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Bactericidal nanospike surfaces via thermal oxidation of Ti alloy substrates

Terje Sjöström, Angela H Nobbs, Bo Su *

School of Oral and Dental Sciences, University of Bristol, Lower Maudlin Street BS1 2LY, UK

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ABSTRACT

With the aim to fabricate bio-inspired antibacterial nanotopography surfaces, nanospikes with varying dimensions were grown on Ti alloy surfaces using a thermal oxidation method. By controlling the acetone vapour concentration inside the tube furnace, the resulting oxide surface changed from nanocolumn shapes to nanospikes with approximately 20 nm diameters. The nanospikee growth was demonstrated to work on 3D Ti alloy bead surfaces, which means translation of the method to implant surfaces would be possible. Microbiology studies using *Escherichia coli*. showed that the nanospikes on the Ti alloy surfaces has potential to reduce bacterial viability. More dead bacteria were present on the nanospike surfaces compared to a smooth control and a 40% reduction of viability was noted in bacterial suspensions incubated with a nanospike surface. It was shown that by annealing the Ti alloy surfaces prior to thermal oxidation, it is possible to grow vertically aligned nanospikes. This could be highly valuable when designing implant surfaces with antimicrobial properties.

1. Introduction

Biofilm formation on surgical implants and the resulting infection is a major challenge in the biomaterials and medical devices field. Infection can result in traumatic and costly revision surgery [1–3], and the prevention of such infection, without impeding the function of the implant, is of high importance. Antimicrobial Ti and Ti alloy implant surfaces have been achieved with binding of antibiotics [4], antimicrobial peptides [3] or nanoparticles that have bactericidal effects [1], to the surface of the implant. A more favourable method of preventing Ti implant infection that could help combat the use of antimicrobials and thus the risk of driving development of antimicrobial resistance, is to use surface topography that actively discourages bacterial adhesion or has inherent antimicrobial properties, without the addition of antibiotics or other chemicals.

It is thought that nanostructures can cause damage to bacteria by piercing the cell membrane [5,6]. Recently, Ivanova et al. [7–9] have demonstrated that nanopillar features on cicada wing surfaces have a bactericidal effect, which prevents biofilm formation by physically damaging bacterial cell walls. This effect was reproduced with similar topographies on both black silicon [8] and PMMA [10]. To achieve antimicrobial nanopillar surfaces on Ti and Ti alloy implants, a fabrication technique is needed that can mimic the dimensions of such surface features found in nature, ideally on arbitrary shaped and porous materials. To achieve nanostructures on Ti–6Al–4V (Ti64) surfaces with dimensions similar to those on the cicada wing, this study utilises a thermal oxidation technique that allows for tuning of the dimensions of the resulting nanospikes.

2. Materials and methods

2.1. Thermal oxidation

Ti64 samples were placed in the centre of a horizontal alumina tube furnace (1500 mm long, 95 mm inner diameter). After purging the tube with Ar the temperature was increased to 850 °C at 15 °C/min. After reaching 850 °C the Ar flow was diverted through a bubbler bottle containing acetone at 25 °C with the Ar flow rate adjusted to a rate in the range of 50–300 sccm. The temperature was kept at 850 °C for 45 min, after which the tube was allowed to cool under a flow of Ar at 500 sccm. To remove the carbon from the as-synthesised nanospikes, the samples heated to 600 °C at a rate of 10 °C/min. An X'pert Philips Diffractometer with a CuK_α source and step size 0.02° was used for the X-ray diffraction (XRD) analyses. A JEOL JSM 5600LV scanning electron microscope (SEM) was used to image the oxidised Ti64 surfaces.

* Corresponding author.
E-mail address: b.su@bristol.ac.uk (B. Su).

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2.2. Bacterial growth conditions

E. coli (K12) was inoculated into Tryptic Soy Broth (TSB) (Oxoid) and grown at 37 °C with agitation (220 rpm) for 16 h. Cultures were diluted to $OD_{600}=0.1$ in fresh TSB and further incubated until mid-exponential phase was reached. Bacteria were then harvested (5000 g, 7 min) and the cell pellet was re-suspended in 5 ml TSB. This procedure was repeated twice and the cell suspension was then adjusted to $OD_{600}=0.3$ for all experiments.

2.3. LIVE/DEAD staining

Aliquots (0.5 ml) of bacterial suspension were added to individual wells of a 24-well plate, each containing a 10 mm square Ti64 sample. Plates were then incubated for 2 h at 37 °C in 5% CO_2. LIVE/DEAD Baclight L7007 component A (Invitrogen) was added to each well (3 μl/ml) and plates incubated in the dark for 15 min. Suspensions were then aspirated and the Ti64 samples gently rinsed with 10 mM Tris–HCl buffer at pH 7 (Sigma) to remove excess stain and non-adherent bacteria. Samples were then visualised by fluorescence microscopy (Leica DMLB).

2.4. Viability assay

Ti64 samples were incubated with bacteria as above for 2 h. Aliquots (90 μl) of bacterial suspension were transferred from each plate to individual wells of a 96-well microtitre plate and 10 μl PrestoBlue (Invitrogen) was added. Plates were incubated for an additional 30 min, and the fluorescence intensity of each well was then measured using a Tecan plate reader with excitation filter at 560 nm and emission filter at 590 nm.

3. Results and discussion

3.1. Fabrication of nanospikes

The SEM images in Fig. 1 shows the synthesised nanospikes after removal of the outer carbon shell. The as-synthesised nanospikes consist of a TiC core with a C shell [11], with the C shell removed and the TiC core converted to TiO_2 when heated in an atmospheric condition [12]. The nanospikes became thinner with increased acetone vapour in the tube furnace. By tuning the flow rate through the acetone bubbler, and thereby increasing the amount of acetone vapour in the tube furnace, the geometry of the nanospikes could be tuned from column-shaped structures to very thin wires with diameters in the region of 20 nm. With an increased carbon layer on top of the TiC, diffusion of Ti species from the substrate is reduced and therefore the growth of the inner TiC core is reduced [13], ultimately resulting in thinner spikes after removal of the carbon layer.

The XRD results in Fig. 2 shows high intensity peaks for the Ti–6Al–4V substrate. These peaks correspond to the α-phase of the alloy which is expected since annealing took place below the α–β transition temperature. The as-processed sample shows TiC peaks, in agreement with previous studies which have used acetone vapour to fabricate nanowires [13]. The TiC peaks were not present for the sample heat treated at 600 °C in air, suggesting that the carbon was successfully removed from the surface and the nanowires converted to oxide. Both the samples showed rutile TiO_2 peaks, with stronger intensity after annealing at 600 °C in air. There was also a weak anatase peak, suggesting the nanowire consists of a mixture of rutile and anatase.

Apart from controlling the diameters of the TiO_2 nanospikes, we further attempted to grow vertically aligned nanospikes on the Ti64 substrates. After pre-annealing under vacuum at 1100 °C the nanospikes were aligned in small bundles (Fig. 3a). When the annealing temperature was increased to 1200 °C the nanospike

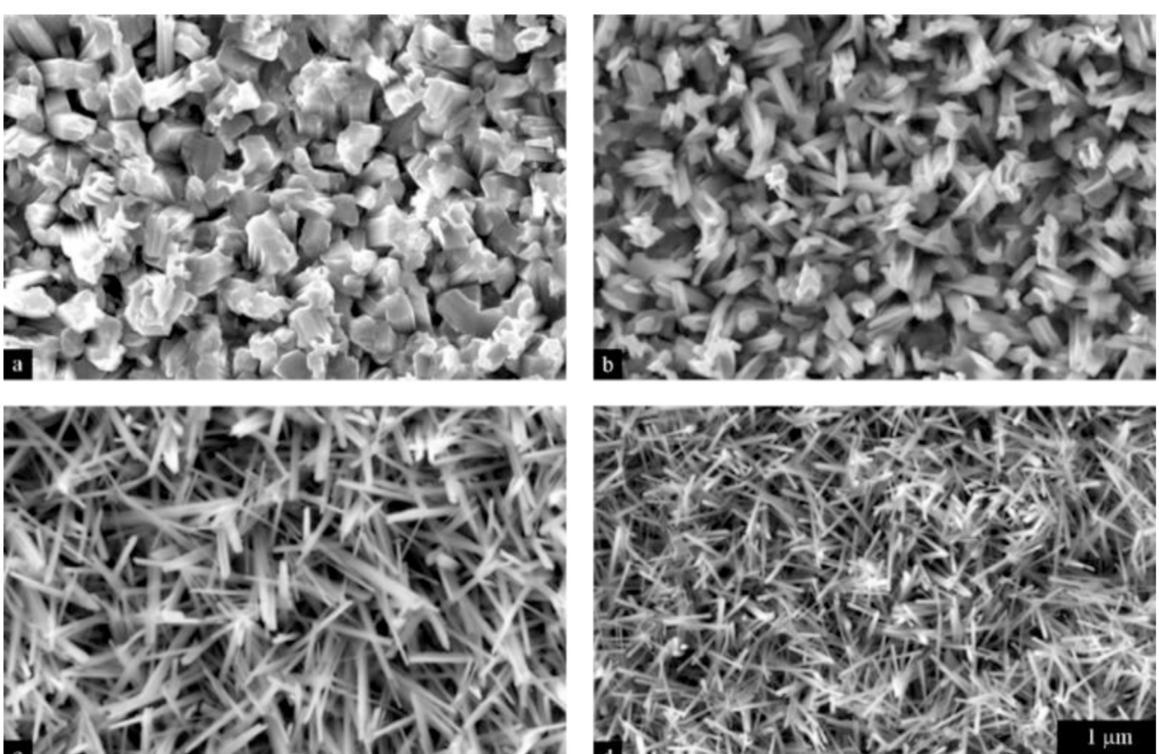

Fig. 1. SEM images of nanospikes on Ti64 substrates after thermal oxidation at various Ar flow rates through the acetone bubbler. (a) 50 sccm, (b) 100 sccm, (c) 200 sccm and (d) 300 sccm. All surfaces were imaged after removal of carbon at 600 °C.