Texture-related biological properties of severely deformed titanium

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Abstract. Pure titanium manufactured by hydrostatic extrusion has been investigated with the aim to find the clear origin of superior biological properties observed in the materials processed by various severe plastic deformation techniques. In so doing, physicochemical properties of the surface were characterised as well as protein adsorption tests and cell culture examinations were thoroughly carried out. It has been found that, irrespective of the grain size, the basal planes exposed to the surface favour protein adsorption, whereas it is prismatic planes that experience pronounced cell activity on the surface of hydrostatically extruded titanium substrates. Biological behaviour of severely deformed titanium-based materials should be attributed to crystallographic orientation of grains, chemical composition of the surface as well as the presence of surface irregularities in the form of various nano-peaks or nano-grooves, while the effect of grain size might be of less importance.

1. Introduction

Stainless steel, aluminium and titanium represent the most commonly strengthened materials by means of severe plastic deformation (SPD) techniques [1]. A remarkable increase in mechanical characteristics is accomplished while using the SPD approaches and a variety of other functional properties are also enhanced [2]. It has been well-documented that early cell response on the surface of microstructurally refined titanium is more pronounced with a comparison to its coarse-grained counterpart [3,4]. In addition, improved in vitro bioactivity of normally inert titanium surface, enhanced protein adsorption and substantially higher alkaline phosphatase activity have also been reported for severely deformed titanium-based materials [5]. The origin of such extraordinary behaviour remains inadequately
understood, yet is typically associated with grain refinement or changes in the physicochemical properties of the surface, i.e., wettability and roughness [3–6]. Recently, the effect of a significant share of high-angle grain boundaries (HAGB) in the volume of a material as well as crystallographic orientation of grains on the biological properties of the SPD-obtained materials have also been considered [7,8].

Equal channel angular pressing (ECAP), accumulative roll-bonding (ARB) and high-pressure torsion (HPT) make up for the most frequently practiced and extensively researched SPD methods, although a great deal of novel manufacturing techniques have come to the fore over the past years [1]. Hydrostatic extrusion (HE), an unconventional SPD process, has been shown to enhance the strength of pure titanium so exceptionally that it reaches the level characteristic to that of the Ti-6Al-4V alloy [9,10]. The HE method is known to be especially advantageous in reinforcing the mechanical properties of hard-to-deform materials, such as zinc or tungsten, yet it tends to produce lengthened wires and rods, therefore its use is somehow limited [11,12]. The possible application of HE includes small-sized medical equipment, parts of advanced appliances and tools, as well as dental implants.

Within the present study, titanium fabricated by the multi-stage HE process has been analysed with respect to its biological behaviour, using cell culture studies and protein adsorption tests. The physicochemical properties of the substrates, varying significantly in the crystallographic orientation of grains, have been additionally examined so the origin of superior biological properties observed in titanium-based materials processed by SPD approaches is to get fathomed. The entire analysis is supported by a thorough characterisation of the material’s microstructure.

2. Materials and experimental details

Pure titanium (grade 2) was manufactured by the HE technique realized in four consecutive steps without the interstage and stress-relief annealing, yet cooling with a stream of cold water was performed every time the material exited the die extruder. Prior to the deformation process, the billet was homogenized at 700°C for 2 hours and left air-cooled. Different cross-sections taken from a final rod were cut, then ground with a set of silicon carbide papers and electronically polished, using the A3 Struers electrolyte. Afterwards, the specimens were cleaned in isopropanol and dried. Overall, mirror-finished specimens were prepared.

2.1. Microstructure and texture

FEI Quanta 3D FEG SEM was used to execute the microstructural and textural characterisations by means of the electron backscatter diffraction (EBSD) technique. Texture of the investigated materials was additionally examined by using X-ray powder diffractometry (Bruker D8 diffractometer). Moreover, FEI TECNAI SuperTWIN G2 FEG (200 kV) combined with HAADF/ STEM/ EDAX attachments was used in order to support the analyses of the deformed microstructures conducted employing the EBSD method.

2.2. Biological properties

Adsorption of bovine fibronectin protein and bovine serum albumin was characterised by using the Qubit® Protein Assay Kit and Qubit® fluorometer, following the manufacturer’s instruction. Statistical significance of the received protein concentrations was evaluated. Human umbilical vein endothelial cells (HUVECs) were studied in the cell-substrate interactions completed wholly in 72 hours. A confocal laser scanning microscope (CLSM) Exciter 5 AxioImager was implemented in order to reveal the cells’ morphology and the extent of proliferation processes.

2.3. Physicochemical properties

An Innova commercial instrument, DSA 100 Krüss contact angle goniometer and a PHI VersaProbeII Scanning XPS system were utilized to carry out roughness, wettability and chemical composition measurements, respectively. The data obtained was analysed in regard to its statistical significance.
3. Results and discussion

3.1. Texture and microstructure

Generally, by undergoing plastic deformation via HE, the texture of a material is changed from the weak one to (101̅0) fiber [10]. Within the present study, the grains analysed in the transverse cross-section of the final rod were oriented parallelly to the (10̅10) planes, as seen in the inverse pole figure illustrated in Fig. 1a. On the other hand, the grains were distributed between (0001) (main maximum) and (21̅1̅0) planes, while considering the samples sliced so the longitudinal cross-section was observed (see Fig. 1e and f). Therefore, for convenience, Ti-(101̅0) and Ti-(0001) designate the surfaces parallel to the transverse and longitudinal cross-sections of the investigated substrates, respectively.

The EBSD orientation map gathered from the Ti-(101̅0) specimen is depicted in Fig. 1b. The microstructure consisted of wavy grains, both coarse and refined. The mean grain size was calculated to be 6.5 μm ± 5.7 μm, implying that a fraction of ultrafine-grains existed within the volume of a material. Contrarily, as seen from the EBSD orientation map collected from the Ti-(0001) sample and shown in Fig. 1f, the micro-grains were elongated in the extrusion direction, marked by a black arrow. Their mean grain size was estimated to be 12.8 μm ± 10.3 μm. A worth-mentioning discrepancy between the substrates was found in the value of high-angle grain boundaries (HAGBs) as the Ti-(101̅0) demonstrated 20% higher density of HAGBs in comparison to the Ti-(0001). It indicates the Ti-101̅0 surface to be more greatly enriched with structural defects.

The bright field (BF) images, seen in Fig. 1c and g, revealed that the Ti-(0001) and Ti-(101̅0) were characterised by the presence of small grains, whose size lied within the sub-microcrystalline range. In addition, large grains were composed of sub-grains and exhibited high density of dislocations. The boundaries between adjacent grains were barely distinguishable, what implies a significant share of low-angle grain boundaries (LAGBs) in the material. The anisotropic microstructure of the investigated substrates is typical of most hydrostatically extruded metals. It is obvious that such property reflects on the mechanical strength of a material, what has already been proved [9,10].

![Fig. 1.](image)

Fig. 1. (a), (b), (c) and (d) shows {001} IPF, EBSD map, TEM image and CLSM image, respectively, for the Ti-(101̅0), (e), (f), (g), (h) shows {001} IPF, EBSD map, TEM image and CLSM image, respectively, for the Ti-(0001).
3.2. Biological properties

It should be expected that the Ti-(10\bar{1}0) samples, having microstructures distinctly more refined with comparison to the Ti-(0001) ones, are those demonstrating superior biological properties. The CLSM image, taken for the Ti-(10\bar{1}0) covered with HUVECs is displayed in Fig. 1e. The cells grew vividly on the entire surface, forming a few confluent monolayers. Both polygonal and flattened shapes of the cells may be easily viewed. The morphology of HUVECs attached to the Ti-(0001) is illustrated in Fig. 1h. Interestingly, the overwhelming majority of cells were positioned in a preferential manner and the active processes of filopodia forming could be observed. Possibly, the HUVECs were lined up with the [10\bar{1}0] crystallographic direction, overlapping with the extrusion direction. In addition, the elongated shape of cells differs greatly from those matured on the Ti-(10\bar{1}0) specimens. Overall, the extent of proliferation is more noticeable for the Ti-(10\bar{1}0) than for the Ti-(0001) substrates.

![Proteins adsorption on the surfaces of the investigated cross-sections](image)

**Fig. 2.** Proteins adsorption on the surfaces of the investigated cross-sections. The asterisk sign stands for statistical significance between the probes (p < 0.05).

The adsorption of bovine fibronectin protein and bovine serum albumin on the surfaces of the analysed cross-sections is displayed in Fig. 2. Rather unexpectedly, the Ti-(0001) samples exhibited a clearly greater adsorption of both the examined proteins, although their cell-substrate response was less pronounced. The difference between the analysed probes was statistically significant (p < 0.05).

3.3. Physicochemical properties

Roughness and wettability data for the analysed substrates is listed in Table 1. The effect of surface properties on the biological performance of the hydrostatically extruded titanium should be fully discarded as the prepared specimens demonstrated mirror-finished surfaces of comparable nano-roughness and hydrophobicity (statistical insignificant differences, p < 0.05).

| Cross-section | Water contact angle [°] | Roughness [nm] |
|---------------|-------------------------|----------------|
| Ti-(10\bar{1}0) | 76.9 ± 1.4 | 1.5 ± 0.4 |
| Ti-(0001)     | 77.8 ± 0.7 | 1.8 ± 0.2 |
Table 2. The elements present on the characterised surfaces.

| Energy [eV] | C       | O       | Ca      | Ti       |
|------------|---------|---------|---------|---------|
|            | 284.8   | 286.8   | 289.1   | 530.3   |
| Groups/    | C-C     | C-O     | O=C-O   | O₂⁻     | OH⁻     | Ca²⁺     | Ti(0)   | Ti(II)  | Ti(III) | Ti(IV) |
| Oxidation  |         |         |         |         |         |         |         |         |         |        |
| state      |         |         |         |         |         |         |         |         |         |        |
| Ti-(1010)  | 28.2    | 3.6     | 3.1     | 34.2    | 12.2    | 0.5     | 1.9     | 1.0     | 2.6     | 12.7   |
| Ti-(0001)  | 36.1    | 4.0     | 3.6     | 27.9    | 13.0    | 1.0     | 0.5     | 0.3     | 1.8     | 11.8   |

A slight difference in chemical composition, shown in Table 2, could substantiate various protein adsorption among the examined cross-sections. It is also worth noticing that the thickness of the titania layer on the Ti-(1010) surface was lower in comparison to that on the Ti-(0001) surface, although the same grinding and polishing procedures were implemented. The presence of carbon and calcium on the examined surfaces might stem from various contamination compounds, e.g., calcium carbonate or calcium oxide.

3.4. Texture-biological properties relationship

It is well-known that titanium strongly reacts with oxygen forming a thermodynamically stable, thick and well-adherent film on its surface. Thus, while investigating the biological performance of titanium and its alloys, it is the passive layer of titanium that is characterised, not the metal itself as designed biological environments are oxygen-containing [3].

In the present study, although the Ti-(1010) substrates experienced superior grain refinement and cell proliferation than the Ti-(0001) ones, the adsorption of proteins was enhanced for the latter. Hence, it seems reasonable to state that the downscaled microstructure of titanium-based materials fabricated by the SPD techniques may not be the major factor governing their biological properties. However, its significance on cell-biomaterial interactions cannot be completely overlooked or rejected as biocompatibility of coarse-grained materials is generally unsatisfactory, therefore they cannot be readily used for medical applications.

The processes of protein adsorption, cell attachment and proliferation are dependent on the physicochemical nature of the surface [13]. Herein, the discrepancies in wettability and nanoscale roughness between the examined cross-sections were found to be negligible. Therefore, varied endothelial cell response may be attributed to the crystallographic orientation of grains as well as to diversified composition and conformation of the adsorbed proteins entangled with differences in the richness of nanostructured defects on the surface. As a matter of fact, proteins tend to conform differently if a surface of a material is covered with various structural defects, in the form of nano-grooves or nano-peaks [14]. Moreover, grain boundaries have shown to strongly influence preosteoblast cells activity on the surface of pure titanium produced by ECAP [7]. In addition, it has been demonstrated that the prismatic planes tend to be more susceptible to etching [13], thereby the surfaces of markedly greater biological activity could be produced.

On the other side, various adsorption of both the bovine fibronectin protein and bovine serum albumin on the characterised cross-sections could result from surface chemistry. It has been reported that the hydroxyl groups tend to modulate proteins to get adsorbed on the surface of a material [15]. In addition, the basal planes have shown to promote the accumulation of the OH⁻ moieties on the surface [8]. Within the present study, the amount of hydroxyl groups was substantially higher on the Ti-(0001) substrates and so was the protein concentration.

As shown, not only does titanium fabricated by the HE process demonstrate anisotropy of mechanical properties, but also its biological behaviour is anisotropic. To the authors’ knowledge it is unclear, whether late-cell events, such as differentiation, are also affected by crystallographic orientation of grains, therefore such studies will be performed in the future.
4. Conclusions

Crystallographic orientation of grains combined with defects-enriched surface and its chemical composition has shown to strongly impact the biological behaviour of HE-manufactured pure titanium. The substrates having the prismatic planes exposed to the surface experienced superior grain refinement as well as more pronounced endothelial cells activity. Contrarily, the basal planes mediated the accumulation of hydroxyl groups on the surface of a material and favoured the adsorption of albumin and fibronectin, yet they were less refined.

References

[1] Bagherpour E, Pardis N, Reihanian M and Ebrahimi R 2019 An overview on severe plastic deformation: research status, techniques classification, microstructure evolution, and applications Int. J. Adv. Manuf. Technol.

[2] Valiev R Z, Estrin Y, Horita Z, Langdon T G, Zehetbauer M J and Zhu Y 2016 Producing Bulk Ultrafine-Grained Materials by Severe Plastic Deformation: Ten Years Later Jom 68 1216–26

[3] Kubacka D, Yamamoto A, Wieciński P and Garbacz H 2019 Biological behavior of titanium processed by severe plastic deformation Appl. Surf. Sci. 472 54–63

[4] Faghihi S, Azari F, Zhilyaev A P, Szpunar J A, Vali H and Tabrizian M 2007 Cellular and molecular interactions between MC3T3-E1 pre-osteoblasts and nanostructured titanium produced by high-pressure torsion Biomaterials

[5] Nie F L, Zheng Y F, Wei S C, Wang D S, Yu Z T, Salimgareeva G K, Polyakov A V. and Valiev R Z 2013 In vitro and in vivo studies on nanocrystalline Ti fabricated by equal channel angular pressing with microcrystalline CP Ti as control J. Biomed. Mater. Res. - Part A 101 A 1694–707

[6] Reshadi F, Faraji G, Mghtaderi H and Faghihi S 2020 Surface and Bulk Modification of Titanium Grade 2 Substrates for Enhanced Biological Activity Jom 72 721–9

[7] Lowe T C, Reiss R A, Illescas P E, Davis C F, Connick M C and Sena J A 2020 Effect of surface grain boundary density on preosteoblast proliferation on titanium Mater. Res. Lett.

[8] Hoseini M, Bocher P, Shahryari A, Azari F, Szpunar J A and Vali H 2014 On the importance of crystallographic texture in the biocompatibility of titanium based substrate J. Biomed. Mater. Res. - Part A 102 3631–8

[9] Kawalko J, Wroński M, Bieda M, Sztwiertnia K, Wierzanowski K, Wojtas D, Łagoda M, Ostachowski P, Pachla W and Kulczyk M 2018 Microstructure of titanium on complex deformation paths: Comparison of ECAP, KOBO and HE techniques Mater. Charact. 141 19–31

[10] Wojtas D, Wierzanowski K, Chulist R, Pachla W, Bieda-Niemiec M, Jarzębska A, Maj Ł, Kawalko J, Marciszko-Wiąckowska M, Wroński M and Sztwiertnia K 2020 Microstructure-strength relationship of ultrafine-grained titanium manufactured by unconventional severe plastic deformation process J. Alloys Compd. 837 155576

[11] Jarzębska A, Bieda M, Kawalko J, Rogal, Koprowski P, Sztwiertnia K, Pachla W and Kulczyk M 2018 A new approach to plastic deformation of biodegradable zinc alloy with magnesium and its effect on microstructure and mechanical properties Mater. Lett. 211 58–61

[12] Zhaohui Z and Fuchi W 2001 Research on the deformation strengthening mechanism of a tungsten heavy alloy by hydrostatic extrusion Int. J. Refract. Met. Hard Mater. 19 177–82

[13] Baek S M, Shin M H, Moon J, Jung H S, Lee S A, Hwang W, Yeom J T, Hahn S K and Kim H S 2017 Superior pre-osteoblast cell response of etched ultrafine-grained titanium with a controlled crystallographic orientation Sci. Rep. 7 1–10

[14] Lotz E M, Olivares-Navarrete R, Berner S, Boyan B D and Schwartz Z 2016 Osteogenic response of human MSCs and osteoblasts to hydrophilic and hydrophobic nanostructured titanium implant surfaces J. Biomed. Mater. Res. - Part A 104 3137–48

[15] Thevenot P, Hu W and Tang L 2008 Surface chemistry influences implant biocompatibility. Curr. Top. Med. Chem.