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Cytocompatibility evaluation of nano-sintered Ti-15Zr-4Nb-2Ta-0.2Pd alloy produced by spark plasma sintering technique

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Abstract. Nano-sintered Ti-15Zr-4Nb-2Ta-0.2Pd alloy made up of non-toxic elements was produced by mechanical alloying using high energy ball mill and synthesized by spark plasma sintering (SPS) hot furnace with an objective to develop cytocompatible metallic implant. These were done to develop an alloy capable of avoiding adverse body reaction and increase the longevity of human life by replacing the lost or diseased organs to restore form and function. Microstructure obtained shows well defined grain boundary lines for sintered CP Ti with no surface porosity. Sintered Ti-6Al-4V microstructure showed well defined sinter-bonding of grain matrix with white rings on grain boundary lines and sintered Ti-15Zr-4Nb-2Ta-0.2Pd alloy showed no definite orientation of grains on the surface and had obvious visible grain boundary lines with a few observable surface pores across the area of the specimen. Cytocompatibility test was conducted by culturing CP Ti, Ti-6Al-4V and Ti-15Zr-4Nb2Ta-0.2Pd alloys separately with murine fibroblast L929 at a concentration of 70 cells/well, and incubated for 7 days at a temperature of 37 °C. Amongst CP Ti, Ti-6Al-4V and Ti-15Zr-4Nb-2Ta-0.2Pd specimen surfaces, the murine fibroblast L929 cells were well attached, grew and formed more colonies on CP Ti and Ti-15Zr-4Nb-2Ta-0.2Pd and least attached to the Ti-6Al-4V surface.

1. Introduction

The success of the biomaterials mainly depends on the reaction of the human body to the implant and this measures the biocompatibility and cytocompatibility of the material [1]. Cell attachment in cytotoxicity analysis is the approach which is used in the comparing of adhesive interactions of cells to the implant in the field of biomedical applications. The main source of the toxic effects that metallic implants have on cells is the metal ion release which is reduced due to high surface integrity as well as corrosion resistance of the implant. The healthy adhesion of cells to the implant surface, as well as their growth and proliferation, are the most important indicators of material cytocompatibility. Little attention has been given to the use of powder metallurgy techniques, mechanical alloying (MA) and

Spark Plasma Sintering (SPS) in the production of nano-grained materials made up of non-toxic and biocompatible elements for orthopaedic applications.

The influence of SPS consolidation on the microstructure of mechanically alloyed Ti-20-13Zr has been previously studied by Hussein and results obtained indicated a mixture of β-titanium in a partially amorphous phase that formed as a result of crystal defect evolution during the mechanical alloying process [7]. Furthermore, the microstructural examination of TNZ alloy displayed grain boundaries together with the secondary structure of dendritic structure. Apart from that, same year Hussein et al., 2015 further studied the effect of sintering parameters on microstructure, mechanical properties and electrochemical behaviour of Nb-Zr for biomedical applications. Results showed that the combination of mechanical alloying and spark plasma sintering could be a promising approach to successfully produce a nano/submicron grain structured Nb-Zr alloy [7]. Specifically, the use of SPS prevents undesirable grain growth within the nano/submicron-grain structured and resulted in a grain size between 100-300 nano-meters.

Surface characterization such as composition and topography exhibit a high influence on the success of implant treatment as it has a direct influence on cell proliferation and differentiation of bone cells, due to this reason the cell adhesion to bone apatite is consequently sacrificed. In o, an implant requires a surface that promotes osteogenic differentiation (i.e. includes human or mouse stem cells functionally defined by their capacity to self-renew and differentiate into multiple cell types such as osteocytes) [2]. Titanium and titanium alloys such as Ti-64 and TiNi have found extensive applications in dentistry and orthopaedics. When the titanium alloy is implanted into a human body, the release of metal ions from the implant may generate an adverse biological effect or allergic reactions. Furthermore, it was observed that both aluminium and vanadium making up the Ti-64 alloy are toxic and can cause mutagenic cytology in addition to triggering allergic reactions. Li et al., 2010 studied the cytotoxicity of titanium and titanium alloying elements such as Ta, Zr, Mo and Si in forms of bulk and powders and different degrees of cytotoxicity was observed. Amongst which Mo and Si exhibited strong cytotoxicity, meanwhile Ta, Zr and Sn exhibited good biocompatibility in both bulk and powder forms. These results indicated that Mo should be limited or rather not be used in designing and developing titanium alloys for dental and orthopaedic device applications [3]. The findings by Li et al., 2010 provided fundamental knowledge of alloying elements that can be used in the future as implants. In this work, cytocompatibilty evaluation of Ti-15420.2 in comparison with CP Ti and Ti-64 was assed using murine fibroblast L929 cells. The assessment was carried out by recording the number of colonies (i.e. a group of cells) attached to the sample surface.

2. Materials and methods

2.1. Materials

High purity elemental powders of titanium, aluminium and vanadium (of -44 microns and 99.0-99.5 % purity); zirconium (of -149 microns, 99.5% purity), niobium (of 1-5 microns, 99.8% purity), tantalum (of 2 microns, 99.9% purity) and palladium (of < 1 micron, 99.9% purity) supplied by Alfa Aesar and Sigma Aldrich were used as starting materials. The powders were mixed in weight percentage as shown in Table 1. The powders were prepared using MB 200B ECO glove box under very high vacuum or an inert atmosphere of argon and mixed for 30 minutes at 49 rpm using the T2F Tabular shaker mixer to obtain a homogeneous mixture. The powders were then mechanically alloyed (MA) for 5 hours at a speed of 500 rpm using a stainless steel pot with <10mm diameter stainless steel balls with an objective to reduce the powder particle size to <100 nanometers (nm) using a powder to ball ratio of 10:1 under argon atmosphere. Mechanically alloyed powders were sintered using the spark plasma sintering (SPS) machine (FCT system-model HHP D25, Germany). The powders were loaded into a 40mm diameter graphite die and punch where a thin graphite foil was placed between the powders and the die to reduce the friction between the die walls and the powders. The test was carried out in a vacuum at a pressure of 50 MPa, the sintering temperature of 1200 °C, heating rate and holding time used was 100ºC/min and 10 minutes respectively [4]. The sintered sample of 40mm

diameter and 2mm thickness were ground using grit 320 and 600 grinding paper and water as a lubricant. First and second stages of polishing were obtained by ultra Pad and Trident polishing cloths, polycrystal 9µm and polycrystal 3µm as lubricants. The metallographically prepared samples were further etched with a mixture of hydrofluoric acid, nitric acid and ultra-pure water prior to microscopic evaluation using JSM 7600F scanning electron microscope (SEM) equipped with energy-dispersive X-ray (EDX) and microscopic evaluation on cytocompatibility was done on Nikon Eclipse ME600L optical microscope (OM).

Table 1. Chemical composition (wt. %) of CP Ti, Ti-6Al-4V and Ti-15Zr-4Nb-2Ta-0.2Pd alloys.

Titanium alloyed powders	Weight percentages of the powder mix
CP Ti	100 %Ti
Ti-6Al-4V	6%Al, 4 %V, & 90%Ti
Ti-15Zr-4Nb-2Ta-0.2Pd	15%Zr, 4%Nb, 2%Ta, 0.2% Pd & 78.8%Ti

2.2. Cytocompatibility test

CP Ti, Ti-6Al-4V and Ti-15Zr-4Nb-2Ta-0.2Pd samples were ultrasonically cleaned using acetone, methanol and ethanol for 5 minutes each. Sterilization on as polished samples was done by autoclaving for two hours at a temperature of 120 ºC in a dry condition in preparation for cytocompatibility test. The cytocompatibility evaluation was performed using the direct contact method, where cell attachment on each sample (three of each alloy) was evaluated by recording the number of colonies (i.e. a group of cells) attached to the samples. A 24 well microplates were used to locate samples separately and cultured with murine fibroblast L929 at a concentration of 70 cells/will in 1mL of the culture medium (E-MEM + FBS). The same number of cells were seeded into an empty well to serve as a control well and incubated for 7 days at 37°C in a 5% vol% C02 atmosphere with 95% relative humidity. After incubation, the cells were fixed and stained using 25% glutaraldehyde solution for 10 minutes and 10 vol% Giemsa's staining solution for 15 minutes, respectively. Fixing was done to kill the cells such that they do not move on the slide or dish and staining was done such that the cells and their organelles holds the stain and appear in purple colour. This helps the observer to see and count cells using their naked eye. Stained cells were observed and captured using a NIKON optical microscope. Results obtained from the counting were presented by calculating the relative plating efficiency (*PE*) using the equation 1:

$$\text{PE}\ (\%) = (Ns\ /\text{As})/(Nc\ /Ac\ ) \quad (1)$$

where N_s and N_c indicate the number of colonies on the sample top surface and in control well, respectively. A_s indicates the sample top surface area and A_c indicates the well bottom area in cm^2. The data obtained was analysed statistically using student *t*-test [5].

3. Results and discussions

3.1. The microstructures of the sintered specimens

Figure 1 shows the microstructure of the sintered specimens obtained from scanning electron microscope (SEM) and elemental mapping of selected areas obtained through energy dispersive spectroscopy (EDS). The chemical analysis of all specimen consisted of only elements included in the compositional design: sintered CP Ti (Figure A & A.1), Ti-6Al-4V (Figure B & B.1) and Ti-15Zr-4Nb-2Ta-0.2Pd (C & C.1). The surface micrograph of CP Ti sintered specimen shows no indication of surface porosity which is associated with incomplete sintering in terms of neck formation. The grain boundaries are well defined with visible homogeneous grain boundary lines. From observation of SEM micrographs of sintered Ti-6Al-4V alloy, the surface has a clear surface topography with a white ring shaped compounds at the grain boundary sites of the matrix. The SEM micrographs of sintered Ti-15Zr-4Nb-2Ta-0.2Pd alloy show a high volume of grain boundary sites, with irregular orientation.

Regardless of the obvious of metallographic finishing on the specimen surface (Figure 1 C.2), it can be observed that the Ti-15Zr-4Nb-2Ta-0.2Pd alloy sintered at 1200oC consists of large surface pores across the area of the sample.

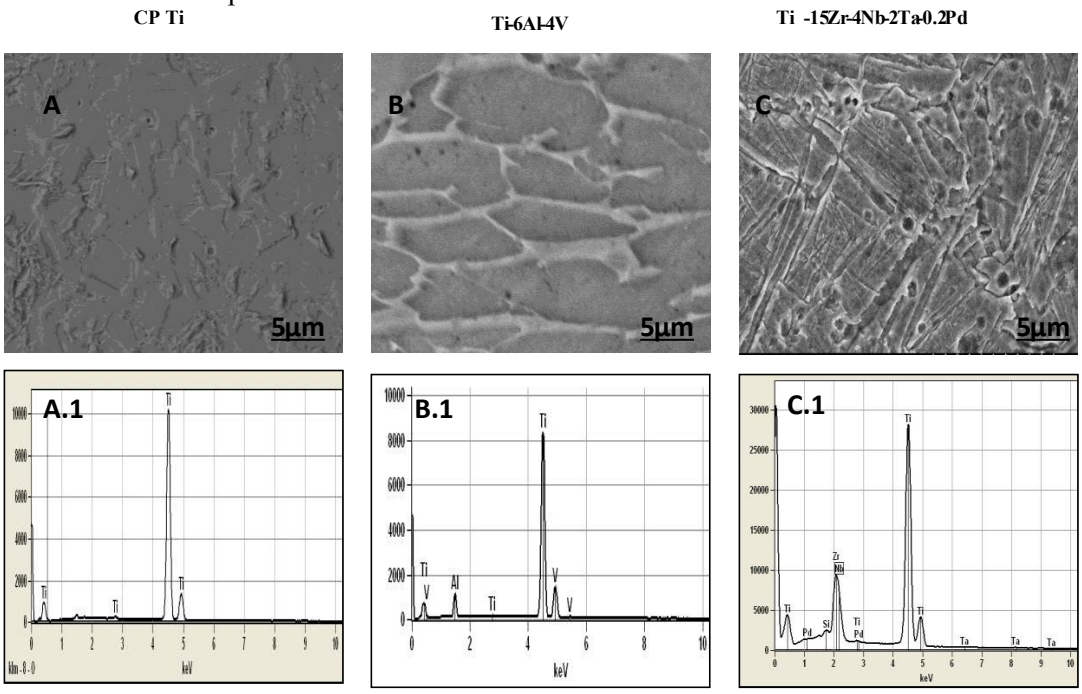

Figure 1. SEM surface micrographs and microstructure characteristics and EDS analysis of CP Ti (A & A.1), Ti-6Al-4V (B & B.1) and Ti-15Zr-4Nb-2Ta-0.2Pd (C & C.1) sintered at 1200oC using the SPS HHPD 25 hot furnace.

3.2. Cytocompatibility test

The evaluation of cytotoxicity was done for Ti-15Zr-4Nb-2Ta-0.2Pd, Ti-6Al-4V and CP Ti which were cultured separately with murine fibroblast L929 at a concentration of 70 cells/well and incubated for 7 days at a temperature of 37 °C. The results of the calculated plating efficiency and morphology of cell attachment to each sample surface are presented in figure 2 and Figure 3 respectively. Both commercially pure titanium and Ti-6Al-4V alloy are known for their development of highly stable surface oxide layer especially when exposed to air or to aqueous media which is responsible for bone bonding characteristics of titanium implants, hence this project used both CP Ti and Ti-6Al-4V as benchmarks in order to study and compare the behaviour of Ti-15Zr-4Nb-2Ta-0.2Pd in comparison with both CP Ti and Ti-6Al-4V.

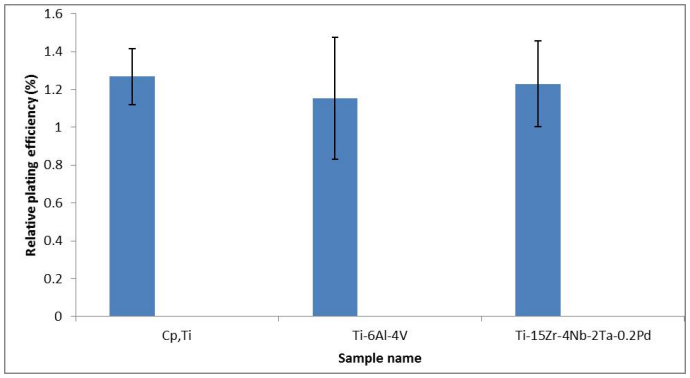

Figure 2. Relative plating efficiency of murine fibroblast L929 cells on the samples.

In the implant and body system, various interactions and reactions may occur including metal ions in the body fluid as well as the transportation to body organs and if the limit of toxicity for the implant is exceeded, this further results in injuries, inflammation and pain for the patient if the implant is found to be toxic rather than compatible. Cell attachment, cytocompatibility or cytotoxicity test serves as an indication as to whether or not an implant used for orthopaedic applications is compatible. Figure 2 shows the relative plating efficiency of all Ti-15Zr-4Nb-2Ta-0.2Pd alloy in comparison with Ti-6Al-4V and CP Ti whereas, Figure 3 shows the morphology of cells attached to each sample surface. No significant difference was observed from Figure 2 due to the fact that cell attachment on all samples as shown in Figure 3 attributed to good cytocompatibility properties with Ti-6Al-4V obtaining the least cell attachment. The relative platting efficiency calculated compliments the results obtained for cell attachment as Ti-6Al-4V still obtained the lowest relative plating efficiency. That said, it is quite obvious that Ti-6Al-4V alloy could be easily substituted with Ti-15Zr-4Nb-2Ta-0.2Pd alloy as it is less toxic and attributed more relative plating efficiency. Furthermore, events after implantation include interactions between biological environments and the artificial material surface or implant, thus, the onset of biological reactions, as well as the particular response by the human body are most critical in orthopaedic device applications.

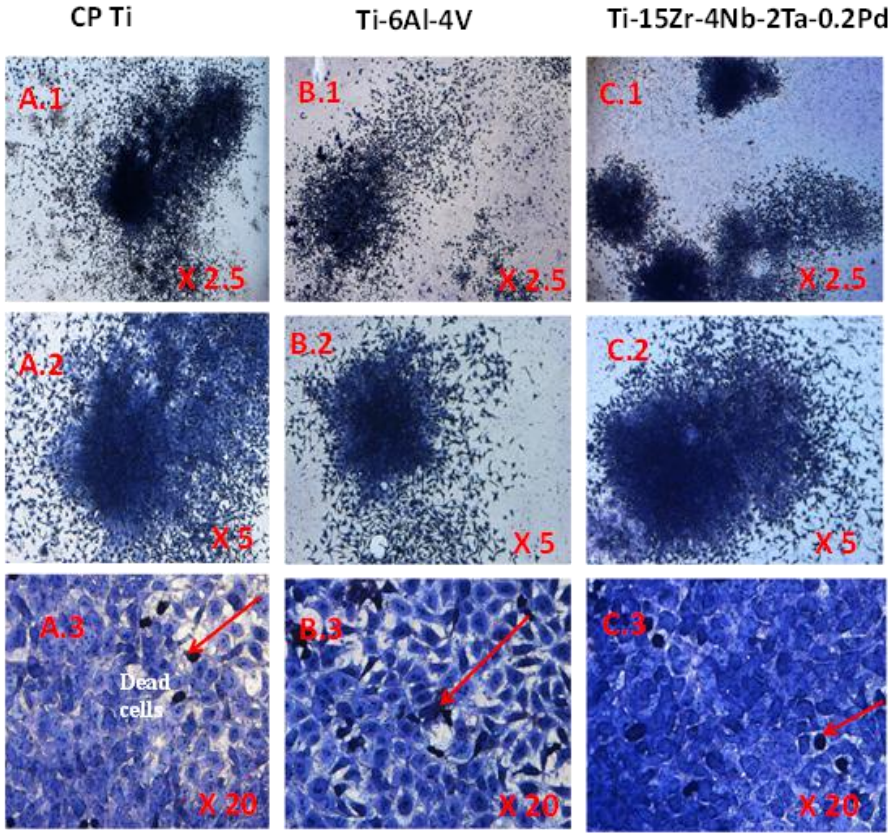

Figure 3. Optical microscope images of colony morphology for the surface of (A) CP Ti, (B) Ti-6Al-4V, (C) Ti-15Zr-4Nb-2Ta-0.2Pd surfaces at X 2.5, 5 and 20 magnification.

The growth of cells on sample surfaces was observed under optical images in Figure 3. Results obtained were in agreement with the calculated plating efficiency presented in Figure 2. Amongst CP Ti, Ti-6Al-4V and Ti-15Zr-4Nb-2Ta-0.2Pd samples surfaces, the murine fibroblast L929 cells well adhered, grew and formed more colonies on CP Ti and Ti-15Zr-4Nb-2Ta-0.2Pd and least attached to the Ti-6Al-4V alloy surface. The cells were well distributed and spread over the samples. Cell attachment relies on the interactions with extracellular substrates in order to maintain the critical cell functions such as cell proliferation. This is further described as the process that results in an increase

in the number of cells defined by the balance between cells that survive verses cell loss as a result of cell death. It was easy to distinguish between cells that survived amongst dead cells as cells that survived appeared shinny and were visible to the naked eye after staining whereas dead cells appeared dark and dull on the surface as shown by arrows in A3, B3 and C3 for CP Ti, Ti-6Al-4V and Ti-15Zr-4Nb-2Ta-0.2Pd respectively. More dead cells were observed on the Ti-6Al-4V surface as opposed to both CP Ti and Ti-15Zr-4Nb-2Ta-0.2Pd. Cell attachment also relies on the expression of structural and adhesive proteins as well as cell viability. A decrease in cell viability and cell proliferation greatly reduces the capacity of the cell to integrate with the implant and this would result in bone growth to the implant. Furthermore, cell adhesion and proliferation sturdily depend on the surface microstructure, chemical composition, wettability and surface roughness [6].Ti-15Zr-4Nb-2Ta-0.2Pd alloy and CP Ti exhibited superior cell adhesion and proliferation than Ti-6Al-4V after 7 days of cell culturing.

4. Conclusion

This pioneering work outlines nano-sintered pure titanium (CP Ti) and titanium alloys (Ti-6Al-4V and Ti-15Zr-4Nb-2Ta-0.2Pd) fabricated by spark plasma sintering for evaluation of cytocompatibility characteristics. The samples resulted in high densification and adequate microstructure due to the complete dissolution of the alloying elements such as zirconium, niobium, tantalum and palladium in the titanium matrix. Furthermore, the complete dissolution of those alloying elements resulted in a good combination of the microstructure of fully densified samples. The sintering parameters and sintering technique (SPS) used for this project resulted in a homogeneous microstructure with low surface porosity and contamination. It is well known that higher sintering temperatures and longer holding times constitute to intensive grain growth. SPS is known to attain finer grain sizes which have proven to exhibit better service properties, hence the microstructural parts of the nanosized matrix obtained through milling are the most eligible for meeting today's industrial and biomedical applications. Results obtained suggested Ti-15Zr-4Nb-2Ta-0.2Pd alloy to be used for biomedical applications due to the fact that the alloy contains non-toxic elements and therefore has the ability to replace Ti-6Al-4V which was found to contain toxic elements; Al and V. Overall results obtained where biocompatibility is concerned, Ti-15Zr-4Nb-2Ta-0.2Pd alloy produced by mechanical alloying and spark plasma sintering showed a potential advantage to be used for biomedical applications. In addition, both tantalum (Ta) and niobium (Nb) making up the Ti-15Zr-4Nb-2Ta-0.2Pd alloy are known for their extremely stable oxide layer formed on the alloy surface which exhibits metal ion release and still shows no cytotoxicity effects. For this reason, Ti-15Zr-4Nb-2Ta-0.2Pd alloy can be regarded as a candidate for biomaterial in orthopaedic device application.

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