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In vitro fibroblast response to ultra fine grained titanium produced by a severe plastic deformation process

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Abstract The in vitro response of the mouse fibroblast cell line 3T3 on the surface of ultrafine grained titanium [produced by a severe plastic deformation (SPD) process] has been studied in this work. SPD Ti showed much higher strength than the coarse grained Ti and equivalent to that of Ti–6Al–4V alloy. Better cell proliferation was observed on SPD Ti compared to conventional Ti and Ti–6Al–4V alloy. This could be attributed to the increased surface free energy by reduction in the grain size and possibly the presence of a large number of nano size grooves at the triple point junctions in SPD Ti sample. There was no significant difference in the results of cytotoxicity tests of fine and coarse grained materials.

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Introduction

High strength-to-weight ratio, good corrosion resistance and excellent biocompatibility properties have made titanium alloys favourable for orthopedics. Ti–6Al–4V has been a widely preferred alloy [1–4] because of relatively poor strength of pure titanium. However, the presence of vanadium ion inhibits the normal differentiation of bone marrow stromal cells into the mature osteoblasts, over a period of time [5, 6]. A number of other Ti-alloys are also used for implant fabrication [7, 8]; however, increasing alloying elements and their complex processing methods increase the cost. If the strength of pure titanium could be increased by reducing the grain size to a nanometric level, it could be a novel substitute for the alloy implants. The suitability of an implant is characterized by the interaction with the cells, which depends on a number of physical and chemical processes. The stages of cell fixing, adherence and spreading are under the direct control of adhesion proteins which depends on the wettability of the substrate [9, 10]. The wettability modulates the biological responses of the tissues in contact with the implant. The cell-material interaction (i.e., protein formation, cellular morphology, proliferation and orientation) was shown to depend on the surface chemistry, surface energy and roughness of the substrate [11–13].

Materials with the finer grain size were reported to show improved biocompatibility over normal materials. It was reported that the osteoblast adhesion on alumina was increased by ~46% when the grain size was reduced from ~177 nm to ~23 nm [14]. Compared to the coarser counterparts, more calcium and phosphorous were deposited when the osteoblastic cells were cultured on the nanophase alloys (Ti–6Al–4V and CoCrMo [15]) and nano ceramics (titania [16, 17], hydroxyapatite [18], carbon nanofibers,

poly-lactic-glycolic acid and polyurethane [15, 19]). The increased cell adhesion was also reported on ultrafine grained titanium [20]. When the grain size was reduced from ~10 μm to ~0.5 μm, the cell adhesion was increased by ~25% on normal titanium and by ~40% on wrought titanium sheet. An enhancement in the biocompatibility of titanium by refining the surface grains to nanosize (by plasma treatment) was shown by Cheng et al. [21].

Contrary to the above observations, the studies of Cai et al. [22] on the effect of the nanometre scale topographies on the proteins (albumin and fibrinogen) adsorption and the cell growth, showed no significant difference than the normal titanium. No significant influence of the surface roughness on the cell proliferation and viability was detected. Guehennec et al. [23] proposed that if the surface is smooth, the attachment of osteoblastic cells can not be enhanced just by refining the structure into fine grains, but it can be enhanced by changing the surface roughness and the pore size. Anselme et al. [24] showed that the cell attachment was influenced by the surface chemistry rather than the surface topography in Ti, Ti alloys and stainless steel substrates. During the in vivo test in zirconia ceramics, the human fetal osteoblast cells adhered better on the rougher surface than the surface with lower roughness [25]. It appears that the above reports are full of contradictory results and the biocompatibility behaviour of nanostructured surface has not yet been clearly understood.

Titanium parts with the nanostructure feature can be produced either through powder metallurgy (PM) route [26, 27] or by SPD process [28–31]. The PM route has serious drawbacks, such as impurity contamination, high reactive nature of nano titanium powder with almost everything, grain growth and complex sintering cycles to achieve full density, etc. On the other hand, SPD route can produce material with relatively higher strength, free from impurity contamination and pores. However, there is hardly any reported study on the biocompatibility of SPD processed titanium, which has been focused in the present investigation. A systematic study on the 3T3 mouse fibroblast cell adhesion and proliferation behaviour on the ultrafine grained titanium produced by SPD process has been made. The cellular response to SPD processed titanium has been compared with that of the conventional titanium and Ti–6Al–4V alloy.

Experimental

Substrate preparation

Commercially available ASTM Grade 2 pure titanium (cpTi) rod and Ti–6Al–4V alloy were used in this work. The cpTi rod was normalized at 705 °C for 30 min in an electric furnace in air and furnace cooled. Ultrafine grained titanium was produced by SPD process (route Bc [28]). The severely deformed sample was annealed at 600 °C for 10 min in the same furnace in air and cooled to get a fine re-crystallized grain structure. Further, the sample surface was ground-off carefully to remove the surface oxides. Hereafter, this sample will be designated as SPD Ti. Three types of materials were taken for the study: (a) cpTi, (b) SPD Ti and (c) Ti–6Al–4V alloy. Two sets of specimens were prepared for each type of materials: (a) for tensile strength test (as per ASTM E8 specifications) and (b) for biocompatibility test (discs of 5 mm diameter and 3 mm thickness). Samples for the biocompatibility test were polished to a mirror finish and cleaned ultrasonically in ethanol for 30 min and subsequently the samples were incubated at 37 °C.

Grain size measurement

The grains sizes of the samples were measured from the optical microscope images using quantitative image analyzer software, for cpTi and Ti–6Al–4V alloy samples, and from transmission electron microscope (TEM) images of SPD Ti sample.

Surface roughness and contact angle measurement

Surface roughnesses of the samples were analyzed by Sloan Dektak Profilometer. Surface wettabilities were determined by measuring the contact angles of deionized water (DI) drops (at three different points) using a contact-angle meter (KRUSS Gmbh, DSA10-Mk2) at room temperature. An auto pipette was used to ensure a uniform volume of DI water droplets (~0.5 μl).

MTT tests for cell adhesion, proliferation and cytotoxicity

Standard protocols of MTT tests [32, 33] were conducted on the samples to determine the cell proliferation, adhesion and cytotoxicity behaviours. Mouse fibroblast cell line 3T3, provided by Sevapharma (Czech Republic) was used. The cells were seeded in the densities of 20×10^3 cells/cm^2 for the test of adhesion and 5×10^3 cells/cm^2 for the test of proliferation. The cells culture, supplemented with 10% fetal bovine serum (ZVOS Hustopece, Czech Republic) was incubated at 37 °C in air containing 3.3% CO_2. Cells were cultured for 2 and 5 days for the proliferation test and 16 h for the adhesion test. Controlled experiments of the cell adhesion and the proliferation were conducted on the conventional 96-well culture plates (Corning, New York, USA) for each set of samples ($n = 3$ for each set). The cells densities on the samples and control (polystyrene plate)

were measured using a UV spectrometer by viable colour change in the cells. The absorbance of 250 µl of blue-coloured solution was measured at 570 nm using Mikroplate Reader EL800 (BIO-TEK, Vermont USA).

Results

TEM micrograph of SPD Ti sample has been shown in Fig. 1. The average grain size was found to be about 238 nm (Table 1). Properties of all three types of materials have been listed in Table 1. SPD Ti sample showed strength of about 60% higher than the strength of the cpTi sample and almost equal to that of Ti–6Al–4V alloy. The surface roughnesses of the samples were found to be almost identical (under identical polishing condition). The measured contact angle of the liquid on SPD Ti sample was found be significantly smaller than that of cpTi sample.

The MTT test yielded relatively better absorbance value for the cell adhesion for SPD Ti sample than cpTi sample and similar to Ti–6Al–4V sample (Table 1). There was a significant increment in the absorbance value for the cell proliferation on SPD Ti specimen compared to cpTi specimen after 2 days. There was a drastic increase in the absorbance value after 5 days on SPD Ti sample compared to both, cpTi and Ti–6Al–4V samples. Data presented in Table 1 for cell adhesion and proliferation has the confidence level of 95% (the confidence interval has been shown with the data as ±error). No statistical difference (Table 1) in the cytotoxicities of the substrates could be seen.

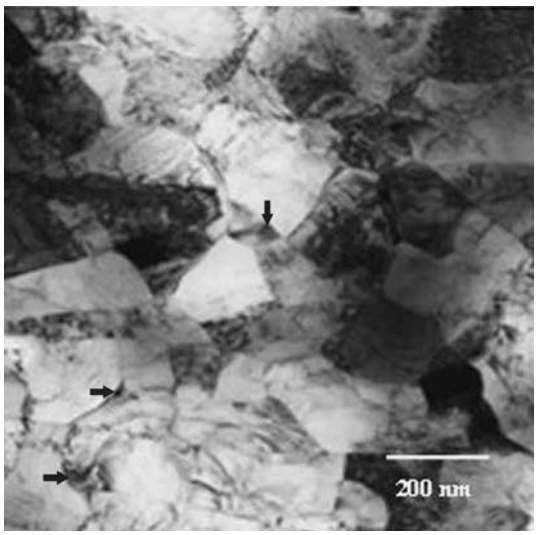

Fig. 1 TEM micrograph of SPD Ti sample. Locations of the grooves have been pointed out by the black arrows

Discussion

SPD Ti sample not only showed submicrometre grains, but also showed enhanced strength comparable to that of Ti–6Al–4V alloy. Relatively smaller contact angle of the tested liquid on SPD Ti sample compared to that of cpTi sample, indicates the enhanced wettability in a fine grain structure, although the contact angle in SPD Ti was still larger than in Ti–6Al–4V alloy. The wettability of the substrate modulates the cell adherence behaviour, thus the trend observed in the cell adherence behaviours of various substrates were corresponding to their respective wettability characteristics. The cell proliferation test shows very interesting results, especially after longer duration of culture (5 days). SPD Ti showed ~61% and ~40% higher absorbance value for the proliferation than cpTi and Ti–6Al–4V alloy samples respectively. A relative increase in the adherence and the proliferation in the submicro-grained material in the present work could be attributed to two factors: (a) the increased surface free energy, and (b) possibly the presence of a number of nano size grooves.

When the material of micrometric grain size (cpTi) was refined into a nanosized grain material (SPD Ti), the number of grains was increased by several thousands for every large grain. Thus, the number of grain boundaries and total surface area would be very high in SPD Ti sample compared to micro grained material. It is well known [34, 35] that the increase in the free energy (ΔE) of the material depends on the increase in the surface area (ΔA), i.e., $\Delta E = \gamma \cdot \Delta A$ (where γ is the surface tension). Hence the surface energy and the grain boundary energy would be increasing drastically. When the grains are at high energy state, they have a tendency to release their energies in every possible way. This encourages the interaction of the material with the liquid or wetting media at the expense of energy, and hence results in higher surface wettability or hydrophilicity [34–36] in finer grained surface compared to its coarser counterpart. When such implant surface was brought into the contact with the body fluids and cells, the extra cellular proteins adsorption rate increased significantly, which was an essential step for the cell proliferation. Proteins, such as extracellular matrix proteins, cytoskeletal proteins and membrane receptors (integrins) are generally involved in the cell-substrate interactions.

Severely deformed material is expected to contain a large number of defects. The atomic densities at the grain boundary regions would be relatively lower and a large number of atoms would be energetically unstable, compared to the inside of the grain. When the deformed material was recrystallized, some voids or grooves remained at the triple grain junctions (Fig. 1), which had not been healed completely by the solid-state mass flow. Sizes of such grooves were very small (in the order of few

Table 1 Properties and cellular response of various specimens

Properties	Materials			
	CpTi	SPD Ti	Ti–6Al–4V	Control
Grain size (μm)	15.2 ± 0.6	0.238 ± 0.05	6.9 ± 0.4	NA
Yield strength (MPa)	418 ± 57	684 ± 75	730 ± 45	NA
Roughness (μm)	0.16 ± 0.05	0.14 ± 0.07	0.18 ± 0.05	NA
Contact angle (°)	74.6 ± 0.6	69.6 ± 1.1	64.63 ± 3.1	NA
Absorbance/cm^2 (Cell adhesion-16 h)	0.801 ± 0.04	0.868 ± 0.05	0.88 ± 0.07	1.12 ± 0.01
Absorbance/cm^2 (Cell proliferation)				
2 days	0.295 ± 0.05	0.504 ± 0.04	0.508 ± 0.08	0.675 ± 0.03
5 days	2.02 ± 0.08	3.26 ± 0.01	2.33 ± 0.03	2.71 ± 0.07
Absorbance/cm^2 (Cytoxicity-5 days)	0.811 ± 0.04	0.836 ± 0.05	0.835 ± 0.05	1.14 ± 0.01

nanometers). In the ultrafine grained material, the number of grains is much larger and obviously the number of such nano size grooves would also be very high. A schematic presentation of possible groove formation has been shown in Fig. 2. The high energy grain boundaries and grooves provide active sites for the osteoblast process. Previous studies [20, 37] about the osteoblast response to the grooved surface (the controlled sized grooves were made on the surface) revealed that the width and depth of the grooves, as well as the number of adjacent grooves on the implant surface, had been the determining factors in establishing a positive reaction with the cells and their orientations. It was found that the cell growth rate was better when the dimensions of the grooves were similar to that of cell dimensions. Jansen and co-workers [38] observed that the cell proliferation in smaller size grooves (~1 μm) was significantly higher compared to the larger size grooves (~5 and ~10 μm). They proposed that the cells have mechano-receptive response to the surface discontinuities, implying the dynamics of the cytoskeleton. The front edges of the cells (the lamellipodia) contain actin microspikes, which could be influenced by the surface discontinuities. In case of cpTi sample, the number of the grain boundaries are relatively very less and possibly the groove size (if present any) are much wider in size (Fig. 2) even though the sample exhibited overall surface roughness similar to that of SPD Ti.

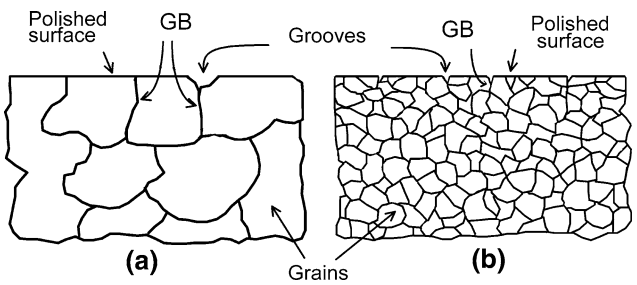

Fig. 2 Schematic presentation of presence of grooves on the polished surfaces of: (**a**) micrometric grains and (**b**) nanometric grains

In case of Ti–6Al–4V alloy, not only the wider grooves, but the presence of vanadium ion also affects the cell proliferation. Eisenbarth et al. [39] observed a drastic decrease in the cytoplasm content of the fibroblast and decrease in the spreading area on vanadium phases in Ti–6Al–4V alloy over a period of 7 days. A similar trend was evident in the present case of Ti–6Al–4V alloy over the period 5 days; the cell proliferation reduced compared to SPD Ti sample.

Conclusions

The effect of microstructure on the strength of the implant and the cell-substrate interaction was demonstrated. The ultrafine grain titanium prepared by SPD route showed improved strength, better biocompatibility in terms of wettability, cell adhesion and proliferation than conventional titanium. Higher cell proliferation was observed on SPD Ti than on Ti–6Al–4V alloy. The high surface energy and the presence of nano sized grooves were the factors for better cellular response to SPD Ti specimen.

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