Bone integration capability of a series of strontium-containing hydroxyapatite coatings formed by micro-arc oxidation

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Abstract: Strontium-containing hydroxyapatites (Sr-HA) combine the desirable bone regenerative properties of hydroxyapatites (HA) with anabolic and anti-catabolic effects of strontium cations. In the present work, a series of Sr_xCa(1-x)TiO_3 coatings on titanium are produced by micro-arc oxidation (MAO), and the effects of the in vivo osseointegration ability of the coatings are investigated by using a rabbit model. All samples are subjected to biomechanical, surface elemental, micro-CT and histological analysis after 4 and 12 weeks of healing. The obtained results show that the MAO-formed coatings exhibit a microporous network structure composed of Sr_xCa(1-x)TiO_3/Sr_xCa(1-x)TiO_3–TiO_2 multilayers, in which the outer Sr_xHA and intermediate Sr_xCA–Sr_xCA1.5TiO_3 layers have a nanocrystalline structure. All Sr-HA coated implants induce marked improvements in the behavior of bone formation, quantity and quality of bone tissue around the implants than the control HA implant and in particular, the 20%Sr-HA coating promotes early bone formation as identified by polyfluorochrome sequential labeling. The bone-to-implant contact is increased by 46% (p < 0.05) and the pull-out strength is increased by 103% over the HA group (p < 0.01). Extensive areas of mineralized tissue densely deposit on the 20%Sr-HA coating after biomechanical testing, and the greatest improvement of bone microarchitecture are observed around the 20%Sr-HA implant. The identified biological parameters successfully demonstrate the osteoconductivity of 20%Sr-HA surfaces, which results not only in an acceleration but also an improvement of bone–implant integration. The study demonstrates the immense potential of 20%Sr-HA coatings in dental and orthopedic applications. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 2465–2480, 2013.

Key Words: Sr-doped hydroxyapatite, in vivo implantation, osseointegration, micro-arc oxidation, coating, titanium

INTRODUCTION
The high success rate of endosseous implantation technique makes it a frequent procedure in health patients with good bone quality. However, with the aging population, the incidence of implant failure will be high in patients with severe alveolar bone absorption and/or poor bone quality1–4; and, the waiting periods for prosthetic loading will take much longer. In these cases, enhanced bone formation and change in intrinsic bone tissue quality around the implant would be the major aim.1,4 This may be achieved by implant coatings that are able to interact actively with the surrounding tissues.

Strontium (Sr), a trace element in human body, has been shown to reduce the risk of vertebral and nonvertebral fractures in postmenopausal osteoporotic patients at low dose.5–9 Evidence from in vitro data has proved that Sr favors bone healing via a unique mechanism by reducing bone resorption while promoting bone formation,10,11 where Sr has been shown to enhance preosteoblasts proliferation and bone collagen synthesis as well as decrease bone resorption by inhibiting the osteoclast resorbing activity and osteoclastic differentiation.11 The dual action of Sr results in a rebalance in bone turnover and improves bone microarchitecture and strength.12 Therefore, Sr salt has been put...
produced into hydroxyapatite-coatings on titanium implants diminishing the adverse reactions by administration of Sr. It seems that the local release of Sr from the coatings can be optimized by altering the electrolyte composition. As no upper limit for the amount of Sr in vivo dosage of Sr depends on complicated environment, not only the chemical resemblance of Ca and Sr. Therefore, Sr has recently been introduced into hydroxyapatite-coatings on titanium implants because of the chemical resemblance of Ca and Sr.

Micro-arc oxidation (MAO) is a promising technology to produce porous and firmly adherent inorganic Sr-containing hydroxyapatite (Sr-HA) coatings on titanium substrates and the amount of Sr introduced into the coatings can be optimized by altering the electrolyte composition. As no upper limit for the amount of Sr that should be incorporated into the hydroxyapatite coatings has yet been defined, it has to be optimized to provide enough to favor bone formation without having deleterious effects on bone mineralization. In addition, the optimal dosage of Sr depends on complicated environment, not only crystal itself but also the adjacent tissue fluid in vivo. Therefore, in this study, a series of Sr-HA coatings are produced on titanium dental implants using MAO, with the substitution degree, respectively, at 0, 5, 10, and 20%. Based on the preliminary analysis of the coating structure, composition, and morphology, a rabbit model is used to evaluate the in vivo biological responses at the bone-implant interface, and the optimal Sr content to substitute in hydroxyapatites (HA) coatings is clarified as well.

**MATERIALS AND METHODS**

**Sample preparation**

Commercial pure titanium rods with dimensions of 3.75 × 6 mm were used as the substrates. Prior to MAO, the samples were ground with abrasive SiC paper and cleaned with acetone and distilled water. In the MAO process, the titanium rod was used as an anode while a stainless steel cylindrical container served as the cathode. The electrolytes used in MAO were composed of calcium acetate (CA,Ca(CH\textsubscript{3}COO)\textsubscript{2}), strontium acetate (SA,Sr(CH\textsubscript{3}COO)\textsubscript{2}), and β-glycerophosphate disodium (β-GPNa\textsubscript{2}, C\textsubscript{3}H\textsubscript{5}(OH)\textsubscript{2}PO\textsubscript{4}Na\textsubscript{2}) and the concentrations were shown in Table I. For the Sr-HA coated group, the titanium rods were treated under an applied voltage of 500 V and the pulse frequency, duty circle, and duration time were 100 Hz, 26%, and 10 min, respectively. While for the non-HA coated group, the samples were prepared at 400 V for 5 min and the pulse frequency, duty circle were fixed at 100 Hz and 20%. After the MAO treatment, the samples were washed with distilled water and dried.

**Materials characterization**

The phase components of the coatings were evaluated by X-ray diffraction (XRD) on the SHIMADZU LIMITED-7000, and surface and cross-sectional morphologies of the coatings were observed with scanning electron microscopy (SEM) on the S-4800 (Hitachi, Japan), respectively. The composition of the surface layer was determined by energy-dispersive X-ray spectroscopy (EDAX) on the scanning electron microscope. The surface roughness of coatings was measured by an Atomic Force Microscope (AFM, SPM400, SEIKO, Japan) in tapping mode with a scan rate of 1 Hz and a scan size of 80 × 80 μm².

**Animal model and implantation procedures**

Thirty adult New Zealand White rabbits weighing 3.5–4 kg were used in this study. This experiment was approved by the Animal Research Committee of the university and conducted in accordance with international standards on animal welfare. The rabbits were assigned either to 4- or 12-week groups. General anesthesia was administered by intramuscular injection of a combination of ketamine hydrochloride (0.35 mL/kg) and xylazine (0.25 mL/kg). The operation was performed under sterile conditions. The distal surfaces of the bilateral femoral condyles were used as the surgical sites. The implants were inserted into the bone cavities under sterile physiological saline irrigation according to the routine surgical protocol. After operation, Cefazolin (0.25 g, IM) was administrated intramuscularly for five days. To evaluate bone healing during the 4 and 12 week time, fluorochrome sequential labeling was followed (Tables II and III). All animals were humanely killed by intravenously

**TABLE I. Composition of the Different Electrolytes**

<table>
<thead>
<tr>
<th>Electrolyte</th>
<th>Concentration of CA (mol/L)</th>
<th>Concentration of SA (mol/L)</th>
<th>Concentration of β-Na2GP (mol/L)</th>
<th>Expected Sr/(Sr+Ca) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.2</td>
<td>0</td>
<td>0.01</td>
<td>0 (HA)</td>
</tr>
<tr>
<td>B</td>
<td>0.2</td>
<td>0</td>
<td>0.02</td>
<td>0.05% (5%Sr-HA)</td>
</tr>
<tr>
<td>C</td>
<td>0.184</td>
<td>0.016</td>
<td>0.02</td>
<td>0.1% (10%Sr-HA)</td>
</tr>
<tr>
<td>D</td>
<td>0.167</td>
<td>0.033</td>
<td>0.02</td>
<td>0.2% (20%Sr-HA)</td>
</tr>
<tr>
<td>E</td>
<td>0.15</td>
<td>0.05</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Note: CA = (CH\textsubscript{3}COO)\textsubscript{2}CaH\textsubscript{2}O; SA = (CH\textsubscript{3}COO)\textsubscript{2}Sr 1/2H\textsubscript{2}O; β-Na2GP = C\textsubscript{3}H\textsubscript{5}(OH)\textsubscript{2}PO\textsubscript{4}Na\textsubscript{2}.
injection of an overdose of ketamine hydrochloride after each observation period followed by removal of the femoral.

### Pull-out tests

The established implant biomechanical pull-out test was used to assess the biomechanical strength of bone-implant integration. Femoral condyles containing a cylindrical implant each (n = 3 for each time point and group) were harvested at weeks 4 and 12 of the healing process and partially embedded in PMMA with the implant top being horizontal. The testing machine (Shimadzu, AGS-10kN, Japan) was used to pull the implant vertically out at a cross-head speed of 1 mm/min. The load-displacement curve was recorded and the maximum pull-out force was then calculated.

### Morphological and elemental analyses of implant-tissue interface

The morphological and elemental analyses of the implant surfaces after the pull-out test were found to be useful in examining the quality of bone-implant integration. After the pull-out test, the implant specimens were carefully exposed, soaked in agitated water for 1 h, and dried under heat and vacuum. After platinum-sputter coating, the specimen was examined by SEM for surface morphology and by EDAX for the elemental composition. The correlation between morphologic and compositional features was also studied.

### Micro-CTs

The femoral condyles including the implants were scanned on a micro-CT system (Inveon CT, Siemens AG, Germany) at 80 kV and 500 μA with an isotropic voxel size of 15 μm. The volume of interest (VOI) was defined as a ring from the implant surface with a radius of 0.8 mm [Fig 4(A)]. The multilevel threshold procedures (thresholds for bone being 455 and implant being 2742) were applied to distinguish bones from other tissues. The following parameters were assessed: bone volume to total volume ratio (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N).

### Histological analysis

The femoral condyles containing the implants were removed en bloc, fixed in 4% neutral buffered formaldehyde (pH 7.2) for 2 days, dehydrated using an ascending series of alcohols (70% to absolute), and embedded in methylmethacrylate. After hardening, blocks were cut parallel to the long axis of each implant and ground without decalcification to make three 30-μm thick sections per implant using a macro-cutting and grinding system (SP1600&SP2600, Leica, Germany). The sections were stained with Ponceau Tri-Chrome for histological observation at a magnification of 100× on an Olympus BX51 microscope equipped with an image analysis system (Japan). For quantitative analysis of the new bone formation around implant, histomorphometric parameters including bone-to-implant contact (BIC%), and mineral apposition rate (MAR) were measured on three different sections per sample.

### RESULTS

#### Composition and morphology of the MAO coatings

Figure 1(A b–d) depicts the XRD spectra of the MAO coatings formed in electrolytes with different concentrations at 500 V for 10 min. Rutile, CaTiO₃, Sr₅(Ca₁₋ₓ)TiO₃(0<x<1), and Sr₅(Ca₁₋ₓ)(PO₄)₆(OH)₂(y=0, 0.5, 1, 2) peaks appear from all samples. The Sr₅(Ca₁₋ₓ)(PO₄)₆(OH)₂(y=0, 0.5, 1, 2) peak shifts to lower 2θ values with Sr addition and the peak intensity also goes up with increasing Sr concentration. In comparison, very little change is observed from the Ca₁₂₃TiO₇(0<z<1) peaks, suggesting that the Sr concentration in the electrolyte is an important factor affecting the formation of Sr-HA coating. Figure 1(A a) shows the XRD spectra of the non-HA coating formed in 0.01 mol β-GPNa₂ and 0.2 mol CA at 400 V for 5 min. Only rutile, anatase and Ca₁₀₃ TiO₃ peaks are detected, which suggests the non-HA coating mainly consists of TiO₂ and Ca₁₀₃ TiO₃.

The surface morphologies of the MAO coatings formed in the different electrolytes are shown in [Fig 1(B,D)] by SEM and AFM examination. All the coatings exhibit porous network structures with rough pore walls. Most of the holes are interconnected which should bode well for bone ingrowth [see the inset images in Fig. 1(B,D)]. At high magnification, many nano-sized crystallites agglomerate on the pore walls of the coatings and the size decreases gradually with increasing Sr concentration, which may be due to the gradual coverage of Sr₅-HA on the TiO₂ matrix. The non-HA coated surface displays a similar porous network structures with smooth pore walls compared with the Sr₅-HA group (Supporting Information Figs. S1 and S2). In quantitative roughness analysis by AFM, the five group coatings show no significant difference in Ra, RMS, and Rz (Table IV; p > 0.05).

The cross-section morphology and elements profiles of the coatings are shown in Figure 1(C). It is clear that the coating is about 32 μm in thickness. No apparent

### TABLE II. Sequences of Polyfluorochrome Sequential Labeling at 4-Weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Substance</th>
<th>Doses</th>
<th>Injection (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td>Alizarin</td>
<td>30 g</td>
<td>3 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>14 days</td>
<td>Calcein</td>
<td>10 g</td>
<td>1 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>21 days</td>
<td>Tetracycline</td>
<td>60 g</td>
<td>6 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>28 days</td>
<td>Exitus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE III. Sequences of Polyfluorochrome Sequential Labeling at 12-Weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Substance</th>
<th>Doses</th>
<th>Injection (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 days</td>
<td>Alizarin</td>
<td>30 g</td>
<td>3 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>32 days</td>
<td>Alizarin</td>
<td>30 g</td>
<td>3 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>42 days</td>
<td>Calcein</td>
<td>10 g</td>
<td>1 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>53 days</td>
<td>Calcein</td>
<td>10 g</td>
<td>1 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>63 days</td>
<td>Tetracycline</td>
<td>60 g</td>
<td>6 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>74 days</td>
<td>Tetracycline</td>
<td>60 g</td>
<td>6 g/100 mL + 2 g Na₂HPO₄</td>
</tr>
<tr>
<td>84 days</td>
<td>Exitus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 1. A: XRD spectra acquired from the MAO coatings formed in different electrolytes shown in Table I: (a) Electrolyte A, (b) Electrolyte B, (c) Electrolyte C, (d) Electrolyte D, and (e) Electrolyte E (H: HA, sH: Sr$_y$Ca(10$y$/C0$y$)(PO$_4$)$_6$(OH)$_2$, R: Rutile, T: CaTiO$_3$, sT: Sr$_x$Ca$_{(1-x)}$TiO$_3$ (0 $< x$ $< 1$), A: Anatase). B: High-magnification surface morphology of the coatings. The inset images in (B) show the low-magnification surface morphology of the coatings. C: Cross-section morphology of the HA-Ti coating and the 20%Sr-HA-Ti coating. The elements distribution corresponds to the white line drawn in (C). D: AFM images of different coating surfaces. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
discontinuity between the coating and the titanium substrate is observed, indicating that the coating can tightly bond to the substrate. From the element profiles, the coating consists of three layers, all of which contain O and P. Ti keeps a high content in inner layer; tends to decrease with the distance from the substrate. The contents of Ca and Sr tend to increase with the distance from the substrate, and reach higher values in the outer layer. Taken the XRD and SEM results together, it is concluded that the MAO formed Srₙ-HA coatings have a multilayered structure composed of an inner SrₓCaₙ(1−ₓ)TiO₃–TiO₂ layer, an intermediate nanocrystalline SrₓHA–SrₓCaₙ(1−ₓ)TiO₃ layer, and outer nanocrystallized SrₓHA layer.

The Ca and Sr concentrations determined by EDS from the Srₓ-HA-coated groups are summarized in Table V and the actual molar ratio of Sr/(Ca + Sr) in each group can be calculated. The atomic ratio of Sr/(Sr + Ca) in the coatings increases with increasing concentration of the SA solution.
in the electrolyte. According to EDS, the molar ratios in the four groups of coatings are 0.17, 7.60, 13.00, and 22.70%, respectively, implying that the elements in the electrolyte can be introduced into the coatings by MAO and the ion concentrations in the electrolytic solutions have a big influence on the elemental composition of the coatings. Although no Sr is added to electrolyte B, the HA has a measured Sr content of 0.17% due to a trace amount of Sr in water and contamination from other sources in the MAO apparatus. The molar ratios of Sr/(Ca + Sr) in groups C, D, and E are higher than the expected value due to the Sr_{x}HA/Sr_{y}HA-Sr_{x}Ca_{1-x}TiO_{3}/Sr_{x}Ca_{1-x}TiO_{3}-TiO_{2} multilayered structure disclosed by XRD and SEM. That is, formation of Sr_{x}Ca_{(1-x)}TiO_{3(0<x<1)} during MAO contributes to the result deviation.

**Verification of advantages of HA coated surfaces over non-HA coated surfaces**

As mentioned in the Introduction, the purpose of this study is to determine the potential significant role of MAO formed Sr\textsubscript{y}-HA coating and to select an optimal Sr content within the four Sr\textsubscript{y}-HA coatings. We believe that it is a reasonable rationale to prove the advantages of Sr after confirming and verifying the advantages of HA coated surface over non-HA coated surfaces.

The biomechanical strength of bone-implant integration evaluated by the pull-out test is consistently higher for HA coated surfaces than for non-HA coated surfaces at 4 and 12 weeks of the healing process (Fig. 2). The increase is as much as 90–155%. The results indicate that implant surface properties on Sr\textsubscript{y}-HA coated titanium implants have an important role in bone-implant integration that is the formation of HA from the HA coated surface results in the increased overall strength as compared with the non-HA coated surfaces.

**TABLE IV. Measurement of Surface Roughness Using AFM**

<table>
<thead>
<tr>
<th>Group</th>
<th>Ra (µm)</th>
<th>RMS (µm)</th>
<th>Rz (µm; 10 points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-HA-Ti</td>
<td>1.01 ± 0.10</td>
<td>1.23 ± 0.13</td>
<td>5.59 ± 0.66</td>
</tr>
<tr>
<td>HA-Ti</td>
<td>1.25 ± 0.19</td>
<td>1.54 ± 0.23</td>
<td>5.82 ± 0.33</td>
</tr>
<tr>
<td>5%Sr-HA-Ti</td>
<td>1.43 ± 0.26</td>
<td>1.72 ± 0.29</td>
<td>6.56 ± 0.65</td>
</tr>
<tr>
<td>10%Sr-HA-Ti</td>
<td>1.46 ± 0.12</td>
<td>1.78 ± 0.15</td>
<td>5.85 ± 0.97</td>
</tr>
<tr>
<td>20%Sr-HA-Ti</td>
<td>1.34 ± 0.17</td>
<td>1.62 ± 0.21</td>
<td>5.92 ± 0.87</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD, n = 3 per group. No statistical difference was found between groups by one-way ANOVA and S-N-K post hoc test (p > 0.05).

**TABLE V. Expected and Measured Elemental Compositions of the Sr-HA Coatings**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected Sr/(Ca + Sr) ratio (%)</th>
<th>Ca (mol %)</th>
<th>EDS Results Sr/(Ca + Sr) Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Ti</td>
<td>0</td>
<td>1.1933</td>
<td>0.0018</td>
</tr>
<tr>
<td>5%Sr-HA-Ti</td>
<td>5</td>
<td>0.7690</td>
<td>0.0629</td>
</tr>
<tr>
<td>10%Sr-HA-Ti</td>
<td>10</td>
<td>0.5832</td>
<td>0.0839</td>
</tr>
<tr>
<td>20%Sr-HA-Ti</td>
<td>20</td>
<td>0.5984</td>
<td>0.1756</td>
</tr>
</tbody>
</table>

Biomechanical strength and bone-implant interfacial tissue properties on Sr\textsubscript{y}-HA coated titanium implants

After confirming the advantages of HA coated surface over non-HA coated surfaces, we determine the difference of bone-implant integration in Sr\textsubscript{y}-HA coated implants. The biomechanical results [Fig. 3(A)] show that the pull-out value for the HA and 5%Sr-HA at 4 and 12 weeks are similar. However, compared to the HA group, the maximal pull-out values determined from 10%Sr-HA and 20%Sr-HA increase by 97.8 and 134.9% (p < 0.01), respectively at 4 weeks and by 59.5 and 103.1% (p < 0.01), respectively at 12 weeks.

To obtain more information at the implant–tissue interface, SEM followed by EDAX analysis is performed on implant surfaces retrieved 12 weeks after implantation. Typical SEM images together with EDAX elemental spectra analysis are shown in Figure 3(B,C). All four groups show some remnant biological structures at the implant surfaces, where the EDAX spot scans are performed. The biological structures with higher content of Ca, P, C, O elements represent to be bone tissues, whereas high peaks of C, O with rarely detectable of Ca or P demonstrate to be unmineralized osteoid matrix or connective soft tissue. The HA surface is largely exposed with few biological structures high in C, O signals [Fig. 3(B a,C)]. This indicates that no mineralized bone formation occurs, or that mineralized bone formed around the area is completely detached during the pull-out test. The 5%Sr-HA implant surface is almost entirely covered by the biological tissues, however, the spectra shows high O, C peaks together with low Ca, P, and Ti peaks, which means osteoid matrix or soft tissue rather than the osseous tissue mainly forms on the implant [Fig. 3(B b,C)]. 10%Sr-HA implant surface shows similar results with that of 5%Sr-HA implant, while half of the surface is covered by a biological structure low in Ca, P peaks contents [Fig. 3(B c,C)]. In contrast with the other three surfaces mentioned above, the
FIGURE 3. A: Pull-out force analysis of HA, 5%Sr-HA, 10%Sr-HA, and 20%Sr-HA implants after 4 and 12 weeks of implantation. The data are expressed as means ± SD. *p < 0.05 versus the HA group, †p < 0.05 versus the 5%Sr-HA group, and ‡p < 0.05 versus the 10%Sr-HA (by one-way ANOVA and S-N-K test). B: Spot elemental analyses for biological structures formed on 0, 5, 10, and 20%Sr-HA coated implants at late healing stage of week 12. The implants are retrieved after pull-out test, the tissue interfaces are exposed and energy dispersive spectroscopy spot elemental analyses are performed within biological structures. The scans were performed at three different areas on each of the implant interface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
surface for 20%Sr-HA coated implants shows a completely masking of the biological tissue structures with significant increase of Ca, P, C, O signals and even rarely detectable of Ti [Fig. 3(B d,C)]. This indicates that a thick layer of mineralized bone tissue has been formed on the 20%Sr-HA implant surface, which bonds so tightly with the coating that the fracture occurs in the bone tissue instead of at the bone-implant interface.

**FIGURE 4.** A: Region-of-interest is defined as a ring with a radius of 0.8 mm from the implant surface. B: 3-D micro-CT images showing newly formed bone within a circle of 0.8 mm around the four groups of implant 12 weeks after implantation. (Bar = 1 mm). C: Microstructure parameters measured by Micro-CT. The data are expressed as means ± SD. *p < 0.05 versus the HA group, †p < 0.05 versus the 5%Sr-HA group, and ‡p < 0.05 versus the 10%Sr-HA (by one-way ANOVA and S-N-K test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Enhanced bone quality around 20%Sr-HA coated implants

The high-resolution three-dimensional (3D) images obtained by micro-CT clearly reveal the difference in the peri-implant trabecular bone architecture for the whole VOI among the four groups [Fig. 4(B)]. As expected, the HA group over the duration of the study has the lowest values in both BV ratio (38.57%) and associated morphological parameters (Tb.N = 2.82 mm⁻¹ and Tb.Th = 0.14 mm), the highest Tb.Sp (0.22 mm), and the minimal bone formation around the implants [Fig. 4(B,C)]. Compared to HA, 10%Sr-HA exhibits a marked treatment effect in restoring the 3D-BV, with a significantly increased BV/TV (1.4-folds) (p < 0.01), Tb.Th (1.3-folds) (p < 0.05), and reduced Tb.Sp (27.6%) (p < 0.01) [Fig. 4(B,C)]. No significant difference is found from Tb.N between the HA group and 10%Sr-HA group. The 5%Sr-HA group shows a smaller treatment effect, which possesses a slightly increased BV/TV (1.1-folds) (p < 0.01) and moderately reduced Tb.Sp (16.4%) (p < 0.01), whereas both Tb.N and Tb.Th are not significantly affected in the VOI [Fig. 4(B,C)]. The 20%Sr-HA group exhibits the highest enhancement in BV/TV (1.6-folds) (p < 0.01), Tb.N (1.1-folds) (p < 0.01), and Tb.Th (1.5-folds) (p < 0.01) as well as marked reduction in Tb.Sp (32.9%) (p < 0.01) compared to the HA group, [Fig. 4(B,C)].

Fluorescent microscopy analysis

Under fluorescent microscopy, newly formed bone is characterized by polyfluorochrome sequential labeling [Fig. 5(A,B)], while the old bone is characterized by a dark green without label. Polyfluorochrome bone markers Alizarin has a red color with a diffuse pattern whereas calcein is represented by green bands and tetracycline by thin yellow/orange lines. After 4 weeks, little bone formation is observed in the space surrounding the HA-coated surfaces with minimum bone-implant contacts [Fig. 5(A a)]. The 5%Sr-HA coated implants show very similar features to that of the HA-coated surfaces. Thin trabeculae of regenerating bone occur from the walls of the implants, however, only with some small punctual contacts [Fig. 5(A b)]. In contrast, the implants coated with 10 and 20%Sr-HA coatings exhibit...
FIGURE 5. A: Micro-fluorescent images of the 0, 5, 10, and 20%Sr-HA implants 4 weeks after implantation. B: Micro-fluorescent images of the 0, 5, 10, and 20%Sr-HA implants 12 weeks after implantation. Bone formation, molding and remodeling are shown by the fluorescent markers: calcine (Ca), tetracycline (T) and alizarin (A). BM: bone marrow. Original magnification: ×100. Bar = 200 μm. C: Quantitative results of bone apposition 12 weeks after implantation showing no significant difference among the various tested groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
higher degrees of bone regeneration from the burr hole walls [Fig. 5(A c,d)], particularly at the edges of the 20%Sr-HA implant that are in contact with the local old bone. Early labels (calcein green) of new bone have closely approached the 20%Sr-HA implant surface [Fig. 5(A d)], which indicates intense bone remodeling has started from the second week after implantation.

After 12 weeks, bone regeneration is increased around all types of implants. On the HA-coated implants, a thin layer of peri-implant bone has been formed, which is in intimate contact with the implant surface [Fig. 5(B a)]. 5%Sr-HA implant also exhibits an increase in peri-implant bone formation. However, the tetracycline label indicates delayed new bone formation [Fig. 5(B b)]. The 10%Sr-HA samples show moderately increased new bone deposition on the implant surface, which is mainly formed in the calcein and tetracycline phases, whereas the weak fluorescence reveals its low maturity. Intensive new bone formation characterized by close contact between the bone tissues and implant surface is observed from the 20%Sr-HA [Fig. 5(B d)]. The new bone is highly fluorescent thus reveals its maturity. Alizarin is on the endosteal surface where a diffuse pattern is found, which indicates that new bones on the implant surfaces have already formed between days 21 and 32 after surgery. To quantify the growth of the newly formed bone around the four kinds of implants, the dynamic histomorphometric indices, that is, bone MAR, are presented in Figure 5(C). No significant difference can be observed from the calculated bone MAR among the four groups, suggesting that the rate of bone matrix synthesis is not affected.

**Bright field analysis**

At 4 weeks after implantation, all implants in the control and experimental groups show direct contact with surrounding bone. No signs of inflammation occur at the bone-implant interfaces [Fig. 6(A)]. Little bone formation is observed from the space surrounding both the HA implants and 5%Sr-HA-coated surfaces [Fig. 6(A a,b)]. Only a few areas of osteoid matrix not yet mineralized are present at the interface of the cancellous bone region in the HA group, whereas a thin layer of regenerated bone forms on the walls of the 5%Sr-HA implant. However, there is still minimal direct bone-implant contact with the bone-implant contact percentage (BIC) being 35.3 ± 3.36% and 45.7 ± 5.90%, respectively [Fig. 6(B)]. In the trabecular bone area of the 10 and 20%Sr-HA group, newly formed bone are found to extend onto the surface of the Sr-HA coatings to achieve good osseointegration. On the surface of the 10%Sr-HA coating, the bony trabeculae appear to be immature with osteoid and osteoblasts still undergoing mineralization, indicating active bone formation. In comparison, on the 20%Sr-HA surface, thicker woven bone tissues are evenly distributed and the thickness is about 200 μm [Fig. 6(A c,d)]. The BIC is 52.0 ± 8.69% and 55.2 ± 8.68%, which increases 47.25 and 56.22% (p < 0.05), respectively [Fig. 6(B)], compared to the HA group.

After 12 weeks, bone regeneration increases on all the implants. The new bone is in direct contact with the implant surface without the presence of intervening layers of fibrous tissues. Thin new bone extends along the HA implant surface from endosteum, which appears to be denser than after week 4, with BIC being 54.0 ± 9.92% within group [Fig. 7(A a,B)]. The 5%Sr-HA surface shows good regeneration of bone along the implant surface, where continuous bone lamellar runs from the endosteum of the lateral cortical bone [Fig. 7(A b)]. The BIC is 68.9 ± 9.49% in the 5%Sr-HA group. The more extensive areas of direct bone apposition on the 10%Sr-HA implant surface with typical osteoblast layers and mature lamellar osteocytes indicate improved osteocompatibility of the coating [Fig. 7(A c)]. A larger amount of cancellous bone formation is observed on the 20%Sr-HA coating, where almost complete bone contact is formed through osteoconduction. The newly formed lamellar bone is dense and well organized with a large quantity of mature osteocytes [Fig. 7(A d)]. The results suggest that the 20%Sr-HA coatings have excellent osteoconductivity. Besides, the BIC of 10 and 20%Sr-HA significantly increases 34.95 and 46.22% (p < 0.05), respectively, compared to the HA group [Fig. 7(B)].

**DISCUSSION**

This study reports the successful creation of a series of Sr,y-HA coatings on titanium implant through MAO-treatment. The feature of the coatings is characterized by its Sr,y-HA/Sr,xCa(1−y)xTiO3/Sr,xCa(1−y)xTiO3/TiO2 multi-layered structure in which the outer Sr,y-HA layer and intermediate Sr,y-HA/Sr,xCa(1−y)xTiO3 layer have a nanocrystalline structure, and the details has been described in our previous study. More importantly, the Sr,y-HA coated implants exhibit better osseointegration than the HA coated implant and in particular, the 20%Sr-HA coatings substantially accelerate bone neo-formation in the early stage of healing as identified by a set of comprehensive analyses employing biomechanical, micro-CT, fluorescent and bright field histomorphometric assays.

The in vivo implant fixation is the most important factor in determining the clinical capacity of implants as load-
The biomechanical pull-out test shows a significant increase in implant fixation by doping 10 or 20% Sr to the HA surfaces during week 4 and 12 of the healing process. The result also identifies an exclusive advantage of 20%Sr-HA coating over 10%Sr-HA coating, which is a continuous and substantial effect up to week 12 in the healing process. This is illustrated by the improved trabecular bone microarchitecture together with polyfluorochrome labeled early bone-formation and increased BIC around the implant in vivo.
3D evaluation with micro-CT provides the information of trabecular bone formation around the implant surface from both qualitative and quantitative perspectives. The data indicate that bone mass or volume, 3D bone distribution such as Tb.N, Tb.Th, and connective density are significantly increased on the 20%Sr-HA surface; and, we assume that these improvements in the quantity and quality of bone generation collectively result in a substantial increase in the biomechanical fixation of 20%Sr-HA coated implants.

FIGURE 7. A: Micrographs of the 0, 5, 10, and 20%Sr-HA implants 12 weeks after implantation. (Ponceau Tri-Chrome stain, original magnification ×100, BM: bone marrow, NB: new bone, CB: cortical bone). B: Percentage of bone-implant contact for different surface treatments after 12 weeks of implantation. Data are shown as the mean ± SD (n = 3). * p < 0.05 indicates significant difference versus the HA group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Polyfluorochrome sequential labeling is an important tool in the identification of bone remodeling after implant insertion. These tracers indicate the site, time, and amount of bone deposition from bone specimens. Data obtained by light microscopy and fluorescent microscopy demonstrate that deposition of new bone on 20%Sr-HA occurs much earlier than on Sr-free, 5, and 10%Sr-HA, which have already started from day 14 after surgery. The continuously formed bone with large lamellar osteocytes, as disclosed by strong fluorescence, is evenly distributed on the 20%Sr-HA coated implants. Based on histomorphometrical analysis, the greatest degree of BIC is found on 20%Sr-HA 4 and 12 weeks after implantation. However, the trend of increased bone neoformation is not related to a deleterious effect on the mineralizing process. The MAR is similar within the groups, meaning that the bone mineral chemistry or bone matrix mineralization is not affected in the case of low strontium concentration. The results are in line with previous in vivo and in vitro studies showing that treatment with low doses of strontium increases the BV and imposes no deleterious effects on bone mineralization.

Another notable finding is the increased Ca, P while decreased Ti content within biological structures on 20%Sr-HA titanium. The decreased Ti content may represent more complete masking of the coating surface by new bone tissue, implying that the bone formed on 20%Sr-HA surfaces may be thicker than that formed on other groups. The increased Ca, P content suggests that the degree of mineralization is greater in bone around 20%Sr-HA surfaces. This is probably due to the strong bonding between the new bone and 20%Sr-HA surfaces, thus biomechanical breakage occurs within bone tissue rather than at the bone–implant interface. Collectively, from the results of biomechanical, micro-CT, fluorescent and bright field histomorphometry and the elemental analyses at the implant interface, we can conclude that the tested 20%Sr-HA titanium surfaces not only enhance bone–implant integration but also substantially increase osteoconductivity. The results of all the osteoconductivity parameters that are increased at both the early and late stages of healing also suggest that the effect is not only to accelerate but also to enhance osteogenesis.

The positive results above can be explained by the following reasons. First, it can be attributed to the changes of surface chemical composition after different content of Sr doping into HA. The XRD diffraction plots show the peaks of Sr2+ shift to lower 2θ values and the peak intensity increases with strontium incorporation. This is expected as Sr is larger and heavier than Ca. The SEM images show that the size of the nanocrystals on the coating surface decreases gradually with Sr addition and the results are consistent with previous observations showing that for five Sr-substituted HA, (SrCa2x)(PO4)3OH, (x = 0.00, 0.25, 0.50, 0.75, and 1.00), the crystallite size decreases to a minimum as Sr substitution approaches 50% and the Sr-Ca apatite is the most disordered. In this study, the changes in the crystal size and crystallinity of Sr-HA may arise from the gradual incorporation of Sr which destroys the crystal symmetry producing distortion and destabilizing effect on the Sr2+ structure, which in turn alters the solubility of the minerals. Therefore, Sr dose is an important parameter to adjust the release rate the Sr2+ coatings in vivo. Moreover, Sr2+-HA-related bone formation is believed to follow a dissolution-reprecipitation type mechanism, which begins with surface dissolution of the HA followed by the release of calcium and phosphate ions into the space around the implant. Reprecipitation of the apatite nanocrystals in the presence of collagen fibers then occurs on the coating surface. Subsequently, this modified surface rapidly absorbs more protein and promotes cell adhesion, particularly osteoblasts, and then bone formation ensues. Therefore, the increased dissolution rate of Sr2+-HA may result in increased local release of Ca++, P, and Sr2+, which readily induces apatite precipitation and leads to increased new bone formation.

Nevertheless, Biomaterials research faces the challenge of demonstrating the specific contributions of surface chemistry. Surface modification of materials, even if it is intended to modify surface chemistry, may change their surface morphology. It is therefore highly necessary to isolate the pure and independent effect of surface chemistry or morphology, the SEM and AFM images for the non-HA coated surfaces and Sr2+-HA coated surfaces showed similar porous network structures, and the quantitative assessment by AFM fails to detect a significant difference in Ra, RMS, and Rz. Thus, it is unlikely that the increased bone–implant integration is due to the surface morphology. Conversely, we have added the nonHA coated control to the Sr2+-HA group to demonstrate the exclusive effects of Sr2+-HA on osteoconductivity. The pull-out value of HA surface significantly increases by 155 and 90%, respectively, at week 4 and 12, compared to the nonHA group. Besides, micro-CT, and bone morphometry analyses (Supporting Information Figs. S3–S8) also collectively rule out the osseointegration effects of rutile and those titanate in these samples.

Second, the influence of Sr released from the Sr2+-HA surface cannot be excluded. Strontium is suggested to play an important role in chemical bonding between the newly formed bone and the modified dental implant surface. Previous reports indicated that Sr promote bone fracture healing via a unique mechanism by reducing bone resorption while promoting bone formation boasting a broad range of anti-fracture efficacy at vertebral as well as peripheral sites. Nonetheless, the mechanism of the role and action of strontium is not well understood. Recent in vitro studies suggest that strontium can increase replication of preosteoblastic and pluripotent mesenchymal cells via a CaSR-mediated mechanism. Sr has been reported to act as an agonist of the CaSR and may directly interact with CaSR triggering mitogenic signals connected to this receptor to promote osteoprogenitor cells replication. A signal-regulated CaSR/ERK1/2 cascade is a potential pathway for mediating cell replication induced by strontium. Recent in vitro data also indicate that strontium ions can either inhibit osteoclast differentiation or prevent mature osteoclasts from eroding the coating via the CaSR. It is possible that there is an optimal Sr content in the Sr2+-HA coating and constant delivery of Sr
Sr$^{2+}$ from the coating surface will further raise the local Sr$^{2+}$ concentration in the bone tissue adjacent to the implant. As a result, the relatively low but increased level of Sr$^{2+}$ in the defined area (i.e., the bone-implant interfacial area) activates the CaSR in osteoblastic cells and bone narrow stromal cells in the vicinity of the implant surface in the presence of a physiological serum Ca concentration.\textsuperscript{49,50} Consequently, new bone apposition and osseointegration of the Sr$_x$HA implants are enhanced.

From the analysis above, we can conclude that the Sr dose is an important parameter to adjust the solubility of Sr$_x$HA and consequently the osteoconductivity of the Sr$_x$HA coatings, and the release rate of 20%Sr-HA must be more compatible with the bone deposition rate, and the concentration of locally released Sr$^{2+}$ from the 20%Sr-HA surfaces must favor new bone formation. Thus, local application of strontium may be an alternative method to enhance bone osseointegration and to avoid the potential adverse reactions induced by systemic administered strontium.\textsuperscript{14–16} Our study reveals the potential of 20%Sr-HA coatings in dental and orthopedic applications.

CONCLUSION

The MAO-formed Sr$_x$-HA coatings possess better biological characteristics than conventional HA coatings and the Sr dose has an important effect on the in vivo properties. In particular, the 20%Sr-HA coating promotes early bone formation as well as substantially increases bone–implant integration identified by in vivo biomechanical test, histological analysis, and micro-CT evaluation. The promising results successfully demonstrate the immense potential of 20% Sr$_x$-HA coatings in dental and orthopedic applications.

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