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Bioactivity and mechanical stability of Ti–6Al–4V implants superplastically embedded with hydroxyapatite in rats

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Abstract

In this in vivo study, Sprague Dawley (SD) rats were used to investigate the bioactivity as well as the microstructural and mechanical properties of Ti–6Al–4V samples embedded with hydroxyapatite (HA) using two different coating methods—superplastic embedment (SPE) and superplastic deformation (SPD). The HA layer thickness for the SPE and SPD samples increased from 249.1 ± 0.6 nm to 874.8 ± 13.7 nm, and from 206.1 ± 5.8 nm to 1162.7 ± 7.9 nm respectively, after 12 weeks of implantation. The SPD sample exhibited much faster growth of newly formed HA compared to SPE. The growth of the newly formed HA was strongly dependent on the degree of HA crystallinity in the initial HA layer. After 12 weeks of implantation, the surface hardness value of the SPE and SPD samples decreased from 661 ± 42.8 HV to 586 ± 249.1 HV respectively. The decrease in surface hardness values was due to the newly formed HA layer that was more porous than the initial HA layer. However, the values were still higher than the substrate surface hardness of 321 ± 28.8 HV. Wear test results suggest that the original HA layers for both samples were still strongly intact, and to a certain extent the newly grown HA layers also were strongly bound with the original HA layers. This study confirms the bioactivity and mechanical stability of the HA layer on both samples in vivo.

1. Introduction

The need for orthopaedic and dental implantations has increased significantly in recent years owing to osteoporosis, bone damage from car/sport accidents and cancer, together with the growing needs of dental/facial reconstruction [1]. Thus, artificial hard tissue replacement implants should integrate well into the musculoskeletal system without causing fibrosis of the connective tissue of the system [2]. For this reason, since the early 1960s concurrent studies have focused on the development of bioactive materials used bare (without a coating) or sometimes with a coating layer [3, 4]. The ultimate goal of the successful fixation of cementless implants used for joint reconstruction is to obtain life-long, secure implant anchoring in the native surrounding bone [5].

Titanium (Ti) and its alloys have been utilized as medical devices, with Ti–6Al–4V being among the most commonly employed. This is due to its excellent combination of biocompatibility and good mechanical properties [6–8]. Nevertheless, the incidence of bone implant interface failure due to ineffective osteointegration with the host tissue is still high [9, 10] and the accumulation of metal ions in the surrounding tissue is reported in long-term implantation, which adversely affects bone tissue growth [11]. Bioactivity is widely viewed as an essential requirement for an artificial biomaterial to exhibit chemical bonding to living host tissues upon bone formation, like apatite layer formation on the material’s surface in any simulated body environment [12, 13]. A bioactive surface can be achieved by coating the metal surface with a thin film of bioactive ceramic such as hydroxyapatite (HA). HA exhibits extended responsiveness as a coating material on titanium surfaces because its roughness increases the surface contact five-fold compared to smooth implants, and it closely resembles the natural minerals in bones and teeth [14–16]. HA coating has been applied extensively for metallic prostheses with the aims of improving bone apposition, implant fixation and reducing healing time [7, 13, 17].
Numerous methods have been established to enhance the surface compatibility of implants with bone. Plasma spraying is very commonly used because of its versatility, ability to attain coating fixation and economic feasibility \([10, 16, 18, 19]\). However, it suffers some drawbacks, such as difficulty with even HA coating and high-temperature processing. Furthermore, it has been reported that the mechanical stability of the interface between the HA coating and titanium alloy substrate could be problematic either during the surgical operation or after implantation \([20, 21]\). Plasma spraying is also not effective for producing a bioactive surface on the inner surface of implants, such as cages or other complex structures. Several ideas to overcome these drawbacks have been suggested \([17, 22–24]\).

The superplastic phenomenon of materials has enabled the formation of complex part geometries requiring particularly high levels of ductility with minimal internal stress \([25]\). The superplastic deformation (SPD) in titanium alloy has widely been exploited in many industries, including biomedical, automotive, electronics and aerospace. The application of SPD in industry offers advantages mainly related to the possibility of producing elements with complex shapes, since its polycrystalline materials can exhibit very high strain values. In medicine, SPD is now an interesting alternative method for forming biomaterials and producing implants. In our previous study, an HA layer with good bonding strength was successfully produced when HA was superplastically embedded into Ti–6Al–4V alloy. The superplastically deformed titanium substrate can strongly support the embedded HA layer \([22, 26–29]\). Other studies on this subject indicate that the superplastically embedded HA layer remains strongly intact on the substrate surface even after the substrate is further deformed (SPD) at high temperatures without HA structure deterioration \([23, 24]\).

The HA layers after SPE and SPD in an \textit{in vitro} condition were also evaluated in our prior study. Results from the \textit{in vitro} study of the HA layer after SPE and SPD suggest that newly formed apatite layers can grow within a week of immersion in simulated body fluid (SBF). The HA layer can also withstand a relatively high load applied to the surface \([24, 29]\) However, the performance of SPE and SPD implants in an \textit{in vivo} condition has not yet been studied. Therefore, in this work, SPE and SPD samples are implanted subcutaneously on the backs of SD rats in order to evaluate their performance \textit{in vivo}. The embedded HA layers after the \textit{in vivo} study were further exposed to a wear test to evaluate the level of their mechanical stability.

2. Materials and methods

2.1. Sample preparation (SPE and SPD)

Ti–6Al–4V alloy (dimensions: 17.5 mm \(\times\) 3 mm \(\times\) 3 mm) was used as the substrate material. HA powder of a spherical particle shape and size of 45 \(\pm\) 0 \(\mu\)m (Taihei Chemical Co. Ltd, Tokyo, Japan) was used as the embedment material. To obtain superplastic formability, the as-received Ti–6Al–4V was solution-heat treated above \(\beta\)-transus temperature (1100 °C) for 30 min and ice-quenched to room temperature, as shown in figure 1. Prior to the embedment process,
the sample surfaces were polished using 1000-grit emery paper with alcohol to remove the oxide layer and irregularities.

The heat-treated Ti–6Al–4V was heated to the embedment temperature (927 °C) and maintained for 1 h before being compressed under $1 \times 10^{-4}$ s$^{-1}$ strain rate until the material’s thickness was reduced by 54%. The samples were cooled to room temperature in a furnace at the end of the process. This process is termed superplastic embedment (SPE).

To prepare the SPD sample, the SPE sample was then deformed at 927 °C under a strain rate of $1.6 \times 10^{-3}$ s$^{-1}$ to achieve a 30% thickness reduction. The sample was cooled to room temperature. Here, the experimental setup was similar to that for the SPE process, except no HA powder was involved. Both processes were performed using a compression testing machine (Instron Corporation, Norwood, MA, USA) equipped with a high-temperature furnace and argon gas atmosphere (controlled gas). A special die and punch were fabricated for the compression test. The material used for the die and punch was high heat resistance steel, where this material is appropriate for hot forming of Ti [30]. Figure 2 illustrates the schematic diagram of the experimental setup for the SPE and SPD processes.

2.2. Animals
Forty five male albino rats of SD stock (weight: 250–300 g) were used in this study. The rats were housed in an internationally accredited facility at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Standard conditions were followed (23 ± 1 °C temperature and a 12 h light/dark cycle) and the rats were fed with a standard pellet diet (Brand: Altromin) and reverse osmosis (RO) water. The guidelines for handling the rats were in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (2011), and the protocols were approved by the Faculty of Medicine, Institutional Animal Care and Use Committee (IACUC).

2.3. Implantation
Samples were implanted subcutaneously on the backs of the albino rats (dorsum area) for 1, 5 and 12 weeks. For each period, five as-received Ti–6Al–4V, five SPE samples and five SPD samples were available for the analysis, giving a total of 45 implant samples. The as-received Ti–6Al–4V was used as a control sample. The animals were anaesthetized by intraperitoneal injection with a mixture of Ketamine HCl (50 mg kg$^{-1}$ body weight) and Xylazine (5 mg kg$^{-1}$ body weight). The rat fur was shaved in the dorsum area and then betadine solution (10%) was applied. An incision approximately 1cm long was made at about the mid-portion of the back and the subcutaneous layer was spread to create a pocket. Subsequently, the respective sample was inserted into the incision, as shown in figure 3. Each incision was closed with intermittent sutures 0.5 cm apart, using surgical nylon thread and a No. 4 suture.

2.4. Analysis
The animals were sequentially euthanized with CO$_2$ at the specified time interval after surgery. X-ray diffraction (XRD, Bruker D8 Advance) was used to analyse the HA crystal structure stability after implantation. Field
emission scanning electron microscopy (Zeiss; FESEM) was employed to characterize the surface morphology, and a Vickers microhardness tester was used under an applied load of 2N and a loading time of 10 s to measure the surface hardness. Measurements were taken five times for each evaluation.

2.5. Binding testing
The binding strength of the SPE and SPD HA layers after 12 weeks of implantation were tested using a wear test against polishing cloths saturated with 500 ml 10% neutral-buffered formalin (NBF) solution at room temperature for 30 min. The tests were carried out using a rotational speed of 165 rpm under pressure of 0.5 MPa.

2.6. Statistical analysis
Statistical analyses on the data of the thickness of the newly-formed HA layer of SPE and SPD samples were performed using a t-test. The significance level was set at $p < 0.05$. Calculations were performed in Microsoft Excel 2010.

3. Results and discussion
In this study, the as-received Ti–6Al–4V substrates were solution-treated prior to the embedment process. Since the heat treatment results are similar

Figure 5. Surface morphologies and cross-sectional view of the embedded HA layer for (a) SPE sample and (b) SPD sample.

Figure 6. X-ray diffractograms of: (a) pure HA powder, (b) SPE sample and (c) SPD sample.
to previous studies [22–24, 27–29], the evaluation is not discussed here.

3.1. SPE and SPD processes

Figure 4 represents the stress versus strain curves obtained from the SPE and SPD processes. A typical increase in stress with strain is observed for both processes. According to the figure, the SPE process shows higher maximum stress (154 ± 14.9 MPa) than the SPD process (112 ± 7.7 MPa). The sample thickness reduced to 1.98 ± 0.2 mm and 1.37 ± 0.1 mm after SPE and SPD, respectively. Table 1 summarizes the properties of each process. No cracks were identified after either process, indicating that the stress level experienced during the procedures was below the failure region. This also suggests that superplasticity occurred and had an important role in both processes through grain boundary sliding.

The surface morphologies of the embedded HA for the SPE and SPD samples and their cross-sectional images are depicted in figure 5. Following the SPE process, an HA embedded layer was obtained of approximately 249.1 ± 0.6 nm (figure 5(c)), with well-melted splat morphology (marked by black arrows) and very fine globular morphology of un-melted HA (marked by white arrows) (figure 5(a)). In contrast, after the SPD process (figure 5(b)), the surface was relatively rough, contained cracks and experienced delamination, and the HA layer was completely removed from the substrate’s surface in certain areas. The SPD sample further experienced 30% deformation where the embedded HA layer’s thickness diminished, as shown in figure 5(d). After SPD, the HA layer was approximately 206.1 ± 5.8 nm thick. The obtained results are quite similar to our previous works, where the embedded HA layer decreased when exposed to higher compression stress [23, 24]. However, according to the cross-sectional view of SPD, the HA nano-layer remained intact and uniform on the substrate, even after experiencing such large deformation.

The XRD observation in figure 6 illustrates that the HA structure was retained even after experiencing deformation. Moreover, the HA crystallinity improved with further heating at 927 °C during the deformation process, which is indicated by the strong HA line peaks, especially at around 40°. Some studies confirmed that HA crystallinity increases when HA coatings are heat-treated [31, 32]. This will be discussed further in a later section.

Figure 7. Cross-sectional views and surface morphologies of the SPE sample at different time points after implantation. (ii) Magnification at ×5000 (iii) magnification at ×10 000. (a) 1 week of implantation. (b) 5 week of implantation. (c) 12 week of implantation.
3.2. Implantation analysis

3.2.1. Macroscopic observation
All rats tolerated the operative procedure well. The as-received, SPE and SPD implants remained intact and subcutaneously united at all post-implantation time points. From a general observation of the rats, no operative site infections and no behavioural changes or visible signs of physical/systematic impairment were recognized in the experimental animals.

3.2.2. SPE and SPD: morphology and HA layer stability
The HA layer thickness and surface morphology of the SPE samples after implantation for various periods of time are illustrated in figure 7. After 1 week
of implantation, the HA layer thickness increased to approximately 537.9 \pm 12.9 \text{ nm} (figure 7(a) (i)). A combination of needle-shaped crystal clusters and bone-like structure (spongy bone) appeared on the entire surface as a newly grown HA layer (figure 7(a) (ii)). It was found that some areas were only covered with spongy or needle-shape structures while other areas were covered with a mixture of both structures. Micro pores are also recognizable more on the spongy area and a well-interconnected macro-porous structure is identified only on the needle-shape structure. The spongy structures appeared where the new HA layer growth was initiated [33]. The HA layer thickness increased to approximately 737.6 \pm 84.6 \text{ nm} (figure 7(b) (i)) and 874.8 \pm 13.7 \text{ nm} (figure 7(c) (i)) when the implantation time increased to 5 and 12 weeks, respectively. The SPE surface morphology evoked abundant rough apatite formation structures after 5 weeks of subcutaneous implantation (figure 7(b) (ii)). In week 12, the overall structure was the same as in week 5, but apatite formation structure growth was apparent, as illustrated by the rougher globular apatite structure with very fine micro pores (figure 7(c) (ii)).

Cross-sectional and surface morphology images of the SPD samples after implantation in weeks 1, 5 and 12 are shown in figure 8. At 1 week after implantation, the surface morphology of the sample (figure 8(a) (ii)) was similar to the morphology of the SPE sample after 5 weeks of implantation. At this stage, the needle-shape structure is observed after 1 week of implantation of SPE is not in the appearance. The HA layer thickness increased drastically to 731.8 \pm 50.1 \text{ nm} (figure 8(a) (i)). By 5 weeks of implantation, the surface morphology was generally the same as in earlier weeks, but the spongy structure was still quite noticeable as marked by the white arrows (figure 8(b) (ii)). This suggests that the HA layer growth continued. The HA layer thickness here was approximately 1139.8 \pm 50.1 \text{ nm} (figure 8(a) (i)). By 5 weeks of implantation, the surface morphology generally the same as in earlier weeks, but the spongy structure was still quite noticeable as marked by the white arrows (figure 8(b) (ii)). This suggests that the HA layer growth continued. The HA layer thickness here was approximately 1139.8 \pm 50.1 \text{ nm} (figure 8(a) (i)). After 12 weeks of implantation, the surface morphology showed no sign of a spongy structure (figure 8(c) (ii)). The HA layer thickness also only shows marginal growth, as seen in figure 8(c) (i) (1162.7 \pm 7.9 \text{ nm}). This indicates that HA layer growth stabilized, and no more growth was expected. Higher magnification images (\times 10000) (figures 7 and 8(a)–(c) (iii)), give a better look of the surface morphology condition. The surface is seen to have a certain degree of roughness that would make cells react easier [34].

Figure 9 summarizes the HA layer thickness growth with implantation time for both SPE and SPD samples. It can be clearly distinguished that despite having a thinner initial HA layer before implantation (206.1 \pm 5.8 \text{ nm}), the SPD sample experienced much faster growth than the SPE sample (initial HA layer thickness of 249.1 \pm 0.6 \text{ nm}). It is interesting to note that even after the new HA layer formed and totally covered the initial HA layer surface of both SPE and SPD samples, the HA layer growth in the following weeks was not of the same rate. From figure 9 it can be determined that the HA layer growth in SPE from week 1 to 5 was about 200 \text{ nm}, whereas for SPD, the growth was much greater at about 408 \text{ nm}. \( p < 0.001 \) for SPE and SPD in week 1, 5 and 12. This suggests the importance of having more HA in the initial condition in order to achieve rapid growth of crystallized HA during implantation. The results also revealed that even after the new crystallized HA layer formed and covered the initial HA layer, the growth of the new HA layer was strongly dependent on the HA amount in the initial condition (before implantation). In this regard, some studies have reported that HA coatings with high degrees of crystallinity demonstrated more direct contact in vivo [35, 36].

Figure 10 illustrates the XRD patterns of the SPE and SPD samples after 1 and 12 weeks of implantation. Obviously, the HA peaks significantly increased after implantation. This is due to the formation of newly grown HA on the HA layers after implantation [24, 29, 37]. The peaks increased with the growth of newly grown HA layers. The HA line peaks for the SPD samples are stronger than for SPE. The results confirm that the initial (deformed) HA layer was able to enhance the bioactivity of the samples.

Figure 11 shows the surface hardness values for the substrate and samples before and after implantation.
The substrate hardness was about $321 \pm 28.8$ HV. After the SPE and SPD processes, the hardness increased to $661 \pm 0.4$ HV and $585 \pm 6.6$ HV respectively. For both samples, the hardness decreased with implantation time even though the HA layer thickness increased (figure 9). This is because the new HA layer that grew on the initial HA layer was more porous than, and not as dense as, the initial layer. The decrease in hardness was more apparent in the SPD samples.

3.2.3. Mechanical stability after in vivo study

Figure 12 shows the cross-sectional view of the SPE and SPD samples (after 12 weeks implantation) after the wear test. Based from the figure, the HA layer thickness ($415.3 \pm 41.1$ nm and $444.3 \pm 46.1$ nm for SPE and SPD respectively) results suggest that the original embedded HA layers for both samples were still strongly intact, and to a certain extent the newly grown HA layers also were strongly bound with the original HA layers.

3.2.4. Stability of as received Ti–6Al–4V in an in vivo condition

For comparison purposes, as received titanium alloy samples were exposed to the same in vivo condition for 5 and 12 weeks. As shown in figure 13(a), after 5 weeks, the surface of the as-received sample was almost like its original condition. After 12 weeks, formations of dark patches on the titanium alloy surface (figure 13(b)) were observed. Higher magnification of the patches (figure 13(c)) revealed a bone-like cell structure, which is similar to the spongy-like microstructure that formed in the SPE and SPD samples discussed earlier in section 3.2.2. However, the formation of the bone-like cell structures took about 12 weeks and they were not homogenized throughout the surface.

From the preceding results, both SPE and SPD samples functioned appropriately in an in vivo condition throughout the testing. New HA layers grew in both samples even after just 1 week of implantation as compared to 12 weeks for the as-received sample, indicating that the HA embedded layers reacted positively with the body system. The bioactivity rate measured in terms of new HA layer growth was better in the SPD sample than SPE because the HA crystallinity in the initial layer was higher. Based on binding strength analysis in both samples, the strengths within the newly grown HA layers and HA/Ti interface were also mechanically stable throughout the testing period. This finding is expected...
to provide valuable information for the application of HA-based implants in the medical field.

4. Conclusions

In the present study, the raw and as-received Ti–6Al–4V samples embedded with HA in two different coating methods—SPE and SPD has been implanted subcutaneously on the backs of SD rats for 1, 5 and 12 weeks. Through in vivo testing, dense HA layers 249.1 ± 0.6 nm and 206.1 ± 5.8 nm thick and with surface hardness values of 661 ± 0.4 HV and 585 ± 6.6 HV formed on the SPE and SPD samples, respectively. For the SPE and SPD samples, the newly formed HA layer was able to grow on the initial HA layer even after one week of implantation. The growth of the newly formed HA layer was strongly dependent on the degree of crystallinity of the initial HA layer. The SPD sample showed much faster growth of the newly formed HA layer compared with SPE, because the degree of HA crystallinity in the initial layer was higher. The surface hardness values for SPE and SPD samples after 12 weeks of implantation decreased to 586 ± 1.3 HV and 425 ± 86.9 HV respectively. This is because the newly formed HA layer was basically more porous than the initial HA layering. However, the values were still higher than the substrate alloy (321 ± 28.8 HV). After a wear test, the original HA layers for SPE and SPD samples were still strongly intact, and to a certain extent the newly grown HA layers also were strongly bound with the original HA layers.

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