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A bioactive elastin-like recombinamer reduces unspecific protein adsorption and enhances cell response on titanium surfaces

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ABSTRACT

We present the immobilization on synthetic substrates of elastin-like recombinamers (ELR) that combine a bioactive motif for cell adhesion with protein antifouling properties. Physical adsorption of the recombinamers and covalent-grafting through organosilane chemistry were investigated. The biochemically-modified surfaces were thoroughly characterized and tested for protein absorption in serum by fluorescence-labelling, XPS, Ellipsometry, and OWLS. The ELR were successfully grafted and stable, even upon mechanical stresses; being the covalent bonding favourable over physical adsorption. The coated metal surfaces exhibited excellent reduction of serum protein adsorption (9 ng/cm^2) compared to the bare metal surface (310 ng/cm^2). Non-specific protein adsorption may mask the introduced bioactive motifs; therefore, the bioactivated surfaces should display serum-protein antifouling properties. Finally, improved hMSCs response was assessed on the bioactivated substrates. In summary, the coatings simultaneously displayed anti-fouling and bioactive properties. These studies investigated key factors to enhance tissue material interactions fundamental for the design of bioactive devices and future biomedical applications.

1. Introduction

Ti and its alloys are biocompatible materials that have been widely used in many biomedical applications such as bone regeneration and dental implants [1]. However, these materials are bioinert, thus leading in some cases to long osseointegration processes, loosening and inflammation that may eventually result in rejection of the implant [2–4]. In order to overcome these drawbacks, a growing interest has been devoted to biochemical surface modification. This strategy is commonly adopted to manipulate traditional surface implants and generate bioactive substrates maintaining the bulk properties of the material. The extracellular matrix (ECM) has often inspired many authors for a biomimetic approach. ECM plays a key role in regulating and controlling cell activity and is responsible for the tissue morphogenesis, homeostasis and repair [5,6]. With this in mind many researchers in recent years have focused their attention on mimicking the ECM features to develop biofunctional synthetic materials that exert control on cell functions, tissue structure, repair and regeneration. Several ECM inspired bioadhesive molecules have been used for biomimetic purposes and probably the most exploited motif is the RGD sequence present in various ECM proteins and recognized by about half of the 24 integrins [7]. RGD on its own has been reported to increase the number of cells adhered and improve proliferation [7,8], even on glass coverslips and tissue culture polystyrene [9–11]. Several studies have demonstrated that RGD is very effective to influence cell adhesion and their response on synthetic surfaces [10,12,13]. However, Schliephake et al.

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[14] reported that no bone–implant contact enhancement was observed when simple RGD was present at titanium surface, where probably the non-specific adsorption of plasma protein covered the RGD signals. In fact, the employed systems are mainly short oligopeptides that present adhesive sequences but lack protein antifouling properties, as a result their interactions with cells may be masked or affected by non-specific adsorption of proteins present in the culture media. Therefore, an ideal system should present a biointerface capable to minimize protein adsorption and induce cell adhesion. A typical approach is to incorporate at the material surface a molecule with anti-fouling properties, such as polyethylene glycol (PEG) or alginate, along with a bioadhesive sequence. For instance, some authors have demonstrated that poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) coated titanium surfaces reduce the adsorbed mass of protein to 5 ng/cm^2 [12,15]. The PLL-g-PEG co-polymer was subsequently chemically modified to incorporate an RGD containing oligopeptide to introduce bioactivity at the metal surface. This represents one of the few examples where a molecule exhibits simultaneously resistance to protein adsorption and bioactivity. Tosatti et al. [12] also reported a reduction of the osteocalcin and alkaline phosphatase activity to the control levels on anti-fouling PLL-g-PEG coated Ti surfaces presenting an RGD containing peptide. Although widely studied in the last decade, the RGD sequence still represents an open debate and further investigation is needed to elucidate many unclear aspects. In this work, we cultured human mesenchymal stem cells on RGD modified Ti interfaces to investigate their bioactivity in terms of cell adhesion, differentiation and proliferation. Ti surfaces have been biomimetically modified with an elastin-like recombinamer (ELR) [16]. ELRs are initially inspired by elastin, the extracellular elastic protein of higher animals and were first discovered by Urry [17] and synthesized through genetic recombination techniques. These techniques allow introducing into the polymers new functionalities such as bioactive domains thus conferring a smart behavior and a high potential for several applications including biomedical devices [17–20]. The ELR used in this work provides specific extracellular matrix features and previous studies in our group on a Ti–Nb–Hf alloy proved that this ELR-modified surfaces improved adhesion and spreading of the osteoblast cell type [21]. The recombinamer sequence {[(VPGIG)$_2$–(*VPGKG*)–(VPGIG)$_2$]$_2$–(AVTG**RGD**SPASS)–[(VPGIG)$_2$–(*VPGKG*)–(VPGIG)$_2$]$_2$}$_n$ is made of three fundamental building blocks: (1) the commonly used extracellular matrix protein motif containing arginine–glycine–aspartate (**RGD**) for specific binding of cells, (2) the elastin-like penta-peptide sequence (VPGIG) to assure the desired mechanical behavior and outstanding biocompatibility and (3) a variation of the previous building block where an isoleucine is substituted by a lysine (*VPGKG*).). This peptide unit is present alternatively along the chain with the previous sequence (VPGIG) and additionally exhibits a primary amine that may serve for covalent immobilization through a nucelophilic attack or aid for physisorption processes by providing electrostatic interactions (e.g. ionic interactions, hydrogen bonds, van der Waals forces).

As this recombinamer sequence is repeated six times along the polymer it was named H-RGD6. Biopolymer immobilization at the surface was achieved through two different strategies: physical adsorption and covalent chemical binding through silane chemistry. These approaches allowed optimizing the immobilization of the elastin-like genetically-engineered protein-based polymers. Physical adsorption is a convenient method to easily anchor molecules at a material surface; however, it presents some shortcomings as the biomolecules may bind in an inactive form. In addition, physisorbed molecules bind through interactions (e.g. hydrophobic, hydrophilic, van der Waals, ionic forces) that at the material surface may show poor stability and may desorb or exchange with other proteins when in contact with body fluids. A valid alternative to this method is covalent chemical binding that requires a more complex chemistry but offers the possibility to achieve robust supports for tethering biomolecules and simultaneously presents the potential to exert a control over molecule orientation at the material interface. To the best of our knowledge this is the first example of an ELR immobilized on a titanium substrate. In summary, the main objectives of this study were to determine how these novel biomodified Ti surfaces minimize adsorption of proteins and simultaneously influence cell response. To that purpose the RGD peptide is used here as a model bioactive motif with known cell adhesion activity. We have characterized the surfaces with several analytical techniques (XPS, Ellipsometry, OWLS) and evaluated the effect of these biointerfaces on human mesenchymalstem cells (hMSCs) in vitro. hMSCs offer the potential to differentiate in various types of cells including osteoblasts, chondrocytes, adipocytes, fibroblasts, marrow stroma and other tissues of mesenchymal origin. In this work, human mesenchymal stem cells were cultured on these systems in the presence of an osteogenic medium with the aim to differentiate them into the osteoblast linage. Cell adhesion, morphology, proliferation and differentiation were evaluated by using fluorescent microscopy technique and measuring lactate dehydrogenase (LDH), alkaline phosphatase activity (ALP).

2. Methods

All chemical reagents, unless otherwise noted, were purchased from Sigma-Aldrich. Silicon wafers (WaferNet GmbH, Germany) were coated with TiO_2 (20 nm) by physical vapor deposition using reactive magnetron sputtering (PSI, Villigen, Switzerland) [22]. Metal oxide coated wafers were subsequently diced into 1 cm × 1 cm pieces for XPS, ex-situ ellipsometry measurements and cell culture studies. Optical waveguide chips for OWLS measurements were purchased from Microvacuum Ltd. (Budapest, Hungary) and consisted of an AF45 glass substrate (8 mm × 12 mm × 0.5 mm) and a 200 nm-thick $Si_{0.25}Ti_{0.75}O_2$ waveguiding surface layer. An 8 nm TiO_2 layer was deposited on top of the waveguiding layer under the same conditions described above for the silicon wafers. A set of new Ti substrates was achieved by modifying their physical and chemical surface properties. A list of the nomenclature used throughout the text for each sample type is shown in Table 1.

2.1. Elastin like protein-based polymers (H-RGD6 and H-RGD(-))

ELR were provided by Technical Proteins Nanobiotechnology S.L., product code TP20254 (Valladolid, Spain). Synthesis, expression, purification and characterization are reported elsewhere [23]. Briefly, gene expression of a recombinant *Escherichia coli* strain BLR (DE3) containing the expressing gene of H-RGD6 was induced in a 12 L Applikon fermenter under controlled conditions of temperature (37 °C) and pH (7.00). Subsequent to fermentation, the culture was harvested by centrifugation, resuspended and lysed by ultrasonic disruption. The cleared lysate was subjected to several cycles of cold and warm centrifugations, of 4 and 40 °C, respectively. All the purification steps were carried out in a sodium chloride (NaCl) solution. The polymer in solution was then frozen and lyophilized.

2.2. Surface preparation.

Prior to chemical modification, TiO_2-coated silicon wafers and waveguide chips were sonicated in cyclohexane, 2-propanol, and ultrapure water (5 min each solvent) then rinsed with acetone, and dried under a stream of nitrogen followed by a 3 min exposure to