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Dual-topography electrical discharge machining of titanium to improve biocompatibility

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ABSTRACT

Surface modifications of titanium are widespread in an effort to improve the osseointegration capabilities of the metal for orthopaedic and dental applications. Here, electrical discharge machining (EDM) was used to create modified, notably, dual-topography surfaces on titanium. By swapping conventional copper electrodes for a titanium electrode and water dielectric, modified surfaces free of trace element contaminants were produced. Three surfaces were produced by varying the peak currents at 10 A, 29 A and a uniquely hierarchical multi-current combination of 29 A followed by 2.4 A. The physicochemical properties of these surfaces were analyzed by scanning electron microscopy (SEM), Energy Dispersive X-Ray Spectroscopy (EDX), and Auger Spectroscopy. These revealed the topography of the modified surfaces and a titanium oxide layer that was markedly thicker on the EDM samples compared to controls. *In vitro* cell testing was carried out with osteoblast-like MC3T3-E1 cells. Cell differentiation was increased in all EDM modified surfaces compared to controls and early differentiation was promoted on the dual-topography surface. The present study suggests the promise of dual-topography surfaces created using EDM for implant applications.

1. Introduction

Due to its widespread use in the medical industry as an implant material, titanium has been modified extensively with the goal of improving osseointegration, where both the surface chemistry and topography are known to contribute to the attachment of bone tissue to implanted devices [1]. In particular, roughened or porous surfaces are known to stimulate cellular attachment, differentiation and bone growth [2–4], while the naturally occurring titanium oxide surface layer provides biocompatibility. Numerous techniques have been examined for these purposes, such as sandblasting, acid-etching treatments, ion coating, laser-modification and plasma spraying for applications in dentistry and orthopaedics [4–11].

Electrical discharge machining (EDM), a non-conventional machining technique, is one such method that has previously been applied to titanium with the goal of improving its osseointegration capabilities [12–15]. EDM is a precision material removal process that utilizes the heat generated from controlled, rapid and repetitive spark discharges to remove small volumes of material by melting and vaporization, altering the surface topography and roughness in the process. The tool and workpiece electrodes are separated by a small gap of several micrometers filled with a dielectric fluid, across which a high-frequency pulsed voltage is applied to strike the discharges. While metallic materials, such as titanium, can be difficult to cut due to their hardness and strength, the lack of direct contact between the electrode and workpiece allows for effective machining by EDM [16–20].

Die sink EDM has been previously used to change the surface composition and roughness of titanium-based implants for bone applications [12,17,21,22]. However, previous studies utilized copper or graphite electrodes with hydrocarbon oil or deionized water dielectrics for EDM surface modification for bone applications [19,20]. These studies found that significant quantities of copper and/or carbon compounds were incorporated into the titanium surfaces [19,20,23]. In addition to being undesirable from a biological standpoint, these carbon deposits can form a mechanically detrimental hard layer of TiC on the EDM modified surfaces [13,23–25].In addition to a biocompatible surface oxide, the length scale of surface topographies is crucial for bone integration. Multi-length scale surfaces with features ranging from micron, to sub-micron, and to the nano-scale have been shown to enhance implant-bone biomechanical stability and integration [26–28]. Until presently, hierarchical EDM surfaces for bio-applications were yet to be explored.

In this study, EDM modification of Grade II commercially pure titanium was employed with a titanium electrode and distilled

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water dielectric fluid to create a micro-roughened surface with a contaminant-free titanium oxide surface chemistry, ideal for bone interfacing implant applications. Notably, a dual-topography surface was explored, where sub-micron features were imposed on micron features by using a sequential multi-current discharge machining protocol. Surface characterization and the effect of EDM modification on the biocompatibility and cellular response were evaluated.

2. Materials and methods

2.1. Electric discharge machining

Grade II commercially pure titanium (ASTM B265) was used as the workpiece (the surface to be modified). Specimens were cut to 25 mm × 25 mm × 4.76 mm for surface characterization, while15 mm diameter discs, 0.889 mm thick, were cut for cell testing and Auger Spectroscopy. The samples were machined using die sinking EDM (AgieTron Impact 2 Ram-type EDM System, Agie Ltd, Illinois, USA). The electrode (positive polarity) material was a cylindrical rod (200 mm × Ø 9.53 mm) of Grade II titanium (ASTM B348). While keeping pulse on-time constant at 21 μs at a duty factor of 0.33 and a working voltage of 40 V, two different single peak currents (10 A and 29 A) and a multi-current (29 A followed by 2.4 A) were used in a water dielectric. Within the parameters of the equipment, these were the minimum and maximum currents that could be produced, and thus selected as a starting point for dual-topography machining.

2.2. Surface characterization

Following machining, samples were ultrasonically cleaned in acetone, then ethanol for 15 min respectively and air-dried before any characterization. Sample surfaces were observed using scanning electron microscopy (SEM; JSM-6610LV, JEOL) at an accelerating voltage of 10 kV. In addition, cross sections of each EDM modified surface were prepared by cold mounting, cutting with a precision cutter, grinding with 500, 800, 1200 and 2400 grit SiC paper and polishing with 9 μm diamond suspension and, lastly, colloidal silica with 10 vol% of hydrogen peroxide before viewing in the SEM. A chemical analysis of the surface and cross section was conducted using energy dispersive X-ray spectroscopy (EDS). Auger electron spectroscopy (AES; JAMP-9500F, JEOL) was used for elemental depth profiling of the untreated sample and a representative modified surface, the 10 A EDM surface, performed at a sputtering rate between 0.33 nm/s up to 240 nm/s. X-ray diffraction (XRD) measurements were obtained using a CuKα| rotating anode X-ray generator with parallel-focusing monochromatic optics (Rigaku RU 200) with a CCD area detector (Bruker SMART 6000). The detector distance was 16.74 cm with an exposure time of 1200 s per frame. Microhardness measurements were performed on cross sections by a Vickers hardness tester (MMT-X7, Matsuzawa, Japan) under 50 g load and 15 s dwell time.

2.2.1. Cell culture

The growth and differentiation of osteoblast-like cells on the titanium samples was thoroughly evaluated by seeding the cells on the surface of the material (direct contact assay). Mouse pre-osteoblast-like MC3T3-E1 cells (Subclone 14, ATCC) were used as the cell model. The cells were maintained in cell culture flasks in an incubator with a humidified atmosphere of 5% CO_2 in air at 37 °C. AlphaMEM medium free of ascorbic acid (Life Technologies, Gibco, ref. n. A10490-01, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin (Sigma Aldrich, ref. n. P4333, St. Louis, Mo, USA) and 10% fetal bovine serum (Thermo Scientific, HyClone, ref. n. SV30160.03, Logan, UT, USA) was used as culture medium. The medium was renewed every second day. Upon confluence, cells were detached with a minimum amount of trypsin 0.25% in EDTA (Thermo Scientific, HyClone, ref. n. SH30042.02) and it was inactivated with supplemented medium after 10 min.

Ti samples were sterilized with isopropanol 70% for 2 h with gentle shaking after which the samples were washed 5 times with autoclaved distilled water (5 × 10 min agitation). The samples were placed in 24 well plates and 19 100 cells were seeded on each disk (10 000 cells/cm^2). Wells without cells were used as blank readings. The media employed for the experiment was Alpha MEM Modification (Thermo Scientific, Hyclone, ref. n. SH30265.01) and it was replaced (500 μl) every two days. From day 3 onwards an osteogenic media was prepared by supplementing the MEM Alpha Modification with 1% of 2.5 mg/ml L-sodium ascorbate (Sigma, ref. n. A-7631) and 1 M glycerophosphate (Sigma, ref. n. G-9422) just before adding the media to the cells. After 3, 7 and 14 days, the cell growth and cell differentiation was quantified. Triplicates were used and the experiment was performed twice.

2.2.2. Cell viability

Cell viability was evaluated using an AlamarBlue® assay (Invitrogen, ref. n. DAL1100, Carlsbad, CA, USA). AlamarBlue®, or resazurin, is a known indicator of cell viability which is irreversibly reduced by metabolically active cells to its oxidized form resorufin which fluoresces. In each 24 well plate, the old media was replaced by 350 μl of 5% Alamar Blue/MEM (Life Technologies, Gibco, ref. n. 51200). After incubation in the dark for 1 h at 37 °C, fluorescence was measured on a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland) at 560 nm excitation and 590 nm emission. The blank fluorescent value was subtracted from each data point which allowed the fluorescent signal to directly correspond to cell number. Afterwards, wells were washed with a phosphate buffered saline solution (PBS) and 300 μl of a lysis solution (0.1% triton in PBS) was added. The well plate was frozen at −20 °C and three freeze-thawing cycles were performed to further quantify total amount of proteins and alkaline phosphatase.

2.2.3. Protein quantification

The total amount of proteins was quantified by a microBCA® assay (Thermo Scientific, ref. n. 23235). 25 μl of cell lysis and 25 μl microBCA reagents (prepared as indicated by the manufacturer) were incubated in dark at 37 °C for 2h. Finally, 50 μl of distilled water was added in every well and absorbance was measured at 562 nm in the microplate reader. A standard curve was prepared with different concentrations of bovine serum albumin (BSA, provided in the microBCA® kit). The blank reading was subtracted from every data point and absorbance was transformed to concentration of BSA.

2.2.4. Cell differentiation — alkaline phosphatase activity assay

The cell differentiation was quantified by measuring the alkaline phosphatase (ALP, Sigma Aldrich, ref. n. P7998) activity, an early marker of osteoblast differentiation that is expressed just before matrix mineralization begins [29]. Cell lysis (50 μl) and ALP reagent (100 μl) were incubated together in dark at room temperature. The reaction was stopped after 20 min with 3M NaOH and absorbance was measured at 405 nm. A standard curve was prepared with different concentrations of p-nitrophenol (Sigma, ref. n. N7660). Blank was subtracted from every data, absorbance was transformed to concentration of p-nitrophenol and data were normalized by the total number of proteins and the reaction time.

2.3. Statistical analysis

Statistical analysis was done using IBM SPSS Statistics 19 software (IBM, Chicago, IL, USA) using one-way ANOVA at a significance level of $\alpha = 0.05$. Scheffe's post-hoc test was used in the case of homogeneity of variances (Levene's test); otherwise, Tamhane's post-hoc test was chosen.