Thin Solid Films 596 (2015) 111–117

Contents lists available at ScienceDirect

Thin Solid Films

journal homepage: www.elsevier.com/locate/tsf

Antibacterial activity and cell compatibility of TiZrN, TiZrCN, and TiZr-amorphous carbon coatings

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ARTICLE INFO

Article history:
Received 18 March 2015
Received in revised form 10 September 2015
Accepted 10 September 2015
Available online 21 October 2015

Keywords:
Titanium zirconium nitride
Carbon nitride
Amorphous carbon
Coating
Antibacterial
Cell compatibility

ABSTRACT

A cathodic-arc evaporation system with plasma-enhanced duct equipment was used to deposit TiZrN, TiZrCN, and TiZr/a-C coatings. Reactive gases (N_2 and C_2H_2) activated by the Ti and Zr plasma in the evaporation process was used to deposit the TiZrCN and TiZr/a-C coatings with different C and nitrogen contents. The crystalline structures and bonding states of coatings were analyzed by X-ray diffraction and X-ray photoelectron spectroscopy. The microbial activity of the coatings was evaluated against *Staphylococcus aureus* (Gram-positive bacteria) and *Actinobacillus actinomycetemcomitans* (Gram-negative bacteria) by in vitro antibacterial analysis using a fluorescence staining method employing SYTO9 and a bacterial-viability test on an agar plate. The cell compatibility and morphology related to CCD-966SK cell-line human skin fibroblast cells on the coated samples were also determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay, reverse-transcriptase-polymerase chain reaction, and scanning electron microscopy. The results suggest that the TiZrCN coatings not only possess better antibacterial performance than TiZrN and TiZr/a-C coatings but also maintain good compatibility with human skin fibroblast cells.

1. Introduction

Due to advantages of biocompatibility and excellent material properties including low modulus, high corrosion resistance, and lightness, titanium (Ti) and its alloys are considered important metallic biomaterials and have been applied in orthopedic joints, screws, and plates, in orthodontic screws, and in dental implants [1,2]. Although Ti is in widespread clinical use, its poor wear resistance prevents its application in some medical fields. Adding alloying elements to Ti to produce Ti alloys can improve this shortcoming; for example, Ti-aluminum-vanadium alloy has good corrosion resistance and biocompatibility, but some concerns remain about its clinical use because of the possibility of toxic aluminum being released [3].

Zirconium (Zr) has also been considered as an excellent biomaterial. Not only has Zr outstanding corrosion resistance [4], bending strength, and fracture toughness [5], but Zr-based implants also exhibit good osseointegration [6,7] and a high degree of bone-implant contact in animal studies [8]. The increasing use of computer-aided design and computer-aided manufacturing techniques in recent decades has led to the increased use of Zr-based materials in dental applications [9], including oral implants [10]. Additionally, some scientists have developed Zr as a coating material for medical implants [11–13].

Titanium (Ti) and zirconium (Zr) have been considered as excellent biomaterials for medical applications, and TiZr-alloy dental implants have been applied commercially in clinical applications. Ti and Zr have similar chemical properties because they are both Group IVB elements in the periodic table. Zr has been used as an alloying element to improve the properties of Ti in the creation of new types of TiZr-based alloys [14]. Its excellent biomechanical properties compared with Ti results in acceptable performance in oral implants [15]. Several researchers have confirmed the osseointegration-enhancing ability of a TiZr implant in both animal tests and clinical trials [16,17].

While Ti and Zr have outstanding biocompatibility compared with other metals, clinical applications add some further requirements. In order to prevent infection and thereby ensure a high success rate of medical devices and implants made from Ti and/or Zr, bacterial colonization might be inhibited by coating the surface with inorganic antibacterial agents such as silver, carbon (C), zinc, copper (Cu) [18–20], or certain oxide- and nitric-related coatings, e.g. zinc oxides (ZnO), titanium nitrides (TiN), and tantalum nitrides (TaN) [21,22].

Even though the mechanical advantages of TiZr alloy and the bacterial inhibition effects of C and N coatings have been confirmed, no study has developed new types of TiZr coatings that include C and/or N and evaluated their material properties, biocompatibility, and antibacterial

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http://dx.doi.org/10.1016/j.tsf.2015.09.082
0040-6090/

activity. The purpose of this study was to determine the effect of adding C and/or N to TiZr coatings on the cytocompatibility and antibacterial performance (including against Gram-positive and Gram-negative bacteria) in medical applications. The cell viability and gene expression were investigated on the coatings by applying the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to CCD-966SK cell-line human skin fibroblast (SKF) cells.

2. Experimental details

2.1. Preparation of coatings and microstructure analyses

Four experimental conditions were employed: (1) pure-Ti plates (20 mm × 20 mm × 1 mm, surface roughness Ra = 0.1 mm, biograde 2; Uniti Titanium, Moon Township, PA, USA), (2) TiZrN-coated Ti plates, (3) TiZrCN-coated Ti plates, and (4) TiZr–amorphous-C (TiZr/a-C)-coated Ti plates. Each condition was tested by performing experiments on ten samples. Coatings were applied to the Ti plates using a cathodic-arc evaporation system. Circular Ti and Zr targets (150 mm in diameter) were arranged on the chamber wall to deposit the TiZrN, TiZrCN, and TiZr/a-C coatings. The samples were placed on a rotational substrate holder for the deposition, and a DC arc current of 70 A was applied between the anode and cathode. The base pressure prior to deposition was less than 1×10^{-3} Pa. Argon and reactive gas (N_2 and C_2H_2) were introduced through a conducting duct around the target to enhance the reaction of the plasma and reduce the amount of macroparticles produced during the deposition process. The temperature of the sample during the deposition was measured by a thermocouple located near the sample to be within the range of 350 ± 20 °C. A substrate bias voltage of −100 V was used. The TiZrN coating was deposited at an N_2 pressure of 2.7 Pa.

For the deposition of TiZrCN and TiZr/a-C coatings, a graded TiZrN interlayer was deposited to reduce the internal stress and improve the adhesion strength of the composite TiZrCN and TiZr/a-C coatings. The transition layer from TiZrN to TiZrCN and TiZr/a-C was formed by gradually changing the reactive gas from pure N_2 to a mixture of N_2 and C_2H_2. At a total gas pressure of 2.7 Pa, a mixture of reactive N_2 and C_2H_2 with a C_2H_2 flow rate of 30 sccm was introduced into the chamber to form the TiZrCN. The TiZr/a-C coatings were deposited with a C_2H_2 flow rate of 95 sccm at a total gas pressure of 2.7 Pa. The total thickness of the coatings was 1.0–1.2 μm after a deposition time of 30 min.

The composition and chemical binding characteristics of the deposited coatings were determined using X-ray photoelectron spectroscopy (XPS; PHI5000 VersaProbe ULVAC-PHI Inc., Japan) with nonmonochromatic Mg Kα radiation. XPS was performed with argon ions to sputter the surface oxide layer for 1 min and reveal the chemical composition of the deposited coatings. A survey spectrum covering the range of 0–1000 eV was recorded for each sample, followed by high-resolution spectra over different elemental peaks, from which the composition was determined. The spectral ranges of 455 ± 10 eV, 182 ± 12 eV, 400 ± 10 eV, and 285 ± 12 eV corresponded to the binding energies of Ti2p, Zr3d, N1s, and C1s, respectively. The energy was calibrated by reference to the Au $4f_{7/2}$ peak from a clean gold surface at 83.8 eV. Curve fitting was performed after Shirley background subtraction by Gaussian fitting [23]. A glancing-angle X-ray diffractometer (X'pert Pro, PANalytical Co., The Netherlands) with a high-resolution ψ goniometer and Cu radiation was employed for phase identification. The diffractometer was operated at 40 kV and 30 mA with a glancing angle of 1–2°.

2.2. Hydrophilicity analysis

The hydrophilicity of the surface of samples was measured by a contact-angle analyzer (FTA-125, First Ten Angstroms, Portsmouth, VA, USA). Each specimen was alternately washed in ethanol and deionized water in an ultrasonic cleaner for 30 min and then dried in a clean dry oven at 55 °C for 6 h. Distilled water (10 ml) was then dropped on the sample surface using a micrometric syringe at room temperature. The sample was immediately imaged, and the contact angle was measured automatically by a spherical fitting approach. Each contact angle reported here is the mean of 10 independent measurements.

2.3. Antibacterial analyses

Samples (500 μl) of *Staphylococcus aureus* (Gram-positive bacteria) or *Actinobacillus actinomycetemcomitans* (Gram-negative bacteria) suspension (2×10^7 cfu/ml) were added to the surfaces of the TiZrN-, TiZrCN-, and TiZr/a-C-coated samples. After incubation for 6 h at 37 °C under a relative humidity of 96% and avoiding light exposure, the sample surfaces were rinsed three times with phosphate-buffered saline (PBS). The retained bacteria were then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and stained with 10 μM SYTO 9, which is a green fluorescent nucleic acid to stain live and dead bacteria, for 30 min at room temperature. The bacteria that had adhered to the samples were quantified by measuring the fluorescence at 488 nm using an enzyme-linked immunosorbent assay reader (Synergy HT, BioTek Instruments, Winooski, VT, USA).

The bacterial-viability tests of the uncoated and coated specimens were also evaluated on agar plates. LB agar (200 ml, comprising 5 g of LB broth, 1.6 g of agar, and 193 ml of glass-distilled deionized water; Difco Laboratories, Detroit, MI, USA) was mixed with 0.2 ml of *S. aureus* or *A. actinomycetemcomitans* (1×10^6 cfu/ml). The uncoated and coated samples were overlaid and immersed in 8 ml of LB agar containing *S. aureus* or *A. actinomycetemcomitans* on sterile plates. They were first kept at room temperature for 30 min and then incubated at 37 °C for 16 h, after which the visible bacterial colonies on the uncoated Ti specimen and the coated samples were imaged and counted for analysis.

2.4. Biocompatibility tests of cell viability and proliferation

The cell viability of SKF cells was examined using the MTT assay (Sigma-Aldrich, St. Louis, MO). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt used in the MTT test turns into a purple formazan product when viable mitochondria in living cells are present. A 3-ml solution containing SKF cells was first seeded at a density of 2×10^4 cells/ml onto the TiZrN-, TiZrCN-, and TiZr/a-C-coated samples, and then incubated at 37 °C in 5% CO_2 for 48 h, at which time the proliferation was assumed to be complete. The absorbance [quantified as the optical density OD)] of the purple formazan was quantified at 570 nm by a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with SoftMax Pro 5.2 241 software (Molecular Devices). The OD of the formazan reflected the level of cell viability, with higher OD values indicating a larger number of living cells on the sample and hence better biocompatibility.

The cell morphology and attachment were observed using scanning electron microscopy (SEM). Prior to SEM, a 3-ml aqueous solution containing cells at a density of 2×10^4 cells/ml was seeded onto the samples and incubated at 37 °C in 5% CO_2 for 24 h. The tested samples were rinsed twice with PBS and immediately fixed in 2.5% glutaraldehyde for 30 min. The tested samples were then rinsed again with PBS, immersed in distilled deionized water for 10 min, and then dehydrated in an ethanol series (35%, 50%, 75%, 85%, 95%, and 100%; each for 10 min). The tested samples were fixed and subsequently dried using critical-point drying with CO_2 (for which the critical point is at 31.1 °C and 7.39 MPa) using a Samdri-PVT-3D (Tousimis Res. Corp., USA) apparatus. The chamber was precooled (10 °C) to make it easier to fill with liquid CO_2 from a gas cylinder. The chamber was then heated to just above the critical temperature, with critical pressure subsequently being achieved. Immediately after critical-point drying, samples were