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Titanium surface bio-functionalization using osteogenic peptides: Surface chemistry, biocompatibility, corrosion and tribocorrosion aspects

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ABSTRACT

Titanium (Ti) is widely used in biomedical devices due to its recognized biocompatibility. However, implant failures and subsequent clinical side effects are still recurrent. In this context, improvements can be achieved by designing biomaterials where the bulk and the surface of Ti are independently tailored. The conjugation of biomolecules onto the Ti surface can improve its bioactivity, thus accelerating the osteointegration process.

Ti was modified with TiO_2, two different spacers, 3-(4-aminophenyl) propionic acid (APPA) or 3-mercapto-propionic acid (MPA) and dentin matrix protein 1 (DMP1) peptides. X-ray photoelectron spectroscopy analysis revealed the presence of carbon and nitrogen for all samples, indicating a success in the functionalization process. Furthermore, DMP1 peptides showed an improved coverage area for the samples with APPA and MPA spacers. Biological tests indicated that the peptides could modulate cell affinity, proliferation, and differentiation. Enhanced results were observed in the presence of MPA. Moreover, the immobilization of DMP1 peptides through the spacers led to the formation of calcium phosphate minerals with a Ca/P ratio near to that of hydroxyapatite. Corrosion and tribocorrosion results indicated an increased resistance to corrosion and lower mass loss in the functionalized materials, showing that this new type of functional material has attractive properties for biomaterials application.

1. Introduction

Titanium and its alloys are widely used in dental and orthopedic implants due to their adequate biocompatibility, high corrosion resistance, and high strength-to-weight ratio. This combination of properties is required for load-bearing applications (Alves et al., 2015). Unfortunately, despite the positive bulk performance, there is still an undesirable number of implant failures. Dental implants failures are around 1–20% (Alves et al., 2017), whereas for orthopedic implants this number is higher than 35% (Tobin, 2017).

To overcome such concerns, implant longevity could be achieved by designing biomaterials where the bulk and the surface are independently tailored with regenerative capabilities. The biocompatibility, as well as the biocorrosion resistance of titanium, is closely related to the properties (e.g., structure, morphology and composition) of a surface oxide layer (Li et al., 2004). Once the surface of the synthetic device is in direct contact with a living organism, biocompatibility becomes a critical requirement in any biomaterial (Silva-Bermudez and Rodil, 2013; Silva-Bermudez et al., 2013).

Strict attention must be paid to the surface of a material system, as its reaction with the host tissue is often the key factor in the success or failure of implantation (Bauer et al., 2013). Various physical and chemical modifications of the Ti surface have been proposed in order to obtain the most biocompatible implant surface (Li et al., 2004; Rafieerad et al., 2015; Wang et al., 2016a, 2016b; Sun et al., 2016; Jemat et al., 2015). Nanostructured metal oxide coatings, such as TiO_2, have shown good results in the protein interaction procedure. Consequently, this is the first step in the initial process of the material's interaction with the biological environment (Kumari et al., 2010).

Reactive functional groups can easily adhere to the titanium (coated with metal oxides), thus yielding surfaces with greater stability and functionality. The introduction of polymer-grafted surfaces or self-assembled monolayers (SAM) produced advances in this field (Tanaka et al., 2007, 2008; Suh et al., 2004; Hanawa, 2010; Zhang et al., 2010; Ma et al., 2013; Balasundaram et al., 2008; Cai et al., 2006). The metal oxide-functionalized surfaces affect the adsorption of biomolecules, like

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proteins and peptides. These surfaces also impact some important cell behavior, like cell spreading and migration (Silva-Bermudez and Rodil, 2013; Advincula et al., 2005).

Proteins from the extracellular matrix (ECM) provide instructional cues for cellular differentiation, migration, wound healing, and immune response (Ravindran and George, 2014). Specifically, functional extracellular matrix proteins contribute to the formation and maintenance of mineralized tissues – like dentin and bone. Dentin matrix protein 1 (DMP1) is an acidic, non-collagenous phosphoprotein, that plays a crucial role in osteoblast/odontoblast differentiation and mineral nucleation events (Ravindran and George, 2014; He et al., 2005; Bhatia et al., 2012). Certain specific extracellular matrix proteins, such as DMP1, act as nucleators for hydroxyapatite formation.

Acidic clusters in matrix proteins play a precise molecular role in mineral surface recognition. He et al. (2003), investigated the acidic domains of DMP1, and determined that the peptides ESQES and QESQSEQDS induce the formation of plate-shaped apatite crystals. Notably, ESQES and QESQSEQDS are both derivatives of DMP1. Once DMP1 peptides stimulate the acceleration and enhancement of osteointegration, osteoblast differentiation, and matrix production, an osteo-inductive effect may be promoted on the implanted material (Frosch et al., 2003).

The following describes improvements in the DMP1 peptides' adhesion process onto functionalized metal oxide surfaces. This process might improve the osteointegration of an implanted material. The spacers employed consist of two bifunctional molecules: 3-(4-aminophenyl) propionic acid (APPA) and 3-mercaptopropionic acid (MPA) (Trino et al., 2018). The interactions between the oxide/organic molecules and organic molecules/peptides were evaluated, and the samples were characterized by X-ray Photoelectron Spectroscopy (XPS) and Atomic Force Microscopy (AFM). These metal oxide surfaces have functional groups that may interact with amino or carboxyl groups from DMP1 peptides.

To analyze the material cytotoxicity, cell proliferation, and differentiation, in-vitro tests with human mesenchymal stem cells were developed. Corrosion and tribocorrosion tests validated the importance of mechanical properties in implants, because once metal starts to degrade, inflammation and implant failure can follow. The use of a functionalized material with DMP1 peptides, which may act as a translator between the surface properties of the material and the cell receptors, is a strong factor in the performance and overall survivability of the implant in the biological environment.

2. Materials and methods

2.1. Titanium dioxide deposition

The titanium dioxide was synthesized by sol-gel method (Oskam et al., 2003). The oxide deposition was performed by spin coating technique (2000 RPM per 60 s) under ambient conditions. Titanium dioxide was annealed at 850 °C for two hours in a heating rate of 1 °C min^{-1} in order to obtain a rutile crystalline polymorphic phase. The obtained TiO_2 film was uniform with thickness around 500 nm (Fig. S1). TiO_2 surface was functionalized with two different bifunctional molecules 3-mercaptopropionic acid (MPA) and 3–4 aminophenyl propionic acid (APPA) by immersion method as described in previous work (Trino et al., 2018).

2.2. Peptides deposition

Peptides pA (ESQES) and pB (QESQSEQDS) derived from dentin matrix protein 1 (DMP1) were synthesized via solid phase. The peptides were diluted in the ratio 1 of pA to 4 of pB in PBS and carbonate buffer solution, in order to have a concentration of 1 mg/mL. The samples were placed in a 24 well plate and covered with the peptides solution. In order to form the crosslink between the peptides, the samples were maintained overnight under UV-light in steric conditions.

2.3. Cell culture

Human mesenchymal stem cells containing the gene for Green Fluorescence Protein (hMSCs-GFP) were seeded (2×10^4 cells) upon the samples in an incubator under 37 °C and CO_2 concentration of 5% for 24 h and seven days, until they were confluent. The complete media was prepared with α-MEM, 20% of FBS, L-glutamin and antibiotic.

2.4. Cell attachment test

To analyze the attachment of the cells upon the substrate after 24 h and 7 days of culture, hMSC-GFP cells were fixed with buffered neutral formalin 10% and imaged by confocal fluorescence microscopy (Zeiss LSM 710) applying a magnification of 20 × .

2.5. Cell proliferation assay

A colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays was performed using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (PROMEGA) kit. The cells were cultured for 1, 3 and 5 days in triplicates. The plates were read in a Synergy 2 multi-mode plate reader at 490 nm. The quantity of formazan product measured by absorbance at 490 nm was directly proportional to the number of living cells in culture.

2.6. In vitro nucleation test

Nucleation was carried out under high concentrations of calcium and phosphate. The samples were immersed in a 1 M calcium chloride solution for a period of 30 min. They were then washed extensively in water to remove any nonspecifically bound calcium and then immersed in a 1 M sodium phosphate solution for a period of 30 min. Finally, they were washed with water and dehydrated with 20%, 30%, 40%, 50%, 70%, 80%, 90% and 100% of ethanol solution and then dried with hexamethyldisilazane (HMDS).

2.7. Quantitative real-time polymerase chain reaction (QRT-PCR) studies

Purification of total RNA from hMSC-GFP cells using spin technology was performed for 2.5×10^4 cells seeded upon the substrates per 7 days. Two sets of samples was analyzed, one with an osteogenic media and a control with standard media. The cells were disrupted in a lysis buffer and homogenized. Ethanol was then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy kit membrane (Qiagen). The sample was then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants were efficiently washed away, and high-quality RNA was eluted in RNase-free water. All bind, wash, and elution steps were performed by centrifugation in a microcentrifuge. The solutions were placed in a 96 well plate to be read in triplicates in a real-time PCR Reader (StepOnePlus, Applied Biosystems).

2.8. Corrosion tests

The electrochemical test was conducted using a standard three-electrode corrosion cell, PBS as electrolyte and a potentiostat (G700, Gamry Inc.) to perform the corrosion experiments on the samples in triplicates. More details can be found at the Supporting information.

2.9. Tribocorrosion tests

The samples were subjected to tribocorrosion testing using a tribometer (Advanced Linear Reciprocating Tribometer, Ducom Instruments). A standard three-electrode corrosion cell and a