Biocompatibility Study of Ti-based Alloys Fabricated by Spark Plasma Sintering

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\textbf{Abstract.} Titanium (Ti) alloys often find their way in orthopaedic applications owing to their superior mechanical properties, biocompatibility, corrosion resistance and low Young’s modulus. Nano/sub-micron grain structured commercially pure titanium (CP Ti), Ti-6Al-4V (Ti-64) and Ti-34Nb-25Zr (TNZ) alloys were fabricated via mechanical alloying (MA) of elemental powders followed by spark-plasma sintering (SPS). The powders were milled at 500rpm for 5h and sintered at a temperature of 1200°C, heating rate of 100°C/min, dwelling time of 10min and a pressure of 50MPa. The sintered samples were metallographically prepared to a mirror finish surface in 5:2 ratio of colloidal silica and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) mixture. Biocompatibility tests were conducted by water soluble tetrazolium and 1-methoxy phenazinium methylsulfate (WST-1 PMS) with sarcoma osteogenetic-2 (SaOS-2) human cell line. High cell proliferation on Ti and Ti-64 was observed after 1 and 4 days of incubation. After 7 days, proliferation on Ti-64 was the highest followed by Ti. However, TNZ alloy have less cell proliferation through the entire period of incubation.

Fibronectin adsorption is less for Ti and TNZ compared to and Ti-64. In decreasing order albumin adsorption was highest on Ti than TNZ alloy followed by less adsorption on Ti-64. Surface wettability of Ti and Ti-64 showed a high contact angle of between 93-98° compared to 86° TNZ alloy. The surface wettability results correlated well to less fibronectin adsorption on Ti-34Nb-25Zr alloy and highest for Ti-6Al-4V. The Ti-34Nb-25Zr showed less cell proliferation which is believed to be linked with fibronectin adsorption results.
1. Introduction

Biometallic materials are increasingly used as implants in medical applications [1]. Titanium alloys are the mostly used metallic biomaterials, specifically for biomedical applications [2]. Owing to their set of suitable properties for those applications, such as low specific weight, high corrosion resistance and biocompatibility [3]. Corrosion resistance is a property of utmost importance in biomedical applications [4]. Biomedical implants are subjected to physiological fluids which are chloride containing and maintained at 37°C. Additionally, corrosion products are responsible mainly for poor biocompatibility and may cause undesirable reactions to the implant-adjacent tissues [5], and corrosion resistance helps in deciding whether the alloy is a best candidate for use as a biomaterial. Some biomaterials that have been used had low biocompatibility and this has encouraged research to improving materials with excellent mechanical behaviour and biocompatibility [5-6].

Ti-6Al-4V is the first titanium alloy used for implant materials, and it is mostly used in implant materials for dental, orthopedic and osteosynthesis applications [7]. However, studies have indicated that vanadium, a beta-phase stabilizer, produces oxides that are harmful to the human body [7-8]. The toxicity associated with vanadium has encouraged the research for alternative materials to replace Ti-6Al-4V [8]. In order to replace Ti alloys containing vanadium, Ti-6Al-7Nb was developed and it is available commercially [9]. Niobium is a beta-phase stabilizer. However, there has been a concern also associated with Al and Alzheimer’s disease [10]. Ti-13Nb-13Zr has been recently developed, a near-beta phase alloy [11-12], the Ti, Nb and Zr elements are not toxic and display excellent biocompatibility [13-15].

The addition of Nb to Ti stabilizes the β phase and it improves the mechanical properties [2]. Some of the Ti-based alloys have been produced using arc melting and casting techniques [2, 3; 6–12]. However, when the melting temperatures between the alloy constituents broadly range, the use of conventional casting techniques in the synthesis of Ti-based alloys does not seem to be an optimal route due to the absence of homogeneity in the final alloy [2]. Powder metallurgy (PM) processing is also considered to be an effective in reducing the exorbitant machining costs of Ti alloys. It has the advantage of producing a homogenous alloy compared to other techniques, when the alloying elements possess a higher melting temperature such as Nb [4].

Mechanical alloying (MA) has also been successfully used in the development of nanostructured materials [1]. This technique is known to be simple, multifaceted, and feasible economically [13]. The mechanically alloyed powders are consolidated by spark plasma sintering. Spark plasma sintering is a non-conventional method for consolidating solid powders into a metallic compact at shorter times and low temperatures to prevent melting [1, 2]. It is considered more suitable for the production of nanomaterials, since it does not induce undesirable grain growth in comparison to other conventional sintering techniques such as hot isostatic pressing (HIP). It also minimises the formation of undesirable secondary phases that deteriorate the properties [3]. The objective of this study is to evaluate the biocompatibility behaviour of Ti, Ti-6Al-4V and Ti-34Nb-25Zr in SaOs-2 human osteosarcoma cells using WST-1 colorimetric method.

2. Experimental methods

2.1. Biocompatibility evaluation

The cytotoxicity tests were conducted following the procedure for cytotoxicity test methods using a direct contact method for biological safety of medical devices. The sample disks were sterilized using an autoclave for 2h at 120°C. The samples of the 3 alloys were separately placed into the bottom of a 24-well microplate. SaOS-2 osteogenic cells were cultured on each disk at the concentration of 60000 cells/ml of culture medium, Dulbecco’s minimum essential medium with 10% fetal bovine serum and 0.2% penicillin streptomycin (D-MEM+10%FBS+0.2%PS). The sample cells were seeded into the well without a sample disk as a control.
The cells were incubated at 37°C in a 5vol% CO₂ atmosphere with relative humidity of 95% for 1, 4 and 7 days. The experiments were performed in triplicates. After incubation, WST-1 PMS in phosphate buffered solution [PBS (-)] colorimetric assay was conducted and the cells were fixed using 25vol% glutaraldehyde solution for 10min and stained using 10vol% of Giemsa’s staining solution for 15min. The stained cells were observed by an optical microscope and their images were captured using a closed couple device camera. The stained cells were observed by an optical microscope and their images were captured using a closed couple device camera. The absorbance was then measured using a microplate reader (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Waltham, USA) at 450nm. Relative cell viability (RCV) was calculated using Equation 1.

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RCV = \left( \frac{Abs. \ 450 \ cell \ well - Average \ Abs. \ 450 \ medium \ well \ sample \ top \ surface \ area}{well \ bottom \ area} \right)
\]

Equation 1

2.2. Protein adsorption analysis
Protein adsorption analysis was performed for albumin and fibronectin. The surface of the sample was polished until mirror finish and sterilized by an autoclave. After sterilization by an autoclave for 2h at 120°C, the samples were placed in the 24-well microplate and immersed in 1ml of Albumin and 1ml of fibronectin. The samples were placed into an incubator for 3h at 37°C. Afterwards, the samples were rinsed with PBS (-) and fixed using Coomassie plus reagent and mixed in a microplate mixer (AS-ONE, NS-P) for 30s. The samples were left for 10 min at room temperature and adsorption was determined through absorbance reading at 595nm in a microplate reader (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Waltham, USA).

2.3. Surface wettability
Surface wettability tests were performed using a dispensing equipment (Kyowa AD-30, Japan) and ultra-super pure (USP) water (milli-Q, millipore) as a medium for wettability. Contact angle measurements were conducted on the surface of Ti, Ti-6Al-4V and Ti-34Nb-25Zr sample disks. The wettability of each sample was determined by sessile drop measurements of the water contact angle at a contact angle goniometer.

3. Results and discussions

3.1. Biocompatibility
Cells are sensitive to the physical and chemical properties of the materials they interact with. Simple modifications of the material, variations in surface texture, topography can significantly affect changes in cellular response [5]. Cell adhesion to implant materials which are artificial is affected by properties such as surface roughness, surface charge and chemical functionalities [9]. Biocompatibility is the most important property for biomaterials to render them suitable for biomedical applications. The calculated relative cell viability and morphologies of cell adhesion on each substrate are shown in Figure 1 and Figure 2, respectively.
Figure 1. Biocompatibility test for Ti, Ti-6Al-4V and Ti-34Nb-25Zr alloys on SaOS-2 osteosarcoma human cells after 1, 4 and 7 days of incubation.

Figure 1 shows that Ti and Ti-6Al-4V has increasing cell proliferation as observed after 1 and 4 days incubation. After 7 days, cell proliferation on Ti-64 was the highest followed by Ti. However, Ti-34Nb-25Zr alloys have less cell proliferation through the entire period of incubation. This can be attributed by variations in physico-chemical properties of the different substrates in which the cells were cultured as well as the processing technique [5, 11]. Figure 2 shows morphologies of the cells cultured for 1, 4 and 7 days on the three kinds of alloys. There was a notable difference observed in Figure 1 since cell proliferation and adhesion on the samples shown in figure 2 attributed to poor biocompatibility as Ti and Ti-34Nb-25Zr had least cell adhesion. Although Ti-6Al-4V alloy had more cells adhering to the surface and it is promising in terms of biocompatibility which was least expected due to the toxicity of vanadium. However, the non-toxic Ti and Ti-34Nb-25Zr alloys showed a complete opposite in terms of biocompatibility. The calculated relative cell viability compliments the results obtained for cell adhesion as Ti-6Al-4V still showed many cells that grew and spread through the surface.

The main cause for the retardation of the growth pattern of the cells in biomaterial surfaces of the studied alloys are still unknown and will be further investigated.

The microscopic images of cultured cells show that after 1 and 4 days of incubation, the cells visually appeared as scattered stained dots. After 7 days of incubation, the cells grew and spread to cover almost the surface area of Ti and Ti-6Al-4V, while a wide area of empty spaces were observed on the disk of Ti-34Nb-25Zr. Cells on Ti and Ti-34Nb-25Zr showed some sickening rather than the ones which grew and spread smoothly on the surface of Ti-6Al-4V.
Figure 2. Optical microscopy images of the cultured cells on the disks of Ti, Ti-6Al-4V and Ti-34Nb-25Zr.

3.2. Protein adsorption
The adsorption of cell adhesive proteins such as fibronectin, plays an important role in cell adhesion to the biomaterial [7]. During the formation of an adsorbed protein in early stages, albumin is expected to adsorb preferentially onto the surfaces upon exposure. When albumin which acts like a surfactant, adsorbs to the interface there is a large interfacial tension between water and a hydrophobic surface, which eventually reduces the interfacial free energy is reduced [7, 14]. Albumin adsorbs strongly on hydrophobic surfaces (with contact angles above 100º) [15]. When a metallic substrate is exposed to cells suspended in a culture medium supplemented with FBS, protein in the serum are adsorbed rapidly onto the surface before the cells adhere.
The adsorbed proteins determines the subsequent cell adhesion behaviour [14]. The amount of proteins adsorbed also depend on the water contact angles and also varies with the kinds of surfaces of the substrates. In this study, the amount of proteins adsorbed showed little variations for all the substrates [15]. Ti, TNZ (Ti-34Nb-25Zr) substrates showed good albumin adsorption than Ti-6Al-4V. However, Ti-6Al-4V shows good fibronectin adsorption (Figure 2). Contrarily, there is no obvious correlation between the proteins adsorbed and the number of adherent cells was observed on the studied substrates.

3.3. Surface wettability

Generally surface wettability is referred to as hydrophobicity and hydrophilicity. It is one of the most important parameters for materials. Wettability affect protein adsorption, activation, platelet and cell adhesion. Hydrophobic surfaces are generally considered to be more protein adsorbent than hydrophilic surfaces due to the interactions occurring on the surface which has strong hydrophobicity. Ti and Ti-6Al-4V show high contact angle values as compared to Ti-34Nb-25Zr as depicted from Table 1. The alloys showed a hydrophilicity behaviour, since their contact angle values are below 100º.

Table 1. Surface wettability of Ti, Ti-6Al-4V and Ti-34Nb-25Zr using USP water.

| Alloy       | Contact angle (º) | Type         |
|-------------|-------------------|--------------|
| Ti          | 93                | Hydrophilic  |
| Ti-6Al-4V   | 98                | Hydrophilic  |
| Ti-34Nb-25Zr| 86                | Hydrophilic  |

4. Conclusion

Biocompatibility of the spark plasma sintered titanium-based alloys (CP Ti, Ti-6Al-4V and Ti-34Nb-25Zr) was studied by immersion in a simulated body fluid for 1, 4 and 7 days. Surface wettability of water and protein adsorption of albumin and fibronectin was also studied. The results obtained show that all the alloys had fairly poor cell proliferation of SaOS-2 osteosarcoma human cells. The surface wettability results correlated well to less fibronectin adsorption on Ti-34Nb-25Zr solid alloy and highest for Ti-6Al-4V. Ti-34Nb-25Zr showed less cell proliferation which is believed to be linked with fibronectin adsorption and poor cell adhesion. It can be concluded that biocompatibility of SaOs-2 osteosarcoma human cells was not established in the studied alloys, although Ti-6Al-4V proved to be a better candidate in terms of cell adhesion after 7 days incubation. It is recommended that a study in processing technique and surface properties of the alloys be carefully monitored and the biocompatibility tests be conducted using a different kind of cells (i.e. osteoblast (L929) and murine fibroblast).
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