INTRODUCTION

The use of implants for the replacement of missing teeth were properly documented for number of years. Titanium (Ti), which possess magnificent mechanical properties and biocompatibility is widely applied for dental implant materials 1. Nevertheless, titanium is considered as bioinert material without satisfactory osteoconductivity. As a result, many efforts are being devoted to approaches for the modification of Ti surface in order to attain desirable biological responses 2-5. The incorporation of bioactive ions into modified titanium surface obtained by surface modifications is a promising approach for achieving strong and rapid implant osseointegration.

In the human body, strontium (Sr) is a vital trace element primarily exited in bone 6. Many studies revealed that Sr can promote bone formation and inhibit bone resorption 7. In recent researches, Sr has been experimentally incorporated into various bone implantation biomaterials. Various researches have proven that strontium-doped hydroxyapatite (HA) increased the mineralization capacity and alkaline phosphatase (ALP) activity 8-10. The other research revealed that Sr-incorporated coating on the surface of titanium using electrostatic immobilization methodology showed great osteogenic activity and biocompatibility 11. Furthermore, Sr-incorporated Ti oxide surfaces obtained by hydrothermal techniques promoted osteoblast activity and enhanced bio-mechanical fixation of implants in a rabbit model 12.

The preparation of the Sr-incorporated Ti oxide layer can be obtained through different ways, for instance, plasma spraying, laser deposition, hydrothermal process, electrochemical deposition, magnetron sputtering as well as sol-gel methodology 13-18. As one way to employ bioactive ions in the surface modification of Ti implants, magnetron sputtering is not just able enough to make Sr ions deposition with foreseeable release values, it is also capable of maintaining a high mechanical stability 19.

In recent times, we coated Sr ions on surface of SLA by magnetron sputtering methods and predicted an improved bioactivity.

MATERIALS AND METHODS

Preparation of Ti disks
Grade 2 commercially pure titanium disks (15 mm in diameter, 1 mm in thickness) were prepared by machining to fit snugly into 24-well plates. The pure titanium disks were roughed by sand blasting and acid etching (WEGO Jericom Biomaterials, Weihai, Shandong, China). The detailed surface modification has been previously described in other studies 20. Magnetron sputtering treatment has been used for obtaining Sr-incorporated ions onto SLA surface. Magnetron sputtering was performed with an industrial physical vapor deposition system (School of Mechanical...
Engineering, Shandong University), with material deposited from a sputtering target consisting of SrTiO₃ powder with a purity of 99.99% (ZhongNuo Advanced Material Technology, Beijing, China). The films were deposited under the following conditions: argon with 0.5 Pa gas composition, 80W power density, 2,700 s deposition time, and 5.5 cm target-substrate distance. SLA combined with magnetron sputtering were treated as experimental group and untreated plates were used as controls. These disks were ultrasonically cleaned using acetone, ethanol, and deionized water, in an order, followed by sterilization with the cobalt-60 irradiation.

Surface characterization
The surface of specimens was examined using scanning electron microscopy (SEM; Sigma 300, ZEISS, Germany). The chemical structure of two types of surface were examined using X-ray energy dispersive spectrometry (EDS; SU70, Hitachi, Tokyo, Japan).

Cell culture
MC3T3-E1 cells (purchased from Cell Bank of the Chinese Academy of Sciences, China) were cultured in α-MEM (HyClone, USA), comprising 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, and 100 mg/L streptomycin and maintained at temperature of 37°C with humid atmosphere comprised of 5% CO₂ and 95% of air. After every three days the medium of culture was changed until the cell attained a confluence between 80–100%.

Cell morphology
MC3T3-E1 cells were seeded on the Ti disks for 2 h in 24-well culture plates at an seeding density of 2×10⁴ cells per well. The morphology of cell on test surfaces was observed at 24 h by SEM (SU8010, Hitachi). Before the SEM assessment, disks with attached MC3T3-E1 cells were fixed with 2.5% glutaraldehyde in cacodylate buffer, dehydrated in a sequential series of ethanol, and then critical point dried and coated with Au/Pd. Before the SEM assessment, disks with attached MC3T3-E1 cells were fixed with 2.5% glutaraldehyde in cacodylate buffer, dehydrated in a sequential series of ethanol, and then critical point dried and coated with Au/Pd.

Cell attachment was assessed by the actin staining. At 24 h following seeding the MC3T3-E1 cells were in 24-well culture plates at a seeding density of 2×10⁴ cells per well on test surfaces, the Ti disks were cleaned using PBS, later on disks were fixed in 4% of formaldehyde nearly for 30 min at normal room temperature. Thereafter, 0.1% triton X-100 was used for permeabilizing cells for 10 min and then two PBS rinses were used. Non-specific binding sites were blocked with 1% BSA in PBS for 1 h. The fixed cells were incubated with 200 μL rhodamine-phalloidin in a humidified chamber for 40 min at room temperature. The staining of cell nuclei was conducted through incubating with 0.1 μg/mL DAPI for around 5 min followed by three PBS rinses. Cells were examined using confocal laser-scanning microscopy (Olympus BX40, Olympus, Tokyo, Japan).

Cell proliferation assay
In order to evaluate cell proliferation, cells were cultured on Ti disks in 24-well culture plates at a seeding density of 2×10⁴ cells per well. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was employed for assessing cell proliferation. After 1, 3, 5, and 7 days, MTT (Sigma, St. Louis, MO, USA) was added (final concentration 0.5 mg/mL) and incubated at 37°C for 4 h to form formazan, which was then dissolved by using 150 mL dimethyl sulfoxide. One hundred microliter aliquots of this solution were collected into a 96-well plate, absorbance values were read at 570 nm wavelength using an automated microplate reader (Bio-Rad, Hercules, CA, USA).

ALP activity and mineralization assay
The ALP activity was examined at 7 and 14 days by evaluating the transformation of p-nitrophenyl-phosphate (pNPP; Sigma) into p-nitrophenol (pNP). At the fixed time points, the cells were lysed with 0.1% triton-X-100 for 4 h of incubation at 37°C. A 50 mL sample was mixed with 50 mL of freshly prepared pNPP substrate (1 mg/mL) and incubated in 37°C water bath and timed. The reaction was stopped by the addition of 0.4 M NaOH and the amount of pNP released was estimated by measuring the absorbance at 405 nm. The activity of ALP was calculated from a standard curve after normalizing to the total protein content, which was calculated by using a Pierce BCA Protein Assay Kit (Sigma).

Alizarin red staining (ARS; Sigma) was employed for measuring the mineralization of cells at 14 and 28 days. Also, cells were cleaned three times in the PBS and fixed with 4% paraformaldehyde (Sigma) for 30 min. Thereafter, the mixture was stained with 2% alizarin red-S (pH 4.2) for 20 min at room temperature. The non-specific stain was removed through repeated washes with distilled water. In order to quantify, the bound stain was dissolved using 10% of (wt/vol), cetylpyridinium chloride (Sigma). The rinse was gathered in 96-well plate and values of absorbance were studied at 570 nm using an automated microplate reader (Bio-Rad).

Statistical analyses
Each experiment was repeated for 3 times. Statistical examination was conducted by SPSS (20.0 version for Windows). The means and standard deviations of data were measured. One-way variance plus LSD analysis was used for examining differences between groups. p<0.05 was considered significant statistically.

RESULTS
Physicochemical properties of strontium on Ti surface
The morphology of SLA surface and Sr-SLA surface were examined clearly using SEM test. The SEM analysis showed that both SLA surface and Sr-SLA demonstrated typical structure contained craters 100 μm in diameter overlapped through pits with 1–3 μm in diameter. Whereas, in between both surfaces no any optical variances were found (Fig. 1). The chemical structure of surfaces analyzed via EDS revealed that Sr-SLA surface mainly featured O, Ti, and Sr components, whereas, the
SEM micrographs showing the surface roughness of SLA (A) and Sr-SLA (B) surfaces at magnifications of 1,000×. SEM shows there were some irregular pores on the surface of SLA. The diameter of the pits with diameter of about 100 μm was about 1–3 μm. The surface of Sr-SLA is similar to that of SLA.

Fig. 2 EDS of SLA (A) and Sr-SLA (B). O, Ti and Al elements appeared on the surface of SLA, Sr element appeared on the surface of Sr-SLA in addition to the original O and Ti elements in SLA group.

Table 1 Result of the Ti, O, Sr and Al element concentration determined by X-ray energy dispersive spectrometry which was expressed as atomic percentage (%)

| Groups  | Ti     | O      | Sr   | Al     |
|---------|--------|--------|------|--------|
| SLA     | 80.23±3.55 | 19.26±1.23 | —   | 0.51±0.32 |
| Sr-SLA  | 67.29±2.98 | 30.64±2.01 | 2.07±0.14 | —      |

(n=8)

SLA surface featured O, Ti, and Al components (Fig. 2). The findings revealed that proximately 2.07±0.14 at% of Sr was incorporated into the Sr-SLA surface following the treatment by magnetron sputtering (Table 1).

Osteoblast morphology and adhesion
The morphology of cell was examined through SEM. With the seeding of 24 h, differences in cell morphology could be noticed. The cells on the Sr-SLA surfaces were more extended than those on SLA surfaces. As shown in Fig. 3, Cells on Sr-SLA surface exhibited a polygonal shape, whereas those on SLA surface exhibited a bipolar spindle-like morphology.

The fluorescence microscopy images of MC3T3-E1 cells following 24 h cultivation has been examined to analyze the cell growth states on different samples. As demonstrated in Fig. 4, on both of the surfaces the growth of MC3T3-E1 cells is good. While comparing with surface SLA, it can be observed that density and spreading of MC3T3-E1 cells increased significantly. The results are consistent with the SEM.

Growth of osteoblasts
The result of different surfaces on MC3T3-E1 cells proliferation was analyzed at 1, 3, 5 and 7 days. As exhibited in Fig. 5, the number of cells raises with time on both surfaces. No significant difference was observed between two surfaces at 1 and 7 days. At 3 and 5 days, cell numbers on Sr-SLA surface significantly enhanced compared with that of SLA surface.
Fig. 3  SEM images of MC3T3-E1 cells 24 h after seeding on SLA (A) and Sr-SLA (B) surfaces.

Fig. 4  Confocal microscopic images of MC3T3-E1 cells 24 h after seeding on SLA (A) and Sr-SLA (B) surfaces.

Fig. 5  MC3T3-E1 proliferation on SLA and Sr-SLA surfaces after 1, 3, 5, 7 days of culture (values are mean±SD, n=5). *Refers to \( p < 0.05 \) 3 days SLA versus 3 days Sr-SLA,  #Refers to \( p < 0.05 \) 5 days SLA and 5 days Sr-SLA.

Fig. 6  Alkaline phosphatase (ALP) activity of MC3T3-E1 cells on SLA and Sr-SLA surfaces after 7 and 14 days (values are mean±SD, n=3). *Refers to \( p < 0.05 \) versus 7 days SLA,  #Refers to \( p < 0.05 \) versus 14 days SLA.

**ALP activity and mineralization of osteoblasts**

The ALP activity was employed for evaluating early osteoblastic differentiation. As exhibited in Fig. 6, the ALP activity of MC3T3-E1 cells cultured on surface Sr-SLA was significantly higher compared with those on surfaces of SLA at days 7 and 14. However, for two groups, the activity of ALP at 14 days were markedly lower compared to those at 7 days. Calcium deposition of MC3T3-E1 was used to analyze the mineralization of osteoblasts. As exhibited in Fig. 7, the Sr-SLA group showed significantly increased values compared to those on surfaces of SLA at 28 days, although at 14 days, no difference was found between both groups.
improving osteoblast activities can be analyzed by the atomic ratio of Sr coating due to the low total Sr concentration in the surface oxide biomaterials, especially in the surface of oral implants, systemic effects of Sr occur at an acceptable level in the benefits for bone development. Furthermore, the adverse outcomes, while lower Sr dose has been observed with showed that higher Sr dose may result in toxic systemic development as an alternative methodology. Local use can avoid many potential negative results, for instance, deleterious epidermal necrolysis, together with drug rash with eosinophilia as well as systemic symptoms disorder. In addition to the systemic application of strontium, the local application of strontium is considered as an alternative methodology. Local use can avoid many potential negative results, for instance, deleterious epidermal necrolysis, together with drug rash with eosinophilia as well as systemic symptoms disorder.

Sr is a natural component of food and beverages. Current studies have also showed that Sr not only possess the capacity to promote mesenchymal stem cell (MSC) commitment to lineage bone, it also has the ability for suppressing the MSC commitment to chondrocytes and adipocytes. In addition to the systemic application of strontium, the local application of strontium is considered as an alternative methodology. Local use can avoid many potential negative results, for instance, deleterious epidermal necrolysis, together with drug rash with eosinophilia as well as systemic symptoms disorder. It has been demonstrated that the local application of strontium incorporated into Ti surface can improve bone development in vitro as well as in vivo test. Studies also showed that higher Sr dose may result in toxic systemic outcomes, while lower Sr dose has been observed with benefits for bone development. Furthermore, the adverse systemic effects of Sr occur at an acceptable level in the case of the surface modifications of bone-implantation biomaterials, especially in the surface of oral implants, due to the low total Sr concentration in the surface oxide layer. In our research the atomic ratio of Sr coating on surface of SLA was controlled with 2%. Generally, the effect of Sr incorporation into bioactive layer for improving osteoblast activities can be analyzed by the preliminary amount of Sr in weight, in atomic or in molar concentration. In this study, the amount is detailed in atomic percentage. Although we showed the atomic ratio of Sr coating on surface of SLA, the thickness of Sr layer was not examined. Li et al. developed a hierarchical hybrid micro/nanorough strontium-loaded Ti (MNT-Sr) surface also fabricated by magnetron sputtering. They found that the strontium ion content on the MNT-Sr surface were 2.73±0.55 and 3.6±0.28 at% Sr on the NT-Sr sample, which was the similar with our results. They investigated the cross section of NT-Sr and MNT-Sr samples from SEM images and indicated that the thicknesses of Sr layers were approximately 20–40 nm. This may have some implications for us.

Magnetron sputtering is widely applied in different industrial applications with great success. The coating was prepared with the use of this treatment that had high bonding strength at the interface and various inorganic components could be deposited on the materials by changing the targets. With these methods, we created a new surface different from surface of classical SLA in chemistry component. Nonetheless, no clear differences were found in between topography of both surfaces before and after the process, probably because porous composition of surfaces was significantly unaltered and quantity of Sr ions was modified little after the treatment or formation parameters was quite dissimilar. Based on differences from this research, Offermanns et al. developed the fabrication of titanium based coatings comprising Sr and oxygen surface based on a magnetron co-sputtering process, structural and chemical composition of the coatings differences were all detected. Li et al. presented a Sr-loaded Ti surface prepared by hydrofluoric acid etching, following treatment with magnetron sputtering that not just altered the chemistry element of material surface, it also gained a nano-structured evidence. In comparison, the other research compared biological results of Sr-SLA surface fabricated by hydrothermal method and SLA surface, which also observed that Sr-SLA surfaces demonstrated more densely dotted nano-structures composed of heterogeneous grains having approximately 50 nm size in diameter. The difference from these studies may be explained by the specific preparation parameters.

The first step of cellular interaction with underlying substrates is cell adhesion. Researches have suggested that the improvement of activities in the early osteoblast attachment and spreading is significant for next differentiation of osteoblasts. In this research, Sr-SLA surfaces considerably enhanced the early osteoblasts spreading and improved osteoblast development. Our findings are in consistent with other researched demonstrating the effects of Sr on osteoblast response. Park et al. proposed that Sr-incorporated oxide layer (SrTiO₃) fabricated by hydrothermal treatment remarkably enhanced attachment, spreading, focal adhesions of mouse BMSCs than those with an untreated grit blasted microrough Ti surface. Liu et al. observed that Sr-modified Ti layer enhanced the osteoblasts

![Fig. 7 The mineralization of MC3T3-E1 cells on SLA and Sr-SLA after 14 and 28 days (values are mean±SD, n= 3). *Refers to p<0.05 versus 28 days SLA.](image-url)
spreading, promoted the early adherent and improved osteoblast development. Panzaovolta et al. also found that Sr has a slight positive effect on the initial adherent cell number. Previous studies have revealed dose-dependent Sr effects on osteoblasts. Tian et al. showed that strontium ranelate-loaded chitosan film enhanced the development of osteoblasts at lower concentration (2 and 20 mmol/L), but suppressed the development of osteoblasts at higher concentration (40 and 80 mmol/L). In this research, the atomic ratio of Sr-coatings on surface of SLA was balanced with 2%, which is low dose and the result was consistent with the above research. In the present research, we also examined the effects of Sr-SLA surface on osteoblast differentiation in vitro. The ability of ALP and mineralization were widely used as markers for early and late osteoblasts differentiation. In this research, the surfaces of Sr-SLA exhibited considerably increasing activity of ALP and increasing deposition of calcium. Nonetheless, the degree of ALP activity reached its peak at 7 days and subsequently declined little after 14 days, which was an interesting view. It can be explained that ALP as is known as early osteoblastic differentiation marker, and with the mineralization process, decline of ALP activity can be found. Being consistent with our findings, Zhang et al. showed that Sr-substituted hardystonite (Sr-HT) ceramic coating with a hierarchical topography enhanced the ALP activity and in vitro mineralization ability. Qiu et al. observed that Sr-doped calcium polyphosphate (SCPP) scaffolds enhanced proliferation and the ALP activity of osteoblastic cells as well. Capuccini et al. also revealed that Sr-doped HA thin film on titanium substrates obtained by pulsed-laser deposition considerably increased the attachment, proliferation, and ALP activity of osteoblast-like MG63 cells. Being consistent with in vivo studies, the in vitro results confirmed the enhanced bioactivity of Sr coatings as evident in the enhanced ALP activity and mineralization ability of osteoblasts. According to these findings, it is expected that surface of Sr-SLA surface prepared through magnetron sputtering approach will enhance osteoblastic cell activities in vitro. However, potential molecular mechanisms were not discussed. As yet, strontium’s mechanisms of action on osteoblasts are not fully understood. Strontium is believed to have more than one mechanism of action. Several studies have proved that strontium can stimulate the calcium-sensing receptor, CaSR, which triggers mitogenic signals leading to proliferation, differentiation, and activation of the osteoblasts. Fromigué et al. indicated that strontium ranelate-induced activation of osteoblast replication and survival is mediated, at least in part, by ERK1/2 and Akt signalling and ‘PGE2 production’, independent of CaSR expression. In future, detailed research needed to perform for attaining convincing conclusions as well as specific molecular mechanisms should be taken into consideration on these effects in vitro.

CONCLUSION

A Sr-incorporated coating on a Ti surface was successfully fabricated by magnetron sputtering treatment. MC3T3-E1 cells were cultured on Sr-SLA surface that revealed improved attachment, proliferation and osteoblast differentiation compared to those on SLA surface. These results suggest that presence of strontium in moderately rough surface can enhance the positive effect of osteoblast behaviors and this may develop a new method for treating titanium implant.

ACKNOWLEDGMENTS

This research was supported by Shandong Medical science and Technology Development Program (Grant No: 2016WS0694) and Yantai major research and development projects (Grant No: 2017YD026).

REFERENCES

1) Niinomi M. Mechanical biocompatibilities of titanium alloys for biomedical applications. J Mech Behav Biomed Mater 2008; 1: 30-42.
2) Jinno T, Kirk SK, Morita S, Goldberg VM. Effects of calcium ion implantation on osseointegration of surface blasted titanium alloy femoral implants in a canine total hip arthroplasty model. J Arthroplasty 2004; 19: 102-109.
3) Cheng H, Xiong W, Fang Z, Guan H, Wu W, Li Y, et al. Strontium (Sr) and silver (Ag) loaded nanotubular structures with combined osteoinductive and antimicrobial activities. Acta Biomater 2016; 31: 388-400.
4) Wu C, Chen Z, Wu Q, Yi D, Friis T, Zheng X, et al. Clinoenstatite coatings have high bonding strength, bioactive ion release, and osteomunomodulatory effects that enhance in vivo osseointegration. Biomaterials 2015; 71: 35-47.
5) Tsui YC, Doyle C, Clyne TW. Plasma sprayed hydroxyapatite coatings on titanium substrates. Part 1: mechanical properties and residual stress levels. Biomaterials 1998; 19: 2015-2029.
6) Marie PJ, Felsenberg D, Brandi ML. How strontium ranelate, via opposite effects on bone resorption and formation, prevents osteoporosis. Osteoporos Int 2011; 22: 1659-1667.
7) Dahl SG, Allain P, Marie PJ, Mauraus Y, Boivin G, Aumann P. Incorporation and distribution of strontium in bone. Bone 2001; 28: 446-453.
8) Moghanian A, Firoosz S, Tahirii M, Sedghi A. A comparative study on the in vitro formation of hydroxyapatite, cytotoxicity and antibacterial activity of 58S bioactive glass substituted by Li and Sr. Mater Sci Eng C Mater Biol Appl 2018; 91: 349-360.
9) Li J, Yang L, Guo X, Cui W, Yang S, Wang J, et al. Osteogenesis effects of strontium-substituted hydroxyapatite coatings on true bone ceramic surfaces in vitro and in vivo. Biomed Mater 2017; 13: 015-018.
10) Yin P, Feng FP, Lei T, Zhong XH, Jian XC. Osteoblastic cell response on biphasic fluorhydroxyapatite/strontium-substituted hydroxyapatite coatings. J Biomed Mater Res A 2014; 102: 621-627.
11) Yuan N, Jia L, Geng Z, Wang R, Li Z, Yang X, et al. The incorporation of strontium in a sodium alginate coating on titanium surfaces for improved biological properties. Biomed Res Int 2017; 2017: 9867819.
12) Park JW, Kim HK, Kim YJ, Jang JH, Song H, Hanawa T. Osteoblast response and osseointegration of a Ti-6Al-4V alloy implant incorporating strontium. Acta Biomater 2010; 6: 2840-2851.
