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A novel microwave recipe for an antibiofilm titanium surface

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ABSTRACT

A microwave based method for the surface modification of titanium was demonstrated for biomedical applications. The surfaces were characterized using XRD, HR-SEM and Goniometer. The absence of rutile, anatase and brookite phases and the presence of an amorphous near-native oxide film were confirmed. The microwave oxidized (MWO) surfaces exhibited a significant antibiofilm activity against *Escherichia coli* and *Staphylococcus aureus*. In the presence and absence of the water pot, the oxidation times of 60 and 20 min demonstrated a high antibiofilm property respectively. The surfaces turned more hydrophobic with increasing oxidation time. The viability of L6 cells remained unaffected on the MWO oxidized surfaces, signifying no loss in biocompatibility. This systematic study presents MWO as a promising technique for solving the biofilm problem faced by the otherwise robust titanium.

1. Introduction

Titanium is widely used as an implant material, as well as, an industrial pipe and tubing material because of the specific combination of outstanding properties including: excellent biocompatibility, high strength to weight ratio, good fatigue resistance and superior corrosion resistance [1]. Its excellent biocompatibility not only makes it an ideal candidate as an implant material but also renders the surface prone to bacterial attachment [2].

A biofilm is an assemblage of surface-associated microbial cells that are enclosed in an extracellular polymeric substance (EPS) matrix. The latter accounts for 50 to 90% of the total organic carbon [3,4]. Non-cellular materials including mineral crystals, corrosion particles, clay or silt particles, or blood components are also found within as biofilm matrix, depending on the environment in which it is found [2]. Biofilm formation on titanium based condenser tube leads to the problem of biofouling [5–7], which decreases the heat transfer efficiency and the need for fouling control strategies increasing the cost of energy production in chemical and nuclear industry.

On the other hand when used as a medical implant, biofilm causes severe prosthetic infections [2]. The biofilm generally enables bacteria to escape the host defences and antibiotic attack. Moreover, the increased competence suggested for biofilm-embedded bacteria results in a higher degree of horizontal transfer of genes including antibiotic resistance markers and the occurrence of persistent cells. As a consequence, antibiotic treatment fails in eradicating biofilm-related implant infections, leading to debridement (surgical removal of infected tissues) and in most cases, premature replacement of the entire implant [8]. So far no conclusive method or technique has been evolved, which would effectively control biofilm formation.

Researchers have been diverting their focus towards biofilm mitigation measures, through surface modification strategies in order to make the materials refractory [9–11]. Coating the surface with antibiotics and antibacterial compounds is popular, but another approach which is more promising is the construction of biofilm-resistant materials by nanostructuring the surface [12,13]. Coatings may have limited life or could lead to antibiotic resistant strains.

Vinita et al. have reported the use of a copper–nickel bilayer [14] and copper coatings [15] on titanium surfaces for the inhibition of bacterial fouling. Statz et al. [16] have demonstrated the marine antifouling and fouling-release performance of titanium surfaces coated with a bio-inspired polymer. Investigators [17] have also studied the effects of repeated pickling and polishing of titanium surfaces leading to reduction in their microroughness resulting in a significant decrease in biofouling. Few other authors have also developed antibiofilm surfaces through surface modifications such as: chitosan coated surfaces [18], zwitterionic surfaces [19] and quaternized chitosan loaded PMMA [20,21].

In the current work, efforts are made to use a rapid, cost-effective, microwave based method for modifying the titanium surface, to exhibit an antibiofilm property. The use of a microwave irradiation based surface modification on titanium for repelling bacterial adhesion is

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novel and not reported previously. The oxide film formed by this method is characterized and its biofilm control property investigated with the help of two pathogenic biofilm forming bacteria. The uniqueness in this approach is that it is a stand-alone technique which can provide antibiofilm properties without the need for light irradiation or any other external factors.

2. Materials and methods

2.1. Preparation of antibiofilm titanium substrates

Commercially (Titanium Tantalum Products, Chennai, India) pure titanium (CP Grade 2) grade-2 coupons (10 mm × 10 mm) were pickled in an acid bath containing nitric acid (400 g/L) and hydrofluoric acid (40 g/L) for 5 min and then ultrasonically (PCi Analytics, 3.5L100H) cleaned using a soap solution to remove the remnant acids from their surfaces [22]. The chips were washed in running water, then finally rinsed in distilled water and air dried. All chemicals used in the study are of analytical grade (purchased from Merck, Himedia and Sigma, India). Millipore water was used for all media preparations. Fig. S1 shows the schematic of the workflow in the current paper.

2.1.1. Surface engineering of titanium by microwave oxidation

The freshly cleaned titanium substrates were subjected to microwave oxidation (IFB 25PG1S, power source — 230 V–50Hz, power consumption 1400 W, MWO output power 900 W, heater output power 1000 W and microwave frequency 2450 MHz), in the presence and absence of water, at varying time intervals. The energy released by the microwave was calculated (using $E = h\upsilon$ (where E is the energy, h is the Plank constant and υ is the frequency) to be $6.634 \times 10^{-34} \times 2450 \times 10^{6} = 16{,}253.3 \times 10^{-28}$ J. The energy (E)/test surface was calculated to be $16{,}253.3 \times 10^{-28}/10^{-4} = 16{,}253.3 \times 10^{-24}$ J/m^2 and the total microwave energy in eV was found to be $16{,}253.3 \times 10^{-24}/1.6 \times 10^{-19} = 0.101$ eV/m^2.

In the case of water pot mediated microwave oxidation (WPM-MWO) a beaker containing 400 mL of distilled water was placed in the centre of the glass turntable and the titanium substrates were kept surrounding this water pot. MWO oxidation was conducted at 5 min, 10 min, 20 min, 30 min and 60 min time intervals. Microwave oxidation (in absentia water pot microwave oxidation (IAWP-MWO) was also conducted in the absence of water for 5, 10 and 20 min. Then, the samples were air cooled, ultrasonically cleaned in ethanol for 15 min and then stored for further studies. Samples without microwave oxidation were maintained as control.

2.2. Characterization of test surfaces

These surfaces were characterized using Diffrac Plus XRD (Bruker Discover D8 diffractometer), HR-SEM (EI Quanta FEG 200 — High Resolution Scanning Electron Microscope) and Goniometer (KRUSS FM41, Easy Drop (30 W), GmbH Germany).

3. Evaluation of biofilm-phobic properties

Staphylococcus aureus (NCIM 5021) and *Escherichia coli* (NCIM 2931) bacterial cultures were purchased from the National Collection of Industrial Microorganisms (NCIM), Pune, India and used for these studies. One millilitre of either of the organism containing approximately 2.25×10^9 and 1.45×10^9 cfu/mL of *S. aureus* and *E. coli* cells was inoculated into 20 mL of sterile nutrient broth taken in a 250 mL conical flask and incubated at 37 °C in an orbital shaker cum incubator (ORBITEK, Scigenics Biotech, India) at 110 rpm for 6 h. This culture broth was then dispensed (6 mL) into 15 mL falcon tubes and one metal coupon each was suspended in each tube. The tubes were held upright in a 500 mL beaker and the entire set-up was placed at 37 °C in an orbital shaker cum incubator at 80 rpm for 48 h.

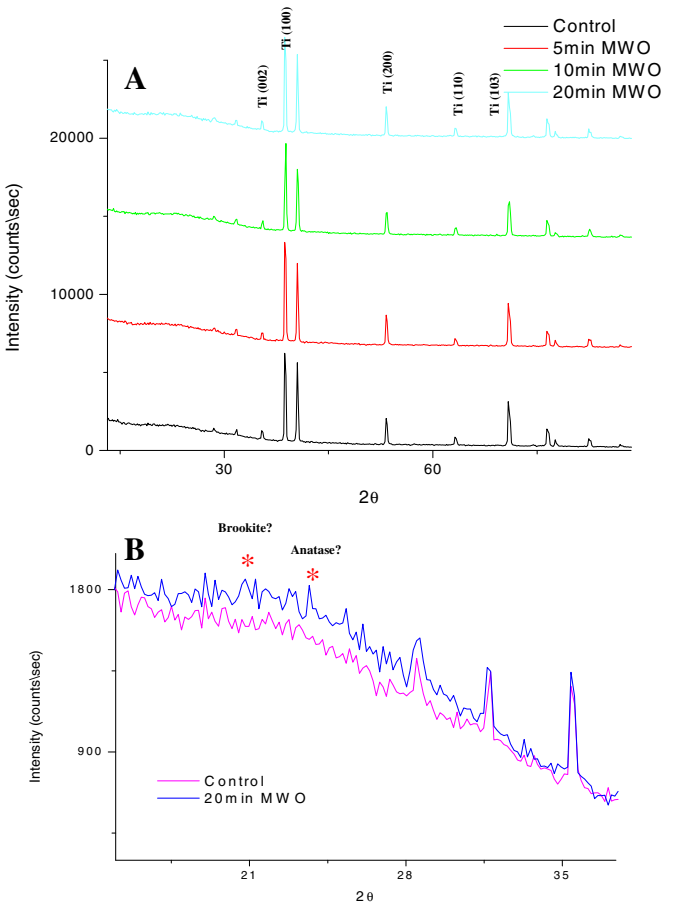

Fig. 1. XRD patterns of in absentia water pot MWO titanium substrates (A) prepared at varying time intervals. (B) Enlarged view showing changes (peaks marked with the symbol *) in the 20–40° region of the 20 min MWO substrate.

3.1. Biofilm quantification — total viable count

The samples were removed from the medium and gently washed (by immersing them using sterile forceps in a beaker of sterile water) to dislodge the loosely adhering cells into 1 mL sterile phosphate buffer (0.0425 g KH_2PO_4, 0.19 g $MgCl_2$ per litre) by ultrasonication for 10 min[5]. The biofilm suspension was serially diluted and 10 μL of each dilution was plated onto a nutrient agar. Then they were incubated for 24 h at 32 °C and the number of colonies was counted. Mean total viable counts were calculated from three coupons and the results were expressed as colony forming units (cfu/mL).

3.2. Biofilm quantification — epifluorescence studies

Another set of the test surfaces after exposure to bacterial culture were observed under a fluorescent microscope to determine the biofilm formation on the titanium surfaces. After gently washing with sterile water and air in a sterile chamber, they were flooded with acridine orange (0.1% solution in distilled water). After 2 min, the excess stain was drained off, washed in sterile water, dried and observed under a Leica CTR 5000 (Leica Microsystems, Chennai, India) inverted epifluorescence microscope. Acridine orange, a fluorescent dye, differentially stains single stranded RNA and double stranded DNA, fluorescing orange when intercalated with the former and green while complexing with the latter. Thus, the number of orange fluorescing cells depicts the actively metabolizing cells on the surface [23]. Photographs were taken with a Leica CTR 5000 digital microscope equipped