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Original Article

Effect of biomineralization on the proliferation and differentiation of MC3T3-E1 cells grown on a titanium surface

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Abstract: The purpose of this study was to investigate the effect of sand blasting/acid etching and biomineralization on the proliferation and differentiation of MC3T3-E1 cells grown on a titanium surface. In this study, a surface of pure titanium was pretreated with sand blasting and acid etching (SLA), and simulated body fluid (SBF) was used as a biomineralization solution to deposit inorganic minerals. Scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS) was used to observe the titanium surface morphology and analyze the surface element composition. MC3T3-E1 cells were seeded on the titanium surfaces, and adhesion, proliferation, and differentiation of the cells were evaluated by fluorescent staining, MTT, and ALP activity assays. SEM results demonstrate that sponge-like structures with uniform distribution were present on SLA-treated surfaces, and large areas of small, needle-like nanoparticles were observed on the biomineralized surfaces. It was evident from cell culture of the different samples that adhesion, proliferation, and differentiation of MC3T3-E1 cells on biomineralized surfaces was significantly higher ($P<0.05$) than on the SLA-treated surfaces. All experimental results in this study indicate that the porous nanosphere structure on the titanium surface demonstrates excellent biocompatibility for biomedical implant applications. Furthermore, the surface treatment of SLA and biomineralization on Ti implants may be useful in further research on titanium surface treatments.

Keywords: Biomineralization, SBF, MC3T3-E1, porous nanosphere

Introduction

Implant surface morphology is one of the most important factors in implant osseointegration [1]. A micro-scale rough surface is widely considered to be favorable for the attachment, proliferation and differentiation of osteoblasts [2, 3]. Recent studies have shown that the best implant-body interface adherence is achieved using hybrid micro-nano and multimodal surfaces [4].

Calcium phosphate coatings have the advantage of facilitating interactions between cells and biomaterials. Many techniques have been used to prepare calcium phosphate coatings, such as plasma spraying, ion implantation, sol-gel, electrophoretic deposition, sintering, biomineralization, and electrochemical deposition. Among these techniques, plasma spraying is the most widely reported method, and performance of these coatings compared with other titanium surfaces has been reported both *in vivo* and *in vitro* [5]. Ong et al. [6] reported a significantly higher bone contact length for hydroxyapatite (HA) implants compared with titanium plasma spray implants in normal bone for both the periods they considered (12 weeks after implantation and 1 year after loading). However, the results from long-term studies of plasma spraying are less encouraging, because of the lack of absorbability, and the thickness [7, 8]. Therefore, recent studies have attempted to develop calcium phosphate coatings that are thinner [9] and absorbable at near-physiological pH and temperature [10].

Simulated body fluid (SBF) was first reported by Kokubo et al. The the *in vivo* bone bioactivity of a material can be predicted by examining apa-

tite deposition on its surface in SBF [11]. SBF has also been widely used in biomineralization on titanium surfaces *in vitro*. After immersion in SBF in a biomimetic environment (37°C; 5% CO_2 + 95% O_2) for a period of time, a layer of HA coating with a thickness of 10-15 μm and crystallinity of ~55% was found on the titanium plates [12].

Biomineralized HA combines the advantages of metal and HA, and therefore, has excellent mechanical properties and biological activity. Moreover, biomineralization can produce nano-HA, which has favorable biological properties on titanium surfaces [10]. However, biomineralization also has certain disadvantages, such as the requirement for the surface treatment of titanium specimens. Pretreatment, such as alkali-heat treatment, acid etching or electrochemical treatment, is needed to activate the sample surfaces, and to ensure the deposition of calcium and phosphorus.

The goal of this study was to develop a surface treatment method combining SLA and biomineralization to construct a micro/nano-HA coating on a titanium surface, which shows greater bioactivity, as well as biocompatibility, in *in vitro* culture of MC3T3-E1 osteoblasts.

Materials and methods

Sand blast and acid etching (SLA) treatment

Pure titanium disks of 8-mm diameter and 1-mm thickness were used. The roughened surface was produced as follows: the surface was polished, sandblasted with TiO_2 (diameter, 178 μm), washed in acetone, 75% alcohol, and distilled water in an ultrasonic cleaner for 20 minutes, soaked in a mixed solution of HCl/H_2SO_4 for 18 minutes at 60°C, washed with distilled water, and dried at 90°C.

Biomineralization treatment

The preparation of SBF was as follows: dissolving reagent-grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$ and Na_2SO_4 in ion-exchanged and distilled water, followed by adjusting the pH of the solutions by adding 1M-HCl and Tris to pH 7.40 exactly, at 36.5°C, as previously described [12].

To produce the biomineralized HA coating, the roughened specimens were soaked in SBF solution at 37°C for 2 weeks with the SBF solution replaced daily, then removed from the solution, rinsed with ion-exchanged, distilled water, and dried at 37°C.

Surface analyses of samples

Scanning electron microscopy/energy-dispersive spectroscopy (SEM/EDS) was used to assess the surface morphology and perform the elemental analysis of the samples (S4800, Hitachi, Ltd.).

In vitro cell culture and mineralization induction of MC3T3-E1 osteoblasts

MC3T3-E1 cells are an immortalized cell line derived from mouse calvarium tissue [13]. MC3T3-E1 cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL vitamin C and 10 mM β-sodium glycerin phosphate, and incubated at 37°C in a humidified atmosphere with 5% CO_2 v/v. The cell suspension (density, 1×10^5 cells/mL) was seeded onto the surface of a sterilized coverslip and cultured for 14 days to induce mineralization. Subsequently, the cells were stained with 1% $AgNO_3$ and 2% sodium thiosulfate using the Von Kossa method [14] to detect phosphate deposits. Meanwhile, the plates were fixed with ice-cold acetone and stained with Alizarin red to detect calcium nodules.

Alkaline phosphatase (ALP) activity was evaluated by ALP staining. The cell slides were fixed in acetone, incubated at 37°C for 6 hours in a solution composed of 6 mL 3% β sodium glycerin, 6 mL 2% sodium pentobarbital, 9 mL 2% calcium chloride, 6 mL 2% magnesium sulfate, and 3 mL distilled water. The slides were soaked in 2% cobalt nitrate for 5 minutes and 1% ammonium sulfide for 2 minutes, then dehydrated using graded ethanol, cleared in dimethylbenzene and sealed with neutral gum.

Cell adhesion and spreading

Cell morphology was observed by confocal laser scanning microscopy (CLSM) after 2 days of culture. The cell suspension (density 1×10^5 cells/mL) was seeded onto the titanium plate surface and then cultured for 48 hours to induce mineralization. The specimens were then retrieved and subjected to immunofluorescence staining with 5 μg/mL fluorescein

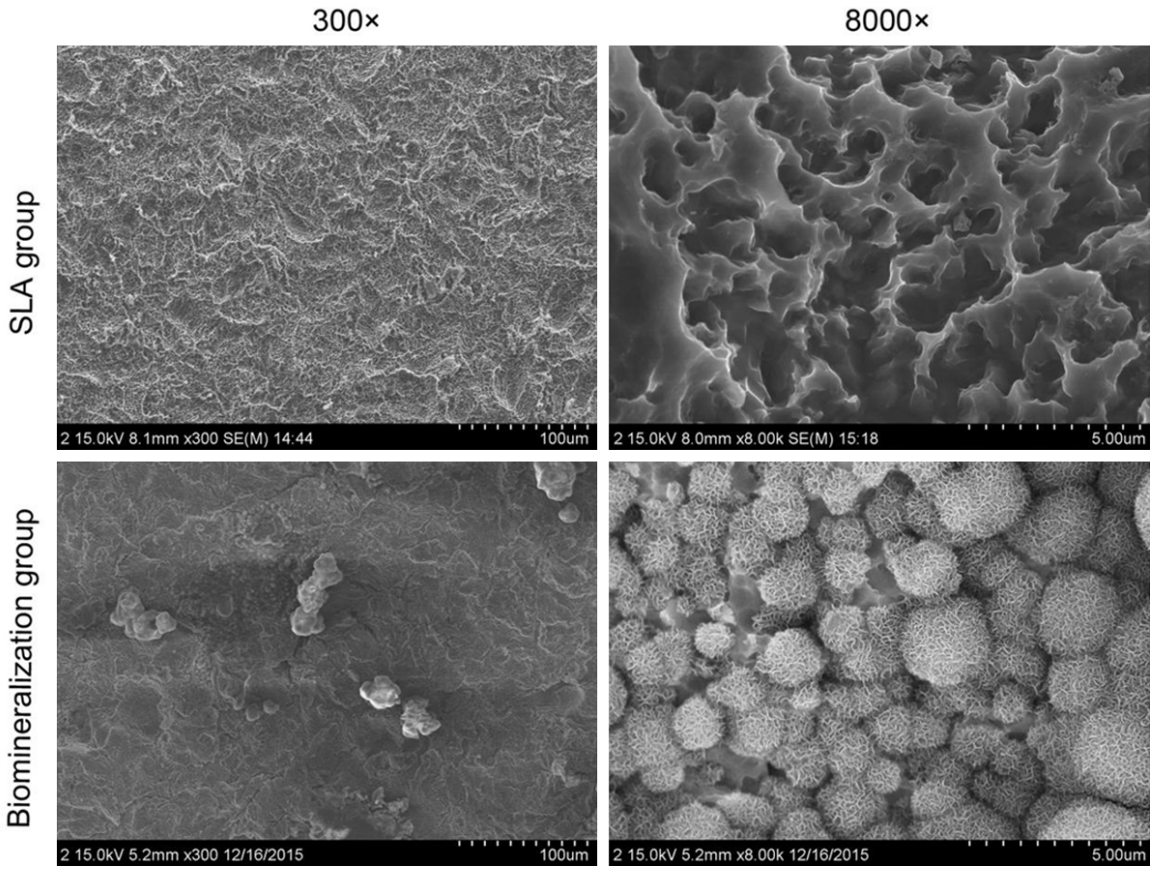

Figure 1. Surface micrograph of SLA and biomineralization groups (SEM, 300 × and 8000 × magnification).

isothiocyanate-phalloidin and propidium iodide, and examined using a confocal laser scanning microscope (FV1000, Olympus Corporation).

MTT cell proliferation assay

Cell suspensions (density 5 × 10^4 cells/mL) were seeded onto the titanium plate surfaces and cultured for 1, 3, 5, and 7 days. After the addition of 100 μl MTT (5 mg/mL), the cells were cultured for a further 4 hours. During this time, viable cells reduced MTT to formazan pigment, which was subsequently dissolved in dimethyl sulfoxide after removal of the culture medium. The absorbance was recorded at 490 nm with a PowerWave XS2 microplate reader (BioTek Instruments, Inc.). The cell growth curve was drawn according to the measured absorbance.

Detection of cell differentiation by ALP activity

Cell suspensions (density 1 × 10^5 cells/mL) were seeded onto the titanium plate surfaces, and cultured for 4, 7 and 14 days for the induction of mineralization. Next, the cell membranes were lysed with 0.2% Triton X-100, and the supernatants were collected and centrifuged at 4°C for 15 min at 12000 × g to precipitate the cell debris. ALP activity of the supernatant extract was detected with an alkaline phosphatase detection kit. Total protein in the sample was measured with a BCA protein assay kit, and then the enzyme-specific activity was calculated according to the following formula: enzyme-specific activity (μmol/min·mg) = enzymatic activity (μmol/min)/mass of total protein (mg).

Statistical analysis

The MTT and ALP activity assay results are presented as the mean ± standard deviation (SD), and were analyzed with SPSS 21.0 software using Student's t test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Surface morphology of titanium plates

SEM images of the samples treated by SLA techniques are shown in **Figure 1**. Observed at

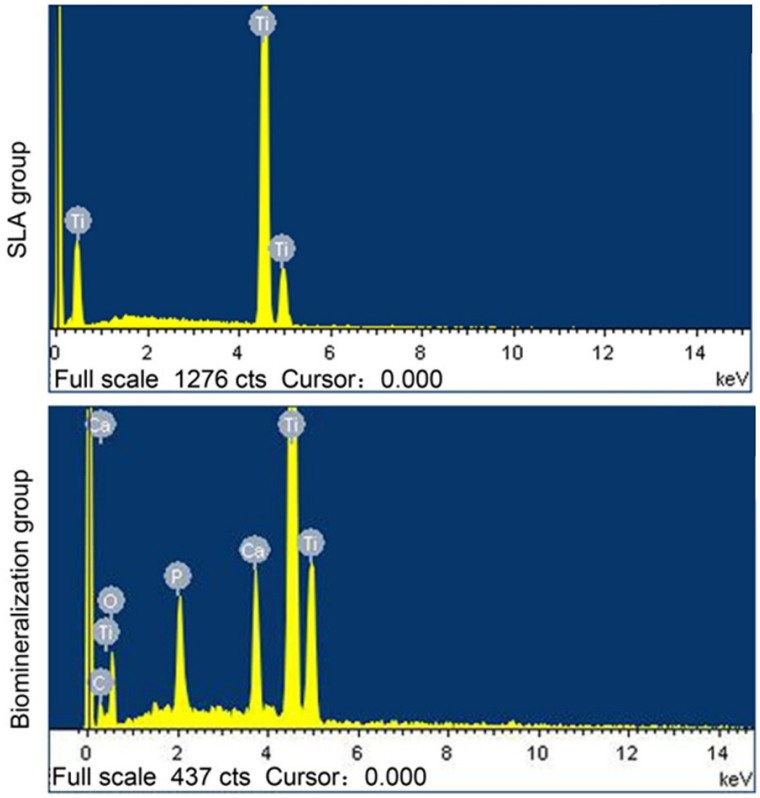

Figure 2. EDS spectrum of SLA and biomineralization groups.

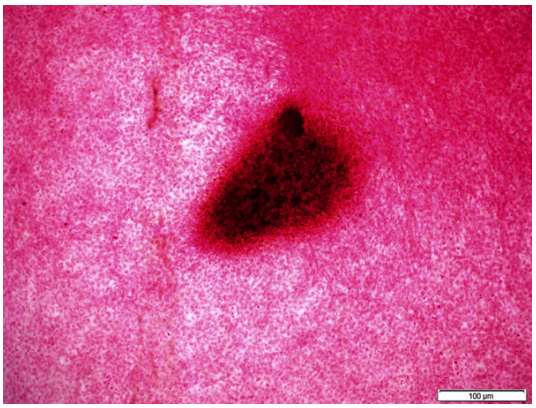

Figure 3. Calcium deposition of nitric acid argentum, and HE staining (light microscope, 40 × magnification).

higher magnification, micropores with diameters of 2-4 μm were formed by sand blasting on titanium surfaces, and secondary cellular micropores with diameters of 0.2-0.5 μm were formed by acid etching. The sponge-like structures, with uniform distribution and blunt edges, also had intercommunicating pores at the bottom of the micropores.

The globular sediment structure of the apatite layer, which had a size range of 1-3 μm, was deposited on the surface of the biomineralized titanium plates. On the micro-nanosphere surfaces, there were numerous needle-like nanoparticles with dimensions of 100 × 40 nm, forming rich porous structures.

Elemental analysis of titanium plates

EDS analysis showed that the surface element of the SLA titanium plates was Ti, and the surface elements of the biomineralized titanium disks were C, O, P, Ca and Ti. The atomic percentage was 9.73% for Ca and 6.24% for P. The Ca/P ratio was 1.56 (**Figure 2**).

Induction of in vitro mineralization in MC3T3-E1 osteoblasts

Inverted fluorescence microscopy showed that, after culture for 14 days, the cells spread in a polygonal shape and covered the entire bottom surfaces of the culture flasks. The results of the Von Kossa method and Alizarin red staining revealed that mineralized nodules had formed on the surface of cells (**Figures 3**, **4**), while ALP staining showed a large number of black granules in the cytoplasm (**Figure 5**). Therefore, a mineralized osteoblast model by inducing MC3T3-E1 cell mineralization *in vitro* was successfully established.

Cell spreading, proliferation and differentiation

CLSM analyses were performed to observe the morphology of MC3T3-E1 cells grown on the surface of SLA or biomineralized titanium disks after culture for 2 days (**Figure 6**). The cells spread in a polygonal shape on the titanium disks, and intercellular connections could be frequently observed on both surfaces. On biomineralized surfaces, actin filaments appeared with less organization than on SLA surfaces, and more cytoplasmic prolongations could be seen.

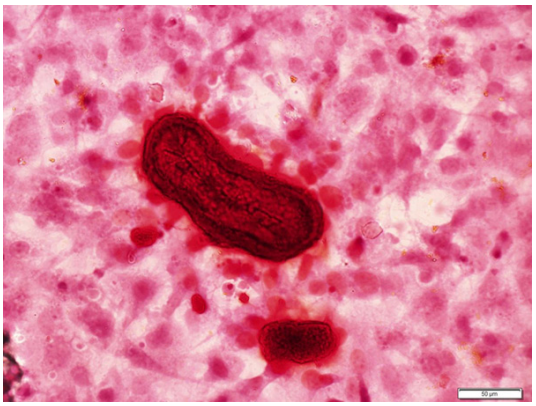

Figure 4. Calcium deposition of Alizarin red staining (light microscope, 100 × magnification).

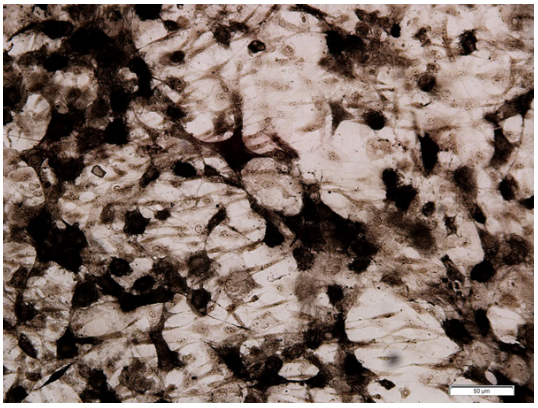

Figure 5. Alkaline phosphatase staining of F-actin (light microscope, 100 × magnification).

According to the growth curve (**Figure 7**), the number of cells on the surface of the titanium disks increased gradually with the duration of culture. It was shown by t-test that the proliferation rate on the biomineralized disks was significantly higher than that on SLA disks on days 1, 3, 5, and 7 ($P<0.05$).

ALP kinetics revealed a significant increase in the enzyme-specific activity on all titanium plates at each time point up to day 14 (**Figure 8**). Cells adhering to the biomineralized titanium surface exhibited significantly higher enzyme activity than those on the SLA surface on days 4, 7, and 14 ($P<0.05$).

Discussion

Sand-blast/acid etching treatment, which can promote the nucleation and growth of crystals during the biomineralization process compared with smooth surfaces, is one of the most widely used surface treatment technologies for titanium implants. Many studies have demonstrated that cell adhesion and proliferation on titanium substrates with micro-scale pores are increased compared with that on polished surfaces [15]. During the acid-etching treatment, the surface passive TiO_2 layer partially dissolves into the solution due to the corrosive effect of the acid:

$$2Ti + 6HCl = 2TiCl_3 + 3H_2\uparrow$$

$$2Ti + 3H_2SO_4 = Ti_2(SO_4)_3 + 3H_2\uparrow$$

The processes of apatite formation on the SLA surface in SBF can be described as follows. The improvement of wettability promotes the hydroxylation of titanium oxides in solution, leading to the formation of Ti-OH groups. Next, the Ti-OH functional groups attract Ca^{2+} cations [16]:

$$TiOH + Ca^{2+} + H_2O \rightarrow TiO^{-}Ca^{2+} + H_2O.$$

Subsequently, the negatively charged PO_4^{3-} and CO_3^{2-} anions are absorbed on the surface to form apatite nuclei [17]:

$$10Ca^{2+} + 6PO_4^{3-} + 2OH^{-} \rightarrow Ca_{10}(PO_4)_6(OH)_2$$

Once the apatite nuclei are formed, they grow spontaneously by consuming Ca^{2+} and PO_4^{3-} ions from the SBF, a highly Ca-P supersaturated solution, thereby developing a bone mineral-like composition of apatite. In this study, the Ca/P ratio of the deposited sediment was 1.56, which is very close to HA (1.67).

The Ostwald ripening theory [18] argues that small crystals or sol particles dissolve, and are re-deposited onto larger crystals or sol particles. This may explain the formation of porous micro-nanosphere structures in this study: the smaller crystals are more likely to dissolve than larger ones, and the dissolved crystals are deposited on the surface of larger crystals, thereby forming nano needle-like particles on the globular surface. Such porous structures are expected to facilitate optimal cellular activities and bone tissue regeneration *in vivo*.

The immortalized MC3T3-E1 cell line, which is derived from mouse calvarium tissue, has a high level of differentiation and ability to induce mineralization, making it an excellent model for studying differentiation *in vitro* [19]. *In vitro*

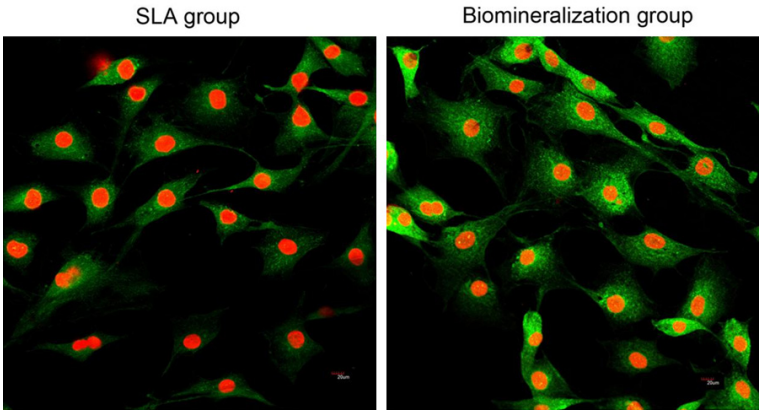

Figure 6. Cell adhesion and spreading of SLA and biomineralization groups after 2 days of culture (CLSM, 200× magnification).

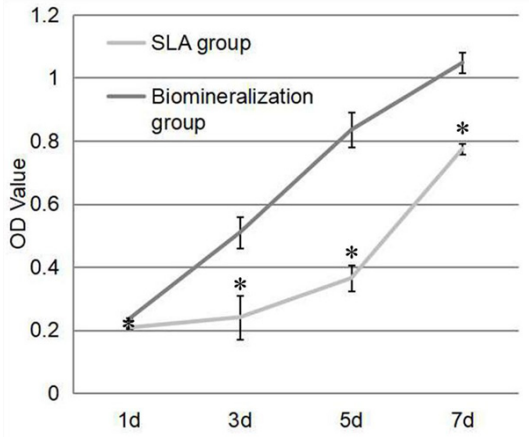

Figure 7. Growth curve of the SLA and biomineralization groups. *P<0.05 vs. biomineralization group.

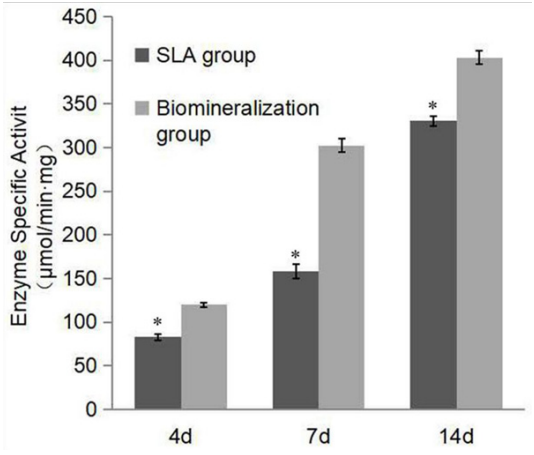

Figure 8. ALP activities of SLA and biomineralization groups. *P<0.05 vs. biomineralization group.

experiments are an important means to evaluate the surface properties of materials. In this study, *in vitro* culture of MC-3T3-E1 cells with 50 g/mL ascorbic acid and 10 mM β glycerophosphate was performed to induce mineralization, and we observed the morphological changes during the process of cell differentiation into osteoblasts with Alizarin red, silver nitrate and ALP staining. We successfully established an osteoblast model by inducing MC3T3-E1 cells *in vitro*, and laid an experimental foundation for subsequent experiments.

Implant-bone osseointegration involves a series of cell adhesion, proliferation, differentiation and matrix mineralization, the initiating factors of these behaviors being cell adhesion and spreading [20]. Irregular, rough surfaces exhibit a larger surface area, which increases the number of attachment sites for cells, promoting the adhesion of fibronectin and thereby enhancing cell adhesion.

Rough surfaces can induce the contact guidance of cells, meaning that cells on discontinuous surfaces show deformation, elongation and directional migration of actin filaments, and ultimately form an equilibrium, which is conducive to cell adhesion. Ca^{2+} on surfaces of biomineralizated HA coatings can provide active sites with a positive charge, which is beneficial to the adhesion of osteoblasts, and leads to the growth of cells on the porous surface [21]. The HA nano-morphology provides a biomimetic environment for cell growth, which can affect the structure of RGD peptides, regulate the signaling of cell integrins, and alter the biological behavior of cells [22]. Nano structures can also adsorb calcium and phosphorus ions from the surrounding environment, promote the deposition of calcium and phosphorus, and promote bone growth and healing. The result of our research was consistent with these studies, which suggested that the biomineralization surface treatment is more favorable for cell adhesion and extension.

The proliferative activity of cells is a key index to determine the biocompatibility of the material. Cells adhering to the surface must have strong proliferative activity, so that they are

able to grow to produce larger numbers of cells on the material-tissue interface. Therefore, the proliferative ability reflects the overall biological characteristics of cells, to a certain extent. Factors affecting cell adhesion also affect proliferation. In this research, the MTT results showed that the proliferation rate of cells seeded on biomineralized disks was significantly higher than those seeded on SLA disks, which indicated that the porous micro-nano HA deposited on the biomineralized surfaces may play a significant role in promoting cell proliferation.

ALP activity is an early indicator of osteoblast proliferation and maturation; ALP can hydrolyze various phosphate monoester compound substrates, and crystal growth inhibitors, such as ATP, increase the concentration of local inorganic phosphorus, initiate and continue the process of calcification, and promote the maturation and calcification of cells under alkaline conditions. Therefore, it is often used to identify osteoblast cells, to evaluate the osteogenic function of osteoblasts and to analyze the differentiation ability of cells growing on scaffolds. The micro-nanostructure can directly affect the formation of bone by changing the cell growth microenvironment [23], and plays an important role in the adsorption, extension, proliferation, and differentiation of osteoblasts [24]. This study demonstrates that the activity of ALP in the biomineralization group is higher than in the SLA group, which was consistent with the previous findings that showed that the biomineralization surface with deposited porous micro-nano HA had good bioactivity, and could promote the differentiation of osteoblast cells.

In summary, a novel hydroxyapatite coating composed of micro/nano globular sediment apatite on a titanium substrate was successfully constructed through a combination of sand-blast/acid etching and biomineralization treatment. All biological responses indicated that the porous nanosphere structure was of excellent biocompatibility for biomedical implant applications. Additional animal experiments are required to confirm the results.

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Disclosure of conflict of interest

None.

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