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dMultidirectional effects of Sr, Mg and Si-containing bioceramic coatings with high bonding strength on inflammation, osteoclastogenesis and osteogenesis

Chengtie Wu[1, 2], Zetao Chen[2, 3], Deliang Yi[1], Jiang Chang[1, 2]*, Yin Xiao[2, 3]*

1. State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, People's Republic of China

2. Australia-China Centre for Tissue Engineering and Regenerative Medicine, Queensland University of Technology, Brisbane, 60 Musk Ave, Kelvin Grove, Brisbane, Queensland 4059, Australia

3. Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, 60 Musk Ave, Kelvin Grove, Brisbane, Queensland 4059, Australia

* Corresponding authors.

Email: jchang@mail.sic.ac.cn (J. Chang)

Tel: +86-21-52412804; Fax: +86-21-52413903

E-mail: yin.xiao@qut.edu.au (Y. Xiao).

Tel.: +61 7 3138 6240; fax: +61 7 3138 6030.

C.W and Z.C are co-first authors.

ABSTRACT

Ideal coating materials for implants should be able to induce excellent osseointegration, which requires several important parameters, such as good bonding strength, limited inflammatory reaction, balanced osteoclastogenesis and osteogenesis, to gain well-functioning coated implants with long-term life span after implantation. Bioactive elements, like Sr, Mg and Si, have been found to play important roles in regulating the biological responses. It is of great interest to combine bioactive elements for developing bioactive coatings on Ti-6Al-4V orthopedic implants to elicit multidirectional effects on the osseointegration. In this study, Sr, Mg and Si-containing bioactive $Sr_2MgSi_2O_7$ (SMS) ceramic coatings on Ti-6Al-4V were successfully prepared by plasma-spray coating method. The prepared SMS coatings have significantly higher bonding strength (~37MPa) than conventional pure hydroxyapatite (HA) coatings (mostly in the range of 15-25 MPa). It was also found that the prepared SMS coatings switch the macrophage phenotype into M2 extreme, inhibiting the inflammatory reaction via the inhibition of Wnt5A/Ca^{2+} and Toll-like receptor (TLR) pathways of macrophages. In addition, the osteoclastic activities were also inhibited by SMS coatings. The expression of osteoclastogenesis related genes (RANKL and MCSF) in bone marrow derived mesenchymal cells (BMSCs) with the involvement of macrophages was decreased, while OPG expression was enhanced on SMS coatings compared to HA coatings, indicating that SMS coatings also downregulated the osteoclastogenesis. However, the osteogenic differentiation of BMSCs with the involvement of macrophages was comparable between SMS and HA coatings. Therefore, the prepared SMS coatings showed multidirectional effects, such as improving bonding strength, reducing inflammatory reaction and downregulating osteoclastic activities, but maintaining a comparable osteogenesis, as compared with HA coatings. The combination of bioactive

elements of Sr, Mg and Si into bioceramic coatings can be a promising method to develop bioactive implants with multifunctional properties for orthopaedic application.

Key words: SMS coatings; bonding strength; osteogenesis; inflammatory reaction; osteoclastogenesis, macrophages, bone marrow derived mesenchymal cells (BMSCs)

1. INTRODUCTION

Plasma-sprayed bioactive ceramics on Ti-6Al-4V, which combines the bioactivity of bioceramics and the mechanical properties of titanium alloy, have been widely used for orthopaedic implant application.[1-5] Due to its high similarity of the bone inorganic composition, hydroxyapatite (HA) has been coated on the surface of Ti-6Al-4V and achieved certain clinical success. However, there are still some drawbacks arousing great attentions, such as relatively low bonding strength, stability and osseointegration ability, which affect its long-term clinical performance and success rate.[6] Bioactive glass and their composite coatings have been investigated, which show good bioactivity.[7-11] However, it is still very challenging to develop ideal bioactive ceramic-coatings on Ti alloy with excellent mechanical properties (*e.g.* high bonding strength and stability) and positive biological effects (*e.g.* limited inflammatory reaction, well balanced osteogenesis and osteoclastogenesis) after implantation, since the osseointegration of bioactive materials with host bone tissues is affected by a series of important factors, such as bonding strength, inflammatory reaction, osteoclastogenesis and osteogenesis.[12-17]

The bonding strength of the coatings with substrates is an important property of coated implants. Although coating materials, like HA, can well integrate with the surrounding bone tissue, they also prevent the bone tissue from contacting with the titanium surface directly. It

means that the bonding strength between the coating and the metal substrate surface represents most of the overall bonding strength between the implants and bone tissue before complete degradation of coating materials. Poor bonding strength may result in the delamination of coatings from Ti alloys and limits their long-term survival after implantation.[18] Therefore, it is of great importance to develop new coating materials which can maintain the good biological behaviours as HA while enhancing the bonding strength between coatings and metal substrates. Previous studies have shown that silicate-based bioceramic coatings generally have higher bonding strength than HA coatings prepared by plasma-spray method,[3, 19, 20] indicating the value of silicate bioceramics to maintain longer life span of orthopaedic implants.

For the successful osseointegration, the implant is supposed to integrate with bone tissue directly without any intervening connective tissue. Inflammatory response is a key factor in determining the formation of fibrous capsule. Excessive inflammation can lead to the formation of fibrous capsule and also separate the bone cells from contacting and integrating with the implants, resulting in the failure of implants.[21] As foreign bodies, implants tend to cause foreign body reaction, which is known to form fibrous capsule. From this point of view, developing orthopaedic coatings with the capability of inhibiting inflammatory reaction could be a potential strategy. Macrophages are known to be one of the most important cells in the material-induced immune response to orthopaedic coatings,[22] thereby they can be used for investigating the interactions between bioactive coatings and immune cells.[23] In addition to their effects on inflammation, macrophages are also known to influence bone physiology and pathology.[24, 25] Macrophages are the precursors of osteoclasts, which participate in the bone remodelling and material degradation. Macrophages also contribute to osteogenesis through the expression and secretion of a wide range of regulatory molecules,[26] such as BMP2, transforming growth factor β (TGF-β), *etc*.[27-29] To our knowledge, there are few studies about

the osteoclastogenesis and osteogenesis induced by orthopaedic coatings with the involvement of macrophages. For these reasons, it is interesting to investigate the inflammation caused by the interaction between the coating and macrophages and the further osteoclastogenesis and osteogenesis induced by orthopaedic coatings with the involvement of macrophages.

Osteoclasts play important roles in degrading the materials and remodelling the new forming bone during osseointegration. The successful osseointegration requires adequate and effective osteoclastic activities. High osteoclastic activities may lead to the bad quality of the newly formed bone tissue (poor bone mass and density), resulting in the bad loading capacity of implants. Such a phenomenon is not uncommon clinically, especially in patients with osteoporosis. The imbalance between bone resorption and bone formation in osteoporosis patients results in high bone resorption. It is thereby of great clinical significance to develop coating materials, which are capable to inhibit osteoclastic activities, especially for patients with osteoporosis.

Bone matrix deposition within the implants is another key step for the osseointegration. Materials enhancing the osteogenesis would be of great significance in improving the quality of new forming bone (high bone mass and density). Osteoblasts are responsible for the deposition of bone matrix. They can also regulate the differentiation and activity of osteoclasts, thereby maintaining the skeletal architecture. In addition to their traditional effects on inflammation, immune cells are increasingly supposed to be indispensable during osteogenesis of biomaterials, with the emergence and development of osteoimmunology.[29] They were found to be closely related with the bone cells, sharing a number of cytokines, receptors, signalling molecules and transcription factors.[30] It is reported that evaluation system for the *in vitro* osteogenesis capacity involving immune cells is more accurate than that only using osteoblastic cells.[29] To our knowledge, there are very few studies on the

orthopaedic coating mediating osteogenesis with the involvement of macrophages. Therefore, it is interesting and more accurate to investigate the osteogenesis of bioceramic coatings by using the evaluation system involving immune cells (macrophages).

For these reasons, it is interesting to coat bioactive ceramic on Ti-6Al-4V with high bonding strength and the ability to inhibit the inflammatory reaction and osteoclastogenesis while maintaining excellent or enhancing osteointergration. More and more evidence has shown that bioactive elements play a key role in influencing the inflammatory reaction, osteogenesis and osteoclastogenesis.[31, 32] Strontium (Sr) as a trace element in human body has been found to enhance osteogenesis while inhibiting osteoclastogenesis, which makes it applied widely in treating osteoporosis.[33-35] In addition, Sr is also found to suppress the expression of the inflammation-promoting cytokine interleukin 6 (IL6) and decrease the production of pro-inflammatory cytokines.[31, 36] Magnesium (Mg) is essential for bone metabolism. The depletion of Mg can affect all the stages of skeletal metabolism adversely, causing cessation of bone growth, decreased osteoblastic and osteoclastic activity, osteopenia and bone fragility.[37, 38] It was also reported to decrease inflammatory cytokine production.[32, 39] Silicon (Si) is another important trace element of bone, which is reported to locate at active calcification sites, involving in the mineralization process of bone growth.[40] Previous studies have shown that the released Si-containing ionic products from bioactive glass, bioceramics and coatings play an important role in stimulating the proliferation and differentiation of bone-forming cells. [41-43]

To our knowledge, although Sr, Mg and Si bioactive ions elicit important effects on inflammatory reaction, osteoclastogenesis or osteogenesis as well as new bone formation, there are few studies to design biomaterials with these properties, especially bioceramic coating materials for orthopaedic application. It is reasonable to speculate that the combination of Sr, Mg and Si in the bioceramic coatings on Ti-6Al-4V may lead to the

development of novel orthopaedic implants with multidirectional effects including the enhancement of bonding strength, inhibition of inflammatory reaction and osteoclastogenesis, and stimulation of osteogenesis. We have previously synthesized $Sr_2MgSi_2O_7$ ceramic powders.[44] In this study, the synthesized $Sr_2MgSi_2O_7$ ceramic powders were coated onto the Ti-6Al-4V surface employing the plasma-spray method. Systematic investigation of the bonding strength of the coating on metal substrates, and the effect of the coating on inflammatory reaction, osteogenesis and osteoclastogenesis as well as the possible mechanisms of the effects was carried out by studying the interactions between the prepared coatings and macrophages, osteoclasts and BMSCs.

2. MATERIALS AND METHODS

2.1 Preparation of SMS coatings on Ti-6Al-4V

The $Sr_2MgSi_2O_7$ (SMS) powders were synthesized by the solid-state reaction process using SrO, MgO and SiO_2 as raw materials according to our previous study.[44] To improve powder flowability, a sinter-crushing method was used. Briefly, the synthesized SMS powder was pressed into tablets, sintered at 1300^{o}C. Then the sintered tablets were crushed and sieved by 200 and 400 meshes to obtain SMS particles with diameter of 40-80 μm for further coating preparation. The prepared SMS particles were characterized by scanning electron microscopy (SEM, JSM-6700, Japan).

The reconstituted SMS particles were sprayed on Ti-6Al-4V (Shanghai Yantai metallic material Co., Ltd, China) substrate with dimensions of 10×10×2 mm. Prior to plasma spraying, the Ti-6Al-4V substrates were grit blasted, ultrasonically washed with ethanol and dried at 60^{o}C. An atmosphere plasma sprayed system (sulzer metco, Switzerland) was applied to fabricate SMS coatings, and the parameters for plasma sprayed SMS coatings were shown as following (argon plasma gas flow rate: 40 slpm, hydrogen plasma gas flow rate: 10 slpm,

spray distance: 120 mm, argon powder carrier gas: 3.5 slpm, current: 650 Å, voltage: 66 V). HA coatings were fabricated by spraying commercial HA powders onto Ti-6Al-4V substrate according to the previous study[3] and used as the control.

2.2 Characterization, bonding strength and apatite mineralization of SMS coatings

The surface microstructure of the prepared SMS coatings was observed by SEM (JSM-6700, Japan). The crystal phase composition of the prepared SMS coatings was characterized by x-ray diffraction (XRD, D8 advance, Bruker, Germany) using Cu Kα radiation with scanning range of 10-80^{o} and step size 0.02^{o}. The average linear thermal expansion coefficient of the SMS-coated titanium and un-coated titanium was tested by using push-rod technique according to the standard testing method GB/T 16535-2008 (Linseis L75 Platinum Series), from room temperature to 800 ^{o}C.

The bonding strength between SMS coatings and Ti-6Al-4V substrate was measured by a mechanical tester (Instron-5592, SATEC, USA) in accordance with American Society for Testing and Materials (ASTM) C-633 used in previous study.[19] In brief, 10 cylindrical Ti-6Al-4V rods (diameter: 25.4 mm) were prepared. Half of the rods were sprayed with SMS coatings, the other half were grit blasted. High-performance E-7 glue (Shanghai institute of synthetic resin, Shanghai, China) was used to join the two rods (one with SMS coatings and the other grit blasted), a compressive stress was applied to both rods end to assure an intimate contact. The combined rods were then place into a 100 ^{o}C oven for 3 h to solidify the glue. The bonding strength was measured by a mechanical tester (Instron-5592, SATEC, USA) at a crosshead speed of 2 mm·min^{-1}, and the average of five measurement was calculated for the bonding strength of SMS coatings with Ti-6Al-4V substrate.

To investigate the apatite-mineralization ability of the prepared SMS coatings, the coated samples were immersed in acellular simulated body fluids (SBF) [45] and kept under shaking

conditions at 37 ^{o}C for 2, 4 6, 8, 10, 12 and 14 days. The ionic concentrations of Sr, Si, Mg, Ca and P ions released from SMS coatings were tested by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Varian 715ES). The formed apatite mineralization on the surface SMS coatings were characterized by fourier transformed infrared spectroscopy (FTIR, Nicolet Co., USA) and SEM.

2.3 Cell culture

Three kinds of cells, including the murine-derived macrophage cell line RAW 264.7 cells, osteoclasts and BMSCs were used in this study. RAW 264.7 cell cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, California, USA) supplemented with 5% fetal bovine serum (FBS, Thermo Scientific, Waltham, Massachusetts, USA), and 1% (v/v) penicillin/streptomycin (Life Technologies, Carlsbad, California, USA) at 37 ºC in a humidified CO_2 incubator. The cells were passaged at approximately 80% confluence by scraping and expanded through two passages before being used for the study.

Osteoclasts were derived from RAW 264.7 cells following the protocol as previously described. [46] In brief, RAW 264.7 cells were seeded to the T25 flask and cultured in complete medium (DMEM supplemented with 5% FBS and 1% (v/v) penicillin/streptomycin) at 37 ºC in a humidified CO_2 incubator. After three days, the medium was replaced with fresh complete medium consisting of DMEM containing 5% FBS and 1% (v/v) penicillin/streptomycin, and supplemented with 35 ng/ml of recombinant human RANKL (Millipore, Billerica, Massachusetts, USA). Media was changed every 3 days and cells were allowed to differentiate into functional osteoclasts over a period of 21 days.

BMSCs were isolated and cultured based on protocols according to our previous studies.[47-49] Briefly, bone marrow was obtained from patients (50-60 years old) undergoing hip or knee

replacement surgery with informed consent given by all donors and the procedure was approved by the Ethics Committee of Queensland University of Technology. Lymphoprep was added to isolate the mononuclear cells from the bone marrow by density gradient centrifugation (Axis-Shield PoC AS, Oslo, Norway). The obtained cells were seeded into the tissue culture flasks containing DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and incubated at 37 ºC in a humidified CO_2 incubator. The culture medium was changed every 3 days until the primary mesenchymal cells reached 80% confluence. The unattached hematopoietic cells were removed through medium change. The confluent cells were routinely subcultured by trypsinisation. Only early passages (p3–5) of cells were used in this study.

2.4 The inflammatory response for macrophage RAW 264.7 cells cultured with SMS coatings

2.4.1 Inflammatory gene expression of RAW264.7 cells

RAW 264.7 cells were seeded on the coating surface at a density of 10^5/coating disk (10×10mm). The cells were incubated for 6 days and the medium was changed on day 3. On day 3 and 6, the conditioned media were collected, and centrifuged at 1500 rpm to gain the supernatants. They were then mixed with complete medium at a ratio of 1:2 for the conditioned-medium experiments. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, California, USA) on day 6 for RT-qPCR detection.

500 ng of total RNA was used for the synthesis of complementary DNA using DyNAmo™ cDNA Synthesis Kit (Finnzymes, Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. RT-qPCR primers (Table S1), which were designed based on cDNA sequences from the NCBI Sequence database. SYBR Green qPCR Master Mix (Life Technologies, Carlsbad, California, USA) was used for detection and the

target mRNA expressions were assayed on the ABI Prism 7500 Thermal Cycler (Applied Biosystems, Foster City, California, USA). Each sample was performed in triplicate. The mean cycle threshold (Ct) value of each target gene was normalized against Ct value of a house keeping gene to gain the relative expression. For the calculation of fold change, ΔΔCt method was applied, comparing mRNA expressions between SMS coating group and HA coating group.

2.4.2 Flow cytometry

To explore the phenotype switch of macrophage, expression of M1 and M2 macrophage cell surface marker CCR7 and CD163, respectively, were determined by flow cytometry. RAW 264.7 cells were seeded in the T25 flask and cultured in complete medium. After 1 day of culture, the medium was replaced by the macrophage-conditioned medium obtained from section 2.4.1. After another 2 days, the cells were detached by scraping. Nonspecific protein binding was blocked by 1% BSA/PBS. Samples were incubated with CCR7 (1:25) (GeneTex, Irvine, California, USA) and CD163 antibody (1:100) (AbD Serotec, Raleigh, North Carolina, USA) for 30 minutes at 4ºC, followed by incubation with Dylight 488-anti-mouse and DyLight 405- anti-goat secondary antibody (DAKO, Multilink, California, USA) for 30 minutes at 4 ºC. After washing with 1% BSA/PBS, cells were analysed on a FC500 flow cytometer (Beckman Coulter, Brea, California, USA). The data were analysed using Flowing Software (www.flowingsoftware.com).

2.4.3 Mechanism of the inflammatory gene expression change

To understand the mechanism of two involved inflammation signalling pathways, Wnt5A/Ca^{2+} (Wnt5A, Fz5, calmodulin-dependent protein kinase II (CaMKII), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α)), and TLR pathways (MyD88, Ticam1/2, IκB-α) were evaluated by RT-qPCR and Western blot. RAW 264.7 cells were seeded on the coating surface at a density of 10^5/coating disk. Total RNA

was extracted using TRIzol reagent (Life Technologies, Carlsbad, California, USA) for RT-qPCR detection as described in section 2.4.1.

The whole cell lysates were collected after 7 and 24 hours of culture for the Western Blot detection of CaMKII and IκB-α. 10 μg proteins from each sample were separated on SDS-PAGE gels and then transferred onto a nitrocellulose membrane (Pall Corporation, East Hills, New York, USA). After being blocked in Odyssey blocking buffer for 1 hour (LI-COR Biosciences, Lincoln, Nebraska, USA), the membranes were incubated with primary antibodies against IκB-α (1:1000, rabbit anti-human/mouse; Cell Signaling Technology, Danvers, Massachusetts, USA), CamKII (pan, 1:1000, rabbit anti-human/mouse; Cell Signaling Technology, Danvers, Massachusetts, USA), and α-tubulin (1:5000, rabbit anti-human; Abcam, Cambridge, United Kingdom) overnight at 4 ℃. The membranes were washed three times in TBS-Tween buffer, and then incubated with anti-mouse/rabbit HRP conjugated secondary antibodies at 1: 4000 dilutions for 1 hour at room temperature. The protein bands were visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska, USA). The relative intensity of protein bands was quantified using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.5 The osteoclateogenesis and osteoclastic activities

RAW 264.7 cells and osteoclasts were seeded on the coating surface at a density of 10^5/coating disk. On day 6, total RNA was extracted using TRIzol reagent for detection of osteoclastic activities related gene expression (TRAP, Cathepsin K (CTSK), Carbonic Anhydrase II (CA 2), receptor activator of NF-kB (RANK) and Calcitonin Receptor (CT), Matrix metalloproteinase-9 (MMP9)) as described in section 2.4.1. On day 3 and 6, the conditioned media were collected, and centrifuged at 1500 rpm to gain the supernatants for the further ions concentration detection.

Given that osteoblastic cells are the important source of osteoclastogenesis regulating cytokines, we further used the macrophage-conditioned media to stimulate the BMSCs to detect the gene expression changes of osteoclastogenesis regulating cytokines. BMSCs were seeded in the 6 well plates and cultured in complete medium. After 1 day of culture, the medium was replaced by the macrophage-conditioned medium obtained from section 2.4.1. After 3 and 6 days, total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, California, USA). RT-qPCR detection was carried out to determine the osteoclastogenesis regulating cytokines (MCSF, RANKL, and OPG) gene expression changes as described in section 2.4.1.

2.6 The osteogenesis for BMSCs cultured with SMS coatings with involvement of immune cells

2.6.1 Alkaline phosphatise activity of BMSCs

To detect the ALP activity of BMSCs, BMSCs were cultured in 24-well culture plates with a seeding density of 20,000 cells per well. ALP activity was assessed at 7 days culture in macrophages conditioned-coating media. The cells were lysed in 100 μL of 0.2% Triton X-100 and then centrifuged at 14 000 rpm for 15 min at 4°C. 50 μL supernatants were mixed with 150 μL working solution and determined using the QuantiChromTM Alkaline Phosphatase Assay Kit (BioAssay Systems, Hayward, California, USA). The total protein content was measured by the BCA Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts, USA). The relative ALP activity was then obtained as the changed optical density (OD) values divided by the total protein content[50].

2.6.2 Bone-related gene expression of BMSCs

BMSCs were plated at a density of 20,000 cells per well in separate 6-well plates. After 24 hours of incubation, the culture medium was removed and replaced by macrophages

conditioned media. Cell morphology was observed under a Nikon's inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan) and figures were taken. Total RNA was extracted using TRIzol reagent after 3 and 7 days of culture for the RT-qPCR detection as described in section 2.4.1.

2.6.3 The mineralization of BMSCs

In order to identify mineralization nodules, Alizarin Red S staining was measured on day 14 after BMSCs grown in macrophages conditioned media in a 96-well plate with osteogenic supplements. The medium was removed and the cells were washed with ddH_2O and fixed in 4% paraformaldehyde for 10 min at room temperature. After gently rinsing with ddH_2O, the cells were stained in a solution of 2% Alizarin Red S at pH 4.1 for 20 min and were then washed with ddH_2O. The samples were air-dried and figures were taken under a light microscope. The glossary of biomedical terms is listed in Table S2.

2.7 Ionic concentrations

SMS and HA coated Ti-6Al-4V were immersed in the complete medium. After 3 and 6 days, the conditioned media were collected, and centrifuged at 1500 rpm to gain the supernatants, which were then mixed with 0.5% HNO_3 at a ratio of 1:2 for the further ions concentration detection. Macrophages and osteoclasts conditioned supernatants obtained from section 2.5 were also mixed with 0.5% HNO_3 at a ratio of 1:2 for the further ions concentration detection. The ionic concentrations of Sr, Si, Mg, and Ca ions in complete culture medium, macrophages and osteoclasts conditioned media were quantified by inductive coupled plasma atomic emission spectrometry (ICP-AES, PerkinElmer, Waltham, Massachusetts, USA).

2.8 Statistical analysis

All the analyses were performed using SPSS software (IBM SPSS, Armonk, New York, USA). Data is shown as means ± standard deviation (SD) and analysed using one-way ANOVA followed by LSD *post-hoc* test. The level of significance was set at $P<0.05$.

3. RESULTS

3.1 Characterization, bonding strength and apatite mineralization of SMS coatings

Figure 1A shows the morphology of reconstituted SMS particles by sinter-crushing method. The size of the SMS particles is about 40-80 μm with an irregular shape. The morphology of the prepared SMS coatings is shown in figure 1B and 1C. The coating has a rough surface built by random staking of fully and partially melted SMS particles (Fig. 1B). A higher magnification image presents that parts of the fully melted coating surface is relatively smooth (Fig. 1C). XRD analysis shows that the main crystal phase of prepared SMS coatings on Ti-6Al-4V is $Sr_2MgSi_2O_7$ (JCPD 15-0016) (Fig. 1D). The average linear thermal expansion coefficient of the SMS-coated titanium and un-coated titanium in the temperature range of 20-800 ^oC was 9.39×10^{-6} and 9.65×10^{-6} $^oC^{-1}$, respectively. The mean bonding strength of the SMS coatings with Ti-6Al-4V substrate is 37.1±3.3 MPa.

After soaked in SBF, there are newly formed apatite clusters on the surface of SMS coatings (Fig. 2A). Higher magnification SEM images shows that the formed clusters are composed of lath-like apatite microcrystals with the diameter of 100 nm (Fig. 2B). There are newly formed P-O characteristic peaks at the wavenumber of 1080, 603 and 562 cm^{-1} in FTIR pattern after soaked SMS in SBF (Fig. 2C). Sr concentrations in SBF solution increase distinctively at the first 2 days of soaking and then decrease with the increase of soaking time. Mg and Si concentrations slightly increase at first 2 days, and then maintain a stable release. The Ca and P concentrations tend to decrease with the increase of soaking time (Fig. 2D).

3.2 The inflammatory response for macrophage RAW 264.7 cells cultured with SMS coatings

Flow cytometry results showed the mean fluorescence intensity of CD163 increased after the material stimulation (Fig. 3A a,b). On the contrary, the mean fluorescence intensity of CCR7 showed no significant changes under the same treatment (Fig. 3A c,d).

Anti-inflammatory genes IL-1ra expression was significantly upregulated by the stimulation of SMS coatings in comparison with the culture on HA coating ($P < 0.05$) (Fig. 3B). On the contrary, inflammatory genes IL-1β, IL-6 and Oncostatin M (OSM) expression were significantly downregulated with the same treatment ($P < 0.05$) (Fig. 3B).

To explore the mechanism of inflammation related gene expression changes, we examined two inflammation signalling pathways (Wnt5A/Ca^{2+}, TLR). Both Wnt5A and Fz5 gene expression were significantly downregulated (Fig. 4A) in comparison with the HA coating group. The downstream molecules CamKII also showed significant decrease in protein expression (Fig. 4C). As to the Toll like receptor pathway, MyD88, Ticam 1 and 2 gene expression were all significantly downregulated (Fig. 4B, $P < 0.05$), while the downstream molecules IκB-α were enhanced in protein expression (Fig. 4C, $P < 0.05$).

3.3 The osteoclastogenesis and osteoclastic activities

Most of the osteoclastic activities related genes (TRAP, CTSK, RANK, CT, and MMP9) by macrophages were significantly downregulated by the stimulation of SMS coatings compared with that of HA coatings (Fig. 5). Similar results were observed in the stimulated osteoclasts, with the inhibitions of TRAP, CTSK, CA2, RANK, and MMP9 genes expression (Fig. 6).

The expression of RANKL, an osteoclastogenesis enhancing gene, by BMSCs under the stimulation by conditioned medium from macrophages on SMS coatings was significantly downregulated on both day 3 and day 7 (Fig. 7, $P <0.05$). MCSF, another osteoclastogenesis

enhancing gene, was also significantly downregulated on day 7 (Fig. 7, $P < 0.05$). Osteoclastogenesis inhibiting gene OPG showed a different pattern. It showed no significant change on day 3, but was significantly upregulated on day 7 (Fig. 7, $P < 0.05$).

3.4 The osteogenesis for BMSCs cultured with SMS coatings with involvement of immune cells

The osteogenic differentiation of BMSCs stimulated by the macrophage-conditioned medium with SMS and HA coatings is shown in Figure 8. Morphology of BMSCs was similar in both SMS and HA coatings groups (Fig. 8A). The ALP activity of BMSCs with SMS-stimulated RAW cell medium is slightly lower than that of HA group (Fig. 8B). Bone-related genes expression (ALP, OPN, OCN, COL1 and IBSP) has almost no significant difference between SMS and HA coatings groups (Fig. 8C) on both day 3 and 7, (Fig. 8D). Alizarin red staining shows that both SMS and HA coatings groups could lead to the formation of mineralisation nodules (Fig. 8E).

4. DISCUSSION

In this study, we successfully prepared Sr, Mg and Si-containing SMS coatings on Ti-6Al-4V by plasma-spray method. For orthopaedic coating applications, the prepared coating materials on titanium alloy should be relatively stable to maintain long-term life span. Although bioactive glass coatings possess excellent bioactivity, their dissolution rate is generally higher than that of crystallized bioceramic coatings. For this reason, we tried to combine the bioactive elements of Sr, Mg and Si into the coatings to induce favourable biological effects, together with higher crystallinity for higher chemical stability. $Sr_2MgSi_2O_7$ is one of the typical crystal phases in the Sr, Mg and Si containing ceramic systems. Our previous study has shown that pure phase $Sr_2MgSi_2O_7$ ceramic can be easily prepared. No phase change was

observed even after high temperature treatment. In addition, $Sr_2MgSi_2O_7$ bioceramics exhibit stimulatory effects on the osteogenic differentiation of BMSCs.[44] Concerning these physiochemical and biological properties, we applied this kind of ceramics as orthopaedic coatings on titanium.

The stability of bioceramic coatings and their bonding strength with Ti alloy is of great importance to maintain their long-term life survival. When the bioceramic coated implants are implanted *in vivo*, host body cells will be in contact with the coating materials first. The coating materials will then experience some degradation either by physicochemical dissolution, cell-medicated dissolution, hydrolysis, enzymatic decomposition, or corrosion. [51] The released ions or degraded particles from the coating are supposed to regulate the local microenvironment, which determines the response and behaviour of host cells. Therefore, it is vital to coat the titanium with bioactive materials, which can create a favourable environment for the new bone formation, like SMS did in this study. To maintain the long-term life span of the implants, bioceramic coatings should release some bioactive ions to assist the osseointegration with host bone tissue and at the same time the prepared coatings should have relatively high stability (or slow degradation). In this study, SMS showed higher osteoclastogenesis-inhibiting capacity than HA, indicating that SMS coatings may have higher biological stability than that of HA coatings. In addition to the degradation, the bonding strength of bioceramic coatings with titanium is another important factor to maintain the long-term life span of the implants, since the high bonding strength will provide a stable coating interface without delamination from titanium to support functional loading before the coating materials are completely replaced by new bone tissue.

The bonding strength of HA coatings is generally in the range of 15-25 MPa.[52] Although the HA coated implants have achieved certain clinical success, the bonding strength of HA coating on titanic alloy is still not strong enough and may lead to the delamination

phenomenon in clinical applications. It is of great clinical significance to develop coated implants with high bonding strength as SMS revealed (37 MPa) in this study. Higher bonding strength of SMS coatings make them more potential for clinical applications, since they may have longer life span with functional loading.

Thermal expansion coefficient of ceramics is one of the main factors to determine the bonding strength between the coating and the metallic substrate. Previous studies have shown that the silicate-based bioceramics possess similar thermal expansion coefficient with Ti-6Al-4V, therefore favouring a higher bonding strength and reducing the residual stress due to the mismatch of the thermal expansion coefficient.[3, 19, 20] In this study, it is found that the SMS-coated titanium and uncoated titanium have similar linear thermal expansion coefficient. In addition, there are no obvious microcracks on the surface of SMS coatings, indicating that SMS coatings have strong bonding with titanium substrate.

In addition to their high bonding strength, SMS coatings could also switch macrophage phenotype into M2 extreme, leading to the inhibition of the inflammatory reaction compared with the HA coatings. These effects may be related to the downregulation of WNT5A/Ca^{2+} and TLR pathways. Wnt5A/Ca^{2+} signalling pathway is known to enhance the inflammation.[53] Wnt5A can bind to Fz5, activating the Wnt/Ca^{2+} signalling pathway via CaMKII and protein kinase C, which culminates the expression of downstream inflammatory cytokine genes via the transcription factor NFκB.[53] After soaked SMS coated implants, the culture medium showed a significant decrease of Ca^{2+} concentration. It is a logical extension to speculate that the decrease of Ca^{2+} concentrations may lead to the inhibition the Wnt/Ca^{2+} signalling pathway, resulting in the anti-inflammatory effects.

Macrophages recognize the foreign bodies via TLR pathway, inducing innate immune response trying to degrade or reject the implants.[54] MyD88 is one of the key components in this pathway. Most of the activated TLRs interact with MyD88, which then activate

downstream cascade.[55] However, TLR3 can only conduct through a MyD88-independent signalling pathways, toll-like receptor adaptor molecule (Ticam), also known as TIR domain containing adapter inducing IFN β (TRIF), while TLR4 can signal through both pathways.[56] Although producing signal through different adapter proteins, both MyD88-dependent and Ticam-dependent pathways eventually activate the NF-κB, resulting in the expression of inflammatory cytokines.[57] In the present study, MyD88, Ticam1 and Ticam2 gene expressions were all downregulated, while the NFκB inhibitor IκB was upregulated. It means that the SMS coatings might also lead to the inhibition of inflammatory response via TLR pathway. After the immersion of SMS coated implants, the culture medium showed a significant increase of Mg^{2+} concentration. Mg was known to suppress inflammatory cytokine production through inhibition of TLR pathway.[32] Therefore, the inhibition of this pathway might be related to the release of Mg^{2+} from SMS coatings. Sr^{2+} is also found to decrease inflammatory cytokine production, which was also increased in the culture medium after soaked SMS coated implants.[31, 36] However, the underlying mechanism is still unknown. Nevertheless, the inhibition of inflammatory response indicates that the SMS coatings are more compatible than HA coatings, which may prevent the formation of fibrous capsule.

The osteoclastogenesis and osteoclastic activities were both inhibited by SMS coatings. The interactions between SMS coatings with pre-osteoclasts (macrophages), and osteoclasts were firstly investigated. Our results showed that SMS coatings significantly downregulated the osteoclast activity-related genes of both pre-osteoclasts (macrophages) and osteoclasts, such as TRAP, CTSK, RANK, and MMP9. Osteoblastic cells are also known to regulate the osteoclastogenesis via the releasing of RANKL, MCSF and OPG. MCSF binds to its receptor, c-fms, on osteoclast precursors and activates signalling through Akt and MAP kinases pathway.[58] RANKL binds to RANK, receptor on the surface of osteoclast precursors, activating signalling through NF-κB, activator protein 1 (AP-1) and nuclear factor of

activated T cells 2 to induce expression of genes for the survive and differentiation of osteoclasts.[59] OPG, a decoy receptor derived from osteoblasts, can bind to RANKL and interrupt its interaction with RANK receptor, thereby inhibit the osteoclastogenesis.[60, 61] Therefore, we further investigated the expression of RANKL, MCSF and OPG genes in BMSCs cultured in the conditioned media. It was found that SMS coatings could decrease the expression of RANKL and MCSF genes, and increase OPG expression of BMSCs. All these results suggest that SMS coatings downregulate the osteoclastogenesis and osteoclastic activities, as compared to HA coatings.

It is found that Sr^{2+} and Sr-containing biomaterials have inhibitory effect on the osteoclastic differentiation and resorptive activity.[62, 63] The underlying mechanism might be related to the suppression of IL6 family of cytokines.[36] IL-6 and OSM are IL6-type cytokines that stimulate osteoclast formation and function, which were both downregulated in this study. IL6 is believed to play a positive regulatory role in osteoclast differentiation by inducing the expression of RANKL on the surface of osteoblasts, activating the RANK signalling pathway on osteoclast progenitors.[64] The inhibition of IL6 receptor can directly block the osteoclast formation.[65] OSM uses the same receptor subunit, gp130, for signaling, and often have similar and overlapping functions with IL6.[66] OSM can help to enhance the osteoclastogenesis in a dose dependent manner, which might be related to its synergistic effects with IL6.[67]

In this study, the concentrations of the released Sr^{2+} in the macrophage-conditioned medium reach 134 ppm for SMS coatings, which are significantly higher than those for HA coatings (only 0.25 ppm) (as shown in Table 1). Therefore, it is reasonable to speculate that the possible mechanism for the downregulated osteoclastogenesis of SMS coatings is mostly relative to the released Sr^{2+} ions from coatings. The inhibition of osteoclastogenesis may help to obtain properly balanced osteoclastogenesis and osteogenesis, extending the applications

of SMS coated Ti-6Al-4V to the patients with harsh bone qualities (low bone mass and density), especially those with osteoporosis.

Previous studies have found that ionic products (Si, Mg, Sr) from SMS powders could enhance the osteogenesis of BMSCs. [44] It is known that the ion release from crystalline materials is mainly due to the dissolution of the materials in the aqueous environment. The dissolution rate of the crystalline materials mainly depends on the chemical composition and crystal structure. In this study, we found that although the dissolution of SMS coatings was slow, it did release Sr, Mg and Si ions, which induces the favourable biological effects. Given to the importance of immune cells during the material stimulated osteogenesis, we further investigated the osteogenesis-inducing capacity of SMS coatings with the involvement of immune cells (macrophages). It was found that the osteogenic differentiation of BMSCs on SMS coatings was comparable to that on the HA coatings even with the involvement of macrophages, indicating that SMS coatings have comparable *in vitro* osteogenesis-inducing capacity to HA coatings. Previous studies for evaluation of the *in vitro* osteogenesis mainly focused on the interaction of osteoblastic cells with bioactive coatings.[68, 69] Current study extends the method by involving the macrophages to investigate the osteogenesis of BMSCs cultured with SMS coatings, which makes the evaluation for the *in vitro* osteogenesis-inducing capacity of SMS coatings more sufficient and accurate.

After implanted, the coating materials will be completely degraded eventually and replaced by new bone tissue. The degradation time differs from the composition and structure of the materials. It means that coating materials will exist temporarily during the integration of titanium substrate with host bone tissue. Titanium materials are not very effective to guide the regeneration of surrounding tissue. Coating with bioactive materials can well solve this issue, endowing titanium with bioactivities. The major function of coating materials is to regulate an osteogenesis-enhancing environment for better integration of the titanium

substrate with host bone tissue. One shortcoming of this strategy is creating one more interface temporarily between titanium substrate and coating materials. The bonding strength should be strong enough for keeping the implants steady before the replacement of coating materials with new bone tissue, which makes the bonding strength between coating materials and metallic substrate a very important property of coating materials. HA has good bioactivities; however, the interface between HA and titanium substrate is not strong enough in bonding strength limiting its application, while the new SMS coatings seems to overcome this problem with significantly higher bonding strength.

5. CONCLUSIONS

In this study, bioactive elements Sr, Mg and Si-containing SMS coatings on Ti-6Al-4V have been successfully prepared by plasma-spray method. The prepared SMS coatings possess significantly higher bonding strength than that of HA coatings. SMS coatings inhibit the inflammatory reaction of immune cells RAW 264.7 possibly via the inhibition of WNT5A/Ca^{2+} and TLR pathways. In addition, SMS coatings could also inhibit the osteoclastic activities and osteoclastogenesis while maintaining good osteogenesis-inducing capacity. These multidirectional effects suggest that the SMS-coated Ti-6Al-4V may be a promising implant material for the orthopaedic applications.

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Associated Content

Supporting Information Available: Glossary of biomedical terms and qPCR primers used in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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		Culture medium immersed				Macrophage conditioned				Osteoclast conditioned			
		HA		SrMg		HA		SrMg		HA		SrMg	
		0-3 d	3-6 d	0-3 d	3-6 d	0-3 d	3-6 d	0-3 d	3-6 d	0-3 d	3-6 d	0-3 d	3-6 d
Ionic concentrations (mg/ L)	Ca	20.19±0.31	17.75±0.53	0.46±0.01	16.69±1.25	29.89±0.21	25.11±0.49	0.32±0.01	18.07±0.02	26.81±0.46	24.93±0.23	0.20±0.02	17.01±0.36
	P	5.31±0.08	5.15±3.08	1.78±0.16	7.11±0.22	4.54±0.26	5.08±0.12	1.51±0.11	7.72±0.09	4.54±0.18	5.08±0.12	1.40±0.09	6.85±0.16
	Si	0.42±0.02	0.22±0.02	24.51±0.85	19.03±0.21	0.43±0.04	0.18±0.01	29.46±0.11	20.02±0.35	0.41±0.02	0.15±0.02	24.88±0.17	19.20±0.49
	Sr	0.26±0.05	0.33±0.11	2.97±0.02	102.06±0.13	0.17±0.14	0.25±0.03	7.49±0.13	134.23±1.38	0.16±0.02	0.21±0.10	5.88±0.19	151.02±3.26
	Mg	1.51±0.03	2.70±0.09	75.97±1.95	21.66±0.34	2.38±0.08	3.53±0.08	73.25±2.16	22.06±0.28	1.90±0.08	3.28±0.05	74.12±0.08	22.66±0.41

Table1. The ionic concentrations of medium for SMS and HA coatings at different culture conditions.

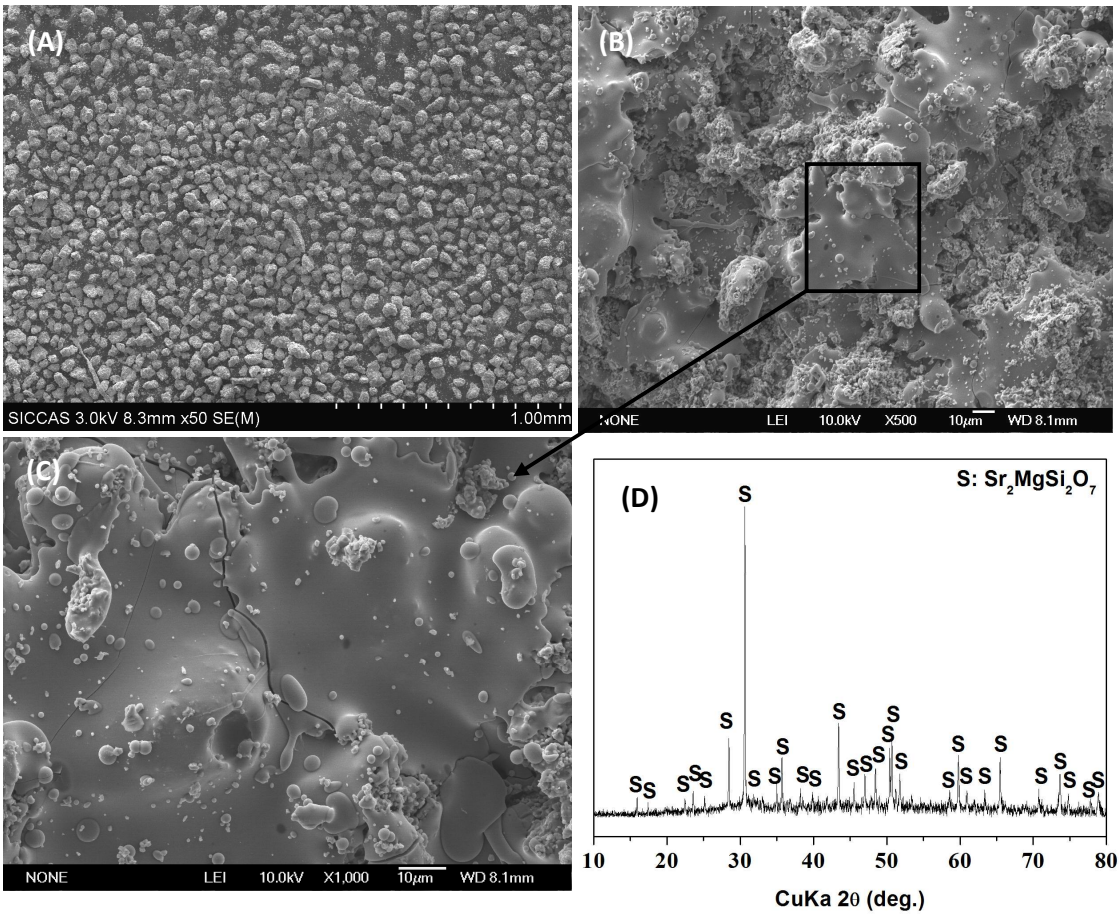

Figure 1. SEM for the prepared $Sr_2MgSi_2O_7$ particles (A) and coatings (B, C). (C) is higher magnification image of (B). XRD analysis for the prepared $Sr_2MgSi_2O_7$ coatings on Ti alloys (D), in which S stands for the characteristic peaks of crystal phase $Sr_2MgSi_2O_7$ in the XRD pattern.

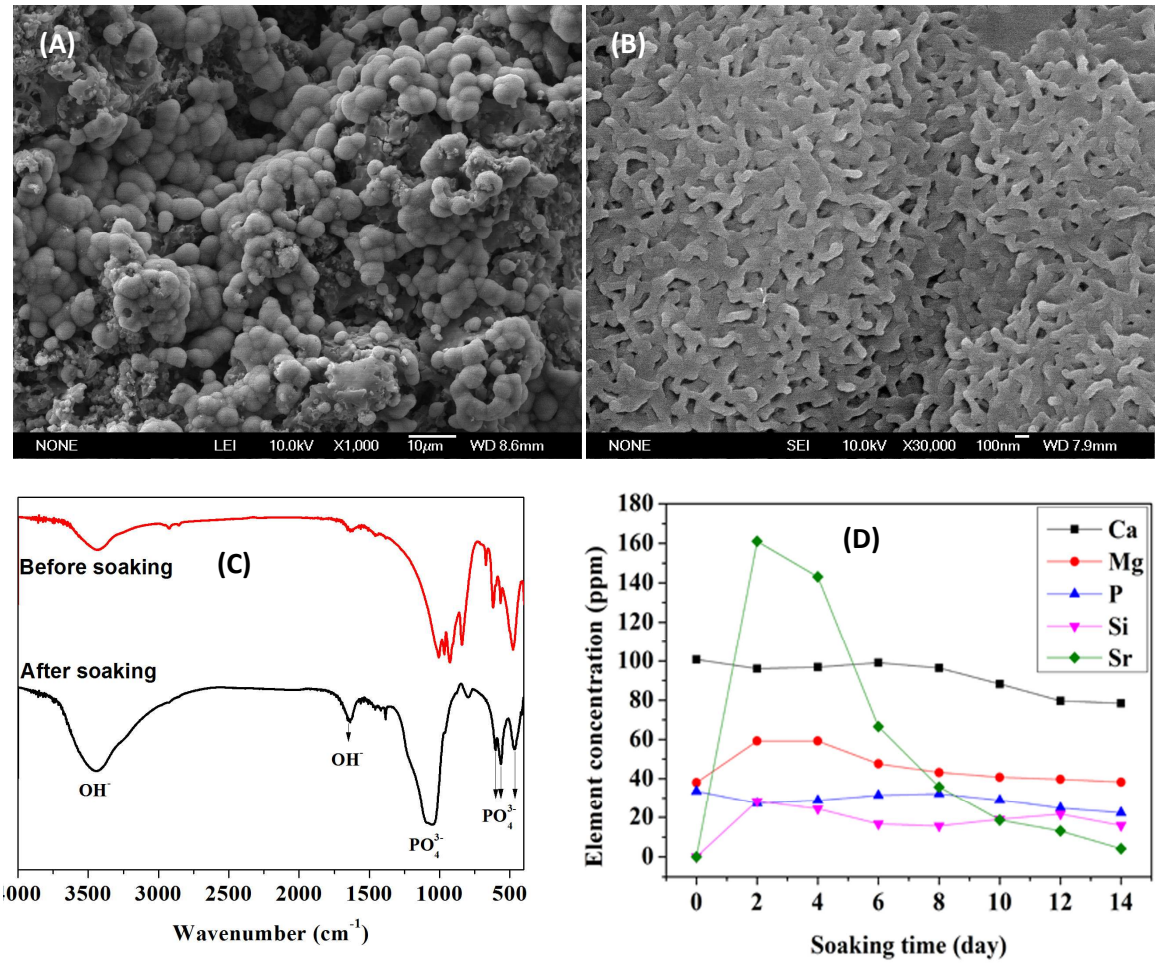

Figure 2. SEM (A, B) and FTIR (C) analysis for the prepared $Sr_2MgSi_2O_7$ coatings on Ti alloys after soaked in SBF for 14 days. ICP-AES analysis for the change of ionic concentrations in SBF soaked with $Sr_2MgSi_2O_7$ coatings (D).

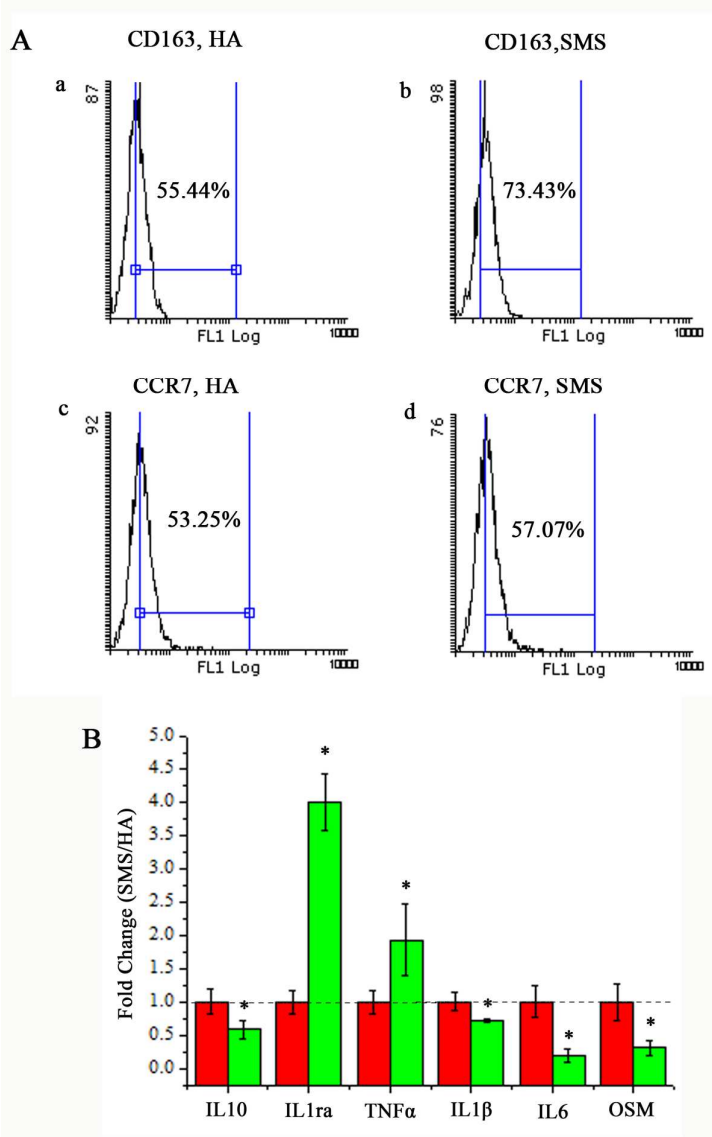

Figure 3. (A). FACS results of RAW 264.7 cells cultured in different coatings. The mean fluorescence intensity of CD163 increased after the stimulation of SMS (a, b); however, the mean fluorescence intensity of CCR7 had only slight increase under the same treatment (c, d). (B). Fold changes of inflammation related genes IL10, IL1ra, TNFα, IL1β, IL6 and OSM, by comparing RAW 264.7 cells cultured in SMS coating with HA (HA group has been standardized as 1, see red bar). *: Significant difference ($P < 0.05$).

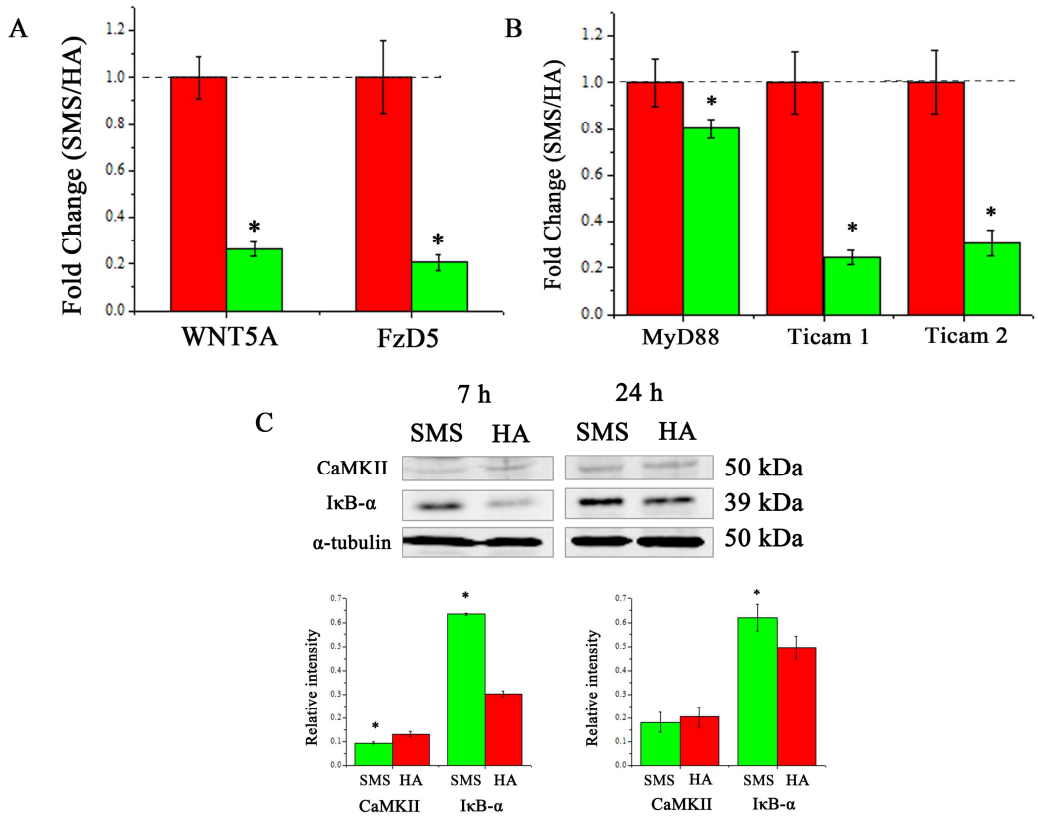

Figure 4. (A) Fold changes of WNT5A/Ca^{2+} pathway related genes: WNT5A and Fz5; (B) Fold changes of Toll-like pathway related genes: MyD88, Ticam1 and Ticam2; (C). Western blotting analysis of CaMKII and IκB-α expression. *: Significant difference by comparing RAW 264.7 cells cultured in SMS coating with HA ($P < 0.05$). (HA group has been standardized as 1, see red bar in Figure A and B).

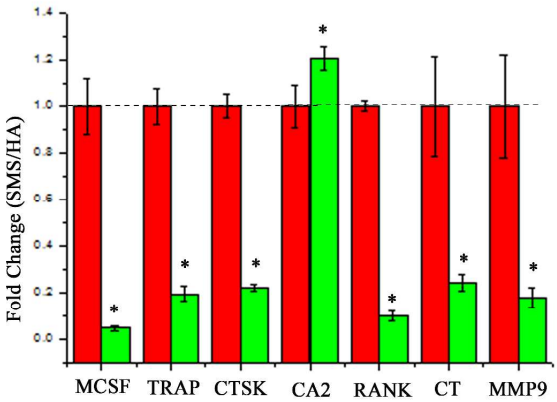

Figure 5. Fold changes of osteoclastogenesis and osteoclast activities related genes: MCSF, TRAP, CTSK, CA2, RANK, CT and MMP9. *: Significant difference by comparing RAW 264.7 cells cultured in SMS coating with HA ($P < 0.05$). (HA group has been standardized as 1, see red bar).

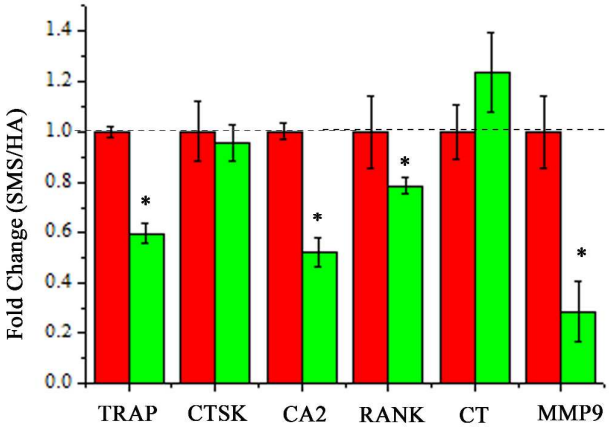

Figure 6. Fold changes of osteoclast activities related genes: TRAP, CTSK, CA2, RANK, CT and MMP9. *: Significant difference by comparing RAW 264.7 cells derived osteoclasts cultured in SMS coating with HA ($P < 0.05$). (HA group has been standardized as 1, see red bar).

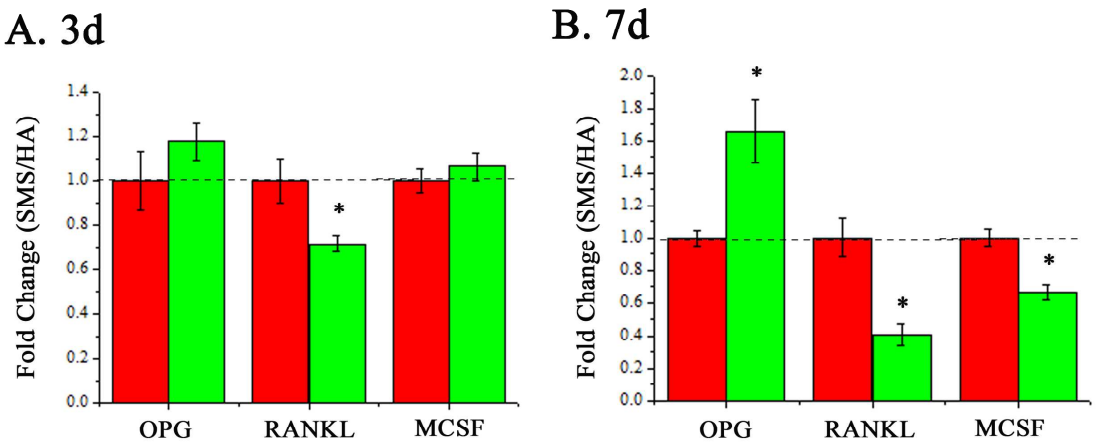

Figure 7. Fold changes of osteoclastogenesis related genes: OPG, RANKL, and MCSF. (A). Day 3, (B). Day 7. *: Significant difference by comparing BMSCs cultured in SMS coating stimulated RAW 264.7 cells conditioned medium with HA coating stimulated RAW 264.7 cells conditioned medium ($P < 0.05$). (HA group has been standardized as 1, see red bar).

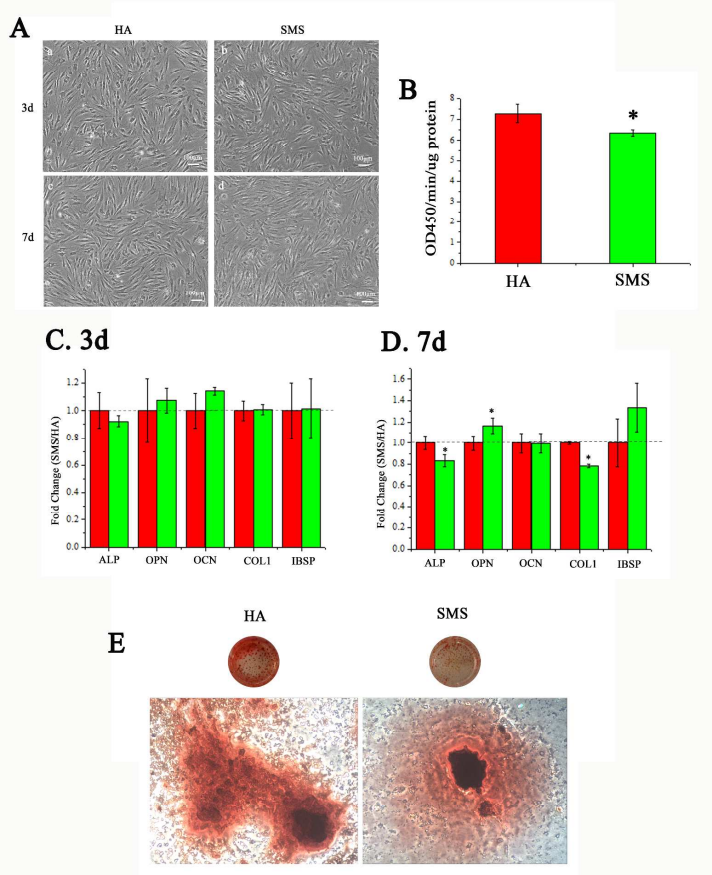

Figure 8. Osteogenic differentiation of BMSCs cultured in SMS and HA coatings stimulated RAW 264.7 cells conditioned medium. (A). morphologies of BMSCs in day 3 and 7; (B). ALP activities; (C&D). osteogenesis related gene expression (ALP, OPN, OCN, COL1, IBSP) by BMSCs in day 3 and 7; (E). alizarin red results. *: Significant difference by comparing BMSCs cultured in SMS coatings stimulated RAW 264.7 cells conditioned medium with HA coatings stimulated RAW 264.7 cells conditioned medium ($P < 0.05$). (HA group has been standardized as 1, see red bar).

Table of contents graphic

Sr, Mg and Si-containing bioceramic coatings possess high bonding strength and osteogenic bioactivity for inhibiting inflammation, osteoclastogenesis