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Assessment of characteristics and cytotoxic effects of 316L stainless steel coated with a new titanium oxide nano-structure coating method

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In order to fabricate the TiO_2 nano-structure coating on the Ti-free stainless steel with good adhesion, commercially obtained 316L stainless steel (316L SS) plates were chemically treated by KOH aqueous solution including TiH_2 powder at 60ºC for 3 days in an oil bath shaker, and subsequently heated up to 550–800ºC under the air atmosphere. The crystal structure, color, adhesive strength and cytotoxic effects of coating were investigated. Fiber-like hydrogen titanate nano-structures were formed on the 316L SS surface and made a fine network. X-ray diffraction (XRD) patterns showed that hydrogen titanate phase converted to anatase and rutile after heat treatment above 550ºC. The heat treated sample at 800ºC for 1 h showed the highest adhesive strength and the lowest cytotoxicity. The alkali treatment method in this study is expected to be used on the other Ti-free metals with further research.

Keywords: Titanium oxide, Nano-structure, Color, Adhesion, Cytotoxicity

INTRODUCTION

TiO_2 coating has attracted many researchers in various fields because of its optical, electrical, photoelectrochemical and biomedical properties[1-6]. Also, it has been used as a coating for dental applications due to its good antibacterial and antiadherent properties[7,8]. In order to fabricate TiO_2 coating, several methods have been employed and developed, such as sol-gel process, atmospheric plasma spraying, and evaporation[9-12]. However, the adhesion of the coated TiO_2 layer to the substrates with those mentioned methods is still expected to be increased. Kim *et al.*[13] shows that titanium could obtain a hydrogen-titanate layer by NaOH alkali treatment, and the layer converted to TiO_2 after heat treatment, besides Ti was coated by soaking into 5 mol/L KOH for comparison between the two types of alkali solution effect on the coating nano-structure[14]. The fabricated TiO_2 coating has good adhesion and biocompatibility *in vitro* and *in vivo* studies, and the method was already clinically used for implants and artificial hip joints[15,16]. However, NaOH alkali treatment method is only efficient on titanium and titanium alloy and the fabrication of TiO_2 coating with alkali method on the Ti-free metal surface has not been reported.

Stainless steel is one of the most widely used metallic materials as screws, wires, and plates in dental fields because of its relatively low cost, good biocompatibility and corrosion resistance with the passive oxide layer on its surface. However, stainless steel and other metals cannot bond directly to the living bone and this characteristic could limit them clinical applications. Therefore, surface modification is required to improve the metals surface. We hypothesized that it is possible to fabricate a hydrogen titanate nano-structure layer on the 316L stainless steel (316L SS) surface by improving the alkali treatment method and obtain the TiO_2 layer with good adhesion after heat treatment. The stainless steel as a Ti-free metal could not supply the Ti ions for titanate coating formation, therefore, TiH_2 was chosen as the source of Ti ions for alkali treatment in this study.

In this study, 316L SS substrates were chemically treated by soaking in the KOH solution including TiH_2 powder. The crystal structure, color, adhesive strength and cytotoxic effects of coating were investigated.

MATERIALS AND METHODS

Sample preparation

Commercially obtained 316L SS plates (Nippon Steel & Sumikin Stainless Steel, Tokyo, Japan) were used in this study. Disk shape, 14.8 mm in diameter and 1 mm in thickness, plates were polished with SiC, #400, waterproof polish papers and then washed in ethanol, acetone and distilled water with an ultrasonic cleaner for 15 min respectively.

Fabrication of the TiO_2 nano-structure

To fabricate the coating, 316L SS plates were chemically treated by 5 mL of 5 mol/L KOH aqueous solution, including 0.4 g TiH_2 powder (Particle size of smaller than 45μm, Wako, Osaka, Japan) and soaked in an oil bath shaker (SB-13, AS ONE, Osaka, Japan) combined with a heater (TR-4, AS ONE) at 60ºC for 3 days. Then the coated samples were gently rinsed with distilled water for 30 s and then soaked in 10 mL of 0.5 mol/L HCl solution for 1 day. Some of the treated specimens were subsequently heated up to 550–800ºC at a rate of 10°C/min under the air atmosphere, kept for 1 h and then followed by cooling to ambient condition in the furnace.

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Scanning electron microscopy (SEM) observation
The morphology and fractured area of the treated surface was observed by SEM (JCM-6000 Plus, JEOL, Tokyo, Japan) and field emission scanning electron microscopy (FE-SEM: JSM-7500 FAM, JEOL) at 15 kV of accelerating voltage after the gold coating.

Phase identification
X-ray diffraction (XRD: SmartLab, RIGAKU, Tokyo, Japan) was used as the radiation source with Cu-Kα (λ=1.5405 A°) operated at 40 kV and 40 mA, at the rate of 2θ=1°/min and the glancing angle of 1 was used to investigate the structure phase of the coating layer before and after heat treatment.

Adhesive strength of coating
The adhesive bond strength of the coating layer to the substrate was measured by using an adhesion test apparatus (ROMULUS IV, Epoxy Resin Adhesive, Quad Group, Spokane, WA, USA) made of the aluminum rivet-shaped stud-pin with 2.7 mm diameter covered by a special epoxy resin adhesive with 50 μm in thickness as the binder. The stud-pin was fixed on the sample by a mounting clip in the oven at 150°C for 1 h. To evaluate the coating layer adhesive strength, universal testing machine (5566S, Instron, Canton, MA, USA) was used. The adhesive strength of the coating layer was measured from the maximum-recorded load. The test was repeated 5 times and the average value and the standard deviation (SD) were obtained.

Color evaluation
The optical property of coated 316L SS samples was evaluated using a computer-controlled spectrophotometer Minolta CM-3600D (Minolta, Osaka, Japan). The color coordinates in CIELAB color space: L^* (lightness), a^* coordinate ($-a^*$: green, $+a^*$: red), b^* coordinate ($-b^*$: blue, $+b^*$: yellow) were instrumentally calculated based on D65 illuminant and 10° observer. A white tile and a black chamber supplied by the manufacturer were used for calibration. Three measurements were made for each sample and the mean value and their SD were calculated. The color difference in the coated samples, ΔE, was calculated using equation (1).

$$\Delta E=[(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2} \quad (1)$$

where ΔL^*, Δa^* and Δb^* represents the color differences between the unheated sample and heated samples for the L^*, a^*, and b^* parameters, respectively.

Cell response
For the cytotoxicity study, MC3T3-E1 mouse preosteoblasts cells were used[17,18]. Cells were cultured in alpha-minimum essential medium (α-MEM) with 10% fetal bovine serum and 1% penicillin/streptomycin (PS) under 37°C, 5% CO_2 environment. Then the cells were seeded on the unheated and heated samples at 600 and 800°C with the density of 5×10^4 cells/well and were cultured under 37°C, 5% CO_2 environment for 3 h. The samples were washed with phosphate buffer saline PBS(–) twice and fixed with 2.5% glutaraldehyde. Then the samples were dehydrated in a graded series of ethanol (50, 70, 80, 90 and 100%) for 30 min, respectively. The samples were dried and coated by the gold sputtering method. The morphology of the adhered cells has been observed by SEM at different magnifications.

The samples before and after heating at 600 and 800°C were placed on a 24-well plate. The MC3T3-E1 cells with 1×10^4 cells/well density initially seeded onto the samples, and the medium was changed every 2 days. The cell viability assay (CellTiter 96AQueous Promega, Promega, Madison, WI, USA) was added to each well dish containing medium after 2, 4 and 6 days proliferation. The medium transferred to a 96-well plate and the absorbance at the 492 nm wavelength was measured by the plate reader (Multiskan™ FC, Thermo Fisher Scientific, Waltham, MA, USA). The difference among the three heating temperature samples was evaluated using one-way analysis of variance (ANOVA), with the statistical significance set at $p<0.05$.

RESULTS

Morphology of nano-structure
The morphology of the coating was shown in Fig. 1. The 316L SS plates were subjected to improved alkali treatment for 3 days. It can be observed that fiber-like nano-structure formed on the 316L SS surface and made a fine network. There is no basically change of nano-structure after the heat treatment up to 700°C, while it was slightly changed in the morphology at the temperature of 800°C. Figure 2 shows the cross-section of coated 316L SS surface before heat treatment after 180° bending. It can be observed that the nano-structure was directly attached to the metal surface and the unheated coating layer was not peeled off by bending. The average thickness of the coating layer was about 3.05±0.63 μm.

Phase constitution
Figure 3 shows the XRD patterns of the TiH_2 powder, 316L SS substrate and surfaces of coated 316L SS before and after heat treatment at various temperatures in the air atmosphere after improved alkali treatment. The nano-structure phase for the unheated sample before soaking into HCl acid was $K_2Ti_2O_4(OH)_2$ (ICDD-PDF 56-0687) and after soaking into HCl and replacing of K with H changed to hydrogen titanate (HT) $H_2Ti_2O_4(OH)_2$[14,19,20]. The fabricated hydrogen titanate on the 316L SS surface has been converted to anatase and rutile after heat treatment above 550°C, peaks already assigned in the XRD patterns. The TiH_2 remained in the coating layer even after improved alkali treatment and heat treatment temperature up to 650°C and then converted to titanium oxide by higher heating temperature.

Color evaluation
Mean values and SD for the color coordinates L^*, a^* and b^* of treated samples are presented in Fig. 4. In the CIE $L^*a^*b^*$ space, a^* represents the red-green color and b^* the yellow-blue color of the sample. The lightness of

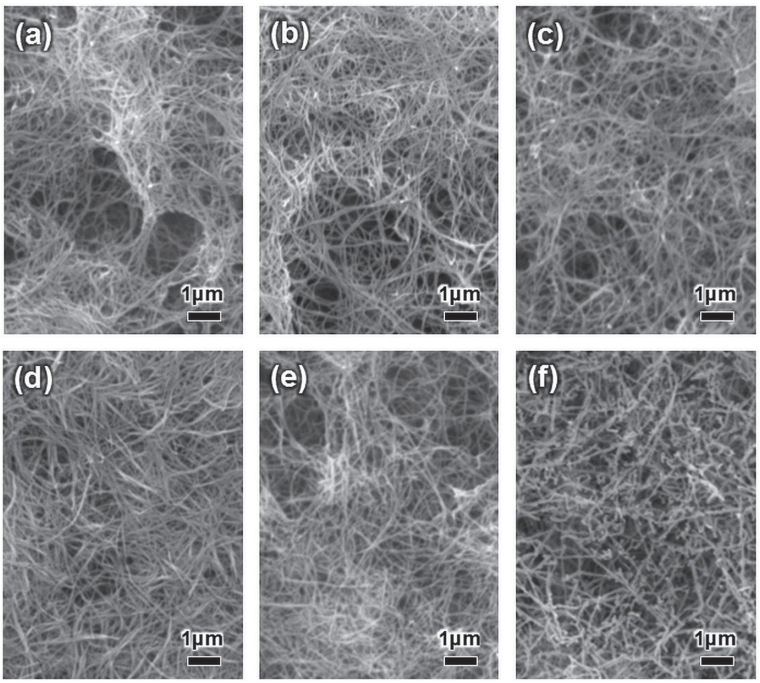

Fig. 1 SEM images of surface morphology of coated 316L SS before (a) and after heated at 550ºC (b), 600ºC (c), 650ºC (d), 700ºC (e), and 800ºC (f) for 1 h.

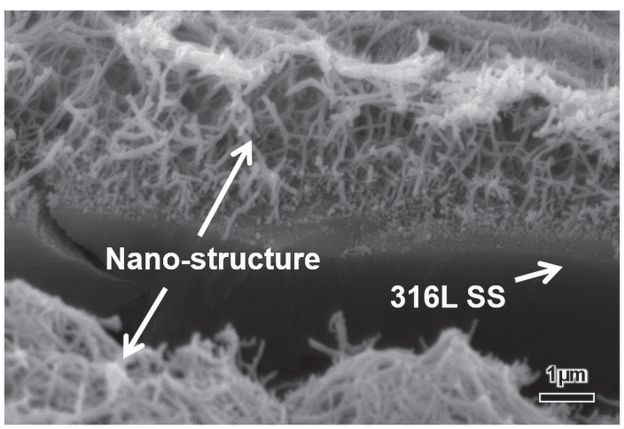

Fig. 2 SEM image of cross-section of 316L SS treated with alkali treatment for 3 days.

316L SS samples was decreased after coating and heat treatment. Also, a^* and b^* coordinates value turned to negative by heat treatment. The obtained ΔE values between the unheated sample and 600ºC heated sample, between the unheated sample and 800ºC sample, and between 600ºC heated sample and 800ºC heated sample were 13.16, 13.15 and 4.88, respectively.

Adhesive strength

The adhesion test results for the unheated and heated samples at 600, 700 and 800ºC were shown in Fig. 5.

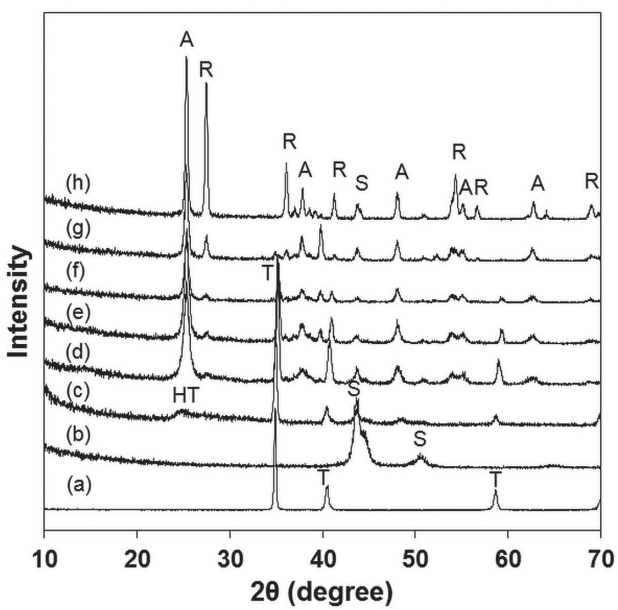

Fig. 3 XRD patterns of TiH_2 (a), 316L SS substrate (b), and the coating layer before (c) and after heated at 550°C (d), 600°C (e), 650°C (f), 700°C (g) and 800°C (h).
R, rutile; A, anatase; S, 316L SS; T, TiH_2; HT, hydrogen titanate.

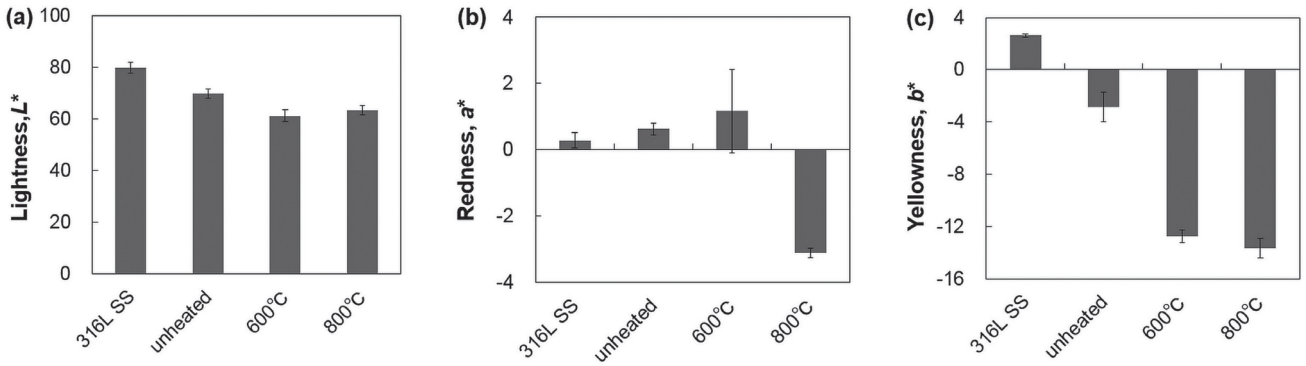

Fig. 4 Chromaticity of 316L SS plate and coated samples.
L^*(a), darkness to lightness; a^*(b), green to red; b^*(c), blue to yellow.

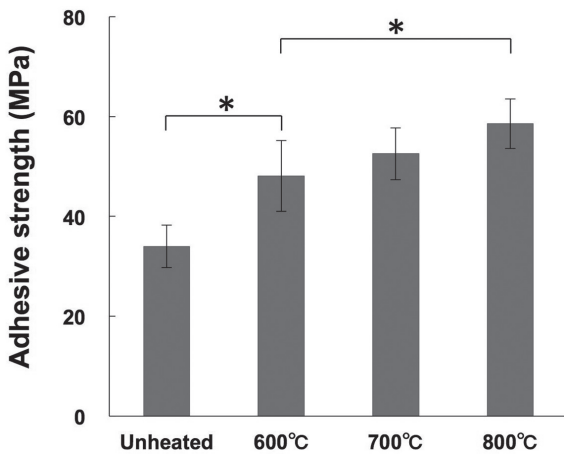

Fig. 5 Adhesive strength of unheated coating 316L SS sample and heated at 600, 700 and 800ºC for 1 h samples.
*p<0.05

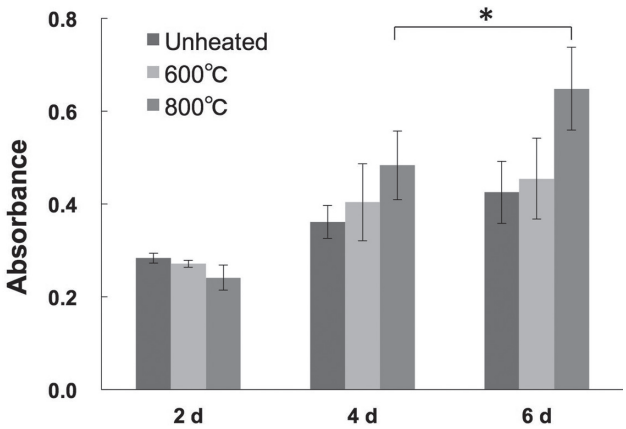

Fig. 7 MTS assay data showing the proliferation of osteoblast cultured on unheated coating layers, heated layers at 600 and 800ºC after 2, 4 and 6 days of incubation.
*p<0.05

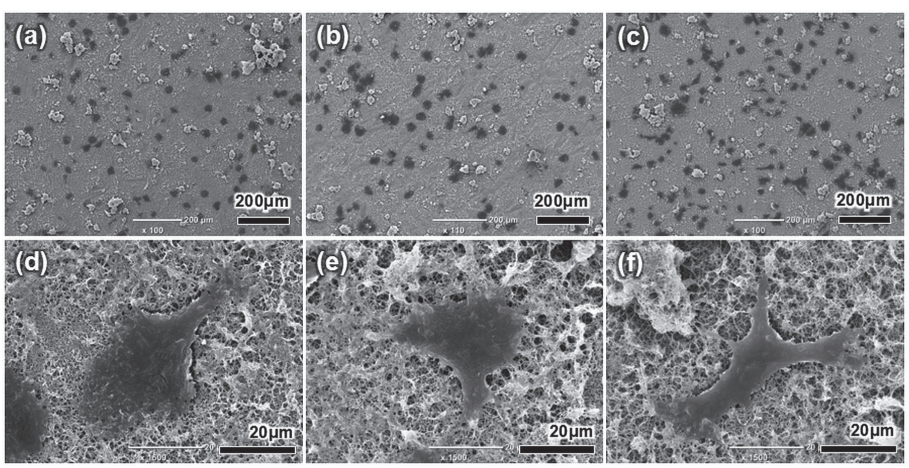

Fig. 6 SEM images of the morphology of MC3T3-E1 cells cultured on unheated coating 316L SS (a and d), heated at 600ºC for 1 h (b and e) and heated at 800ºC for 1 h (c and f).

Among all unheated and heated samples, The highest measured adhesive strength of the coating layer and 316L SS interface was 58.58±4.98 MPa for the specimen obtained by the heat treatment at 800ºC for 1 h ($p<0.05$).

Cell response

For the assessment of cytotoxicity, MC3T3-E1 cell morphology and viability was investigated. SEM images shown in Fig. 6 represent the morphology of MC3T3-E1 cells cultured for 3 h on unheated and heated samples after seeding. Low magnification SEM images cells were extended and adherent well on all of the treated samples surface. MC3T3-E1 cells spread more on 800ºC heated samples, flattened to a spindle shape and showed more filopodia (Fig. 6f). The cell viability of MC3T3-E1 cells cultured on samples heated at different temperatures was evaluated by MTS assay while the unheated sample was used as a control (Fig. 7). The trend of cell proliferation on coating at various heating temperatures was as follows: heated at 800ºC>heated at 600ºC>unheated sample.

DISCUSSION

The NaOH alkali treatment method is only efficient on Ti and Ti alloy, we improved it by using KOH solution mixed with TiH_2 powder and the fiber-like nano-structure coating layer was successfully formed on the 316L SS surface. The nano-structures made a fine network and the morphology is similar to the structures fabricated by Ti powders soaked in 5 mol/L KOH in Kim *et al.* study[14]. The coating on the 316L SS was not peeled off by 180° bending and it could attribute to each nano-fiber fabricated on the metal surface individually and the bending could only break the interval of the substrate between nano-structure and the connection of fiber networks.

During immersion of the stainless steel in the alkali solution passive film becomes more hydrated[21]. It can be presumed that in our study the Ti ions containing in the solution could bond directly to the created OH groups by alkali treatment on the stainless steel surface. After immobilization of the Ti to the surface the $K_2Ti_2O_4(OH)_2$ could be formed by the similar mechanism with the alkali treatment of the Ti substrates. Furthermore, after alkali treatment of the substrates the Fe ions of stainless steel surface undergoes selective dissolution and the existence of the Ti ions in the solution could form stable FeTi intermetallic compounds after heat treatment[22] and improve adhesion of the fibers to the metal surface. The SEM images clarified that the nano-fibers attached to the surface individually, therefore, the coating shows good adhesion. For investigation of the reactions and the coating mechanism on the 316L SS in further detailed thin film characterizations are necessary.

In order to obtain the TiO_2 nano-structure, heat treatment was subjected to the coated samples. TiO_2 has three types of crystalline structures: anatase, rutile, and brookite. Brookite is an amorphous structure, which can be converted into the anatase at low temperatures and the rutile at high temperatures. However, we did not get any brookite peaks in this study. The fabricated hydrogen titanate and TiH_2 on the 316L SS surface have been converted to anatase and rutile above 650ºC, and the peaks intensity increased with increasing heat treatment temperature. The anatase structure was formed from 550ºC, and the rutile structure needed a higher temperature. The 800ºC sample has the high index of anatase and rutile peaks, so we presumed that the change of crystalline structure of nano-structure may lead to the change of the 800ºC sample nano-structure morphology (Fig. 1).

The color of 316L SS specimens was dramatically changed by the coating covered the metal surface after improved alkali treatment. The L^*, a^* and b^* mean values of coated samples showed slight changes in lightness, and wide changing ranges in a^* and b^*. The 800ºC heated sample showed the lowest a^* and b^* values. Also, values of the color difference between unheated and heated samples represent the existence of macroscopic discrimination. Comprehensively, the color of heated samples tends to very light bluish gray. Depends on the nano-structure phase in Fig. 3, the increment of anatase and rutile in the coating layer after heat treatment might result in the changes of L^*, a^* and b^* values. So in this study, we considered that the coating color could be adjusted by controlling the heating temperature.

A good adhesion of the coating is always needed for most of the dental applications. In our study, there are statistically significant differences between unheated and heated samples in adhesive strength result. The difference between the adhesive strength of unheated and heated samples could be attributed to the phase transformation of the amorphous hydrogen titanate to the crystalline anatase and rutile in the nano-structure coating layer. Figure 3 shows the sample heated at 800ºC for 1 h contains the highest amount of anatase and rutile, and it resulted in the highest measurement of adhesive strength. According to the result, the decrease of amorphous peaks and increase of anatase and rutile peaks could enhance the strength of nano-structure, and lead to higher adhesive strength. We also thought the epoxy resin on aluminum stud-pin diffused into the fiber-like nano-structure and made a stronger layer. However, the adhesion strengths of heated samples in our study were higher than the atmospheric plasma spraying for 41.50 N/mm^2 [11].

Materials used in the oral cavity must be nontoxic and biocompatible, have good mechanical properties, and be able to resist corrosion. During the study procedure, the traditional alkali treatment protocol was followed, therefore, the high temperature was applied however the mechanical properties of untreated 316L SS could be decreased by heat treatment[23]. On the other hand, the surface of 316L SS came to be anticorrosion by the formation of the passive film treated with the alkali solution. Besides the complete coverage of the surface with the nano-structure coating layer could protect the metal surface. In addition, the heating time is short

and may has no effect on the metal characteristics. Therefore, for investigation of the coating layer, heating temperature and duration time effects on the mechanical or corrosion properties of the stainless steel further studies required.

The TiO_2 coating with antibacterial and antiadherent properties and good biocompatibility had been reported in many studies. MC3T3-E1 cells were used for cytotoxicity assessment in the dentistry literature[17,18]. A significant difference in MC3T3-E1 cell morphology was observed in this study. Most of MC3T3-E1 cells cultured on unheated sample surface still remained in their original round shape, only few of cells showed polygonal shape (Fig. 6d). From 600ºC heated samples, cells became more flattened compared to the cells of unheated samples and the filopodia could be clearly observed, and the cells on the 800ºC heated samples have better shape and showed more filopodias (Fig. 6). The filopodia indicate a good sign of the biocompatibility for the coating layer. Malkoc *et al.*[24] shows that the orthodontic mini-implant which was made by 316 SS showed significant decrease of MC3T3-E1 cell index from 96 h cell culture compared with other types of mini-implants. In our study, MC3T3-E1 cells on all coated 316L SS samples proliferated greatly over 6 days means that the nano-structure fabricated by improved alkali treatment has less cytotoxicity and the cytotoxic effects also can be decreased by heat treatment. The surface of 316L SS became anticorrosion by enrichment of oxy-hydroxide in the passive film treated with alkali solution[21]. In addition, from the cross-section image of coated 316L SS, it can be observed that the coating covered the 316L SS surface completely therefore it could be presumed that there is no contact between substrate and cell culture medium after coating, and considering with the anticorrosion of passive film, the substrate will not be corroded after coating or even after cell study.

Besides, in the literature, many results show that anatase phase plays an important role in osteoblast proliferation and mineralization. The anatase phase of titania film enhances osteoblast adhesion, proliferation, and differentiation, and the anatase/rutile phase is more efficient for promoting apatite formation[25-27]. In our study, anatase/rutile nano-structures showed accelerated growth of osteoblast than amorphous coating layers. According to Fig. 4, heated at 800ºC sample had larger anatase and rutile peaks and less amorphous peaks than heated at 600ºC sample, and MTS assay showed the sample heated at 800ºC had better osteoblast proliferation. Also, the filopodia of osteoblasts on heated at 800ºC sample showed a good biocompatibility of coating. Thus, the TiO_2 coating of the 316L SS formed by improved alkali treatment and heat treatment not only has less cytotoxic effects for MC3T3-E1 cells, but also the good cell proliferation showed the possibility of use as a coating on the orthodontic stainless steel mini-implant.

Among all results, it is obvious that the anatase and rutile crystal structure played an important role in this study, especially the rutile crystal structure content of the TiO_2 nano-structure. The TiO_2 nano-structure with the highest content of rutile has the highest adhesive strength, better color evaluation and the lowest cytotoxic effects. However, further research is required to investigate the effect of rutile and anatase phase ratio of TiO_2 nano-structure, and more animal studies and human trials are required before the coating can be used *in vivo*.

CONCLUSION

The TiO_2 nano-structure coating was successfully formed on the 316L SS and changed surface color by using KOH solution including TiH_2 powder and heat treatment. The produced nano-structure on the 316L SS contains the anatase and rutile phases when heat-treated at the temperature above 550ºC. The coated sample subjected to heat treatment at 800ºC for 1 h showed the highest adhesive strength and the lowest cytotoxicity. The results indicated that the TiO_2 nano-structure coating of the 316L SS has good adhesion and less cytotoxic effects, and the alkali treatment method in this study is expected to be used on the other Ti-free metals with further research.

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