

Electronic Supplementary Information (ESI) for Nanoscale

**Developing Biomedical Nano-Grained β -Type Titanium Alloys using High Pressure
Torsion for Improved Cell Adherence**

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Experimental

Materials: A hot-forged TNTZ bar, which its chemical composition was previously reported¹⁴, was used to prepare substrates of different microstructures. Solution treated and aged TNTZ samples prepared according to literature methods,¹⁴ were used as CG substrates. In order to obtain UFG/NG, the aged TNTZ was processed using an HPT machine (Toyohashi, Japan) under quasi-constrained condition⁷ (rotation number: 20, rotation speed: 0.2 rpm, load/pressure: 40 ton/1.25 GPa, under air, at room temperature).

Microstructural characterization: Microstructure were observed by optical microscopy (OM) (Olympus BX51, Japan), scanning electron microscopy (SEM) (JEOL FESEM 2100F, Japan), and transmission electron microscopy (TEM) (Topcon 002B, Japan) operating at an accelerator voltage of 200 kV. HRTEM images were obtained using an TEM operating (FEI Titan 80-300, Japan) at 300 kV with a field emission gun and a Cs-corrector for the objective lens and an aperture of ~50 nm.

Mechanical tests: Vickers' Hardness (HV) measurements were carried out using a micro-Vickers hardness tester with a 500 g load and 15 s dwell time. In order to investigate the local HV distribution along the coin-shaped specimen surface of HPTed TNTZ, measurement were performed at 1 mm and 22.5° intervals between measurement positions in the radial direction, as shown in Figure S1a. Local HV distributions were plotted as color-coded contour maps (Figure S1b).

Surface characterization: In order to investigate surface characteristics and cell-substrate interactions, the substrates were wet-polished using waterproof emery papers (<#2400) and buff-polished using a colloidal SiO₂ suspension to obtain a mirror finish. The substrates were then cleaned and sterilized in an autoclave at 394 K and 1 atm for 15 min. The surface roughness and topography of the substrates were evaluated by atomic force microscopy (AFM, Nanocute, Seiko Instruments Inc., Sendai, Japan) in contact mode. The surface

physicochemical characteristics of the substrates were evaluated by contact angle measurements (DMs-401, Kyowa Interface Science Co., Sendai, Japan) at 298 ± 2 K using distilled water ($\gamma_L^d = 21.8 \text{ nm m}^{-1}$, $\gamma_L^p = 51.0 \text{ nm m}^{-1}$) and diiodomethane ($\gamma_L^d = 50.8 \text{ nm m}^{-1}$, $\gamma_L^p = 0 \text{ nm m}^{-1}$). The surface free energy (SFE) was calculated using the Owens-Wendt-Rabel-Kaelble (OWRK) method^{29,30} according to Equation (1) using the values defined above for distilled water and diiodomethane:

$$\gamma_L(1 - \cos \theta) = 2(\gamma_{sd}\gamma_{Ld})^{1/2} + 2(\gamma_{sd}\gamma_{Ld})^{1/2} \quad (1)$$

Cells: Human osteoblast (hOB) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12, containing 10% fetal bovine serum (FBS) and $50 \mu\text{g mL}^{-1}$ penicillin/streptomycin at 307 K under a 5% CO₂ humidified atmosphere. To investigate cell adhesion and proliferation on the substrates, hOB cells were seeded on each substrate surface at $\sim 2 \times 10^4 \text{ cells mL}^{-1}$ and $\sim 2 \times 10^3 \text{ cells mL}^{-1}$ in aliquots of DMEM/F12 (100 μl) containing 10% FBS and $50 \mu\text{g mL}^{-1}$ penicillin/streptomycin. The cell-modified substrates were incubated for cell attachment (6 h) and proliferation tests (6 d) at 307 K under a 5% CO₂ humidified atmosphere. The substrates were then washed with PBS ($\times 3$) to remove dead and unattached cells, and the cells lifted using a trypsin-EDTA solution. Cell numbers were counted using a disposable hemocytometer (C-Chip, NanoEnTek Inc., USA). The actin filament localization of the cells was evaluated immunocytochemically by seeding the cells on each substrate surface at $2 \times 10^4 \text{ cells mL}^{-1}$ in aliquots of DMEM /F12 (100 μl) (10% FBS + $50 \mu\text{g mL}^{-1}$ penicillin/streptomycin) and incubated at 307 K. After 3h, the Actin Cytoskeleton and Focal Adhesion Staining Kit (FAK 100 - Millipore, USA) was used to immunocytochemically stain actin filaments. IF images of cells were visualized using a fluorescence microscope (Olympus IX71 inverted microscopy, Japan).

Statistical Analysis: Data were first analyzed by analysis of variance (ANOVA); when statistical differences were detected. Data are reported at significance levels of $p < 0.05$ and 0.01.

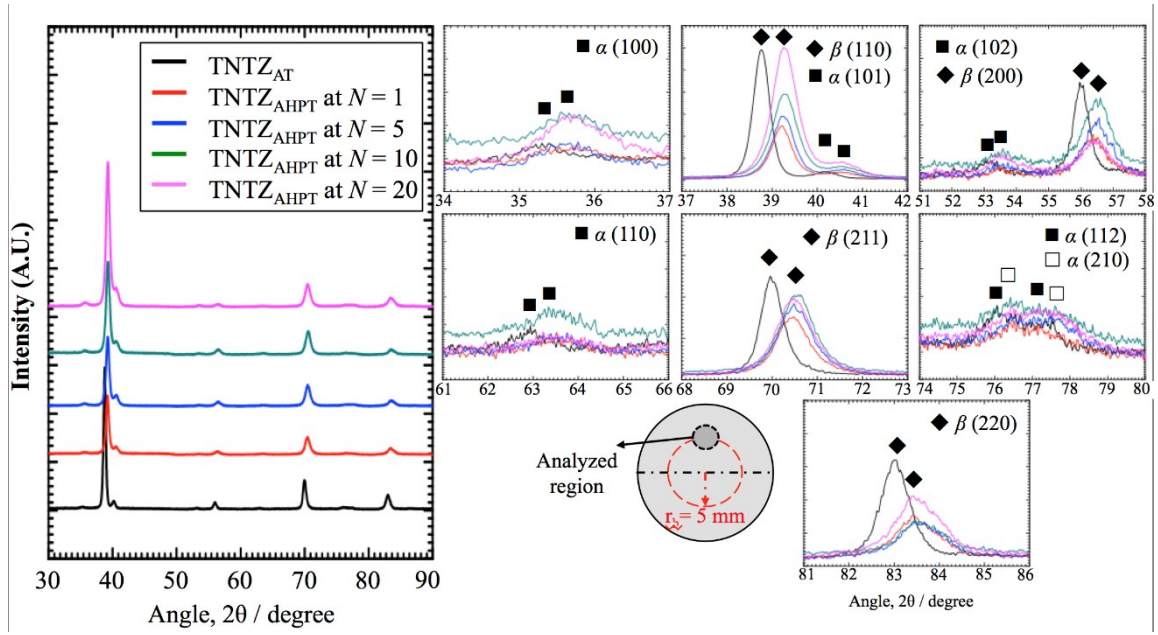


Figure S1. XRD profiles of TNTZ_{AT}, and TNTZ_{AHPT} at $N = 1, 5, 10,$ and 20 at the r_h position with magnified XRD peaks.

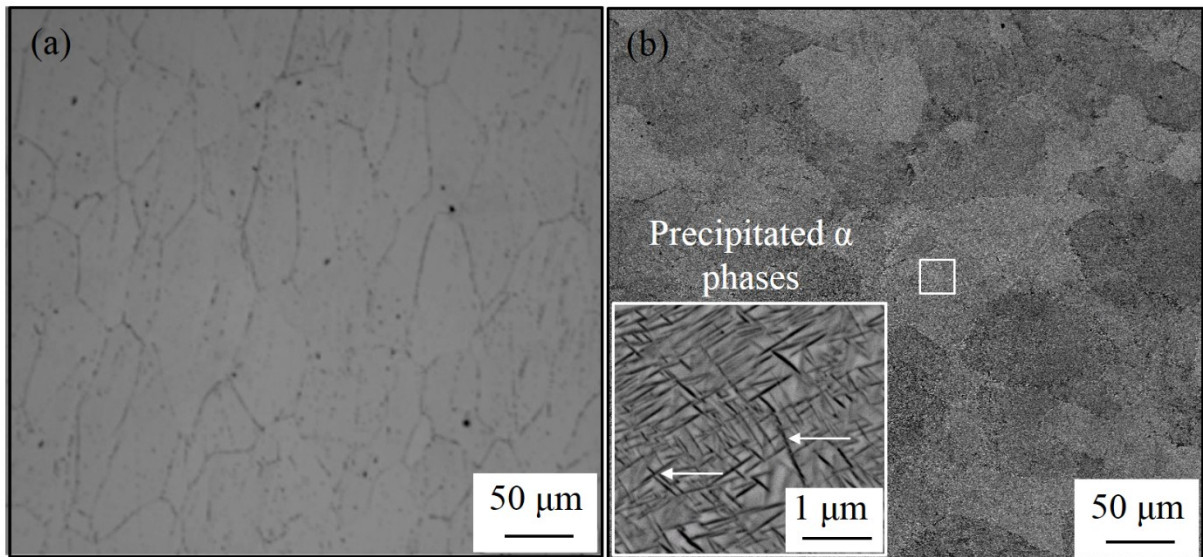


Figure S2 (a) Optical micrograph of TNTZ_{ST} (b) SEM images of TNTZ_{AT}

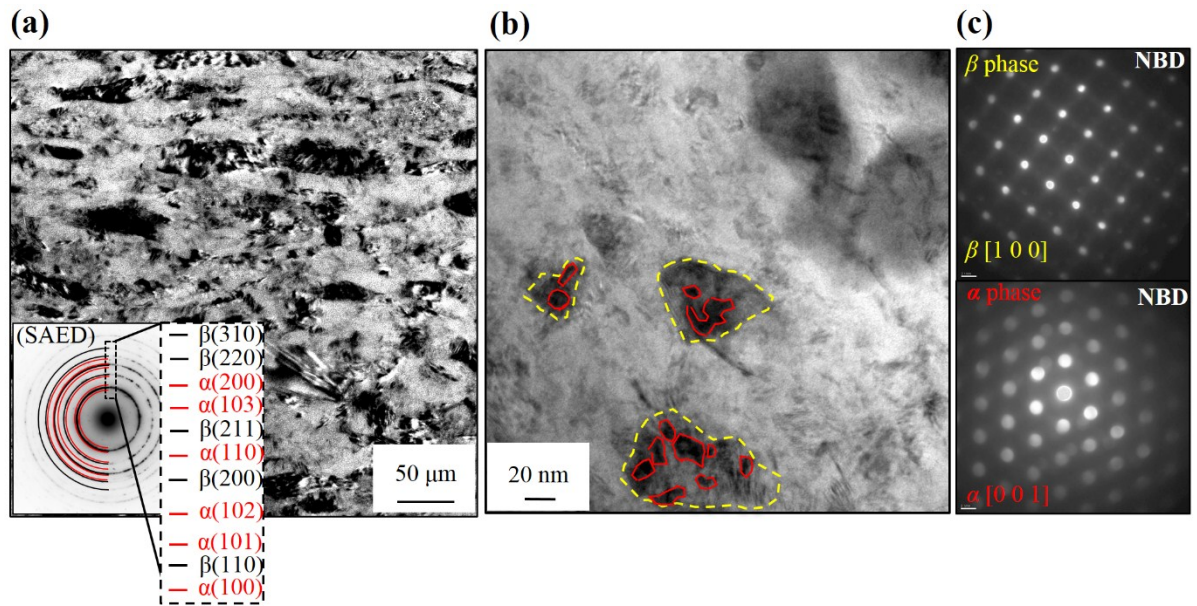


Figure S3 (a) TEM bright image of TNTZ_{AHPT} with selected area electron diffraction pattern (b) HRTEM bright field image with (c) nano-beam diffraction patterns. Precipitated α phases were marked with red line. The strain fields between β matrix and α phases were marked with yellow dashed line in the HRTEM image.

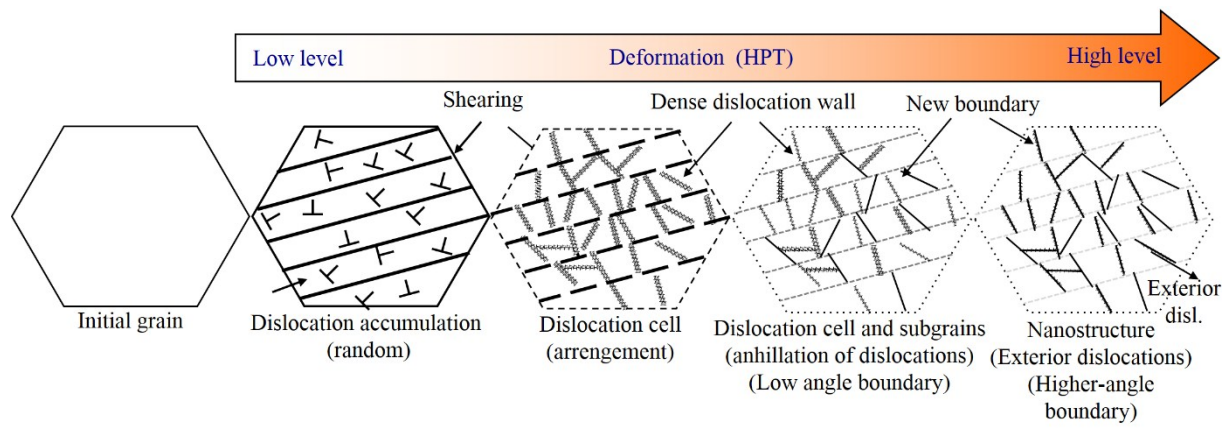


Figure S4 Illustrations of dislocations accumulation, arrangement and annihilation for new grains.

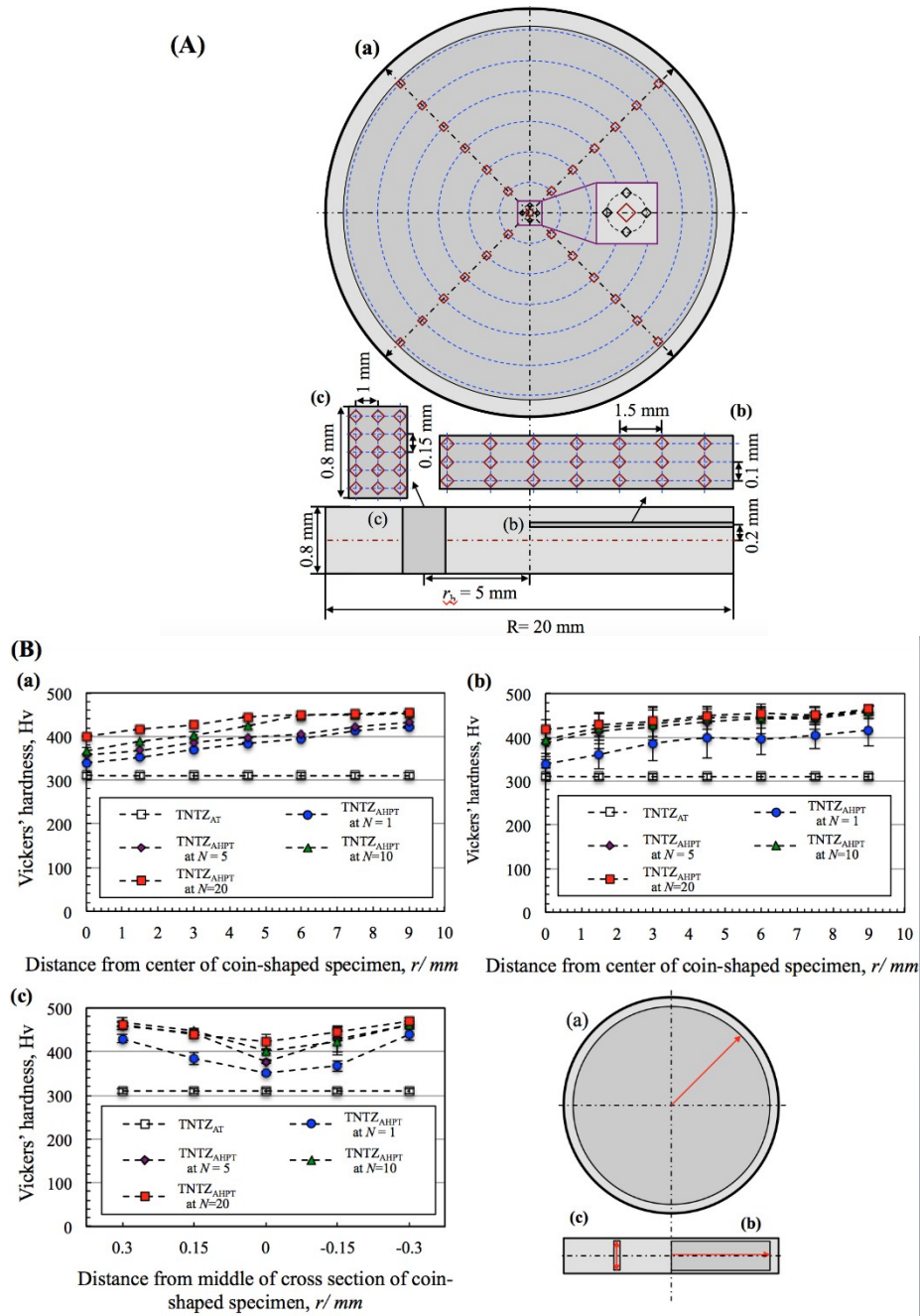


Figure S5. (A) Schematic drawing of hardness measurement along radial direction on (a) surface and (b) cross section and (c) depth direction of coin-shaped specimen with magnified measurements. (B) Hardness distributions along the radial direction (a) on the surface, (b) cross section, and (c) depth directions on the cross section of TNTZ_{AHPT} at $N = 1, 5, 10,$ and 20 . The average hardness of TNTZ_{AT} has been added to the graphs. ^[16]

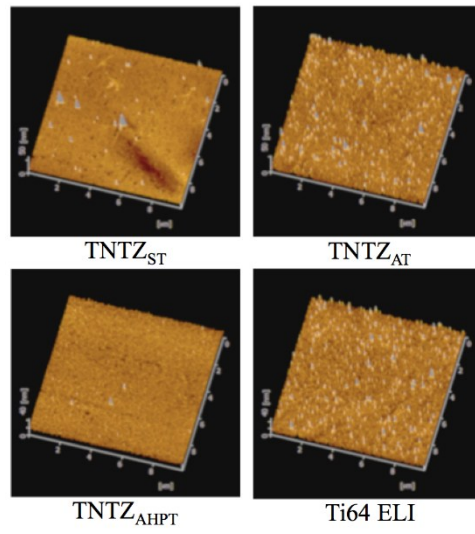


Figure S6. AFM contact mode 3D images of the mirror-like polished surfaces of TNTZ_{ST}, TNTZ_{AT}, TNTZ_{AHPT}, and Ti64 ELI substrates.

Table S1. Roughness values of the mirror-like polished TNTZ_{ST}, TNTZ_{AT}, TNTZ_{AHPT}, and Ti64 ELI substrates.

Sample	Roughness, RA/ nm	Roughness, RMS/ nm
TNTZ _{ST}	1.5 [± 0.12]	2.31 [± 0.16]
TNTZ _{AT}	2.35 [± 0.35]	4.75 [± 0.23]
TNTZ _{AHPT}	1.52 [± 0.2]	2.33 [± 0.13]
T64-ELI	2.7 [± 0.4]	3.85 [± 0.26]

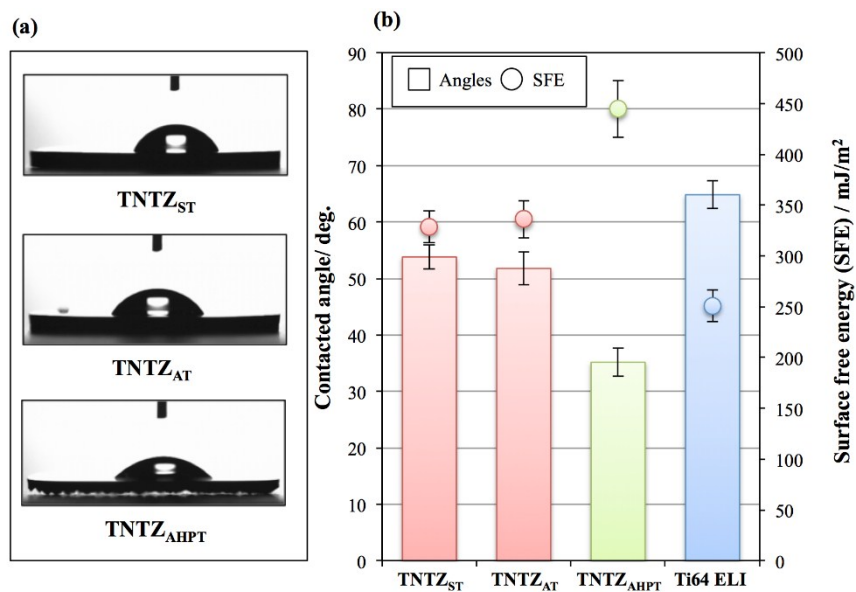


Figure S7. Pictures of contacted angles of distilled water and average contacted angles and SFE for TNTZ_{ST}, TNTZ_{AT}, TNTZ_{AHPT}, and Ti64 ELI.

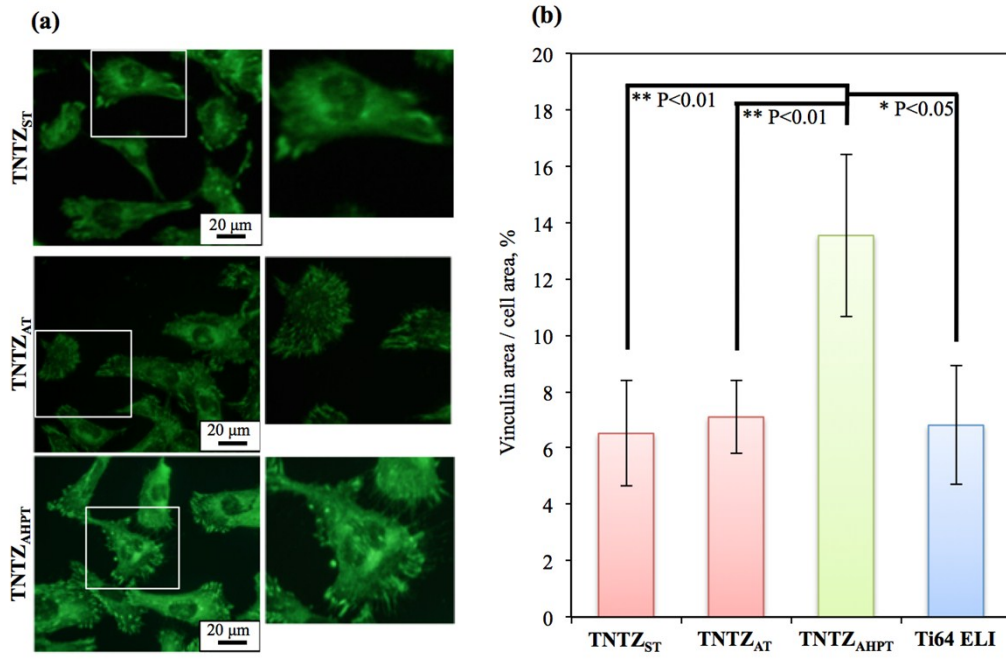


Figure S8 (a) IF micrographs of vinculin focal contact plaques in hOBs attached on the substrates of TNTZ_{ST}, TNTZ_{AT}, and TNTZ_{AHPT} and **(b)** area fraction of vinculin focal contact plaques in hOBs attached on TNTZ_{ST}, TNTZ_{AT}, and TNTZ_{AHPT}, and Ti64 ELI substrates.

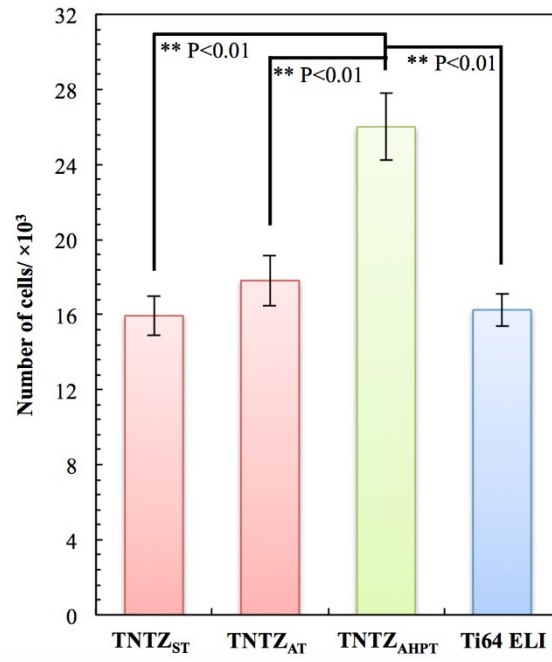


Figure S9 Average numbers of hOBs on the substrates of TNTZ_{ST}, TNTZ_{AT}, TNTZ_{AHPT}, and Ti64 ELI after incubation for 6 days at 307K.