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Effects of hydrothermal sterilization on properties of biological coating fabricated by alkaline-heat treatment on titanium

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ABSTRACT

Alkaline-heat (AH) treatment is a feasible method to prepare Titanium (Ti) based metals with an active ceramic coating for biological applications. Currently, the AH treated Ti substrates are usually sterilized with ethylene oxide gas (EO) or Gamma-ray irradiation (GI) before biological experiments and clinical implantation. However, the EO process is complex and residual gas is toxic for tissue and cells whereas radioactive source is difficult to access for GI and therefore both methods are not practical for dental offices and laboratories. Previously, hydrothermal sterilization (HS) was proposed for all kinds of Ti based endosseous implants and had been proven promising for clinical applications due to its advantage of restoring the osseointegration of Ti implants stored for long-term. In this study, the effects of HS on properties of AH treated Ti were studied in comparison with conventional autoclaving (AC). Both sterilizations were carried out at 121 °C for 20 min in a common autoclave and distilled water was used as medium for HS only. Results showed that, both AC and HS altered the nano-structure of the ceramic coating formed by AH and caused significant Sodium (Na) loss. AC decreased crystallinity of the coating by forming numerous nano-sheets, whereas HS increased the crystallinity, densified the coating and improved its scratch resistance. AC depressed the formation of apatite, in contrast, a complete apatite layer was formed on the HS specimen after being immersed in simulated body fluid (SBF) for 4 days. Both attachment and proliferation of osteoblast-like MC3T3-E1 cells on the HS specimens were better than that on the AC ones. Decontamination, superhydrophilicity and high crystallinity from HS were believed to be the main contributors. In summary, the current results showed that HS can be used as a sterilization method for AH treated Ti implants in biomedical applications.

1. Introduction

Surface modifications are undoubtedly important for biological performances of titanium (Ti) made devices. Various methods have been developed to enhance the bonding of devices to living bone, including simple surface roughening by machining and/or sandblast in the early stage [1,2], numerous coating techniques [3–6] and atomic decorations in recent years [7]. Among them, alkaline-heat (AH) treatment proposed by Professor Kokubo et al. in the 1990s has attracted considerable attentions [8]. Dispensing with sophisticated equipment and violent reactions, this method is simple and feasible for Ti based devices with various shapes and will not change their dimension obviously as the thickness of modification coating is $< 2\,\mu m$. It has also achieved great success in clinic application, for instance, hip joints with titanium stems treated by such method are now used in Japan and showing considerable promise [9]. Nowadays, the AH treatment is still attractive since engineers have few choices in surface modification of custom-made devices by additive manufacturing which usually has high processing accuracy [10,11].

Sterilization is compulsory for tools and implants before biomedical applications. There are several reliable sterilization methods available for Ti implants for purposes of basic research and clinical application, including Ethylene Oxide gas treatment (EO), dry heating, autoclaving, Gamma-ray irrigation (GI), *etc.* [12]. Autoclaving is the most popular and considered to be the gold standard for sterilization procedures. However, studies found that, autoclaving will change the delicate micro-nano hybrid surface surfaces of Ti substrate [13]. Sometimes, the change may be undesired, and even worse, severe carbon-contained contamination is always detected on the surface of Ti substrate after autoclaving [14]. Considering the possible decrease of surface activity, dry sterilization methods are usually applied when handling with sandblast large grit and acid-etching (SLA) and AH specimens such as

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EO and GI [15,16]. EO is effective to kill bacteria, but the process is complex and residual gas will cause cold burn. GI is also powerful enough for thorough sterilization, but the accessibility of radioactive source is quite difficult. Although both methods are preferred in mass production, they are costly and not practical for dental offices and laboratories.

Recently, our research group proposed a novel sterilization method, hydrothermal sterilization (HS), which can be feasibly conducted in dental offices and laboratories. A common autoclave is used as instrument and it is conducted at a normal autoclaving procedure of 121 °C, 20 min as well. The only different to AC is that water was used as medium and glass bottle as package [14,17]. High-resolution field emission scanning electron microscopy (FESEM) observation confirmed that such sterilization only alerted the microtopography in nano-scale and did not change the dimension of specimens. More importantly, it presents overwhelming superiority in term of ensuring a good osseointegration according to our previous studies involving sandpaper polished and SLA treated Ti substrates. The HS was hence recommended as a standard sterilization method that can be widely used alternatively for all Ti based endosseous implants without apatite coating. We therefore expected this novel sterilization method can also bring certain biological benefits to Ti substrate after AH treatment.

In this study, HS was applied to AH treated Ti substrate and its effects on the properties of biological ceramic coating were investigated in comparison with AC sterilization. Results were discussed in terms of crystalline structure, surface chemical composition, apatite inducing ability and initial osteoblast-like cell responses.

2. Materials and methods

2.1. Preparation and sterilization of specimens

Commercially available pure Ti plate of Grade 2 (Baoji Titanium Industry, China) was used as substrate. Specimens were cut into disks of 14.5 mm in diameter by linear cutting. After being degreased, they were abraded with *SiC* sandpaper until 800#. Then they were washed and processed with the well-known alkaline-heat treatment to get the AH specimens. Briefly, specimens were kept in NaOH solution of 5 mol/L at 60 °C for 24 h, then thoroughly rinsed with distilled water and heated afterward at 600 °C for 1 h. After furnace cooling, specimens were sealed into polyethylene (PE) bags with air tight zip and stored for 4 weeks.

After storage, AH specimens were divided into two groups and sterilized separately. The first group were sealed into sterilization pouch (A.R. Medicom, QC, Canada) individually and then autoclaved at 121 °C, 20 min in a clinical autoclave (BXM-30R, Boxun, China). These specimens were code as AC and were kept in the same pouch before characterizations and cell tests. The second group was sterilized with the novel HS method. Specimens were put into reagent glass bottles (Boro 3.3, Shuniu, China) and distilled water of 50 mL peer specimen was added. Water was controlled to be < 1/3 of the volume of the bottle to ensure safety. After being sealed with propene polymer (PP) screw caps tightly, these bottles were also put into the same autoclave and autoclaved at 121 °C for 20 min. Specimens were kept in the same reagent bottle with water after cooling down and were coded as HS. They were dried in pure N_2 gas just before surface characterizations. For cell test, HS specimens were taken out from bottles by sterilized tweezers and transferred into culture wells directly.

2.2. Surface characterization

Surface morphology was observed with field emission scanning electronic microscope (FESEM, S4800, Hitachi, Japan). Crystalline phase was determined by thin film X-ray diffractometer (TF-XRD, D8 Advance, Bruker-AXS, Madison, WI, USA) using Cu K_α radiation. Confocal Raman scattering measurements were carried out on a Laser Raman Micro-Spectroscopy (InVia Reflex, Renishaw, UK) at a single wavelength of 514.5 nm. Surface chemical composition of all the specimens was analyzed by X-ray photoelectron spectroscopy (XPS, ESCALAB 250Xi, Thermo Fisher Scientific Inc., America). The spectra were calibrated by setting hydrocarbon C1s to 284.8 eV. Surface wettability was evaluated by contact angles that measured on a contact angle meter (JC2000D, Zhong Chen Co., China) with sessile drop method. Scratch resistance of the coating were investigated using a thin film scratch tester (CSR-2000, Rhesca, Japan). A stylus with diamond tip, radius = 5 μm, was moved on the specimen surface at a speed of 10 μm/s with applied load of 100 mN/min. To evaluate the *in vitro* apatite formation ability, specimens were immersed in Kokubo's simulated body fluid (SBF) at 36.5 °C for 4 days and then observed by SEM. Non-sterilized AH specimens were also immersed as control.

2.3. Cell culture

Osteoblast-like MC3T3-E1 cells were cultured in α-medium supplemented with 10% fetal bovine serum and 1% antibiotics in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was replaced every 2 days. Before confluence, cells were *trypsinizated* and seeded onto specimens. For initial cell spreading study, 1×10^4 cells were seeded onto each specimen and after 3 h of culture they were washed with phosphate-buffered saline (PBS) and then fixed with 3% glutaraldehyde for 30 min at 4 °C. After dehydration with an ascending series of ethanol solution and removing ethanol with hexamethyldisilazane (HMDS), morphologies of cells were observed by SEM. Cell viability was studied by Alamar Blue assay (Biosource International, Camarillo, CA). 1×10^4 cells were seeded onto the specimen in each well and assay was performed after 2, 4 and 6 days' culture, respectively.

2.4. Statistical analysis

3 specimens were used for each test in physiochemical characterizations. For cell tests, a same batch of osteoblast-like cell was used and 5 specimens were used for each evaluation as well. A *t*-test was performed using Origin 8.5 (OriginLab, USA) for individual comparisons of groups and $p < 0.05$ was considered to be statistically different.

3. Results and discussions

3.1. Surface morphology

Fig. 1 (a) and (d) show the surface morphologies of specimens before sterilization. It could be seen, a typical coating of porous structure was formed on pure Ti after AH treatment. The structure consisted of numerous nano-sized struts that interlaced with each other forming a fine network. Pores in the structure could be divided into macro ones of micron/submicron scale and micro ones of nano-scale. After AC, the morphology of coating did not seemed to be essentially changed at low magnification, Fig. 1 (b). While at higher magnification, it was found the struts in the coating structure disappeared and numerous nano-sheets were formed. And interestingly, there was a ridge on top of each sheet which looked like a horizontal bar holding a flag. Those sheets overlapped and interlaced with each other and assembled a porous structure. Unlike that of AC, after HS treatment, the coating on specimen was densified and appreciable change in the morphology could be observed even at low magnification, as could be seen in Fig. 1 (c), although the layer was still porous. Struts formed in the earlier AH treatment disappeared as well. Crinkly leather-like nano-sheets and plenty of nano-rods of 100–200 nm in length were formed and a porous structure was also formed.

Studies revealed that, autoclave could change the appearance of Ti based materials in nano-scale [13,18,19] due to dissolution-precipitation reactions of TiO_2 during the AC sterilization process in which saturated steam of high temperature acted as a moisture medium. The