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Protein interactions with layers of TiO_2 nanotube and nanopore arrays: Morphology and surface charge influence

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ABSTRACT

In the present work we investigate the key factors involved in the interaction of small-sized charged proteins with TiO_2 nanostructures, i.e. albumin (negatively charged), histone (positively charged). We examine anodic nanotubes with specific morphology (simultaneous control over diameter and length, e.g. diameter – 15, 50 or 100 nm, length – 250 nm up to 10 μm) and nanopores. The nanostructures surface area has a direct influence on the amount of bound protein, nonetheless the protein physical properties as electric charge and size (in relation to nanotopography and biomaterial's electric charge) are crucial too. The highest quantity of adsorbed protein is registered for histone, for 100 nm diameter nanotubes (10 μm length) while higher values are registered for 15 nm diameter nanotubes when normalizing protein adsorption to nanostructures' surface unit area (evaluated from dye desorption measurements) – consistent with theoretical considerations. The proteins presence on the nanostructures is evaluated by XPS and ToF-SIMS; additionally, we qualitatively assess their presence along the nanostructures length by ToF-SIMS depth profiles, with decreasing concentration towards the bottom.

Statement of Significance

Surface nanostructuring of titanium biomedical devices with TiO_2 nanotubes was shown to significantly influence the adhesion, proliferation and differentiation of mesenchymal stem cells (and other cells too). A high level of control over the nanoscale topography and over the surface area of such 1D nanostructures enables a direct influence on protein adhesion. Herein, we investigate and show how the nanostructure morphology (nanotube diameter and length) influences the interactions with small-sized charged proteins, using as model proteins bovine serum albumin (negatively charged) and histone (positively charged). We show that the protein charge strongly influences their adhesion to the TiO_2 nanostructures. Protein adhesion is quantified by ELISA measurements and determination of the nanostructures' total surface area. We use a quantitative surface charge model to describe charge interactions and obtain an increased magnitude of the surface charge density at the top edges of the nanotubes. In addition, we track the proteins presence on and inside the nanostructures. We believe that these aspects are crucial for applications where the incorporation of active molecules such as proteins, drugs, growth factors, etc., into nanotubes is desired.

1. Introduction

Titanium (Ti) and its alloys are ideal implant biomaterials, due to their favorable biocompatibility and corrosion resistance [1]. Additionally, their surface properties influence the biological response and therefore nanoscale surface modifications have been extensively evaluated [2,3].

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TiO_2 nanostructures have found broad interest, as both nanotopography and high surface area significantly influence their use in biomedical applications (e.g. osseointegration, antibacterial activity, mitigate inflammatory response, etc.) [2,4–7]. Self-organizing electrochemical anodization is the preferred method for growing TiO_2 nanotubes (NTs) directly on Ti substrate, as it enables a good control over their geometry, long-range order and ease of application [8,9].

Recently it was shown that cells respond to the nanoscale dimensions of nanotubes, i.e. enhanced adhesion, proliferation and differentiation were observed on 15 nm diameter NTs [10,11], and can be synergistically influenced by NTs morphology and growth factors [12]. Other properties, e.g. charge distribution, materials size and chemistry, can also influence the adhesion of cells [13–15].

In a biological environment, proteins are always present at the material's surface as an intermediate layer further mediating cell attachment and proliferation [3,16] and as the first event occurring at the initial contact between implant's surface and biological environment (tissue, body fluids) is protein adhesion, their adsorption on Ti implants was widely investigated [17–20]. Briefly, it consists of the i) first (fast) adsorption, i.e. direct attachment of molecules arriving at the surface, and ii) second (slow) process – where rearrangement can take place, either by changes in molecular orientation, or by exchange with new arriving ones [17]. Other parameters, e.g. surface charge density or chemistry, topography, hydrophilicity, proteins isoelectric points, solution pH, further influence protein adsorption (for more information see Wilson et al. [20]).

From the above parameters, the electrical force occurring between proteins and surface of implant is crucial [9,15] and is generally evaluated by the isoelectric point (IEP), e.g. native Ti – IEP = 4.0 [21], fibrinogen – IEP = 5.5 [22], albumin – IEP = 5.0 [19], while for TiO_2 NTs values are in the 4.7–5.18 range (depending on NTs morphology) [23]. It was also reported that the difference in protein size contributes to their adsorption sites and thus to adhesion on Ti [19].

The above reviewed literature investigates compact TiO_2, TiO_2 nanoparticles, nanorough Ti or other biomaterials. It is known that proteins adsorb more on TiO_2 NTs (compared to compact layers) due to their higher surface energy [24] and this leads to an increased initial protein adsorption. Thus enhanced cellular interactions occur as proteins mediate the interactions between the cell membrane and TiO_2, both negatively charged [9,15,25,26]. Therefore, the principles elucidated from this work can offer guidance for the modification of the implant surface towards an optimised surface geometry and profile, to best fit the required protein and cell interactions.

Herein, we show the influence of the morphology of TiO_2 nanostructures (nanotubes – NTs and nanopores – NPs) on the adsorption of small-sized charged proteins. We obtain diameter-controlled and at the same time length-controlled anodic nanostructures for 15, 50 and 100 nm diameter series and we evaluate their interactions with small enough proteins (<10 nm) to enter all the investigated structures, as well as different charge i.e. albumin (negative) and histone (positive). The effect of protein characteristics to their adhesion leads to an adsorption trend based on the nanostructures' morphological characteristics, including also their surface charge density. Additionally, surface coverage of proteins is investigated by XPS and ToF-SIMS, whereas adhesion inside the nanostructures is followed with ToF-SIMS sputter depth profiles.

2. Experimental

2.1. Growth of anodic TiO_2 nanostructures

TiO_2 nanostructures are grown by electrochemical anodization of Ti foils (Advent, 0.1 mm thickness, 99.6% purity) that are cleaned by ultrasonication (acetone, ethanol and deionized water, for 5 min each) and dried in a N_2 stream. Anodizations are performed at room temperature (~22 °C) in a two-electrode cell (anode – Ti foil, cathode – Pt mesh, 15 mm working distance) using a two-step anodization approach – see Supplementary material (Fig. S1). The electrolytes used are ethylene glycol (EG) based (with specific water and hydrofluoric acid, 40%, content) – Table 1. After anodization, samples are kept in ethanol for 2 h, washed with distilled water and dried. Ultrasonication was performed only for $NT_{100nm,7\mu m}$ and $NT_{100nm,10\mu m}$, to remove nanograss.

2.2. Surface and chemical characterization

The top and cross-section morphologies of TiO_2 arrays are observed using a field-emission scanning electron microscope (Hitachi FE-SEM S4800). Chemical composition is investigated by X-ray photoelectron spectroscopy (PHI 5600, spectrometer, USA) using AlKα monochromatized radiation (calibrated to Ti2p, 458 eV). Peak fitting is performed with Multipak software.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) surface spectra in positive and negative polarity are recorded on a ToF SIMS 5 instrument (ION-TOF, Münster, Germany). Negative depth profiles are recorded in dual beam mode, with a pulsed 25 keV Bi^+ liquid–metal ion beam (bunched down to <0.8 ns) for spectra generation and a 500 eV Cs^+ (15 nm diameter NTs/NPs) or a 2 keV Cs^+ ion beam (for 100 nm NTs) for sputter-removal, on a 50.8 × 50.8 μm^2 area in the center of 250 × 250 μm^2 sputter crater. Signals are identified according to their isotopic pattern as well

Table 1
Anodization conditions (sample name shows diameter and length of nanostructures).

Sample name	Electrolyte	Potential used (V)	Anodization time
$NP_{15nm,250nm}$	EG + 6 M water + 0.2 M HF	10 V	1 h
$NT_{15nm,250nm}$	EG + 8 M water + 0.2 M HF	7 V	3.5 h
$NT_{15nm,370nm}$	EG + 8 M water + 0.2 M HF	10 V	2.5 h
$NT_{15nm,600nm}$	EG + 6 M water + 0.2 M HF	10 V	6 h
$NT_{50nm,1\mu m}$	EG + 8 M water + 0.2 M HF	20 V	2.5 h
$NT_{50nm,1.75\mu m}$	EG + 6 M water + 0.2 M HF	40 V	1 h
$NT_{50nm,3.1\mu m}$	EG + 4 M water + 0.2 M HF	100 V	45 min
$NT_{50nm,3.7\mu m}$	EG + 4 M water + 0.2 M HF	92 V	1 h
$NT_{100nm,2.4\mu m}$	EG + 10 M water + 0.2 M HF	50 V	2 h
$NT_{100nm,3.7\mu m}$	EG + 8 M water + 0.2 M HF	58 V	2.5 h
$NT_{100nm,5\mu m}$	EG + 6 M water + 0.2 M HF	100 V	1 h
$NT_{100nm,7\mu m}$	EG + 6 M water + 0.2 M HF	60 V	8 h
$NT_{100nm,10\mu m}$	EG + 4 M water + 0.2 M HF	60 V	12 min
	+	+	+
	EG + 4 M water + 0.2 M HF	85 V	3 h