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Research Paper

Improved osseointegration of long-term stored SLA implant by hydrothermal sterilization

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ABSTRACT

The sandblasted, large-grit and acid-etched (SLA) surface is easy to be contaminated during storage and its surface chemical state is usually changed by different sterilization methods. This causes an undesirable increase in surface hydrophobicity and results in osseointegration degradation. To overcome this problem, a low temperature hydrothermal (HT) sterilization method was proposed in this study. Briefly, 4 weeks-stored pure titanium SLA specimens were sterilized using a sealed glass bottle with pure water in an autoclave set at 121 °C for 20 min. Results showed that, stored SLA specimens were superhydrophobic before and after conventional autoclaving, whereas, HT sterilization decontaminated and endowed stored SLA surface with superhydrophilicity. Osteoblast spreading was greatly enhanced, ALP expression was upgraded and bone nodule formation was obviously promoted on HT sterilized specimens compared with autoclaved ones. More bone formation around HT sterilized specimens was observed and HT sterilization increased bonding strength of implant to bone by 95% and 127% after 2 and 4 weeks of healing, respectively. The simple, feasible HT sterilization restored osseointegration of SLA implant while diminishing recontamination as much as possible. Therefore, it is proposed as a standard sterilization method for implant practitioners and researches.

1. Introduction

Sandblasted large grit and acid-etched (SLA) surface is one of the most popular titanium dental implant surfaces which has been successfully applied in clinic for many years. Most literature ascribed such success to three aspects: superimposed micron/submicron-scaled structure that mimicked osteoclast resorption pits, high surface energy retained on the rough TiO_2 surface and firm implant-bone mechanical fixation (Boyan et al., 2003; Zhao et al., 2007; Le Guehennec et al., 2007; Cochran et al., 1996). However, once produced, titanium implant surface will adsorb organic molecules within seconds to 1 min because of high surface energy. The progressive accumulation of organic impurities gradually impairs carefully designed original surface, resulting in time-related chemical composition alteration, hydrophilicity loss

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and osseointegration degradation (Zhao et al., 2007; Att et al., 2009; Park et al., 2012). Most implants will not be implanted immediately after producing and thus constantly accumulated contamination has become a great challenge for all the implant handling steps, including storage and sterilization (Kasemo and Lausmaa, 1988).

Sterilization is the final step before implantation and it is considered as the last step of surface modification since it may change physicochemical properties of implant surface and alter bone cell expression detectably (Kasemo and Lausmaa, 1988; Stanford et al., 1994). Considering surface decontamination, two promising sterilization methods, ultraviolet irradiation (UV) and plasma treatment, have been widely studied.

Both *in vitro* osteoblast behavior and implant-bone binding strength were improved as aged sandblasted, acid etched or electrochemically oxidized titanium underwent UV treatment (Att et al., 2009; Suzuki et al., 2009; Aita et al., 2009; Liu et al., 2008). However, it is not a highly reliable sterilization method, since it is distance dependent and penetration power is weak, causing non-homogeneous sterilization for complex shaped devices. In some studies, UV irradiation was carried out in a clean room and/or γ-ray irradiation was employed to ensure sterilization (Aita et al., 2009; Liu et al., 2008). Besides, decontamination and superhydrophilicity recovering by UV treatment takes time, from 48 to 168 h as reported (Suzuki et al., 2009; Aita et al., 2009; Liu et al., 2008).

Compared with UV treatment, plasma is much more efficient in decontamination and superhydrophilicity can be restored on SLA surface within minutes (Park et al., 2012; Moisan et al., 2002). However, its main limitation is weak penetrating power as well. Complete sterilization could not be achieved even if plasma treatment was conducted for 120 min as UV and other plasma species attacked only the spore surface (Lee et al., 2006). Furthermore, sterilization effectiveness will be severely impaired if implants have complex geometries, such as blind hole.

Therefore, although conventional autoclaving (AC) method has been found to deposit hydrophobic organic contaminants over implants and result in depressed cell responses (Kasemo and Lausmaa, 1988; Stanford et al., 1994; Baier et al., 1982), it is still the most popular sterilization method for titanium implants in clinic and researches due to great convenience, low cost and reliable sterilization effect. Dry heat and ethanol immersion are also used for titanium sterilization and they also change the chemical state of titanium surface to some extent (Hirano et al., 2014). Such changes affect the initial responses of cell and bone tissue on implants and bring difficulties to interpret and compare results from different research groups. To overcome these problems, a reliable standard sterilization for titanium implant is urgently demanded.

Hydrothermal (HT) treatment can decontaminate titanium surface effectively, however, such treatment is usually conducted under 150–250 °C with duration of 6–24 h (Shi et al., 2012, 2013). Although sterilization can be ensured, such treatment is time costing and is not suitable for clinical application. Nevertheless, it was recently reported that, HT treatment at lower temperature could also decontaminate implant surface to some extent (Shi et al., 2015). Inspired by this report, this study proposed low temperature HT sterilization for long-term stored SLA implants. Physicochemical properties of SLA surface after HT sterilization were studied and biological performances were evaluated both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Specimen preparation and storage

Disk-shaped specimens with diameter of 15 mm and thickness of 1 mm were prepared with commercially pure titanium (grade 2). Rod shaped specimens with a diameter of 1 mm and a total length of 3.0 mm were prepared from titanium wire of same purity grade. Specimens were sandblasted with large-grit alumina (0.2–0.5 mm) and then acid etched in 67% H_2SO_4 at 120 °C for 10 min to get the well-known SLA surface. After sufficient washing with ultrapure water and drying in hot air, specimens were put into airtight PE bags individually and kept in a dark box for 4 weeks.

2.2. Sterilization

Specimens were sterilized either by conventional autoclaving, AC, or by HT sterilization. In AC, specimens were packaged in thermal-sealed sterilization pouches (A.R. Medicom, QC, Canada). In HT sterilization, specimens were put into clean reagent glass bottle with blue screw cap and gasket PTFE ring, SEBC bottle, (Boro 3.3, Shuniu, China) and ultrapure water (Resistivity 18 MΩ/cm, MilliQ, Millipore, USA) was added until specimens were completely covered. After that, the bottles were sealed. Both sealed sterilization pouches and SEBC bottles were transferred into autoclave (BXM-30R, Boxun, China) and autoclaving was carried out for 20 min at 121 °C. After cooling down, specimens sterilized in pouches, coded as SLA-AC, were dried in an oven at 60 °C for 30 min. Whereas, specimens sterilized in SEBC bottle with water, coded as SLA-HT, were kept in bottle and dried in pure N_2 flow just before surface characterization.

2.3. Surface characterizations

For both type of sterilizations, surface characterizations were carried out immediately after drying. Surface morphology of specimen was observed with a scanning electronic microscope (SEM, S-3000, Hitachi Co., Japan). Roughness was analyzed by a color 3D laser scanning microscope (VX9000, Keyence Co., Japan). Surface wettability was assessed through contact angle of 2.5 μL distilled water with a contact angle meter (JC2000B, Zhong Chen Co., China). Surface chemical composition was analyzed by X-ray photoelectron spectroscopy (XPS, PHI 5000 VersaProbe, UlVAC-PHI Co., Japan).

2.4. Cell culture

Bone marrow cells isolated from femur of 4-week old male SD rat were suspended with alpha minimal essential medium (α-MEM, SIGMA) supplemented with 15% fetal bovine serum (FBS), 50 mg/L ascorbic acid, 10 mmol/L Na-β-glycerophosphate, 10^{-8}

mol/L dexamethasone, 1% penicillin, 1% streptomycin and 0.292 mg/mL L-glutamine. They were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was renewed every two days until 7 days. Osteogenic phenotype using this protocol has been confirmed by others (Aita et al., 2009; Maniatopoulos et al., 1988; ter Brugge and Jansen, 2002).

Just before cell seeding, SLA-AC and SLA-HT specimens were taken out from sterilization pouches and SEBC bottle, respectively, with sterilized tweezers and transferred into 24-well culture plate.

2.5. Cell morphology

Cells were seeded onto specimens at an initial density of 10^4 cells/well. After 3 h, cells attached to the disks were washed twice with phosphate buffer saline (PBS), fixed with 3% glutaraldehyde and then dehydrated using an ascending series of ethanol-distilled water solution. After removing the ethanol with hexamethyldisilazane (HMDS) and sputtered with Au–Pt alloys, attached cells were observed by SEM.

2.6. Alkaline phosphatase activity

Cells were seeded at an initial density of 10^4 cells/well and medium was renewed every two days. After 7 and 14 days of culture, cell alkaline phosphatase (ALP) activity was determined using a biochemical colorimetric assay kit, LabAssayTM, following instruction of the manufacturer. ALP activity was normalized by total protein content and expressed as nmol/min/μg protein.

2.7. Mineralization assay

Cells were seeded at an initial density of 10^4 cells/well and the culture medium was renewed every two days. After 21 days of culture, specimens were washed twice with PBS and fixed with 10% formalin for 15 min at room temperature. After rinsing with distilled water, fixed cells were stained with 1.5% Alizarin red S solution for 5 min. After removing excess dye, images of stained specimens were documented by camera with a macro-lens.

2.8. Implant-bone bonding strength

Eight-week-old male SD rats were used for implantation. After general anesthesia, the implant site was prepared 10 mm down from the distal edge of the femur on each hind leg by drilling with a 0.8 mm burr and then enlarged using reamers (0.9 mm and 1.0 mm). Rod-shaped specimen was inserted into the prepared bone defect. After 2 and 4 weeks of healing, femurs with implant were harvested and embedded into auto-polymerizing resin on a glass plate. The position and inclination of femurs were carefully adjusted to ensure the center axis of the implant be vertical to the glass plate. Specimens were fixed on the vise of an electro-mechanical MTS universal testing machine (E42, MTS System, Inc., USA) equipped with a 100 N load cell and the implants were pulled out at a speed of 1 mm/min. The bonding strength was determined by picking the peak of the load–displacement curve. Six specimens were tested for each group.

After pull-out experiments, implants were rinsed with distilled water, fixed with 3% glutaraldehyde overnight, dehydrated in ethanol–distilled water solution and were sputtered with Carbon before observed by SEM.

The biological experimental protocol was approved by the Institutional Animal Care and Use Committee of Jiangsu University of Science and Technology.

2.9. Statistical analysis

Surface roughness was evaluated at three sites on three different specimens. Contact angle was measured on three different specimens. Five specimens were used for cell culture studies. 12 animals were used for the biomechanical pull-out test. One-way ANOVA was performed to examine the difference between AC and HT sterilized specimens; $p<0.05$ was considered statistically significant.

3. Results

3.1. Surface morphology

Fig. 1 shows surface morphology of the investigated specimens obtained by SEM. All the specimens showed typical SLA surface with complex hierarchical structure characterized by blasting cavities with diameters ranging from 20 to 40 μm and superposing smaller etching pits of about 0.1–2 μm in diameter. Neither the SLA-AC nor the SLA-HT specimen displayed different topography compared to unsterilized one. All specimens displayed almost identical mean R_a values, that is, 3.60 ± 0.14 μm for SLA, 3.61 ± 0.21 μm for SLA-AC and 3.58 ± 0.13 μm for SLA-HT specimens, respectively. Apparently, surface roughness was not noticeably affected by sterilization methods.

3.2. Surface wettability

Contact angle on 4-weeks stored SLA disk was $133.3\pm1.6°$, which were similar to previous reports (Park et al., 2012; Stanford et al., 1994). After AC sterilization and drying, the drop kept as a sphere on SLA-AC specimens and presented an even higher contact angle of $140.3\pm1.2°$, as shown in Fig. 2. In contrast, water spread very fast once dropped onto SLA-HT surface and wetted almost the whole surface. SLA-HT specimens showed a contact angle of 0° and indicated an extremely hydrophilic surface.

3.3. Surface chemistry

The Fig. 3 shows chemical composition of SLA, SLA-AC and SLA-HT specimens analyzed by XPS survey scans. All the surfaces were mainly composed of Ti, O, C and N. After AC treatment, amount of surface absorbed C increased slightly while the percentages of O and Ti decreased. On the other hand, the HT sterilization altered surface chemical composition notably. The amount of C reduced dramatically, whereas O and Ti increased significantly on the SLA-HT surface.

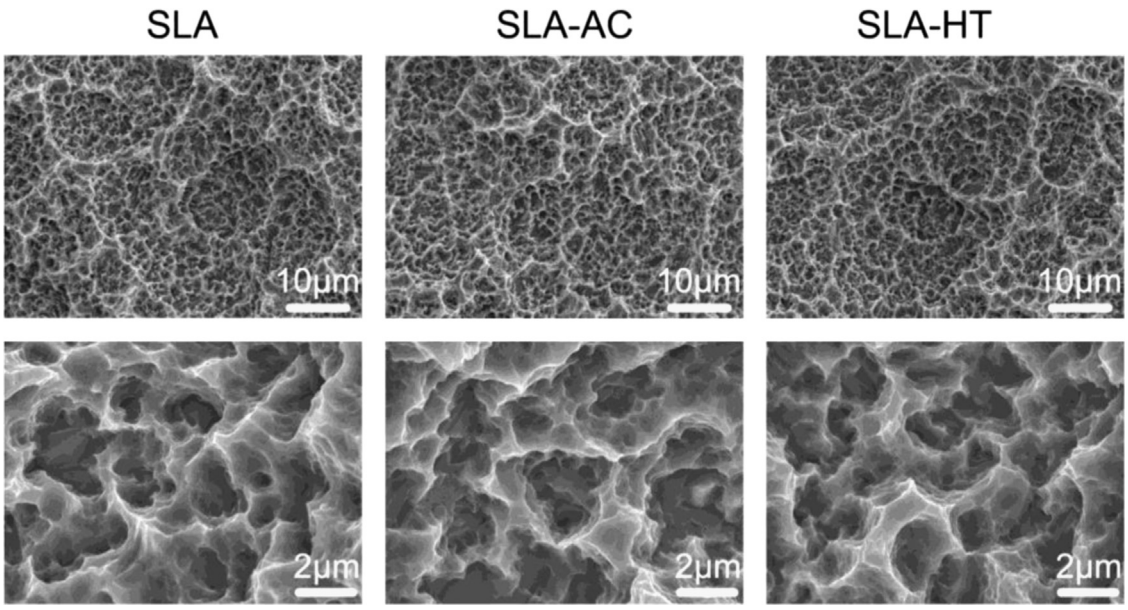

Fig. 1 – SEM images showing the surface of different specimens.

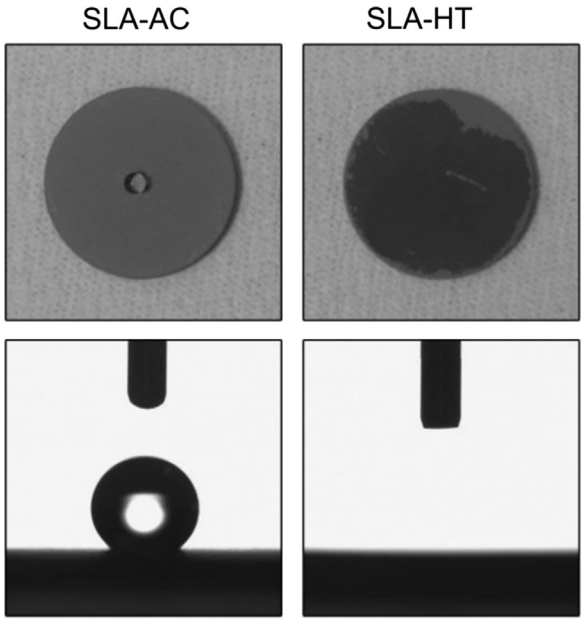

Fig. 2 – Contact angles and water spreading on different specimens.

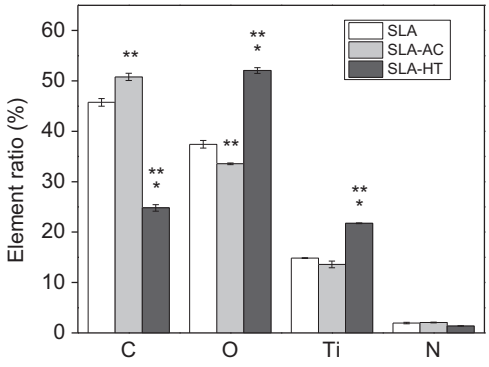

Fig. 3 – Surface element ratio calculated from XPS survey scans.

3.4. *Cell attachment*

3 h after seeding, cells presented quite different morphologies depending on the substrates, as shown in Fig. 4. Generally, cells presented more processed cellular developments on SLA-HT surface than on SLA-AC surface. Cells on SLA-AC surface stayed at bottom of large cavities created by sandblast and kept a relatively restricted spreading configuration with matrix shrunk around the nucleus. Only a few protrusions of pseudopodia from each cell could be observed, indicating that spreading had just started. Apparently, at this time point, cell-substrate contact was not well developed. Whereas, osteoblasts on SLA-HT surface had climbed out of cavities, showed polygonal shape, occupied larger areas, spread and developed more pseudopodia and lamellipodia. Those cells seemed to be flat, stretched over more than one blasting cavity and their protrusions grasped the wall of etching pits firmly. Obviously, a closer cell-substrate contact had been established.

3.5. *ALP activity*

ALP activity was detected to assess the early differentiation of osteoblast on different surfaces. As shown in Fig. 5, cells on SLA-HT surface showed higher ALP activity than that on SLA-AC surface with significant statistically difference 7 days after seeding. At day 14, however, ALP activity of both groups decreased to some extent and no statistical differences were found.

3.6. *Cell mineralization*

As culture period increased, cell mineralization progressed on specimens. Bone-like nodule formed and coated some areas of all disks. Fig. 6 presented the Alizarin red S staining results to visualize calcium deposition. On SLA-AC specimens, blank

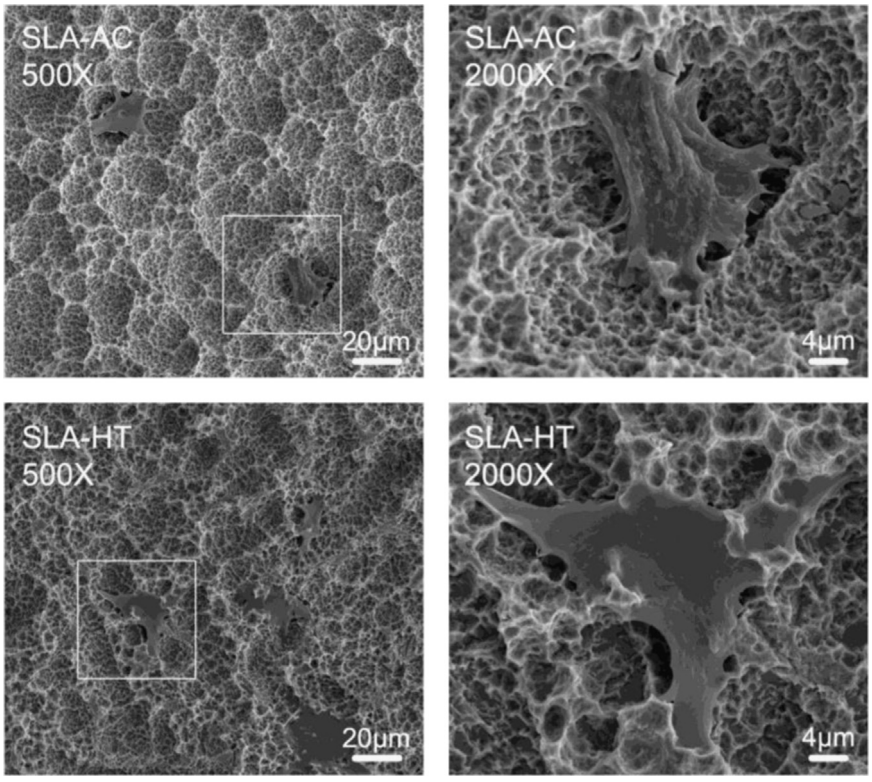

Fig. 4 – Osteoblasts morphology after being cultured on specimens for 3 h.

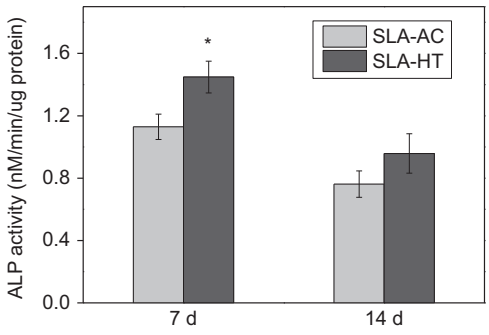

Fig. 5 – ALP activity of osteoblasts cultured on different specimens. $^{*}p<0.05$, statistically different to SLA-AC, $n=5$.

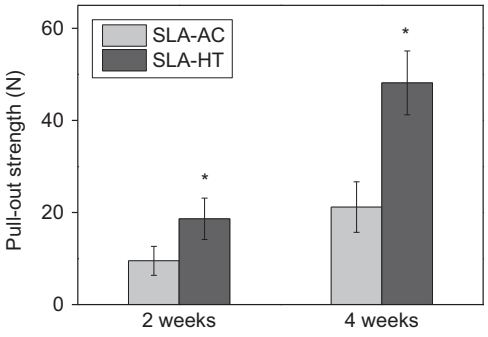

Fig. 7 – Biomechanical strength of implant-bone integration evaluated by pull-out test. $^{*}p<0.05$, statistically different to SLA-AC, $n=6$.

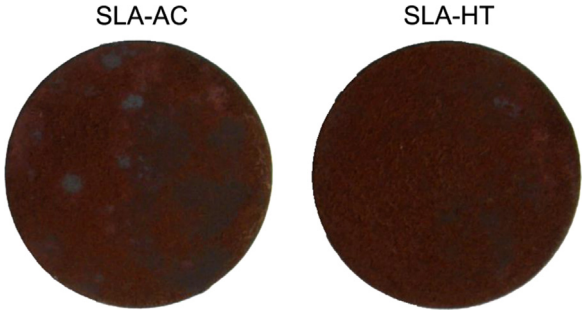

Fig. 6 – Formation of bone-like nodules on different specimens after 21 days culture. Stained with Alizarin red S. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

areas could be observed and bone nodule did not homogeneously cover the disk surface. Cell calcification was greatly increased on SLA-HT surface and bone nodules covered the whole surface much more homogeneously. Mineralization area on two kinds of specimens as percentage of total Ti disk area was 56.8% and 87.3%, respectively. There was significant difference.

3.7. *Implant-bone bonding evaluation*

Implant-bone integration strength was significantly influenced by sterilization methods as shown in Fig. 7. At early healing stage, 2 weeks, the average bonding strength of HT sterilized specimens was about 1.9 times of those subjected to conn AC. After 4 weeks healing, bonding strength of both kinds of specimens had obviously increased and the results

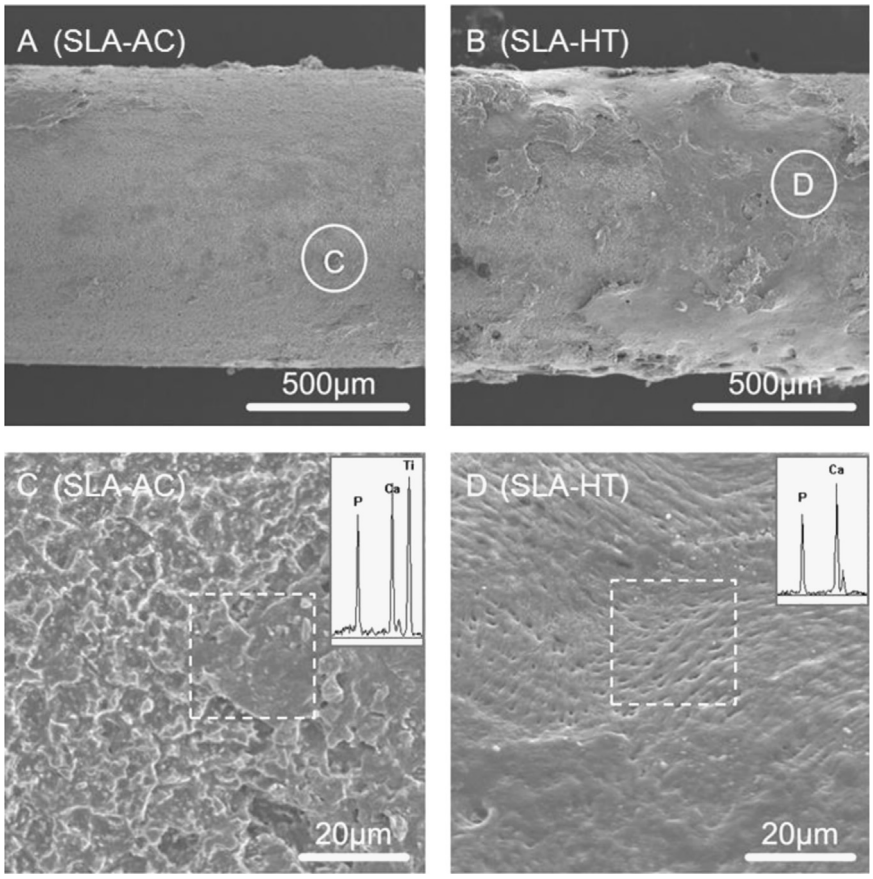

Fig. 8 – SEM observation and EDS analyzes for biological structures formed on UV-treated and untreated implants.

of SLA-HT implants kept their advantage over the SLA-AC implants for about 130%.

SEM observation results of implant surface after pull-out test are presented in Fig. 8. Under lower magnification, it was observed that most of the SLA-AC specimen surface seemed to remain bare. Whereas, abound remnants were observed on SLA-HT surface. Higher magnification observation focusing on dark spots (area C) on SLA-AC specimen found that deposition covered the micropores induced by acid etching. EDS spectra for the deposition area showed Ca, P and together with high Ti peak, Fig. 8(C). Closer observation on remnants on SLA-HT specimen revealed dissipated stripes and EDS spectra for this area showed only Ca and P peaks, as shown in Fig. 8(D). Small holes among stripes were also observed and they could be related to osteocytes lacunas. It can be assumed that new bone covered most area of SLA-HT surface after 4 weeks implantation. From Fig. 8(D) it was also observed that the interface between new bone and old compact bone was not obvious.

4. Discussions

Both long-term storage and improper sterilization will impair the excellent osseointegration ability carefully designed by material researchers. The fact was noticed 30 years ago, but unfortunately, in the past decades most literature ignored the fact, partially due to the lack of a proper sterilization method. This brought difficulties for readers to interpret and compare their results. The demand for rapid osseointegration and early loading has become stronger and stronger and therefore it is urgent to find a proper sterilization method now. UV and plasma showed great benefit for improving osseointegration; however, due to the fatal shortcoming of poor penetrating ability, they cannot be used as reliable sterilization methods yet. To solve the problem, this study proposed a low temperature HT treatment as a novel sterilization method and confirmed its ability to improve osseointegration of SLA implant.

HT treatments for titanium surface modification at low temperature (<150 °C) have rarely been reported. Dissolution-precipitation happened during high temperature HT treatment, whereas, in this study SEM observation did not reveal any morphology change on specimens subject to HT treatment at 121 °C for 20 min. It means that the oxide layer over specimens cannot be remodeled under this condition. Nevertheless, similar to HT at higher temperature, the treatment at 121 °C achieved a decontaminated surface with low C content as well, as shown in Fig. 3. This is a great advantage over AC sterilization conducted at the same temperature and duration. Such decontamination together with complicated surface structure may contribute to the superhydrophilicity as shown in Fig. 2.

Hydrophilic titanium surface can be obtained within short time by radio frequency glow discharge (RFGD), plasma and UV irradiation (Park et al., 2012; Aita et al., 2009; Baier et al., 1984). But the loss of hydrophilicity could also occur quickly

in atmospheric environment, as treated surfaces possess high energy and recontamination can easily happen. In this study, the sterilized implants were stored in pure water inside sealed glass bottle until implantation and therefore, to the greatest extent, avoided recontamination.

Although the superimposed structure of SLA surface mimics hierarchical structure of bone tissue and may facilitate osteoblast anchoring, hydrophobization due to contamination will restrict *in vitro* cell responses and *in vivo* bone formation (Park et al., 2012; Aita et al., 2009; Baier et al., 1982; Buser et al., 2004; Ferguson et al., 2006). Compared with hydrophobic surface, a hydrophilic surface would facilitate protein adsorption in a conformation that presents adhesion motifs enhancing cell attachment and spreading (Le Guehennec et al., 2007; Park et al., 2012; Gittens et al., 2014), as shown in Fig. 4. Moreover, it is interesting to see that such benefits last to the later stages of osteoblast differentiation, as shown in ALP expression and bone nodule formation results, Figs. 5 and 6. The obviously enhanced cell spreading is believed to be the sign of released intracellular tension and it triggered the further promotions. The biological dilemma of an inverted correlation between proliferation and differentiation rates in osteoblasts seemed to be broken through in this way. It is interesting to find that bone-like nodule cover ratio on HT sterilized specimens seems to be higher than UV irradiated ones after 21 days culture (Aita et al., 2009). Besides, in some case cell attachment on plasma treated specimens was not found to be obviously enhanced due to fast recontamination (Park et al., 2012) and thus HT sterilization presents great advantage.

The influence of HT sterilization on early stage healing was also confirmed *in vivo*, as shown in Figs. 7 and 8. Both bonding strength and amount of newly formed bone covering implant were increased. In spite of various surface morphology and roughness, improved hydrophilicity has been reported to enhance both *in vivo* osteoblast differentiation and bone-to-implant contact ratio obviously (Aita et al., 2009; Liu et al., 2008; Eriksson et al., 2004). These reports and our results suggested that better hydrophilicity itself could inspire strong osteoblast responses that improve implant-bone interface healing *in vivo*, especially at the early stages of healing (less than 4 weeks). 48 h UV irradiation can increase implant-bone bonding strength by 50% after 4 weeks (Park et al., 2012); therefore it is satisfied to find that the improvement of implant-bone bonding strength by HT sterilization is comparative to or even better than that of UV irradiation.

This is of great significance as shorter healing time is strongly demanded by patients who expect to restore tooth function as soon as possible. More importantly, as primary stability of implant from mechanical interlocking becomes weak gradually, rapid osseointegration with new bone is the only way to enhance secondary stability of implant to avoid early failure before crown restoration (Raghavendra et al., 2004). The facts discussed above underscore the importance of good hydrophilicity. Researchers may be interested in finding the optimum wettability of implant surface; nevertheless, most currently available data suggest superhydrophilicity could definitely promote osseointegration.

Studies revealed that aseptically packaged titanium implants available on market presented surface Carbon content of around 32–53% (Kang et al., 2009). Therefore, it is attractive for producers and dentists to superimpose packaged implant with superhydrophilicity by a simple, effective and low-cost method. HT sterilization, as proposed in this study, should be preferred as it can sterilize the implant reliably, induce superhydrophilicity and keep such surface feature until implantation. The unchanged morphology also indicated that the application of this technique can be extended to other titanium orthopedic products and thereby can serve as a standard sterilization method for both clinic and research.

5. Conclusions

Hydrothermal sterilization carried out in pure water diminished surface contamination occurred during implant storage effectively. Compared to steam sterilized ones, the hydrothermally sterilized SLA surface was more osteoblast-affinity by enhancing cell attachment, spreading and differentiation. Furthermore, hydrothermal sterilization increased the strength of implant-bone integration by more than 100% after first 4 weeks healing and induced much more new bone formation. Therefore, we have developed a simple and feasible sterilization method for titanium implants that ensures rapid bone-titanium integration. It is worthy further studies for extensive clinical applications as a standard method.

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