Progress in Organic Coatings 111 (2017) 23–28

Contents lists available at ScienceDirect

Progress in Organic Coatings

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

Chitosan patterning on titanium implants

Eduardo Gilabert-Chirivella[a], Ricardo Pérez-Feito[b], Clarisse Ribeiro[c], Sylvie Ribeiro[c], Daniela M. Correia[c,d], Maria Luisa González-Martín[e,f], José María Manero[g,h], Senentxu Lanceros-Méndez[c], Gloria Gallego Ferrer[a,f,*], José Luis Gómez-Ribelles[a,f,*]

[a] Centre for Biomaterials and Tissue Engineering (CBIT), Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[b] Applied Thermodynamics Department, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[c] Centro/Departamento de Física, Universidade do Minho, Campus de Gualtar, 4710-057, Braga, Portugal
[d] Centro/Departamento de Química, Universidade do Minho, Campus de Gualtar, 4710-057, Braga, Portugal
[e] Department of Applied Physics, University of Extremadura, Avda de Elvas s/n, 06006, Badajoz, Spain
[f] Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain
[g] Biomaterials, Biomechanics and Tissue Engineering Group, Department of Materials Science and Metallurgical Engineering (UPC), ETSEIB, Av. Diagonal 647, 08028, Barcelona, Spain
[h] Centre for Research in NanoEngineering (CRNE) (UPC), C/Pascual i Vila 15, 08028, Barcelona, Spain

ARTICLE INFO

Keywords:
Titanium
Microgrooves
Chitosan
Myoblasts
Pre-osteoblasts
Cell adhesion

ABSTRACT

Titanium and its alloys are widely used in medical implants because of their excellent properties. However, bacterial infection is a frequent cause of titanium-based implant failure and also compromises its osseointegration. In this study, we report a new simple method of providing titanium surfaces with antibacterial properties by alternating antibacterial chitosan domains with titanium domains in the micrometric scale. Surface microgrooves were etched on pure titanium disks at intervals of 60 μm using a modified 3D printer and were then coated with chitosan antibacterial polysaccharide. The dimensions of the patterned microgrooves made it possible to fix the chitosan domains to the titanium substrate without the need for covalent bonding. These domains were stable after 5 days of immersion in water and reduced the surface contact angle. Preliminary cell adhesion assays demonstrated that MC3T3-E1 pre-osteoblasts preferentially adhered to the titanium regions, while C2C12 myoblasts were uniformly distributed over the whole surface.

1. Introduction

Titanium and titanium alloys are widely used in medical devices such as dental and orthopedic implants [1] due to their excellent properties, including: chemical stability [2], low Young's modulus [3], low thermal conductivity [4] and biocompatibility [5].

However, despite the superior properties of this material, implant failure may still occur, very often due to bacterial infection [6]. For instance, in a 15-year study Phillips et al. [7] reported that 0.57% of patients with a hip replacement and 0.86% with a knee replacement developed a deep infection. In fact, many studies report the appearance of an implant infection in approximately 0.3-4% of cases [7–10].

Even though the levels of success are relatively high, unsuccessful cases involve a large number of patients, as the percentage of the population given implant surgery increases every year [9]. For example, in the UK, a total of around 40,000 hip and knee replacements are performed annually [7], while in the US in 2006 approximately 800,000 of these procedures were carried out. The economic impact of surgical site infections is quite high as the average length of hospital stays after orthopedic surgery is prolonged with an increased cost per stay of about $15,500 per patient in the US [11].

Several strategies have been proposed to modify the titanium surface to inhibit bacterial infections. Some of these include: adding inorganic molecules capable of killing bacteria to the implant surface, e.g. silver [6] or zinc [12] particles, which prevent the formation of bacterial biofilm by means of cross-linked albumin coatings [13]; using the photocatalytic properties of titanium to create anti-bacterial surfaces [14] or covalent bonding of antibiotics to surfaces [15].

Also of interest is coating the titanium surface with organic, antibacterial materials such as chitosan [16], widely used in the food [17], textile [18] and medical industries [19,20]. One of the reasons for its many uses is its antibacterial properties, which act through different factors including: microorganism species, molecular weight, chelating capacity, charge density and degree of deacetylation, pH and tempera-

* Corresponding authors at: Centre for Biomaterials and Tissue Engineering (CBIT), Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain.
E-mail addresses: ggallego@ter.upv.es (G.G. Ferrer), jlgomez@ter.upv.es (J.L. Gómez-Ribelles).

http://dx.doi.org/10.1016/j.porgcoat.2017.04.027
Received 6 June 2016; Received in revised form 24 February 2017; Accepted 19 April 2017

ture. Although several theories have been proposed to explain chitosan's antibacterial mode of action, this is not yet clear [21]. Nevertheless, the most widely accepted view attributes the electrostatic alterations to the amine groups present in chitosan's structure, which give it cationic characteristics. This means that when chitosan comes into contact with bacterial cell walls, these suffer changes, mainly in their permeability, that cause osmotic imbalances which give rise to the leakage of intracellular substances (such as glucose, proteins and ions), making it impossible for the bacteria to carry out their normal biological processes and causing their death [22–25].

In principle, the ideal implant should fulfill the following criteria [26]: biocompatibility, anti-infective efficiency, uncompromised fixation properties, durable anti-infective effect, good mechanical properties and stability of the antibacterial coating.

Many attempts have been made to functionalize titanium with chitosan, via: silanization [27], deposition of carboxymethyl chitosan [9], by a chitosan/hydroxyapatite composite [28], with a chitosan-lauric acid conjugate [29] or via electrodeposition [30]. These techniques involve coating the entire surface with chitosan, but as far as we know nobody has attempted to functionalize titanium surfaces with chitosan in micrometric domains. The aim of this study was therefore to develop new implant surfaces with improved antibacterial properties by accumulating chitosan in microgrooves etched into the titanium surface. The stability of the chitosan in the sample immersed in aqueous media was assessed and surface wettability (surface energy) was also explored, as it is a key factor in the biological interaction of materials. This preliminary study is an initial approach to determining how well cells adhere to this new type of surface.

2. Materials and methods

2.1. Preparation of titanium surfaces with micrometric chitosan domains

The method we developed to obtain titanium surfaces with alternated antibacterial chitosan domains consisted of two steps: the first consisted of etching parallel microgrooves on smooth titanium surfaces, and the second of filling the grooves with chitosan.

Pure cp-titanium disks 10 mm in diameter were obtained from Technalloy (Sant Cugat del Valles; Spain). According to the supplier, this material contains a maximum of 0.03% nitrogen, 0.3% iron and 0.25% oxygen. They were smoothed up to a surface roughness (R_a) of under 40 nm. Once polished, the samples were cleaned with isopropanol, ethanol, water and acetone by sonication. Parallel microgrooves were etched at intervals of 60 μm by means of a novel method. For this, a G11 scalpel blade was coupled to a 3D-printer and was set to etch parallel microgrooves on the surface of the titanium samples with the required separation through the Python programming language. The blade feed rate was set at 60 mm/min, as this speed provided the cleanest grooves. A circular area of 6 mm in diameter was grooved in three replicates of the titanium samples.

The samples were then placed under a nitrogen flow to remove any shavings. Next, the surface of the samples was gently rubbed with P2000 sandpaper to remove the burrs left behind after the mechanical process. The final cleaning consisted of washing the samples three times for ten minutes in an ultrasonic bath with distilled water, ethanol, and distilled water, respectively. The samples where then allowed to dry at room temperature.

The grooves made in the titanium surfaces were then filled with chitosan. For this, 3% w/v practical grade chitosan solution (Chitosan from crab shells. Practical grade. CAS 9012-76-4 with a deacetylation degree of around 85%, and viscosity average molecular weight 1.5×10^6 g/mol [31]) was prepared using dilute acetic acid (3% w/v). Using a 000 nylon brush, a very small droplet (1–2 μl) of the chitosan solution was spread on the grooved titanium surface. The excess chitosan was removed from the surface by wiping 10 times in the direction of the grooves using a nylon cloth (90 μm pore size). The samples were then submerged for three hours in a 1 M sodium hydroxide solution to neutralize protonated amines from the chitosan. Finally, the samples were washed in distilled water until neutral pH (≈7) was achieved and left to dry in a vacuum oven overnight. The samples with and without chitosan were identified as functionalized (F) and non functionalized (NF).

2.2. Microscopy

Different microscopy techniques were used to determine the actual separation and depth of the grooves. Optical microscopy was performed by an Olympus Lext OLS 3100 instrument. Scanning confocal laser microscopy by Nikon C1 microscope was used to obtain a cross-sectional view of the grooved surfaces. The stripe-like pattern of the chitosan inside the grooves of the titanium was observed with a fluorescence microscope (Nikon Eclipse 80i microscope) at an excitation of 488 nm. Field emission scanning electron microscopy (FESEM − FIB 6 Auriga Compact) was carried out to confirm the presence of chitosan inside the microgrooves of the titanium samples. Prior to observation, the samples were freeze-dried and sputter coated with platinum to ensure the conductivity of the chitosan functionalized surfaces.

To calculate the separation between the grooves, three images were taken by confocal microscopy in different zones; the distance between the grooves was calculated measuring the distance from the middle of a valley to the middle of the following valley and the mean value was calculated. The width of the microgrooves was obtained by measuring the horizontal distance between the walls halfway between the highest point of the adjacent ridges and the deepest point of the valleys. The depth of the grooves was also measured. The results are expressed as the mean ± standard deviation.

2.3. Water contact angle

Contact angle measurements were performed at room temperature in a Data Physics OCA 20 set-up and SCA20 software. Droplets of 2 μl of ultra pure water were dropped onto the dry surfaces. A total of three measurements were taken and an average value was used. Water contact angle was determined for functionalized (F) and non functionalized (NF) samples and was measured parallel (//) and perpendicular (⊦) to the direction of the microgrooves at t = 0 min and t = 5 min. Values were analyzed for t = 5 min, as it was considered that after this time the drop had stabilized.

2.4. Stability of chitosan in the grooves of titanium surfaces

The stability of the chitosan inside the titanium microgrooves immersed in water was analyzed to make sure that the chitosan would not come out of the grooves during cell culture. A chitosan-functionalized titanium sample was immersed in distilled water for five days. The weight of the sample was checked before and after the test (with the sample always dry). Fluorescent microscopy was also used to determine the presence of chitosan once the experiment had concluded.

2.5. Cell culture

To sterilize the supports before cell culture, functionalized and non-functionalized circular titanium samples with chitosan lines were exposed to ultraviolet (UV) light for 1 h each side and washed 3 times in a phosphate buffer saline (PBS) solution for 5 min each wash.

Two different cell lines were used: mouse C2C12 myoblast and mouse MC3T3-E1 pre-osteoblast. C2C12 and MC3T3-E1 cells were grown in 75 cm^2 cell-culture flask and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 4.5 g L^{-1} and 1 g L^{-1}, respectively, supplemented both with 10% Fetal Bovine Serum (FBS, Biochrom) and 1% Penicillin-Streptomycin (P/S, Biochrom). The