) and HPO42− (8.1 mM), while, for the ion-substituted HA surfaces, Sr nitrate and sodium silicate were added to the PBS as sources of Sr and Si. All the chemicals were of analytical grade and were used as received without further purification.

Prior to the biomimetic surface modification process, the threaded implants were heat-treated at 800°C for 1 h and then soaked in 5 M sodium hydroxide solution. The heat treatment oxidizes the surface and produces a crystalline thermal TiO2. The heat- and alkaline-treated implants were washed using ethanol and deionized water separately in an ultrasonic bath before soaking in the PBS.

HA, Sr-HA and Si-HA surfaces were prepared by immersing the pre-treated Ti screws into the PBS with Ca2+, H2PO4−, HPO42− and Sr2+/SiO2− (iPBS). The concentrations of the Sr and silicate ions in the PBS were 0.6 and 4 mM, respectively. The pH value was controlled at 7.4 at the beginning of the soaking process. Ten screws at a time were soaked into 50 ml of PBS in sealed plastic bottles kept at 60°C for two weeks. During soaking, the implants were kept standing, with the top end at the bottom of the vial. The PBS was changed every 4 days. After completed soaking, the implants were removed from the PBS, rinsed with de-ionized water and allowed to dry in air after surface modification. All the implants were placed in sealed containers and finally sterilized with gamma irradiation (Sterigenics Inc., Germany).

2.2. Surface characterization

2.2.1. Surface topography and morphology

The morphology of the implant surfaces was investigated using field emission scanning electron microscopy (LEO 1550; Leo Electron Microscopy Ltd., Cambridge, UK).

2.2.2. Crystallinity of ion-substituted apatite surfaces

Surface modification was performed in an identical manner on plates for the analysis of surface crystallinity. The specimens were analysed using X-ray diffractometry (XRD; Siemens Diffractometer 5000) by Cu K radiation (λ = 1.5418 Å). The diffractometer was operated at 45 kV and 40 mA at a 2θ range of 10–80° with a fixed incidence angle of 2°.

2.2.3. Elemental and chemical composition

The composition of the surfaces was analysed with inductively coupled plasma optical emission spectrometry (ICP-OES). Each surface was dissolved in 0.5 ml HNO3 and 0.5 ml HCl for 2–3 days. When the surfaces were dissolved, the implants were removed from the HNO3/HCl solution and the solution was diluted with MilliQ water to a final volume of 12.5 ml. The resulting solutions were analysed with respect to Ca, P, Mg, Sr and Si concentration with ICP-OES analysis instruments calibrated with standard solutions of known concentrations of the elements in question.
Based on the measured concentrations, the absolute amount of each individual element per implant was calculated. These measurements were also used for calculating the concentration ratios of the elements.

2.3. Experimental design and implantation procedure

Twenty male Sprague-Dawley rats (250–350 g), fed on a standard pellet diet and water, were anaesthetized using a Univentor 400 anaesthesia unit (Univetor, Zejtun, Malta) under isoflurane (Isoba Vet, Schering-Plough, Uxbridge, UK) inhalation (4% with an air flow of 650 ml min$^{-1}$). Anaesthesia was maintained by the continuous administration of isoflurane (2.7% with an air flow of 450 ml min$^{-1}$) via a mask. Each rat received analgesic (Tengesic 0.03 mg kg$^{-1}$, Reckitt & Coleman, Hull, UK) subcutaneously post-operatively and the following 2 days, twice daily. After shaving and cleaning (5 mg ml$^{-1}$ chlorhexidine in 70% ethanol), the medio-lateral aspect of the proximal tibial metaphysis was exposed through an anterolateral skin incision, followed by skin and periosteum reflection with a blunt instrument. Two holes were prepared in each metaphysis (proximally and distally) using subsequent enlarging ( Ø1.4 and Ø1.8 mm burs) under profuse saline irrigation. Four different implants were installed randomly in each animal (80 implants in total for the experiment), using a predesigned placement schedule to ensure maximum rotation for the different implant surfaces and placements. The subcutaneous layer of the wound was closed with resorbable poliglycatin sutures (5-0, Vicryl, Ethicon, Johnson & Johnson, Brussels, Belgium) and the skin was closed with transcutaneously glued upwards on a regular SEM stub using a carbon adhesive tape and coated with a thin layer of gold. The microscopes that were used were a LEO Ultra 55 (surface modification, healing time and end points) equipped with quadrupole backscatter detectors and energy-dispersive X-ray spectrometry (EDS). Backscatter imaging using a 20 kV acceleration voltage was performed with magnifications up to 100 000×. In addition, element distribution maps for the implant–bone tissue interface were obtained using the EDS detector. The main elements investigated at the interface site were Ca, P, Ti, O and the ion-substitution ions (Si and Sr).

2.4. Histology and histomorphometry

The formalin-fixed tissue–implant bloc was prepared for SEM analysis, where the blocs were glued upwards on a regular SEM stub using a carbon adhesive tape and coated with a thin layer of gold. The microscopes that were used were a LEO Ultra 55 FEG SEM and a LEO 440 W-filament SEM (Leo Electron Microscopy Ltd, Cambridge, UK) equipped with quadrupole backscatter detectors and energy-dispersive X-ray spectrometry (EDS). Backscatter imaging using a 20 kV acceleration voltage was performed with magnifications up to 100 000×. In addition, element distribution maps for the implant–bone tissue interface were obtained using the EDS detector. The main elements investigated at the interface site were Ca, P, Ti, O and the ion-substitution ions (Si and Sr).

2.5. Scanning electron microscopy and elemental analysis

The counterpart of the embedded tissue–implant bloc was prepared for SEM analysis, where the blocs were glued upwards on a regular SEM stub using a carbon adhesive tape and coated with a thin layer of gold. The microscopes that were used were a LEO Ultra 55 FEG SEM and a LEO 440 W-filament SEM (Leo Electron Microscopy Ltd, Cambridge, UK) equipped with quadrupole backscatter detectors and energy-dispersive X-ray spectrometry (EDS). Backscatter imaging using a 20 kV acceleration voltage was performed with magnifications up to 100 000×. In addition, element distribution maps for the implant–bone tissue interface were obtained using the EDS detector. The main elements investigated at the interface site were Ca, P, Ti, O and the ion-substitution ions (Si and Sr).

The percentage of bone-to-implant contact (BIC%) was then calculated for both sides of each implant, using the actual BIC length as numerator and the actual measured implant length of the implant as denominator. The relative amount of bone area (BA%) around the implant within the threads was measured for both sides of each implant, using the mineralized B percentage as the numerator and the actual measured whole area within the threads of the implant as the denominator.

2.6. Statistics

A multi-variate analysis (generalized-linear model) was used for statistical analysis, whereas all parameters (surface modification, healing time and end points) were tested in one single test using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). When necessary, a subsequent Bonferroni post hoc test was applied. Statistical significance was indicated by $P$-levels of less than 5 per cent.

3. RESULTS

3.1. Surface characterization

Images obtained by SEM showed that the pre-treated Ti, HA, Sr-HA and Si-HA surface modifications presented significantly different surface morphologies.
The heat and alkaline treatment of the Ti implants prior to biomimetic modification (figure 1a) showed a relatively rough and irregular surface morphology, quite different from the original machined surfaces (not shown). Newly formed Si-HA (figure 1b), Sr-HA (figure 1c) and HA (figure 1d) surfaces were composed of plate-like crystals, with crystal sizes for Si-HA and Sr-HA smaller than for HA.

XRD analysis (figure 2) confirmed that there were specific apatite peaks for the Si-HA, Sr-HA and HA surfaces. The Sr-HA surface showed weaker yet sharper apatite diffraction peaks than the other two apatite samples, indicating that the Sr-HA consisted of a smaller total amount of crystalline material but larger crystallites.

The amounts of Ca, P, Mg, Si and Sr for the Si-HA, Sr-HA and HA surfaces measured by ICP-OES are...
shown in Table 1. Also shown are some different molar ratios that reflect the amount of ion substitution. Judging from the absolute amounts of Ca and P, the amount of apatite on the surface is significantly lower for the Sr-HA than for the other two apatite surfaces. There are approximately 45, 7.6 and 54.5 μg of Ca and 28.7, 8.2 and 32 μg of P in each Si-HA, Sr-HA and HA implant, respectively. The amount of Ca and P in Si-HA and Sr-HA surfaces is close but much higher than that in the Sr-HA surfaces. Si (1.2 μg) and 10.6 μg of Sr have been found in the Si-HA and Sr-HA surfaces, respectively. The Ca−P and substituted Ca−P ratios for all of the surfaces show that they are calcium deficient, when compared with stoichiometric HA (theoretical Ca−P ratio of 1.67). The substituted Ca−P ratio of Si-HA and Sr-HA surfaces is higher than the Ca−P ratio for HA, reflecting that part of the calcium position in apatite has been substituted by Mg and Sr ions. These substituted Ca−P ratios are closer to the real Ca−P in the biomimetic surfaces. The substitution of Sr is much higher than the substitution of Mg and Si, based on the results from ICP-OES. The Sr-substituted Ca−P is approximately 1.4, which is higher than the silicate-substituted Ca−P and Mg-substituted Ca−P from Si-HA and HA surfaces.

3.2. Clinical observation
During implant placement, the surfaces of the entire ion-substituted HA- and HA-modified implants were observed quickly to attract blood, which may be taken as an indication of high surface hydrophilicity (Figure 3).

The healing period was uneventful for all the experimental animals. No operative or post-operative complications were encountered. One implant was excluded from the study due to clinical instability immediately after euthanization.

3.3. Histology and histomorphometry
The implantation sites in the distal and proximal tibia both consist of cortical bone, implying that the two most coronal threads are located within the cortex. The remaining part of the implant protrudes into the marrow cavity without contacting the endosteal surface of the opposite cortex. At the level of light microscopy, the implant modifications were too thin to distinguish in the ground cross sections.

In general, no signs of inflammatory tissue reaction in the different groups were observed as judged by histology. After 7 days following implant installation (Figures 4–8), healing was characterized by the marked formation of new bone in all implant groups. However, no new bone formation or signs of remodelling were seen at the cut edge of the cortex in all implant groups.

In the marrow cavity, some of the bone fragments with empty osteocytic lacunae were still present. This pre-existing bone was not resorbed at this time point. A similar pattern of bone apposition for the ion-substituted HA surfaces (Si-HA and Sr-HA) was observed in comparison with HA and Ti surfaces. This newly formed mineralized tissue extended from the endosteum onto the implant surface of all implant groups, but it also projected along the Si-HA and Sr-HA surface of the implants (Figures 6 and 7).

The newly formed bone, which was distinguishable from the pre-existing bone by its different staining pattern, was deposited on the pre-existing bone. The trabeculae of this woven bone formed a randomly oriented scaffold that confined large and numerous intratrabecular bone marrow areas. The trabecular surface was lined with osteoblasts. Large lacunae containing well-developed osteocytes were observed. At the level of light microscopy, the bone matrix was in close contact with the implant surface.

After 28 days of healing, the ground sections (Figure 8) displayed a similar pattern of bone apposition for all the implant surfaces. The implant surfaces facing the marrow cavity were covered by an endosteal layer of mature lamellar bone, continuous with that of the cortex. The pre-existing bone fragments had been completely resorbed. On the 28th day, the layer of newly formed bone close to the implant surface had become thicker than that observed after 7 days. Bone marrow
filled all the spaces between the bone trabeculae. The osteocytic lacunae were smaller than those from the seventh day and some osteocytes could be observed at the bone–implant interface.

Figure 9 shows the mean percentage of bone–implant contact (BIC), BA% and standard deviations between different implant groups at 7 days and 28 days. The degree of BIC and the amount of bone inside the threads increased for all four implant surface modifications/implant types between 7 and 28 days. A significantly higher amount of BIC was observed for Si-HA when compared with HA ($P = 0.030$), whereas a significantly higher amount of BA was observed around the Sr-HA ($P = 0.034$) when compared with HA. Further, the healing time had a significant influence on both BA and BIC ($P < 0.001$).

### 3.4. Scanning electron microscopy

Scanning electron microscopy revealed a picture similar to the histological evaluation, with an endosteal downgrowth from the cortex. Using the backscatter detector, increased contrast differences could be obtained between the implant bulk, surface layer and bone tissue. A distinct surface layer could be observed for all implants, with an approximate thickness of 3 $\mu$m (figure 10a), which corresponds to the enlarged surface oxide according to EDS analysis. Further, a separation was commonly observed between the bone tissue and implant surface, judged as an artefact where the bone tissue clearly follows the contours of the implant surface. In some regions, direct contact was observed (figure 10b). The HA- and ion-substituted HA layer could not be visualized at large parts of the surface, while, in some smaller regions, a contrast difference with a thickness of 2 $\mu$m could be observed (figure 10c). It is difficult to judge whether this is the original biomimetic surface layer or an apatite that had developed in vivo. Osteocyte lacunae and canaliculi were frequently seen close to the implant surface for all surface modifications (figure 10d).

The element maps acquired by EDS showed bone formation along the surface (figure 10e), as indicated by the presence of Ca and P. The substituted ions (Sr and Si) could not be detected by the EDS and it was difficult to distinguish the surface coating from the bone formation occurring at the implant surface.
4. DISCUSSION

In this study, a biomimetic approach was used for the ion substitution of HA on Ti implants at ambient temperature. The surface modifications were obtained by soaking the implants in a concentrated SBF solution. This method of surface modification was suggested to obtain a more porous and soluble HA surface in contrast to the commercially used plasma-sprayed coatings. Further, Sr and Si ions might be tentatively delivered locally at the implant site potentially to stimulate bone growth and/or bonding.

Sr and Si ions have been incorporated into the calcium phosphate coatings. However, physical adsorption cannot be excluded. Sr ion could replace the position of Ca, and silicate ion could replace the position of phosphate via this deposition process as reported in the literature [25,30–32].

The SEM results from the ion-substituted implants showed that their surfaces were significantly rougher than those of the thermally oxidized machined controls. The chemical analyses revealed the expected elements and the amount of surface varied somewhat between each group. The results for the amount of elements indicated that Ca and P in the Sr-HA were lower than those in Si-HA and HA modifications, but the substitution of calcium was much higher in the Sr-HA. Even if the Si substitution was substantially low, then the crystal size of Si-HA decreased and the thickness of the surface was not greatly influenced. The low Ca–P and substituted Ca–P ratios from the surfaces confirmed that the surfaces were biomimetic, since the natural bone mineral is calcium-deficient and ion-substituted.

The morphologies of the biomimetic surface modification are different from the normal coating derived from the sol–gel and plasma-spray process. Instead of being dense and smooth, they are rough and porous and thus have a morphology that may be favourable for the adhesion and proliferation of cells [33,34]. Pure apatite surfaces produced by the biomineralization process were composed of plate-like particles. The crystal size of the surface was changed by incorporating Si and Sr ions into the apatite structure.

Sr and Si have been extensively investigated both in vitro and in vivo for their dual action of promoting new bone formation and inhibiting bone resorption [21–24,35–37].

However, no in vivo experiment investigating the effects of biomimetic Sr-HA or Si-HA surfaces on the screw-shaped implant fixation in bone has been reported.

The main finding in this study was that the incorporation of small amounts of physiological ions (Si and Sr) in apatite surfaces can significantly improve the surface bioactivity of Ti implants. Interestingly, the histomorphometric calculations of the Si-HA implants showed that, after just 7 days of insertion, the bone–Ti contact accounted for at least 50 per cent of the total bone-to-implant apposition. This result suggests that the incorporation of Si ion in the HA biomimetic surfaces can stimulate bone apposition in the very early stages of bone healing following implant placement. The higher bone formation in the area within the threads for Sr-HA supports...
the concept that Sr could increase bone formation and reduce bone loss. Sr is known to reduce the proliferation and differentiation of osteoclasts, which generally reduces resorption of bone [38,39]. Sr is also known to enhance the proliferation and differentiation of osteoblasts, which leads to a larger pool of active osteoblasts and thus to an increase in new bone formation [40,41].

As Ca and P were the constituents of both the implant surface and bone, it was difficult morphologically and ultrastructurally to interpret the relationship between the implant surface layer and bone based on the presence of these elements. So, emphasis was placed on the distribution pattern of these elements by mapping with SEM–EDS. The results demonstrated that Ca and P were uniformly distributed inside the threads and directly at the implant surface after 7 days of healing, indicating the mineralization of newly formed bone. The low amount of substituted ions, in combination with the limited thickness of the modified surface, as well as the possibility of surface resorption in vivo, is a probable reason for not detecting the ions by SEM–EDS.

Our finding of a synergic effect by adding the Si ion to the HA surface on BIC is in agreement with those results obtained previously with granules in comparisons of the in vivo behaviour of Si-HA versus HA granules [37]. Several interpretations of this effect have been proposed: the higher dissolution rate of Si-HA ceramic than HA ceramic owing to the low connectivity of Si incorporated in HA, which facilitates the release of Si into the contact fluid [42,43]. Furthermore, it has been observed that the dissolution rate of the surface coating is critical to the cell attachment and proliferation [43]. The dissolution rate of the Si-HA is dependent on the Si content but also on the crystallinity. Another explanation of the higher bioactivity of Si-HA is based on the nature of bonding of Si ions into the HA structure, whose rapid hydrolysis is deemed to contribute to the surface hydrophilicity [44].

5. CONCLUSION
Within the limitations of this study, the present results show that Ti implants were successfully modified by

Figure 8. After 28 days of implantation, mineralized bone also follows the contour of the Si-HA implant in the medullary area and is observed in direct contact with the implant surface.

Figure 9. Histomorphometry. (a) Total bone–implant contact (%) after 7 and 28 days. Mean ± s.e.m. (b) Total bone area (%) after 7 and 28 days. Mean ± s.e.m. The markings indicate that the BIC was significantly higher for Si-HA surface than for HA surface (P = 0.030), and the BA was significantly higher for Sr-HA surface than HA surface (P = 0.034). The differences were most pronounced at the early time point.
Figure 10. BSE micrographs of the implants after 28 days of healing. (a) Low-magnification images showing the implant, implant surface and bone tissue. The black line separating the bone from the surface oxide was due to separation (Ti sample). (b) At some locations, direct contact was observed (Sr-HA sample). (c) A contrast difference was sometimes observed between the surface oxide and the bone tissue, indicating remnants of the surface (Si-HA sample). (d) Osteocyte lacunae and canaliculi were frequently observed close to the implant surface (HA sample). (e) Overlapped element maps showing calcium (green), titanium (red) and oxygen (blue), showing bone formation along the HA-implant surface at 7 days healing. The enlarged surface oxide is shown in purple (overlapped blue and red) along the implant perimeter.

The biomimetic Sr- and Si-substituted apatite surfaces. The biomimetic surfaces improved the surface bioactivity and promoted the early bone formation, leading to enhanced osseointegration along the surface of implants. The mechanisms behind these responses may be the release of Sr ions and the nature of bonding of Si ions in the HA structure, whose rapid hydrolysis is deemed to contribute to the surface hydrophilicity.

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