Applied Surface Science 330 (2015) 339–350

Contents lists available at ScienceDirect

Applied Surface Science

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

Two approaches to form antibacterial surface: Doping with bactericidal element and drug loading

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ARTICLE INFO

Article history:
Received 25 August 2014
Received in revised form
17 November 2014
Accepted 20 December 2014
Available online 27 December 2014

Keywords:
Bioactive nanostructured films
Antibacterial activity
Cell proliferation
Drug release

ABSTRACT

Two approaches (surface doping with bactericidal element and loading of antibiotic into specially formed surface microcontainers) to the fabrication of antibacterial yet biocompatible and bioactive surfaces are described. A network structure with square-shaped blind pores of $2.6 \pm 0.6 \times 10^{-3}$ mm^3 for drug loading was obtained by selective laser sintering (SLS). The SLS-fabricated samples were loaded with 0.03, 0.3, 2.4, and 4 mg/cm^2 of co-amoxiclav (amoxicillin and clavulanic acid). Ag-doped TiCaPCON films with 0.4, 1.2, and 4.0 at.% of Ag were obtained by co-sputtering of composite $TiC_{0.5}$-$Ca_3(PO_4)_2$ and metallic Ag targets. The surface structure of SLS-prepared samples and cross-sectional morphology of TiCaPCON-Ag films were studied by scanning electron microscopy. The through-thickness of Ag distribution in the TiCaPCON-Ag films was obtained by glow discharge optical emission spectroscopy. The kinetics of Ag ion release in normal saline solution was studied using inductively coupled plasma mass spectrometry. Bacterial activity of the samples was evaluated against *S. epidermidis*, *S. aureus*, and *K. pneum. ozaenae* using the agar diffusion test and photometric method by controlling the variation of optical density of the bacterial suspension over time. Cytocompatibility of the Ag-doped TiCaPCON films was observed in vitro using chondrocytic and MC3T3-E1 osteoblastic cells. The viability and proliferation of chondrocytic cells were determined using the MTS assay and PicoGreen assay tests, respectively. The alkaline phosphatase (ALP) activity of the SLS-fabricated samples loaded with co-amoxiclav was also studied. The obtained results showed that the moderate bacteriostatic effect of the Ag-doped TiCaPCON films is mainly manifested in the change of bacterial colony morphology and optical densities of bacteria suspensions. In contrast, the SLS-prepared samples showed a very rapid initial drug release resulting in strong bactericidal effect just from the start of the test and for as long as several days. Cytocompatibility and ALP activity tests demonstrated that it is possible to achieve a pronounced antibacterial effect compatible or even higher than that in the control sample (standard disk loaded with the amoxicillin/clavulanic acid mixture (30 μg)) without compromising the material biocompatibility and bioactivity.

1. Introduction

Implant-related bacterial infections remain a problem that medical community has faced for many years but the solution has not been found yet. Doping the implant surface with antibacterial agents is considered as one of the most promising methods of their targeted delivery to the inflammation region [1,2]. The general idea of this approach is that it is necessary to provide high antibacterial efficiency in the first hours after implantation to prevent the formation of bacterial biofilm, and then to maintain a constant and more uniform drug release to prevent bacteria growth. Various metallic, nonmetallic, and ceramics materials with high surface roughness and porosity were successfully used as orthopedic implants with micro- or nanocontainers for medicine [3–7]. The main problem of the drug-loading approach is that all medicine leaches out of the surface too quickly, typically within several hours [3,4]. By varying the size and distribution of pores it is possible to thoroughly control

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http://dx.doi.org/10.1016/j.apsusc.2014.12.119
0169-4332/

the amount of drug loaded into implant surface. Taking into account that the effectiveness of antibiotics is not always proportional to their dose [8], the reduction of their concentration is particularly important from the viewpoint of reducing the adverse effects. It was shown that the amount of loaded drug is directly proportional to the size of pores and inversely proportional to the pore depth [4]. The rate of drug release can also be controlled via the pore size [9]. Note that not all types of drug can be used as container fillers. For instance, high water solubility of many antibiotics, their short half-life, or complex pharmaceutical form may limit their application [10]. Other shortcoming of medicines includes rapid bacteria adaptation to a particular antibiotic [11].

Another approach to minimize the risk of bacterial contamination is the doping with well known bactericidal element, for instance, Ag. In this case, the bactericidal effect is achieved through the damage of cell membranes and change of enzyme functions due to Ag ion adsorption by cells [12,13]. While antibiotics inhibit metabolic processes in bacteria, bactericidal elements, such as Ag, act via different mechanisms. They are segregated on bacterial walls and destroy them, which means that no effect of habituation can be developed to such bacteria killers. Note that the available literature data disagree on the amount of Ag required for the pronounced antibacterial effect. For instance, strong antimicrobial activity was observed for materials with only 2% of Ag [14–16], whereas at 20 at.% of Ag, the antibacterial effect was reported to be reduced [17,18]. Finally it should be remarked that doping with either antibiotics or bactericidal elements should not affect other biological characteristics, such as biocompatibility and bioactivite, as the latter properties determine the character of cell/material interaction [19–21].

The aim of this study is to compare two approaches to the fabrication of antibacterial surfaces, which are based on (i) surface doping with bactericidal element and (ii) loading of drug into specially formed surface relief. It the former case, Ag in the amount of 0.4, 1.2 and 4.0 at.% was added into TiCaPCON film which was previously characterized as bioactive nanostructured film [22,23]. In the latter case, TiCaPCON-coated Ti plates with cellular surface structure were used as containers for drug. Antibacterial activity was evaluated against *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*), and *Klebsiella pneumoniae subspecies ozaenae* (*K. pneum. ozaenae*). To assess the cytocompatibility of the Ag-doped TiCaPCON films, in vitro tests using chondrocytic and osteoblastic cells were performed. The alkaline phosphatase (ALP) activity, which is known as an early marker of osteoblastic cell differentiation influencing mineralization process, was also studied.

2. Materials and methods

2.1. Sample fabrication

2.1.1. Fabrication of surface microcontainers for drug loading

In order to obtain samples with blind porosity for drug loading, the selective laser sintering (SLS) method was employed using a "Phenix PM-100" machine equipped with a CW Yb Fiber Laser YLR-50 from "IPG Photonics", as described elsewhere [24]. A network structure was obtained by laser scanning of pre-deposited powder tracks, 150 μm thick, in mutually orthogonal directions. During SLS treatment, the radiation power and scanning speed of the laser beam were kept constant at 50 W and 120 mm/s, respectively. The network structure was fabricated on the surface of Ti plate (Grade 4, ASTM F 67-00) with a size of 10 mm × 10 mm. The obtained blind pore volume was about $2.6 \pm 0.6 \times 10^{-3}$ mm^3. In order to provide bioactive surface characteristics, some of the SLS-fabricated samples (hereinafter referred to as SLS samples) were covered by multicomponent bioactive nanostructured TiCaPCON films as described elsewhere [24].

2.1.2. Fabrication of Ag-doped TiCaCON films

Ag-doped films were deposited on mechanically polished and Ar ion-beam cleaned Ti substrates (Grade 4, ASTM F 67-00) by co-sputtering of composite $TiC_{0.5}$-$Ca_3(PO_4)_2$ and Ag (99.99% purity) targets. The $TiC_{0.5}$-$Ca_3(PO_4)_2$ target was produced by the self-propagating high-temperature synthesis [25]. The deposition experiments were fulfilled under negative bias voltage −100 V in a gaseous mixture of Ar + N_2 at a nitrogen partial pressure of 15%. The applied magnetron current and voltage were 2 A and 450 V, respectively. The Ag target was sputtered using an additional ion source operating at a current of 40–50 mA. The deposition parameters are listed in Table 1.

2.2. Structural characterization

The surface structure of SLS samples and cross-sectional morphology of TiCaPCON-Ag films were studied by scanning electron microscope JSM-7600F (JEOL). The microstructure of films was examined by TEM in a JEM-2100 (JEOL) microscope. The through-thickness of Ag distribution was obtained by glow discharge optical emission spectroscopy (GDOES) using a spectrometer Profiler 2 (Horiba Jobin Yvon).

2.3. Ag ion release

The rates of Ag ion release were studied by means of inductively coupled plasma mass spectrometry (ICP-MS) using an "X-Series II" unit. The samples with films were immersed in flasks filled with 40 ml of a normal saline at room temperature. To analyze Ag concentration, 1 ml of the solution was collected after 1, 3, 5, and 7 days.

2.4. Bacteria cultures

The screening was performed using test microorganisms isolated from surgical patients: *S. epidermidis*, *S. aureus*, and *K. pneum. ozaenae*. To derive the bacteria cultures, probes of biological material from surgical patients were inoculated on the tested plate, such as blood agar with 5% of sheep erythrocytes, Endo agar, and Mannit-Kochsalz-Agar (all from Pronadisa, Spain) for 18 h. To identify the microorganisms in grown bacteria colonies, smears were Gram stained and investigated for morphological characteristic. Further cultivation of bacteria was fulfilled in the meat infusion agar using single colonies of microorganisms. After 18 h of incubation in a Densimat machine (Becton Dickinson, USA), the bacterial suspension (0.5 McFarland turbidity standard) was placed in the cells of identification panel (Enteric/Nonfermenter ID System or Gram-Positive ID System, BD BBL Crystal GP, USA). The panels were

Table 1
Deposition parameters of the TiCaPCON-Ag films.

Sample	Average Ag content, %	Ion source current, mA	Ion source voltage, kV	Bias voltage, V	Deposition time, min
1	0.4	40	2	−100	30
2	1.2	50	1.5	−100	40
3	4.0	50	3	−100	30