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Characterization and in vitro biocompatibility study of Ti–Si–N nanocomposite coatings developed by using physical vapor deposition

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ABSTRACT

Amongst the Ti alloys used as orthopedic implant materials, Ti6Al4V is one of the widely used alloys. Magnetron sputtering was used to deposit nanocomposite coating of Ti–Si–N on the Ti6Al4V substrate at different power and then the coating structure and surface properties were characterized through contact angle measurement, X-ray diffraction (XRD), scanning electron microscopy (SEM), and atomic force microscopy (AFM). In vitro biocompatibility of the coatings was assessed by using mouse bone marrow mesenchymal stem cells (mBMMSC). Antibacterial studies were performed using *Escherichia coli* (*E. coli*) microorganisms. The osteogenic differentiation was also carried out in order to get gene expressions. The AFM results confirmed that the coatings deposited at 120 W was smoother as compared to other coatings developed at different power, along with optimum contact angle, also these coatings showed good antibacterial results. The fluorescent and viability results of 120 W sample confirmed their good biocompatibility as compared to the coatings deposited 20, 40, 60, and 100 W power. Hence, the coating deposited at 120 W exhibit desirable microstructural characteristics beneficial for surface modification of orthopedic implants.

1. Introduction

Titanium and its alloys have been widely used in many biomedical and industrial applications due to their high specific strength, corrosion resistance. However, titanium and its alloys are still not sufficient for clinical use because the compatibility of these materials must be improved further [1]. Reservation has been expressed concerning the presence of element, vanadium in long-term implants, which is toxic both in the elemental state and in the form of oxides [2]. To enhance the biocompatibility of the titanium implants, many attempts have been made to modify the composition and chemistry of the titanium surfaces including deposition of bioactive coatings [3–7]. But, high coefficient of friction and low wear resistance of these coatings limits their usage in some applications. However, its poor tribologic behavior has limited the extension of Ti6Al4V in application areas related to wear resistance [8]. Transition metal nitrides, especially CrN and TiN coating, have usually been used to enhance the weak surface performance of the substrate as good wear resistant materials [9,10]. However, their low thermal stability and cell adhesion properties limit their application as bioimplants. It has been reported that the addition of amorphous Si increases the biocompatibility of the implants [11]. Therefore, in order to enhance the biocompatibility of these coating with retained wear properties, Ti–Si–N coating has been fabricated on Ti alloys to enhance the cell proliferation, differentiation, and cytocompatibility of the implant, in this work.

Furthermore, numerous advanced surface techniques, such as nitriding [12], ion implantation [6,7], plasma spraying [13] and physical vapor deposition [14–16] have been studied with the aim of enhancing the surface properties of the substrate. Among these, physical vapor deposition (PVD), due to its environmentally friendly characteristic, convenience, and precision in deposition has been one of the favorable techniques [17,18]. The biologic studies of a Ti–Si–N coated Ti6Al4V alloy by using PVD technique are scarce in the literature. However, the tribological properties of Ti–Si–N coating have been recently reported in the literature [19].

Ti–Si–N nanocomposite coatings on Ti6Al4V substrate by magnetron sputtering at different power were fabricated in the present work. Surface characteristics of the coatings were analyzed by SEM, AFM, and contact angle measurement. The biocompatibility

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of the coating has been studied through in vitro tests as well as the antibacterial activity of the coatings for their suitability as bioimplants.

2. Experimental procedure

2.1. Mechanical polishing/etching

Titanium alloy (Ti-6Al-4V) cubes with A (6 mm × 6 mm × 6 mm) dimensions, were mechanically polished using 200, 400, 600, 800, 1000, and 1200 grit SiC sand paper sequentially. The samples were further polished using 1 μm alumina to achieve a mirror finish for the implant surfaces. The samples were then cleaned with ethanol using an ultrasonicator for 10 min. This ensures the removal of the impurities arising due to mechanical polishing. The mechanical treatment was followed by chemical etching using 4% HNO_3 in ethanol for 1 min. The samples were then allowed to dry at room temperature.

2.2. Formation of nanocomposite Ti–Si–N coatings

Ti–Si–N coatings were fabricated on the surface of Ti6Al4V alloy plates by magnetron co-sputtering. 99.99% pure Ti and Si targets (2 in. diameter and 5 mm thick) targets were placed in the magnetron target holders fixed at an angle of 45° to each other. DC sputtering and RF sputtering for Ti and Si targets were used, respectively, during deposition process. The substrate to target spacing was kept fixed at 5 cm. The base pressure of deposition was less than 4×10^{-6} T while sputtering pressure was 10 mT. The deposition process was carried out in $Ar + N_2$ atmosphere with the ratio of 80:20 (40 sccm Ar: 10 sccm N_2). The targets were pre-sputtered for 15 min prior to deposition on Ti6Al4V substrates. Co-sputtering was performed using 150 W DC of power on Ti target with substrate heating at 500 °C. To study variation in nanocomposite coating properties, the power applied to Si target was varied in the range of 20 W to 120 W RF, resulting in the increase in Si content in coatings while the sputtering time was fixed at 2 h.

2.3. Antibacterial activity

For analysing the antimicrobial properties of the Ti–Si–N coatings, the samples were exposed to *E. coli*. The microorganisms were grown in LB broth (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) at 37 °C. The samples were placed into 10 ml. Falcon tubes, covered with 100 ul overnight grown bacterial incubated by gentle rotation for 24 h at 37 °C. Microorganisms were quantified by evaluating the absorbance in a UV–vis-spectrophotometer (Lasany®, UV–vis double beam spectrophotometer) at 620 nm.

2.4. Cell culture study

The mouse bone marrow mesenchymal stem cells (mBMMSC) were used for all experiments. The cells were cultured in DMEM-low glucose in 75 cm^2 flasks with 10% fetal bovine serum (FBS), and 1% antibiotic at 37 °C in a humidified atmosphere with 5% CO_2. The cells were seeded onto the Ti–Si–N coated Ti6Al4V at an equal density of 5 × 104 cells per well for the cell adhesion and other assays.

2.5. Cell attachment (DAPI staining)

For cell adhesion assay, cells adhered to the surface of samples were stained with 4′, 6-diamidino-2-phenyindole (DAPI, Sigma) after incubation period of 1 and 6 days. Prior to addition of dye, samples were washed with PBS. Images were captured with a fluorescence microscope (Axiovert 25, Germany).

2.6. Cell viability (MTT assay)

The viability of cultured cells on each sample was compared by the MTT colorimetric assay as described by Eirich. For MTT assay, MSCs at a density of 2×10^4 were grown on coated surface of each Ti6Al4V in 24-well plate (Corning, Germany). After 1st, 6th, and 12th days of seeding, the cells grown on each sample were incubated with 0.5 mg/ml of tetrazolium salt solution (MTT), 3-[4,5-dimethylthiozol- 2-yl]-2,5 diphenyltetrazolium bromide (Himedia, India) for 4 h at 37 °C inside CO_2 incubator. Only viable cells have the ability to reduce the yellow water-soluble MTT dye into water-insoluble dark blue crystals of formazan precipitate with the help of an enzyme called mitochondrial succinic dehydrogenase. After incubation of 4 h, medium was removed and 1 ml ethanol-dimethyl sulfoxide (DMSO) (Amresco, Solon, Ohio, USA) solution (1:1) was added to each well containing coated samples to completely solubilise insoluble formazan crystals. Then, absorbance of the solution of each well containing coated sample was measured at 570 nm with spectrophotometer. The blank reference was taken from wells containing no cells, also incubated with the MTT solution.

2.7. Osteogenic differentiation of mesenchymal stem cells (MSCs)

The cells were seeded on Ti–Si–N coated titanium alloy samples until they reached confluence. The medium was then removed and replaced with differentiation medium containing 10% FBS and osteogenic supplements (20 mM ß-glycerophosphate, 20 mM of ascorbic acid, 10^{-7} M dexamethasone, and 1% antibiotic solution. Differentiation medium was replaced every 2nd day. After completion of differentiation period, RNA from the cells was isolated as per standard isolation protocol. Reverse transcription was performed according to manufacturer's instruction (Bangalore genie) and was analyzed for the expression of osteoblast related genes *osteoprotegerin*, *osteopontin*, and *osterix*. The relative expression of these genes was determined by normalizing to ß-actin. PCR conditions used include an initial denaturation at 95 °C for 10 min, followed by a 32 cycle amplification consisting of denaturation at 95 °C for 15 s, and annealing at 55–60 °C for 30 s.

2.8. Statistical analysis

The values of crystallite size, contact angle, anti-microbial activity, and cell viability are expressed as mean ±S.D. The statistical significance was evaluated by ANOVA at 5% level of significance. The statistical package used was origin 6.1 (Origin Lab Corporation, USA).

3. Results and discussion

3.1. Structural analysis

The Ti–Si–N nanocomposite coatings were characterized by using XRD (Bruker AXS, D8 Advance) with CuKα (λ = 1.54 Å) radiation. The grain size of the coatings was estimated from the Scherrer equation, (1). In this equation, the grain size '*t*' is along the surface normal direction, which is also a direction of the diffraction vector.

$$t = \frac{0.9\lambda}{B\cos\theta} \quad (1)$$

where '*B*' is the corrected full width at half maximum (FWHM) of a Bragg peak, 'λ' is the X-ray wavelength, and 'θ' is the Bragg angle.

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