Materials Science and Engineering C 35 (2014) 100–105

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Titania nanotubes with adjustable dimensions for drug reservoir sites and enhanced cell adhesion

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ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form 4 October 2013
Accepted 29 October 2013
Available online 9 November 2013

Keywords:
Anodic oxidation
Implant infection
Titania nanotube
Gentamicin
Drug eluting implant
Drug release

ABSTRACT

This study aims to generate a bactericidal agent releasing surface via nanotube layer on titanium metal and to investigate how aspect ratio of nanotubes affects drug elution time and cell proliferation. Titania nanotube layers were generated on metal surfaces by anodic oxidation at various voltage and time parameters. Gentamicin loading was carried out via simple pipetting and the samples were tested against *S. aureus* for the efficacy of the applied modification. Drug releasing time and cell proliferation were also tested in vitro. Titania nanotube layers with varying diameters and lengths were prepared after anodization and anodizing duration was found as the most effective parameter for amount of loaded drug and drug releasing time. Drug elution lasted up to 4 days after anodizing for 80 min of the samples, whereas release completed in 24 h when the samples were anodized for 20 min. All processed samples had bactericidal properties against *S. aureus* organism except unmodified titanium, which was also subjected to drug incorporation step. The anodization also enhanced water wettability and cell adhesion results. Anodic oxidation is an effective surface modification to enhance tissue–implant interactions and also resultant titania layer can act as a drug reservoir for the release of bactericidal agents. The use of implants as local drug eluting devices is promising but further in vivo testing is required.

1. Introduction

Titanium metal and its alloys are considerably biocompatible and have favourable mechanical properties to be used widely in orthopaedic and dental implants [1–3]. However, one of the common disadvantages of titanium is to be non-bioactive for rapid osseointegration and occasionally this feature may led to generate a fibrous capsule around implant itself. The fibrous tissue may further cause implant loosening and finally a failure in the implantation process [4,5].

Anodic oxidation is a well-known procedure to generate a controllable oxide layer on valve metals for the prevention of surface against corrosion and wear. In the past decade, this process attracted much more attention in biomaterial studies thanks to adding a bioactive surface character to titanium and titanium alloys [6–8]. Enhanced apatite formation, increased surface area for adhesion proteins and better integration with osteoblast cells have become primary advantages of anodized titanium surfaces [7–9].

Another complication that may be encountered after the implant operation is infection [10–14]. The infection rate is not very common due to strict aseptic conditions and prophylactic antibiotic therapies, however the results severely affect the implant and surrounding tissues, patient comfort etc. In biofilm forming phenomenon bacteria can build a thick, dense layer on the infection site, which makes the organism much more resistant to conventional antibiotics and invulnerable [11,15]. Preventing the bacterial adhesion in the very beginning of the infection process and also establishing a local resistant area is crucial after the implantation because of the aforesaid situations.

The more recent approach in localized antibiotic release from implants is polymer free techniques, which is possible via titanium oxide nanotubes [16–18]. In this approach a surface layer consisting cylindrical titanium oxide nanotube cavities can be used as drug reservoir. The diameter and length of the nanotubes can be adjusted by anodic oxidation process parameters, which gives an option to have various dimensions [19,20].

In this study, we anodized the commercially pure titanium metal with 0.15 M NH_4F in ethylene glycol solution. A two set of sample groups were prepared first of which are same in length but having different diameters, and a second set of sample which have a surface layer of nanotubes with the same diameter but with different lengths. Gentamicin, a widely used antibiotic against Gram negative organisms was incorporated into nanotubes and the release and bactericidal behaviours were investigated. The effect of nanotube diameter on cell attachment and proliferation was also tested in vitro.

2. Materials and methods

2.1. Preparation of titanium samples

Commercially pure titanium foils (99.7% purity 0.127 mm thickness, Alfa Aesar, USA) were cut into 10 mm × 20 mm dimensions and cleaned with pure ethanol and deionized (DI) water, respectively in

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http://dx.doi.org/10.1016/j.msec.2013.10.033

an ultrasonic agitator. Later on the samples were immersed for 5 min into an aqueous etching solution consisting of 5 M HNO_3 and a few drops of 48% HF for the removal of natural protective oxide layer. After etching procedure samples were washed with DI water and dried in vacuum oven at 70 °C.

2.2. Anodic oxidation process

Anodic oxidation process was performed in a two electrode electrochemical cell where Ti sample was used as anode and a platinum mesh (99% purity, Alfa Aesar, USA) as cathode. The electrodes were kept parallel position with a 4 cm distance. 0.15 M NH_4F in ethylene glycol solution was used as electrolyte and 20, 40 and 60 V of electrical potentials were applied during 20, 40 and 80 min of various anodization periods.

2.3. Analyses of titanium samples

Titanium samples were subjected to analyse morphologically before and after the anodic oxidation process by scanning electron microscopy (SEM, JSM-6700F Japan). The wettability of the surfaces were also tested with goniometer (OCA20, Dataphysics, Germany) in a process of which 2 μL of deionized water droplets randomly analysed through the software ($n = 8$).

2.4. Cell culture tests

Titanium samples anodized at 20, 40 and 60 V potentials for 20 min were subjected for in vitro cell culture studies, since the cells interact with the surface, only nanotube diameter was chosen as a parameter for testing.

As a model of osteoblast cell behaviour, human osteosarcoma cell line (Saos-2/An1, Foot and Mouth Disease Institute, Ankara, Turkey) was used for the biocompatibility of the surfaces. Cells were cultured at 37 °C temperature and 5% CO_2 environment in 25-cm^2 flasks with Dulbecco's modified Eagle's medium (Sigma-Aldrich Co) supplemented with 10% foetal bovine serum (Gibco BRL, Grand Island, NY, USA), 1 mM L-glutamine, penicillin (20,000 U/mL), and streptomycin (20,000 mg/mL) prior to in vitro evaluations. After becoming confluent, cells were harvested and 50 μL of cell suspension (1×10^5 cells/mL) was added onto the autoclaved titanium samples and incubation started. Relative cell proliferation on samples was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the 1st, 3rd, 5th and the 7th days of culture. In a typical MTT assay, samples were washed with pH 7.2 phosphate buffer saline (PBS) and 200 μL fresh culture media, which includes 20 μL MTT reagent solution (5 mg/mL PBS), was pipetted. After 4 h of incubation, medium was removed and 200 μL of isopropanol.HCl (0.04 M) mixture was added to dissolve formed purple formazan crystals. This liquid was read in ELISA reader (ASYS Biochrome, UK) at 570 nm wavelength for the evaluation of relative cell proliferation.

In the meantime, on the 5th day of culture, cells were fixed with 4% paraformaldehyde solution and evaluated under SEM for the morphological characterization.

2.5. Drug incorporation and release

100 μL of gentamicin sulphate (Santa Cruz, USA) at 5 mg/mL concentration was pipetted onto 1 × 1 cm cut titanium samples. The titanium sample groups in this test subjected to 20, 40 and 80 min of anodic oxidation at 20, 40 and 60 V of potential in order to investigate both nanotube diameter and length. Samples were dried at 37 °C in vacuum oven and for the removal of excess drug, the surfaces were cleaned with a piece of paper tissue and specimens were kept at +4 °C.

The quantification of the incorporated drug was carried out colorimetrically as previously described in literature [16] and PBS (pH 7.2) at 37 °C was used as release media. The specimens were completely immersed in release media and left in an orbital shaker at 30 rpm. In a typical procedure, the whole release media (500 μL) was collected and mixed with equal amounts of complexing solution consisting of 2.5 g o-phthalaldehyde, 62.5 mL methanol and 3 mL 2-mercaptoethanol in 560 mL of 0.04 M sodium borate. This reagent was freshly prepared and kept in dark at room temperature before the analyses. The determination of gentamicin amount was performed by measuring the absorbance at 332 nm wavelength and calculating with standard calibration curve.

2.6. Antibacterial efficacy evaluations

Drug incorporated metal samples were subjected to disc diffusion test method for the inhibition of the colony formation of bacteria species. In this study, gentamicin release from samples was investigated against *S. aureus*. In the procedure Müller Hinton agar (Merck, Germany) was dissolved in distilled water with a concentration of 34 g/L and autoclaved at 115 °C for 10 min and then spread on petri dishes for the solidification. Meanwhile, bacteria were grafted into 3% (w/v) Todd Hewitt (Merck, Germany) media and incubated overnight. Bacteria suspension was diluted to 0.5 MacFarland concentration with sterile PBS and well spread onto agar containing petri dishes. Drug loaded titanium samples were placed on petri dishes and incubated overnight and in the following day, inhibition zones occurred on petri dishes that were measured.

3. Results and discussion

3.1. Surface characterization of samples

An organized titania oxide layer emerges in on the surface of the titanium metals after the anodization process. The titania layer consists of ingrowing nanotubular shaped entities and the diameter of the titania nanotubes was found to be increasing with ascending voltage. The average diameters of the nanotubes (mean ± SD, $n = 8$) were 31 ± 5 nm, 56 ± 9 nm and 84 ± 8 nm for the anodization voltages of 20 V, 40 V and 60 V, respectively. Anodic oxidation process alters the wettability character of the metal dramatically, generating a hydrophilic surface whereas the unmodified titanium is fairly hydrophobic ($\Theta = 81.2°$). Surface wettability also increased with ascending applied voltage during anodization and the water contact angle values (Θ) was found to be 56.2°, 42.4° and 21.7° for 20 V, 40 V and 60 V, respectively. The representative SEM images of the titanium and anodized titanium and the water droplet thumbnails were schematized in Fig. 1.

A thin titania layer consisting of nanotube shaped entities successfully formed on metal surfaces after anodic oxidation. The nanotube diameter was found to be increasing with elevated anodization potential as previously reported in the literature [20,21]. This effect can be explained by more oxide dissolution which is caused by improved electrolyte conductivity at higher electric potentials. Use of high voltage resulted larger pore sizes by enhancing field-assisted dissolution [22]. The use of nanotube layers as drug eluting platforms is a quite new approach for localized delivery in recent years [18,23] and the use of ceramic or polymer coatings as barrier layer for controlling the release has become promising for in vitro studies [24,25]. This is an effective strategy to overcome systemic toxicity and low efficacy issues, however the coatings are often open to ruptures due to mechanic dynamics in body fluid and/or tissues.

Wettability studies showed that the water contact angle decreases dramatically after anodic oxidation and the downtrend continues with increased voltage. The increase in water wettability is in parallel manner with the diameters, but also the contribution of chemical components must be considered. If we take account of the reaction during the anodization process we can see that the product is $Ti(OH)_4$, a hydroxide containing compound, which interacts with water via hydrogen bonding. As the solution rate increases with the voltage, more $Ti(OH)_4$ containing