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Combined effect of grain refinement and surface modification of pure titanium on the attachment of mesenchymal stem cells and osteoblast-like SaOS-2 cells

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ABSTRACT

Surface modification is an important step in production of medical implants. Surface roughening creates additional surface area to enhance the bonding between the implant and the bone. Recent research provided a means to alter the microstructure of titanium by severe plastic deformation (SPD) in order to increase its strength, and thereby reduce the size of the implants (specifically, their diameter). The purpose of the present study was to examine the effect of bulk microstructure of commercially pure titanium with coarse-grained (CG) and ultrafine-grained (UFG) bulk structure on the surface state of these materials after surface modification by sand blasting and acid etching (SLA). It was shown that SLA-modified surface characteristics, in particular, roughness, chemistry, and wettability, were affected by prior SPD processing.
Additionally, biocompatibility of UFG titanium was examined using osteosarcoma cell line SaOS-2 and primary human adipose-derived mesenchymal stem cell (adMSC) cultures. Enhanced cell viability as well as increased matrix mineralization during osteogenic differentiation of MSCs on the surface of ultrafine-grained titanium was shown.

1. Introduction

Currently, titanium and its alloys are among the metals most widely used for medical implants. This is attributed to a combination of desirable properties for orthopaedic applications these materials possess. These include lower elastic modulus (compared to stainless steel or Co—Cr alloys), excellent corrosion resistance, and biocompatibility [1]. Of all implant titanium alloys, Ti6Al4V is the most commonly used one due to its extensively studied properties, notably high mechanical strength [2]. However, issues with potential toxicity of its alloying elements have been raised recently. There are concerns that vanadium and aluminium ions released from the implant due to corrosion processes would accumulate in the surrounding tissues and lead to a wide range of adverse health effects [3,4]. These may include DNA damage, cytotoxicity, neurotoxicity and hepatotoxicity [5–7]. Aluminium has been studied by some researchers with respect to its role in neurological disorders, such as Alzheimer's disease [8,9]. Although no conclusive evidence has yet been provided to fully document the risks [10], it seems worthwhile to investigate possible candidates to replace Ti6Al4V alloy to avoid even hypothetical impacts on human health. One such candidate is pure titanium (namely, Grade 2 and Grade 4), possessing properties matching those of Ti6Al4V, yet being free of alloying elements. The major drawback of using commercial purity titanium in medical implants is its mechanical strength, which is well below that of titanium alloys. Inferior mechanical strength of implant material would require larger cross-section of the implant to maintain the load-bearing functionality. As a result, the size of the implant, particularly, its diameter, would have to be increased, leading to a more significant surgical trauma, longer healing periods and, consequently, a higher risk of complication. In addition, larger implants may not be suitable for certain categories of patients, for example, children.

It has been shown, however, that the strength of metallic materials can be greatly improved through grain structure refinement by a number of techniques, commonly known as severe plastic deformation (SPD), particularly by equal channel angular pressing (ECAP) [11]. It has been reported that through SPD processing, the strength level of Ti6Al4V alloy can be reached with commercially pure (CP) titanium

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[12,13]. As a consequence, the diameter of implants made from ECAP-processed pure titanium does not need to be increased, which is potentially beneficial for the patients receiving them.

It is not only the mechanical properties of the material employed that determine a successful biomedical implant, though. Of even higher importance is its biocompatibility – "…the ability of the device to perform its intended function, with the desired degree of incorporation in the host, without eliciting any undesirable local or systemic effects in that host" [14]. It is widely accepted that biocompatibility can be significantly influenced by surface properties of an implant, such as roughness, surface energy, wettability, and crystallographic texture [15–28].

This understanding led to a broad implementation of surface modification of implants in order to enhance their biocompatibility. The most common and widely studied approach is to modify the topography of the surface. This can be achieved by a variety of processes, which make use of one of the following techniques or a combination thereof: plasma-spraying, grit-blasting, acid-etching, and anodization [29]. One special variant of these processes, called SLA ("**S**and-blasting with **L**arge grit and **A**cid-etching"), combines the positive effects of grit-blasting and etching to create a macroscopically rough surface with micro pits [30]. It was shown that SLA-treatment can promote biological performance of the surface resulting in an increased differentiation of stem cells [31] and enhanced osteoblastic cell viability [22]. In vivo tests revealed increased ability of bone anchorage to such surfaces [32]. In addition, a very high survival rate of dental implants with SLA-treated surface was reported both in short term clinical tests (98.7% after 15.2 months) [30] and after 5 years of implantation (95.41%) [33].

In addition to that, it was hypothesised that grain refinement can also influence certain surface characteristics, particularly, roughness [34]. Since most of the surface parameters are interdependent, this may also affect other surface properties, such as wettability, chemistry and crystallographic texture, and alter the overall biological response. Indeed, it was reported that finer grain size, obtained by SPD techniques, can promote adhesion and proliferation of human tissue cells [35–41].

However, it remains to be determined to what extent this effect of grain refinement will be preserved once the surface is mechanically and/or chemically modified. To the best of our knowledge, there is little information to date on the combined effect of grain refinement by means of SPD and subsequent surface modification of implant materials.

Therefore, the purpose of the present work was to determine how certain material/process parameters (purity, microstructural state and surface state) can influence the surface properties of titanium and, in turn, what effect they can have on the interaction of titanium with human tissue cells. With regard to purity, two grades of pure titanium were used for the experiments – Grade 2 and Grade 4. The microstructural states were set by the processing history: as-received coarse-grained (CG) and SPD-processed ultrafine-grained (UFG) conditions. To examine the effect of the surface state, samples were analysed in polished condition and after SLA surface treatment. The surface parameters, including roughness, wettability, chemistry, and crystallographic texture, of the materials considered were assessed. An interaction between the surface of titanium and two types of human tissue cells (osteoblast-like human osteosarcoma SaOS-2 cells and adipose-derived mesenchymal stem cells adMSC) was examined for all studied conditions.

2. Materials and methods

2.1. Material preparation

Commercially pure titanium Grade 2 and Grade 4 rods (Table 1) were received from TIMET and Carpenter Dynamet® (USA), respectively. These materials were subjected to thermo-mechanical treatment according to two schedules: (I) Conventional equal channel angular pressing (ECAP) (at 300 °C with a pressing speed of 1 mm/min via Route B_C employing a die with a 90° angle between the channels) to a total accumulated strain of 460%; and (II) ECAP-Conform followed by additional thermo-mechanical processing (TMP) to a total accumulated strain of 510%. Henceforth, these two processing conditions will be referred to as ECAP(I) and ECAP(II), respectively. The as-received condition will be denoted AR.

Table 1
Chemical composition of titanium used for the experiment.

	Element content, %				
	Ti	C	Fe	O	N
Grade 2	Base	<0.01	0.18	0.12	<0.01
Grade 4	Base	0.04	0.14	0.40	0.01

Disk-shaped specimens were cut with a Buehler low speed saw from the as-received and SPD-processed rods of titanium followed by grinding with a sequence of 800, 1200 and 2400 grit size grinding papers. To achieve a mirror-like surface, the specimens were polished manually using a Struers MD-chem polishing pad with a mixture containing 90% colloidal silica (Struers OP-S) and 10% hydrogen peroxide. Prior to testing, samples were cleaned in an ultrasonic bath with pure ethanol.

An identical set of flat samples was surface treated by SLA at Institut Straumann Ltd. (Switzerland). This treatment included grit-blasting with 0.5 mm diameter particles of Al_2O_3 followed by etching with a mixture of hydrochloric acid (HCl) and sulfuric acid (H_2SO_4).

2.2. Surface characterisation

Topography of the samples was assessed using a Veeco optical profilometer in the PSI regime over an area of 300 μm × 228 μm. Typically, 20 measurements were made per sample processing condition. Five surface characteristics were measured: R_a – average roughness; R_q – root mean square roughness; R_z – ten-point height average over 10 most prominent peaks and valleys; R_{sk} – skewness, which describes the symmetry of the profile about the mean line; and R_{ku} – kurtosis, which provides a measure of the sharpness of the profile. The first three parameters characterise the topography of the surface in terms of mean characteristics, indicating distances of peaks and valleys from the mean level along a scan line. The latter two parameters characterise the sharpness and the size of peaks and valleys on the surface [42].

Surface wettability was measured using the sessile drop method using an FTA1000 (First Ten Ångstroms Inc.) instrument. Static contact angles on titanium discs were determined using three solvents, MilliQ water, formamide (Sigma) and diidomethane (Sigma) [43]. The average over at least five measurements is reported [43]. Each measurement of a particular contact angle was recorded in 50 images in 2 s with a Pelco Model PCHM 575-4 camera and the contact angle was determined as a result of image analysis by the FTA Windows Mode 4 software. The average contact angle for each of the three solvents on each surface tested was used to calculate the surface free energy and its components.

X-ray photoelectron spectroscopy (XPS) was performed on mirror-polished samples using a Kratos AXIS Nova (Kratos Analytical Ltd., UK) spectrometer equipped with a monochromated Al K_α X-ray source ($h\nu = 1487$ eV) having X-ray power of 150 W. The scanning area was 300 μm × 700 μm, and survey spectra were recorded at 1 eV/step up to 160 eV. Peak fitting parameters were adopted from Biesenger et al. [44].

2.3. Cultivation and seeding of SaOS-2 and adMSC

For SaOS-2 cell propagation, cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, Germany) containing 44 mM $NaHCO_3$ (Sigma Aldrich, Germany), 2 mM L-glutamine (PAA, Austria), and 10% fetal calf serum (FCS) (PAA, Austria) at 37 °C in 5% CO_2. After reaching approximately 80% confluency, cells were subcultured by rinsing the cell layer with 0.05% (w/v) trypsin (PAA, Austria) and then incubating at 37 °C for 5 min. Splitting ratios were