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The determining role of nanoscale mechanical twinning on cellular functions of nanostructured materials

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ABSTRACT

Considering that micromotions generated at the bone-implant interface under physiological loading introduce mechanical strain on the tissue and surface of the implant and that strain can be introduced during processing of the biomedical device, we elucidate here the interplay between mechanically-induced nanoscale twinning in austenitic stainless steel on osteoblast functions. Mechanically-induced nanoscale twinning significantly impacted cell attachment, cell-substrate interactions, proliferation, and subsequent synthesis of prominent proteins (fibronectin, actin, and vinculin). Twinning was beneficial in favorably modulating cellular activity and contributed to small differences in hydrophilicity and nanoscale roughness in relation to the untwinned surface.

1. Introduction

Titanium alloys and stainless steels continue to be used as biomedical devices for bone fixation, partial/total joint replacement and spring clips for the repair of large aneurysmal defects (Reclaru et al., 2001; Vigorita and Ghelman, 1999; Dearnley et al., 2004). These biomedical metallic devices for knee and hip joints are envisaged to have life of 15 years or more, but sometimes fail prematurely because of inadequate bone build-up around the implants, making them loose. Furthermore, production of metallic debris introduced by wear, is absorbed near the implant and is attacked by the body's immune system, leading to inflammation, death of tissue, and loss of bone surrounding the implant. Besides orthopedic use of stainless steels, other applications include cardiovascular diseases (Chen and Thouas, 2015), and in the treatment of neurological diseases, such as full or partial paralysis, and Parkinson's disease, where stainless steel electrodes are adopted to stimulate the targeted regions of brain with the help of electric signals (Dymond et al., 1970; Gimsa et al., 2005; Polikov et al., 2005). Another important example is cranioplasty, which involves the utility of a stainless steel mesh to repair large cranial defects, which may come into contact with the outer brain tissue (Datti et al., 1985).

Considering the long term stability of bulk metallic implants and enhancement of cellular activity, we have recently addressed the subject of metallic debris and favorably modulating the cellular activity by developing the ingenious concept of phase-reversion to obtain high strength-high ductility combination in nanostructured biomedical stainless steel (Misra et al., 2009a, 2009b). Studies on osteoblast functions on nanograined stainless steels indicated significant modulation of cellular activity and cell-substrate interactions (Misra et al., 2009b, 2013; Faghihi et al., 2007a, 2007b). Several other researchers have contributed to the influence of microstructure and grain size on protein-material and cell-material interactions to understand the subsequent biological activity and to promote osseointegration (Chen and Thouas, 2015; Run et al., 2013; Bagherifard et al., 2014, 2015; Muley et al., 2016; Guo et al., 2015; Webster et al., 2000a). Furthermore, nanocrystallization on the surface or bulk has proven to decrease the risk of infection by resisting biofilm formation and bacterial attachment (Mei et al., 2009; Yu et al., 2010; Nune et al., 2015). Simultaneously, the high strength of nanograined biomedical device provided the required wear resistance and is in addition to thinner and reduced mass (high strength/weight ratio) for long term stability.

Furthermore, the cellular activity and biological functions on a metallic implant can be tuned by controlling the plastic deformation during processing of implant, and consequent activation of nanoscale deformation mechanisms. The mechanical deformation-induced nano-to-microscale structure can be exploited in tailoring and favorably modulating the biological functions with high cell viability, adhesion, and cell-implant interactions.

It was recently demonstrated by us that in stainless steels, the deformation mechanism depends on the grain size (Challa et al., 2014). For instance, the deformation mechanism during tensile loading or compression loading of high strength nanograined stainless steel was

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twinning (nanoscale twins), while in the low strength coarse-grained counterpart, it was strain-induced martensite. Twinning contributed to the excellent ductility in high strength nanograined stainless steel, while in low strength coarse-grained counterpart, ductility was also good, but due to strain-induced martensite. This change in deformation mechanism can impact adhesion of cells and biological functionality. In the current study, we have fundamentally elucidated the dependence of deformation mechanisms occurring at the nanoscale in nanograined (crystal size in the nanometer scale range) austenitic stainless steels that were subjected to tensile deformation up to a plastic strain of 10% and 20%. The current study opens the venue for utilizing microscopic level tissue-metallic material relationship to facilitate new medical treatments.

2. Experimental procedure

2.1. Material

The experimental material was biomedical grade commercial (AISI 301LN EN 1.4318) austenitic stainless steel of ~ 1.5 mm thickness and having nominal composition (in weight %) of Fe-0.017C-0.52Si-1.3Mn-17.3Cr-6.5Ni-0.15Mo-0.15N. To develop nanograined (NG) structure, the austenitic stainless steel was severely cold deformed (~70% deformation) in a laboratory rolling mill, followed by reversion annealing experiment in a Gleeble-1500 thermo-simulator at 850ºC for 10 s to obtain nanograined (NG) structure. After annealing, the samples were force-air-cooled at a cooling rate of ~200ºC/sec to 400°C, followed by convective air cooling. The annealing condition is specific to the experimental strip material used here. In other words, material with large cross-section, tuning of % cold deformation and temperature-time sequence is required to obtain NG structure at the surface (~top 0.5–1 mm layer) or in the bulk where cellular attachment and tissue growth occurs. During annealing, the cold deformed structure (martensite) reverts to austenite.

The grain structure was examined by transmission electron microscopy (TEM). Thin foils for examining the structure by TEM were prepared by twin jet polishing of 3 mm disks punched from the specimens, using a solution of 10% perchloric acid in acetic acid as electrolyte.

The mechanical properties (yield strength and elongation) were determined by tensile testing samples machined to a profile of 25 mm × 25 mm with 20 mm gage length.

To elucidate the impact of surface deformation morphology as a function of strain, NG tensile specimens were subjected to ~10% and ~20% tensile strain, respectively, through interrupted tensile test, at pre-defined strains and specimens unloaded. Post-mortem analysis of the tensile deformed region was carried out within the gage length by TEM. TEM foils were prepared in a manner identical to that described above for the phase reversion annealed steel.

2.2. Surface topography of tensile strained NG steel

In the context of adhesion of cells and cellular activity, surface topography and roughness is of particular relevance. It is known that interactions between extracellular proteins and focal adhesion points of cells are envisaged to occur at the nanoscale, which activate intracellular molecular signaling pathways influencing cellular activity (Goldberg and Jinno, 1999; Buser et al., 1991; Li et al., 2001). Surface topography may also influence proliferation. Prior to the interrupted tensile test, the samples were subjected to a standard metallographic procedure involving the use of a series of polishing grit SiC papers (200, 400, 600, 800, and 1200), followed by polishing on a micro cloth with 0.05 μm alumina to obtain mirror-finish surfaces with near-similar microscale roughness. Surface roughness of as-polished phase-reversion annealed steel (0% strain) and steel subjected to ~10% and ~20% plastic strain in terms of nanoscale roughness was studied using atomic force microscope (AFM) (Park NX10) in tapping mode. The tip used for the study was tapping mode etched silicon probe (TESP), with a tip height of 3.5 μm and tip radius of < 10 nm, the scan area was 1×1 μm^2.

2.3. Surface wettability (contact angle)

The ability of cells to attach to the substrate surface was measured as contact angle (wettability) of deionized water using goniometer approach (Rame'-Hart Inc, Germany). An autopipetting system was used to ensure a droplet of water with uniform volume (0.5 μL).

2.4. Cell culture

Cell culture studies were performed using mouse pre-osteoblasts cell line MC3T3-E1 subclone 4 (American Type Cell Culture Collection, Manassas, VA, USA) using standard protocol. Alpha minimum essential medium (α-MEM, Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) were used to culture pre-osteoblasts. All the stainless steel samples (0%, 10%, and 20% tensile strained) with dimensions (1 *1 cm^2) were cleaned in an ultrasonic bath with ethanol, followed by washing with deionized water and sterilized in an autoclave. Identical volume of pre-osteoblasts with 80–85% confluence obtained from T-flask cultures were used to seed the samples. In brief, the cells were washed with phosphate buffered saline (PBS), incubated with 0.25% trypsin/0.53 mM EDTA for 5–7 min to detach the cells from Petri dish, dispersed cells in trypsin/EDTA, transferred to a centrifuge tube and centrifuged at 2000 rpm for 5 min. Cell pellet obtained after centrifugation was re-suspended in culture medium and dilution carried out using culture medium to obtain the required cell concentration. Next, the sterilized steel samples were placed in 24-well plate and incubated with cell suspension at 37ºC in a humidified atmosphere with 5% CO_2 and 95% air.

2.5. Cell attachment

The initial attachment of cells on the steel surface as a function of tensile strain (0%, 10% and 20%) was determined as follows. Pre-osteoblasts (10,000 cells/cm^2) were seeded on the surface of substrates and incubated for 7 days at 37ºC in a CO_2 incubator (5% CO_2 and 95% air). The initial cell attachment and viability (after day 1) and proliferation (after day 7) was measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The principle of MTT assay is based on the reductase activity in mitochondria of living cells. The enzymes cleave the tetrazolium ring, which turns the pale yellow MTT into dark blue formazan, the concentration of which is directly proportional to the number of metabolically active cells. The widely adopted procedure is as follows: After 1 d and 7 d incubation, the samples were washed twice with PBS and incubated with fresh culture medium containing MTT (0.5 mg/mL medium) at 37ºC for 4 h in dark. Next, the unreacted dye was removed and dimethyl sulfoxide (DMSO) was added to dissolve the intracellular purple formazan product into a colored solution. The absorbance of this solution was quantified by photospectrometry at 570 nm with a micro plate reader (Bio TEK Instrument, EL307C).

2.6. Cell density and proliferation assays

The protocol was as follows: fluorescent labeling of nucleic acids was performed to assess the number density and proliferation of osteoblasts on samples subjected to different strain using fluorescence microscope (Nikon Eclipse E600 FN). Pre-osteoblasts were grown on steel surfaces up to 7 days at 37ºC in a CO_2 incubator. Culture medium was changed every two days. After 7 days, the cells were stained with the nucleic acid dye, Hoechst 33342. Next, cell-seeded disks were washed twice with PBS and incubated (10 μg dye/mL PBS) for 10 min at