UNIVERSIDAD POLITÉCNICA DE MADRID
ESCUELA TÉCNICA SUPERIOR DE INGENIEROS DE
CAMINOS, CANALES Y PUERTOS

***DESARROLLO Y CARACTERIZACIÓN DE
BIOMATERIALES DE TITANIO FUNCIONALIZADOS
CON RESPUESTA BIOLÓGICA MODULABLE***

TESIS DOCTORAL

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MASTER OF SCIENCE IN MATERIALS SCIENCE AND
ENGINEERING

2018

CENTRO DE TECNOLOGÍA BIOMÉDICA
DEPARTAMENTO DE CIENCIA DE MATERIALES
ESCUELA TÉCNICA SUPERIOR DE INGENIEROS DE
CAMINOS, CANALES Y PUERTOS

***DEVELOPMENT AND CHARACTERIZATION OF
FUNCTIONALIZED TITANIUM BIOMATERIALS
WITH TUNABLE BIOLOGICAL PROPERTIES***

DOCTORAL DISSERTATION

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2018

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تقدیم بہ پدر عزیز و مادر مہربانم

Dedicated to my dearest Father and my kindest Mother

زدانش یکی برتش جوشن است

پس از مرگ روشن بود یاد من

فردوسی

هر آن مغز کور را خرد روشنت

گر افزون شود دانش و داد من

Verses from *Shahnameh* (The book of Kings) by the Persian poet *Ferdowsi* (940-1020 CE), English translation reads:

When wisdom is a light within the brain

The knowledge is the body's coat of mail¹

Then, if my knowledge and my justice wax²

My memory will shine when I am dead

English translation adapted from Warner, A.G., Warner, E., "The Shāhnāma of Firdausi", Kegan Paul, Trench, Trübner & Co., London, 1905.

1- Mail: Armor

2- Wax: Increase, Grow

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ACKNOWLEDGEMENTS

First of all, I would like to express my sincere appreciation to Professor *José Pérez-Rigueiro* for his support, patience, optimism and immense knowledge. For being there with me every step of the way and showing me how to see the full half of the glass.

I am also deeply grateful to Professor *Milagros Ramos*, for introducing me to the vast world of cells, for her knowledgeable and kind words of advice, for her insightful suggestions and for always receiving me in her office with a smile.

I would like to express my gratitude to Professor *Gustavo Guinea* for granting me the opportunity to carry out my doctoral studies and work in a scientifically challenging environment.

I also would like to acknowledge Professor *Daniel González-Nieto* for supplying the mesenchymal stem cells, *Antonio Quílez* for providing the fibronectin, *Miguel Panizo* for his help in performing the nanoscratch tests, *Javier Martínez* for preparing the resin coated samples and Dr. *Mihaela Iordachescu* for providing the Ti-6Al-4V alloy used in this work.

During the years I devoted to completing this dissertation, a lot of people helped and supported me and left a lasting impression on my life to whom I wish to say thank you.

To *Saeed* for reminding me to have fun every once in a while and for making my trips back home more enjoyable; to *Triana* for being a great friend I could always count on and for making my stay in Spain more pleasant; to *Rafa* for the countless hours we spent together working with the AFM and for showing me his stunning hometown; to *Sole* for making the work with cells immeasurably more fun and for teaching me Spanish like no other teacher could; to *Blanca* for making the lab more than just a simple workplace and

for the interesting conversations over lunch; to *Rodrigo* for his clever tips and for showing me how to see the spiritual side of life; to *Maria* for introducing me to the AVS and teaching me every trick there was to it; to *Fran* and *Gustavo* for their valuable advice and for welcoming me into their office, and to all the people of *Centro de Tecnología Biomédica* and *Departamento de Ciencia de Materiales* who made me feel welcome and treated me as one of their own.

I also wish to offer my special thanks to my grandparents, *Jafar*, *Mohtaram*, *Aziz* and *Manizhe*, my aunts and uncles, *Anoosheh*, *Sedigheh*, *Hooshang*, *Behdad*, *Farzin*, *Behin*, *Mehrdad* and *Hosseini*, my brother *Pirooz* and my sister *Parastesh* for their love and support over all these years.

The most important gratitude goes to my parents, *Hassan* and *Sepideh* for always being there for me and supporting me unconditionally. I am truly indebted and thankful. Without you this would have never been possible.

ABSTRACT

Titanium and its alloys are among the most-frequently used biomaterials for the replacement of hard tissue. This choice is due to their good corrosion resistance, superior mechanical properties and excellent biocompatibility. The feature that separates titanium and titanium alloys from the rest of the metallic biomaterials, is their elastic modulus which is lower than that of the rest of the metallic biomaterials and closer to the elastic modulus of bone. These properties make titanium alloys the ultimate choice for fabrication of biomaterials for hard tissue replacement, particularly for load-bearing applications. However, a drawback in using titanium as bone implant is that it lacks the ability to establish a direct contact with the adjacent bone. More often, the implant is surrounded by a capsule of fibrous tissue. The specialized tissue is able to form on top of the fibrous tissue layer, but the fibrous tissue acts as a mechanically weak link between the implant and the newly formed bone. The presence of this fibrous tissue layer might cause infections or loosening of the implant and lead to the need for revision surgery. In this regard, development of biomaterials capable of establishing a close integration with bone tissue is of outmost importance. One strategy to address this problem is to modify the surface of the material by covalently binding proper biomolecules capable of promoting the activity of specialized bone cells on the surface, while preserving the excellent bulk properties of the material.

To do so, it is necessary to consider three distinct but complementing steps. Firstly, the proper functional groups have to be generated on the surface of the substrate material. Secondly, a proper cross-linking chemistry must be employed in order to link the surface functional groups to the target biomolecule and thirdly, a biomolecule has to be chosen which should be able to induce the desired effect in the cells, while being compatible with the cross-linking chemistry.

Thus, the objective of this work is to develop a robust method for the covalent immobilization of various biomolecules on the surface of Ti-6Al-4V alloy. In this regard, a novel technique for the deposition of amine functional groups on the Ti-6Al-4V substrates known as Activated Vapor Silanization (AVS) was used and studied. By varying the deposition parameters of AVS, different layers of amine functional groups were deposited on Ti-6Al-4V substrates and characterized in terms of roughness and surface amine concentration. Based on the findings, two optimal functionalization conditions were selected and further characterized in terms of layer thickness, contact angle, cell response and stability under physiological conditions.

Four different target biomolecules were addressed in this work: Collagen type I, albumin, fibrinogen and fibronectin. Initially, each protein was physically adsorbed on the surface of the bare Ti-6Al-4V alloys and the response of mouse pre-osteoblasts (MC3T3-E1) and bone marrow mesenchymal stem cells (BM-MSC) to the adsorption of these proteins was determined. It was concluded that collagen type I and fibronectin were able to positively influence the adhesion of BM-MSCs on the Ti-6Al-4V samples.

On the other hand, all mentioned proteins were covalently immobilized on the surface of AVS-functionalized Ti-6Al-4V samples using EDC/NHS as cross-linker. The immobilized proteins were characterized by atomic force microscopy (AFM) and fluorescence microscopy. Additionally, the stability of the immobilized proteins was assessed by exposure to detergents and, as expected, it was found that the covalently immobilized proteins were more stable than the physically adsorbed ones.

Finally, the reaction of BM-MSCs to the covalently immobilized collagen type I and fibronectin was investigated by performing cell cultures. In this regard, a cleaning protocol for removal of excess EDC/NHS as a possible cause of toxicity was also developed. Ultimately, it was concluded that the covalent immobilization of either collagen type I or fibronectin leads to an enhanced BM-MSC adhesion on Ti-6Al-4V. This finding may be employed for the development of bone-contacting biomaterials with enhanced biological properties.

RESUMEN

El titanio y sus aleaciones se encuentran entre los biomateriales de uso más frecuente para la sustitución de tejidos duros. Esta elección viene motivada por su elevada resistencia a la corrosión, sus notables propiedades mecánicas y su excelente biocompatibilidad. La principal característica que distingue al titanio y sus aleaciones del resto de biomateriales metálicos es su módulo elástico, que resulta ser inferior al del resto de dichos biomateriales metálicos y, por tanto, más próximo al módulo elástico del hueso. Estas propiedades hacen que las aleaciones de titanio sean la elección preferida para la fabricación de biomateriales para la sustitución de tejidos duros, especialmente en aquellas aplicaciones que requieren soportar cargas mecánicas. Sin embargo, un inconveniente del uso de titanio como implante óseo es la ausencia de un contacto directo del metal con el hueso adyacente. Así, se encuentra que el implante aparece rodeado de una cápsula de tejido fibroso. Aunque puede generarse tejido óseo especializado sobre la capa de tejido fibroso, dicha capa actúa como un eslabón débil entre el implante y el hueso que se haya podido formar posteriormente. La presencia de esta capa de tejido fibroso puede favorecer la aparición de infecciones o el aflojamiento del implante, situaciones cuya solución requiere de una nueva intervención quirúrgica para la revisión del implante. En este contexto, el desarrollo de biomateriales con la capacidad de establecer un contacto íntimo con el tejido óseo constituye un reto de la máxima importancia. Una estrategia para alcanzar este objetivo lo constituye la modificación del material uniendo a su superficie moléculas adecuadas mediante un enlace covalente. Dicho procedimiento debe promover la actividad de células especializadas sobre la superficie del material, al mismo tiempo que preserva las propiedades en volumen del mismo.

La realización del procedimiento indicado requiere considerar tres pasos complementarios. En primer lugar, se tienen que generar grupos funcionales adecuados

sobre la superficie del material. En segundo lugar, se debe emplear una química con agentes entrecruzantes apropiados para unir los grupos funcionales de la superficie del material con las biomoléculas diana y, en tercer lugar, deben seleccionarse unas biomoléculas diana que tengan la capacidad de inducir el efecto deseado en las células del organismo siendo, adicionalmente, compatibles con la química entrecruzante seleccionada.

Por tanto, el objetivo de este trabajo es el desarrollo de un procedimiento robusto que permita la inmovilización covalente de diversas biomoléculas sobre la superficie de una aleación Ti-6Al-4V. Para este fin se utilizó una novedosa técnica que permite la deposición de grupos amino sobre la superficie de los sustratos de Ti-6Al-4V, denominada silanización por vapor activado (AVS, por su acrónimo en inglés). La variación de los parámetros de deposición en la técnica AVS permitió depositar diferentes láminas delgadas funcionalizadas sobre los sustratos de Ti-6Al-4V, estudiándose tanto la rugosidad superficial como la densidad de grupos amino superficiales de las láminas depositadas. Basándose en los resultados obtenidos se han seleccionado dos condiciones de funcionalización óptimas, procediéndose a una caracterización más exhaustiva de las mismas en términos de espesor, ángulo de contacto, respuesta celular y estabilidad de la lámina delgada bajo condiciones fisiológicas.

Se seleccionaron cuatro biomoléculas diferentes con el objetivo de analizar su unión covalente a la superficie funcionalizada: colágeno de tipo I, albúmina, fibrinógeno y fibronectina. En una fase inicial, cada proteína se adsorbió sobre la superficie sin funcionalizar de la aleación Ti-6Al-4V, estudiándose la respuesta a dichas proteínas de células pre-osteoblásticas murinas (MC3T3-E1) y de células mesenquimales de médula ósea (BM-MS), también de ratón. Estos estudios permitieron concluir que tanto la presencia del colágeno tipo I como de la fibronectina ejercía una respuesta positiva a la adhesión celular sobre la superficie del material.

En experimentos independientes, se procedió a la inmovilización covalente a superficies funcionalizadas mediante AVS de las cuatro proteínas mencionadas anteriormente empleando como agentes entrecruzantes EDC/NHS. Se procedió a la caracterización de las proteínas inmovilizadas mediante microscopía de fuerza atómica (AFM) y microscopía de fluorescencia. Además, se evaluó la estabilidad de las proteínas inmovilizadas mediante la exposición de las muestras a diferentes detergentes,

encontrándose que las proteínas inmovilizadas covalentemente presentaban una mayor estabilidad comparadas con las proteínas simplemente adsorbidas sobre la superficie.

Finalmente, considerando que tanto la presencia del colágeno tipo I como de la fibronectina adsorbidas había conducido a una mejora en la respuesta biológica de las células BM-MSc sobre las muestras de Ti-6Al-4V, ambas proteínas fueron seleccionadas para la caracterización biológica de las proteínas inmovilizadas covalentemente. La reacción de las células BM-MSc al colágeno tipo I y a la fibronectina inmovilizados se investigó mediante la realización de cultivos celulares. En particular, fue necesario desarrollar un protocolo de limpieza para eliminar el exceso de EDC/NHS debido a su posible efecto citotóxico sobre las células. En conclusión, se ha encontrado que la inmovilización covalente de colágeno tipo I o, alternativamente, de fibronectina conduce a una mejora en la adhesión de las células BM-MSc a la aleación Ti-6Al-4V. Este descubrimiento puede ser empleado para el desarrollo de biomateriales que tengan que estar en contacto íntimo con el hueso y que presenten una respuesta biológica mejorada con respecto a los materiales empleados para esta función en la actualidad.

1 INTRODUCTION

Over the past few decades, there has been an increasing demand for materials that are able to replace various parts of the human body and mimic their functions. Several causes may lead to the impaired or loss of function of the body organs. In this regard, hard tissue replacement is of outmost importance. Degenerative diseases cause degradation in bones and diminish their functions. It is estimated that 90% of people over the age of 40 suffer from such diseases, a situation that gets worse due to the increase in the aged population over the past few years. Additionally, accidents and trauma are also responsible for the early loss of function in hard tissue. The result of this situation, is that one million hip and knee replacement surgeries are performed in the United States per annum (Q. Chen & Thouas, 2015). Consequently, there is a need for structures that are able to replace damaged bones and perform a function as close as possible to the healthy tissue and, preferably, along the whole lifetime of the patient. In this context, the importance of developing novel biomaterials replacing bone tissue is evident.

However, the development of a biomaterial has proven to be a very challenging task. By definition, a biomaterial is “any biocompatible material, natural or synthetic, which is used to replace or assist part of an organ or tissue, while in intimate contact with it” (Q. Chen & Thouas, 2015). Thus, the prerequisite of biocompatibility should be taken into account. The Food and Drug Administration of the United States (FDA) defines biocompatibility as “the ability of a device material to perform with an appropriate host response in a specific situation” (Webpage: FDA, J. M. Anderson, 1983). Therefore, not only the physical properties of the biomaterial and its ability to perform the designated task should be considered, but also the chemical interactions of the biomaterial with the host physiological system are of outmost importance and should be tailored delicately. In

this regard, various effects of the biomaterial on the host body should be considered and avoided such as: cytotoxicity, carcinogenicity, allergenicity and thrombogenicity.

Different types of materials can be used as biomaterials to be implanted in the body: metallic, ceramic, polymeric, composite and natural biomaterials. In this case, the selection of a given biomaterial depends on the therapeutic approach intended against a pathology. Since one of the main functions of hard tissue (bones) is sustaining mechanical loads, the mechanical properties and, in particular, stiffness and toughness are primary factors to take into account for the selection of the material.

1.1 Structure of bone

Bone is a porous composite material which consists of an organic phase and a mineral phase. The mineral phase is in the form of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) composed of calcium and phosphorus. The organic phase accounts for 35% of the mass of bone and is mainly made of a collagen matrix and non-collagenous proteins, lipids and cells. Two types of bone can be identified in the body: cortical and cancellous bone. Cortical bone is made up of dense bone tissue and comprises about 80% of the total bone mass. Cortical bone is found in load-bearing locations. Cancellous bone is highly porous and filled with bone marrow and forms the vertebrae and the end of long bones (Clarke, 2008).

Bone is a living tissue that is constantly remodeled by the effect of bone cells, which create and maintain bone tissue. There are five main types of bone cells: osteoblasts, osteoprogenitors, osteocytes, osteoclasts and bone-lining cells. Osteoblasts, osteoprogenitors, osteocytes and bone-lining cells are in charge of producing and preserving bone while osteoclasts regulate the absorption of bone tissue (Pablo Rodríguez et al., 2004).

Osteoprogenitors and related mesenchymal stem cells (MSC) are unspecialized cells that are able to differentiate into various cell types. MSCs grow and proliferate in the bone marrow. When a high density of MSCs is reached, these cells start to differentiate into osteoprogenitors and subsequently, into chondroblasts or osteoblasts. This process is highly dependent on the presence of growth factors such as bone morphogenic proteins (BMP) as well as physical stimuli (Pablo Rodríguez et al., 2004).

Osteoblasts are essentially in charge of producing the mineral phase and various proteins such as collagen, sialoproteins and alkaline phosphatase. Thus, their function is vital for

the bone growth and healing processes. When the new bone is formed some osteoblasts are embedded inside the newly formed matrix and differentiate into osteocytes. Osteocytes are the most-commonly found cells in the bone tissue. They control the concentration of calcium and phosphate in the tissue by regulating the crystal size of the bone minerals. Other osteoblasts remain on the surface of the bone and become inactive to form the bone-lining cells (Eriksen, 2010).

Osteoclasts are cells of the macrophage lineage which differentiate from bone marrow stromal cells and their main function is to remove bone in the bone remodeling process, mainly from damaged areas. Osteoclasts attach to the bone defect site and form a membrane that isolates the defect. Subsequently, by acidifying the environment, the calcium phosphate minerals are dissolved (McNamara et al., 2009).

One of the main properties of bone is its capacity of healing and remodeling itself. When an injury or trauma that leads to bone damage occurs, inflammatory cells are transferred to the injury site. Subsequently, fibroblasts and MSCs are recruited to form a tissue consisting of cells and blood vessels around the damage site. Macrophages are also employed for the elimination of damaged tissue fragments. After the inflammation phase, the restoration process initiates. In this step, MSCs differentiate into osteoblasts or fibroblasts. Osteoblasts produce bone tissue while fibroblasts produce cartilage by subsequent differentiation into chondrocytes. However, in some cases the newly formed tissue is accompanied by fibrous tissue which causes an enlargement in the cross-section of the newly formed tissue compared to the tissue before the injury which might result in poor bone healing, and reduced movement of the joints (Eriksen, 2010).

Although in many cases, bone is capable of repairing itself, several diseases and traumatic injuries can disrupt the normal cycle of bone remodeling. Additionally, in extremely large fractures, bone self-healing processes are not activated and external medical intervention and replacement of the lost tissue is necessary. As indicated above, biomaterials intended to replace bone should be stiff and tough. Consequently, metals are the preferred choice for substituting this hard tissue.

Figure 1.1: Schematic representation of cellular activity in bone remodeling (McNamara, 2017).

1.2 Metallic biomaterials

Metals have been widely used as implants in the body, most-commonly for load-bearing applications. The use of metals as biomaterials dates back to the 19th century, when the use of iron, gold and silver for bone and dental repair was started (J. Park & Lakes, 2007). However, most of the attempts for implantation of the metals in the body failed until Lister's techniques for aseptic surgery were developed in the 1860s (Browner et al., 2008). This development was followed by a boom in the use of metallic implants for bone and dental replacement.

Metals exhibit remarkable values of tensile strength, hardness, elastic modulus, corrosion resistance, wear behavior and fatigue strength, that are basic for their consideration as the main option for replacing bone.

Metals used as biomaterials should have significant tensile strength, fracture toughness, and fatigue resistance so as to support mechanical loads without breaking. Since many metals present these properties, corrosion resistance appears as a key factor to define a metallic biomaterial. Since prostheses are in permanent contact with the corrosive body fluids, any kind of degradation due to corrosion that results in a loss of strength of the material (Singh & Dahotre, 2007) should be avoided. Corrosion not only weakens the

material, but also leads to the release of unwanted metal ions in the body. These ions can be accumulated in the adjacent tissue or be transported to other parts of the body and cause several harmful effects (Okazaki & Gotoh, 2005, Hallab et al., 2005). Additionally, a sufficient wear resistance is responsible for preventing the loosening of the implants of long duration, and the release of wear debris that can cause tissue inflammation (Ramsden et al., 2007). The elastic modulus is another crucial factor for the selection of metallic implants and, preferably, should be close to that of bone. The elastic modulus of cortical bone is measured to be between 4 and 30 GPa (Katz, 1980). Since most, if not all metals, show an elastic modulus higher than that of bone, metals with low elastic modulus will be preferred in general. A higher elastic modulus of the implanted material may cause changes in the distribution of stress to the adjacent bone (stress shielding) which in turn, may cause bone resorption and the loosening of the implant (Sumner et al., 1998).

In summary, any metallic biomaterial should possess an optimal combination of the above-mentioned properties. Such implant materials could stay in the host body for longer durations, saving the patient from extra pain and costs of the revision surgery.

Nowadays, the accepted metallic biomaterials that can be used as implants for load-bearing applications in the body include: stainless steels, cobalt-based alloys and titanium and its alloys.

1.2.1 Stainless steels

Stainless steels are iron-based alloys containing other alloying elements such as: chromium (Cr), molybdenum (Mo), manganese (Mn) and nitrogen (N). The stainless steel accepted for medical use is 316L, although to a lesser amount 302 and 304 alloys are also used. 316L stainless steel in particular, possesses good mechanical properties such as high tensile and fatigue strength (Ebara, 2010, Sudarshan et al., 1990). In contrast, stainless steel 316L has an elastic modulus of 210 GPa which is much higher than that of bone (Niinomi, 2003). This difference in elastic modulus results in an unpaired stress transfer to the adjacent bone and may cause bone resorption in long-term implants. Furthermore, the wear resistance of this alloy is reported to be poor (Nielsen, 1987) and the released wear debris can cause allergic reactions and lead to the loosening of the implant.

Additionally, the corrosion resistance of the alloy in physiological conditions is far from optimal. The corrosion resistance of these alloys depends on the formation of a passivated

oxide layer composed mainly by Cr_2O_3 (Yue et al., 2017). This oxide layer is very thin (thickness ~ 2 nm) and is susceptible to scratching and removal during the manufacturing process or implantation. Although in the presence of oxygen the oxide layer is formed again, in oxygen-deficient environments these alloys may undergo corrosion. (Navarro et al., 2008). Moreover, the release of Ni and Cr ions from the material inside the body can cause serious health problems. Cr ions in the body can cause allergic reactions (Colomb et al., 1969), and in high concentrations acute toxicity (Segura-Muñoz et al., 2003) and even DNA damage (Nickens et al., 2010, Oliveira & Oliveira-Brett, 2010). Ni ions can also cause allergic reaction (Santucci et al., 1989), respiratory problems, toxicity and be carcinogenic (Oller et al., 1997).

Consequently, nowadays the application of stainless steel materials as implants in the body is limited to short-term devices such as fracture plates, screws, and hip nails (J. Park & Lakes, 2007), that are to be removed after fulfilling their function.

1.2.2 Cobalt alloys

Cobalt-based alloys are essentially composed of cobalt (Co), chromium and molybdenum. The most common CoCrMo alloy for application as implant is composed of Cr (27-30%), Mo (5-7%) and Ni (2.5%) and has been used as dental material and in artificial joints (Alvarado et al., 2006). These alloys show an enhanced corrosion resistance compared to that of stainless steels (Evans & Thomas, 1986). The mechanical properties of CoCrMo alloys are also superior to those of stainless steel, showing better wear and fatigue resistance. Thanks to these properties, hip, knee and ankle joint replacements and similar movable parts that are subjected to wear, have been made from CoCrMo alloys (Pramanik et al., 2005).

Although these alloys possess some desirable properties for application as implants, there are some factors that have restrained their applications. First of all, CoCrMo alloys are far more expensive than stainless steels and the process of manufacturing and machining is more complicated in the case of these alloys (Kamath et al., 2011). Secondly, the elastic modulus of CoCrMo alloys is roughly 220-230 GPa (Niinomi, 2003), even larger than the elastic modulus of stainless steel.

Thirdly, the corrosion products of CoCrMo alloys are more toxic than those of stainless steel. In addition to Ni and Cr, the release of Co from prostheses inside the body is

reported to cause muscle fatigue, reduced cognitive function, dyspnea, headache and anorexia (Mao et al., 2011). Inflammation in the adjacent tissue and systemic allergic reactions are also observed in prostheses made of CoCrMo (Delaunay et al., 2010).

1.2.3 Titanium-based materials

Titanium (Ti) is a metal with low density, moderate strength and low elastic modulus (Niinomi et al., 2016). Ti has two allotropic forms. At temperatures below 885 °C, Ti has an HCP crystal structure known as the α phase. At temperatures above 885 °C, the crystal structure changes to BCC or β phase. Hence, titanium alloys are classified into three categories according to their crystal structure: α alloys, $\alpha + \beta$ alloys and β alloys. Alloying elements also influence the structure of Ti. Elements such as oxygen (O), nitrogen (N), aluminum (Al) increase the $\alpha - \beta$ transition temperature and stabilize the α phase while, other elements like hydrogen (H), vanadium (V) and molybdenum (Mo) decrease the $\alpha - \beta$ transition temperature and stabilize the β phase. The structure of these alloys can also be tailored by controlling the cooling rate, heat treatments and deformation parameters. Therefore, a wide range of structures and properties can be produced within these alloys (Lütjering & Williams, 2013). Ti and Ti alloys also exhibit superb corrosion resistance which is due to the formation of a TiO_2 passivating layer on the surface of the material. It is stated that the corrosion resistance of Ti in protein solutions is superior to stainless steel (Steinemann, 1998).

Commercially pure Ti (98.9-99.6% Ti) which is almost completely made up of the α phase, has moderately low yield strength and high ductility. The strength of the α alloys can be increased by increasing the O content. Likewise, the amount of interstitial oxygen increases the fatigue strength. Due to the low yield strength of commercially pure Ti, its use as implant is restricted to non-load bearing applications.

In contrast to α Ti alloys, $\alpha + \beta$ alloys possess higher yield and tensile strengths due to the hardening effect of β phase precipitates. Ti-6Al-4V alloy is a well-known $\alpha + \beta$ alloy which accounts for 45% of the total Ti production (Davis, 2003) and is the most-commonly used Ti alloy in medical applications. In addition to high strength and low elastic modulus, this alloy shows extremely good corrosion resistance due to presence of vanadium and aluminum passivating oxides on its surface. Although the release of Al and V ions inside the body can produce adverse effects (Ress et al., 2003, Verstraeten et al.,

2008), the superior corrosion resistance of this alloys limits such a phenomenon considerably (Lemons et al., 1976, Pramanik et al., 2005).

Moreover, the elastic modulus of Ti-6Al-4V alloys is roughly 110 GPa which is closer to that of bone than those of other metallic biomaterials. The effects of the elastic modulus of the implant on bone healing throughout a 22-week period in a rabbit model is shown in Figure 1.2. The use of stainless steel 316L with a higher elastic modulus caused a larger resorption of the bone, while the bone healed naturally when Ti-6Al-4V was used as the implant.

Figure 1.2: X-ray images of healing and remodeling of a fractured rabbit tibia during 22 weeks. a) Ti-6Al-4V and b) Stainless steel 316L. Dashed lines shows atrophic changes in the bone (Niinomi, 2003).

The main mechanical parameters of stainless steel, Co-Cr alloys, Ti and Ti-6Al-4V are shown in Table 1.1 and compared with those of cortical bone.

Thanks to these excellent properties, Ti-6Al-4V has been used in various medical applications such as dental implants and orthodontic surgery parts, bone fixations and joint replacement parts for hip, knee, spine, shoulder elbow and wrist. Figure 1.3 shows several applications of Ti biomaterials in the body

Table 1.1: Mechanical properties of metallic biomaterials compared with those of bone (Patel & Gohil, 2012).

Material	Elastic modulus (GPa)	Yield strength (MPa)	Tensile strength (MPa)	Fatigue limit S_{end} (MPa)
Stainless steel	190	220-1200	590-1300	240-820
Co-Cr alloys	210-250	450-1600	650-1900	200-950
Titanium	110	485	760	300
Ti-6Al-4V	116	890-1000	960-1100	620
Cortical bone	15-30	30-70	70-150	N/A

Figure 1.3: Ti-based biomaterials used in different part of the human body (Liu et al., 2004).

1.3 Challenges in the use of metallic biomaterials

Due to the excellent properties mentioned above, titanium biomaterials are interesting candidates for their application as implants and as replacement of hard tissue. However,

metals in general and Ti in particular, exhibit some drawbacks as they are not able to form an intimate contact with the adjacent bone tissue.

When the metallic biomaterials are implanted into the bone, a series of events occurs: First, water molecules are adsorbed on the surface of the implant. Secondly, proteins from blood and body fluids are adsorbed onto the surface and finally, cells start to adhere to these proteins (Geetha et al., 2009).

Since the implant is recognized as a foreign object by the body, the surface of the implant is checked initially by macrophages. When inflammation starts, these macrophages together with fibroblasts form a matrix of collagen around the implant to isolate it from the surrounding tissues. Thus, the implant becomes encapsulated in a layer of dense connective tissue (Coleman et al., 1974, Ratner et al., 2004), in a process referred to as fibrous encapsulation. Therefore, no direct contact between the implant and bone tissue (osseointegration) is established. These series of events define the biotolerant behavior of a biomaterial. Formation of fibrous tissue around a Ti-6Al-4V implant can be seen in Figure 1.4.

Although this connective tissue is not a pathological condition, the lack of osseointegration of the implant can lead to a number of problems, since a weak connection between the bone and implant may favor infections (Santavirta et al., 1992). Additionally, the fibrous tissue interface is mechanically weak and causes micro-movements of the implant with respect to the adjacent bone. These micro-movements can affect the bone healing process adversely and lead to the loosening of the implant over time (Agarwal & García, 2015) which causes pain for the patient and often requires revision surgery. Thus, it is reported that loosening of the implants is the most frequent cause for revision hip arthroplasty (Wroblewski, 1984).

To counteract this problem and to facilitate the osseointegration of implants, it is necessary to attract osteoprogenitor cells to the surface of the implant and promote their activity while preventing the formation of fibrous tissue. Such an approach would result in formation of the bone directly on the surface of the implanted material. Therefore, recently, the development of novel implants with enhanced properties capable of direct osseointegration has been of special interest.

Figure 1.4: Ti-6Al-4V implanted in the femur of goats. Cross-section of the implant site after 6 weeks (Barrère et al., 2003).

In all the physiological interactions described above, the surface of the material plays a crucial role. In this regard, the paradigm of biocompatibility establishes that the response of the organism to the implant depends on the biomolecules, mostly proteins, adsorbed on the surface of the biomaterial. These adsorbed biomolecules are recognized by various cell lineages that elicit the response to the implant. Incidentally, the paradigm of biocompatibility suggests that a strategy based on the surface modification of Ti might enhance the biological response to the implant, while preserving the excellent bulk properties of Ti biomaterials.

1.4 Surface modification of Ti alloys

The surface modification techniques employed for Ti and its alloys can be categorized into mechanical and chemical routes.

1.4.1 Mechanical surface modification

The main objective of mechanical surface modification techniques is to somehow alter the topography and roughness of the surface in order to achieve a better specific cell response, obtain mechanical inter-locking between the bone and implant or to improve wear and corrosion resistance. The techniques used in this approach involve machining (Henry, 1987, Lausmaa et al., 1990, Sutherland et al., 1993, Lucchini et al., 1996),

polishing (Bowers et al., 1992) and blasting methods (Buser et al., 1999, Baleani et al., 2000, Wennerberg et al., 1996).

1.4.2 Chemical surface modification

In contrast to the mechanical routes mentioned above, chemical surface modification techniques include employment of different techniques to chemically alter the surface of the material. Such techniques include acid (Takeuchi et al., 2003, Sittig et al., 1999, Taborelli et al., 1997) and alkali (H. M. Kim et al., 1996, B. H. Lee et al., 2002, Nishiguchi et al., 1999) treatments, calcium phosphate coating (Brendel et al., 1992, Weng & Baptista, 1999, Gross et al., 1998) and biochemical coatings.

In recent years there has been an increasing interest in the biochemical surface modification pathways. The objective of this method is to control cell-material interactions by inducing specific responses in cells through the immobilization of proteins, peptides or growth factors on the surface of the material. This strategy possesses the ability to allow increasing the adhesion of a particular cell line, inducing cell differentiation and proliferation and, improving the osseointegration of the material, thus reducing the necessary healing time. The immobilization of biomolecules on the surface can be achieved by either physical adsorption or by covalent bonding.

Physical adsorption of biomolecules on different surfaces occurs through several interactions, such as van der Waals, hydrophobic or electrostatic forces. The process of adsorption of biomolecules on the surface however, is reversible and non-specific. In effect, since an equilibrium is established between the liquid medium and the surface, the biomolecules are susceptible to desorption or can be replaced by other biomolecules. The main advantage of this method is its technical simplicity (Roach et al., 2005, Rabe et al., 2011).

On the other hand, covalent bonding is experimentally more complicated to perform but, the covalent bond between the biomolecule and the surface is extremely stable and the biomolecule is much less susceptible to desorption. In this method, usually high levels of surface coverage by the biomolecule are achieved and even small molecules that do not adsorb stably, may be immobilized permanently on the substrate. In covalent immobilization, a total control over the orientation, density and spacing of the immobilized biomolecules, in principle, is attainable.

Covalent binding of biomolecules to a surface demands the presence of complementary functional groups both on the surface of the material and in the target biomolecule. These functional groups include: carboxyl ($-\text{COOH}$), hydroxyl ($-\text{OH}$), thiol ($-\text{SH}$), amino ($-\text{NH}_2$), and vinyl ($-\text{CH}=\text{CH}_2$) groups. While these functional groups usually are naturally present in the structure of the target biomolecules, they have to be generated on the surface of the material (Hermanson, 2013) in a process known as functionalization. Consequently, covalent immobilization of biomolecules on a surface comprises three steps. First, the surface of the substrate biomaterial should be covered with suitable functional groups. Second, a cross-linker molecule is employed which is able to covalently bind to both the functional groups of the substrate and to those of the target biomolecule. Finally, the target molecule presenting compatible functional group must bind the cross-linker. This strategy as described by Bauer et al. is summarized in Figure 1.5.

Figure 1.5: Strategies for the attachment of biomolecules to the surface of metals (Bauer et al., 2013).

In the case of implants for hard tissues, the immobilized biomolecules should regulate cell-implant interactions. Consequently, the surface of the implant may be coated with biomolecules that facilitate the adhesion of osteoprogenitors to the implant surface and induce osteoblast matrix formation and mineralization. Extra cellular matrix (ECM) proteins are a natural choice for this purpose, since their biological functions imply a strong interaction with different cell lineages.

1.5 Functionalization methods for Ti-based materials

Several procedures were proposed for the functionalization of Ti-based materials. All methods employ a molecule with a head group reactive towards the metallic substrate and a functional tail group (such as -NH_2 , -COOH or -SH) that is able to bind to the target molecule either directly or via a cross-linker.

In metallic biomaterials, the surface of the metal tends to be covered with surface oxides. Since these surface oxides in aqueous solutions typically form hydroxyl groups on the surface (Parfitt, 1976), several functionalization processes are based on the reaction of the reactive molecules with the hydroxyl groups. In particular, moieties such as organosilanes, organophosphates, and photosensitive chemicals can bind to the hydroxyl groups and form stable chemical bonds with the surface.

Among these methods, the approaches that employ organosilanes has proven be relatively versatile and simple. The procedure involves grafting the organosilane precursor to the surface of the substrate by siloxane bonds. Depending on the tail functional group of the used organosilane, the surface can be coated by NH_2 , COOH or OH functional groups (Brzoska et al., 1994, Kurth & Bein, 1993). Among the precursors used for silanization, 3-aminopropyltriethoxysilane (APTS) is one of the most widely used reactive molecules for the functionalization of metallic surfaces (Ogasawara et al., 2000). The molecule of APTS is shown in Figure 1.6 and results in the fixation of -NH_2 reactive groups to the surface.

Silanization processes using APTS as precursor can be performed in liquid or in gas phase. The liquid phase functionalization is performed by immersing the substrates in a solution of APTS in an organic solution, such as pentane or toluene (Marín-Pareja et al., 2014, Kurth & Bein, 1995). This method is simple and easy to perform, but lacks reproducibility. Additionally, it results in the formation of a low concentration of functional amine groups which are not homogenously distributed. Another disadvantage of this method is that it is highly dependent on ambient conditions, such as temperature and relative humidity, since the silanization reaction is highly susceptible to the hydrolyzation and oligomerization of the precursor APTS molecules (White & Tripp, 2000, Arkles et al., 1992). Moreover, in this technique the substrate surface OH groups should be activated prior to the silanization, which requires a pre-treatment step usually

performed with an acid (Fraiola et al., 2015, Bagno et al., 2007) or with an oxygen plasma (Herranz-Diez et al., 2015).

Figure 1.6: APTS molecule represented in a) 2D and b) 3D models (Webpage: nanoniele).

1.5.1 Activated vapor silanization (AVS)

Due to the disadvantages of the liquid phase silanization technique discussed above, a novel approach to silanization has been recently developed which performs the silanization reactions in vapor phase and aims at limiting the effect of ambient conditions on the process. This technique is based on chemical vapor deposition (CVD) of APTS from a gas phase onto the surface of the substrates and is known as activated vapor silanization (AVS). In this approach the APTS molecules are activated in vapor phase at high temperatures ($T > 700\text{ }^{\circ}\text{C}$) before impinging on the substrate (Martín-Palma et al., 2004). The separation of activation furnace from the substrates protects the samples from being exposed to elevated temperatures, which allows for the use of substrates than cannot withstand high temperatures.

Additionally, as activation and deposition reactions take place under low vacuum ($P \approx 10^{-2}$ mbar), the reactions generally do not depend on ambient conditions, which tends to increase reproducibility. As a result, AVS functionalized samples show a high concentration of amines which are homogeneously distributed. Besides, the control

exerted on the process through the deposition parameters allows a certain degree of variation in the properties of the functionalized layers. The scheme of the AVS reactor can be seen in Figure 1.7.

Figure 1.7: The scheme of Activated Vapor Silanization (AVS) reactor. 1) Evaporation chamber, 2) Evaporation furnace, 3) Tape furnace, 4) Activation furnace, 5) Activation chamber (Martín-Palma et al., 2004).

In the AVS process, APTS is evaporated in the evaporation chamber and the vapor is transported by an argon flux until it reaches the activation furnace, where the temperature is raised to 700-800 °C. This sudden increase in temperature leads to the breakdown of the APTS molecule, which subsequently impinges on the surface of the substrates located in a chamber immediately below the activation furnace. The process occurs at low pressure maintained by a rotary pump. This process results in the deposition of a thin functional film on the surface of the substrate.

Biofunctional thin films deposited by AVS using APTS as precursor are composed of Si, O, C, H and N, with an approximate empirical formula of $\text{SiO}_{2.5}\text{C}_{2.5}\text{H}_{2.6}\text{N}_{0.5}$, as characterized by X-ray photoelectron spectroscopy (XPS) and Rutherford backscattering spectrometry (RBS) (Arroyo-Hernández et al., 2008). This elemental composition is the result of the decomposition of APTS, which results in a thin layer stabilized by Si-O-Si bonds, while maintaining a relatively high proportion of the other elements, most

importantly nitrogen. The analysis by FTIR of layers deposited with different AVS processing parameters also showed that, although AVS always yields the same decomposition products, their quantitative proportion is highly sensitive to processing parameters. Quantitative variations are especially important regarding the content of amino groups in the functionalized layer (Arroyo-Hernández et al., 2014). Finally, the model shown in Figure 1.8 was proposed for the AVS deposited layer.

Figure 1.8: The model proposed for the AVS-deposited layer (Arroyo-Hernandez et al., 2003).

AVS has been employed to functionalize several substrates such as silicon, porous silicon and sputtered Ti successfully, and showed the formation of functional layers which could bind reactive moieties such as fluorescein isothiocyanate (FITC) (Martín-Palma et al., 2004).

Moreover, the functional layer deposited by AVS on porous silicon substrates is believed to be bioactive. This was demonstrated by soaking the porous silicon samples functionalized by AVS in simulated body fluid (SBF) for 18 days. Moderate levels of calcium phosphate precipitation were detected on the samples after immersion in SBF (Arroyo-Hernández et al., 2007)

One of the major advantages offered by AVS, is the possibility of tuning the deposition parameters. These parameters include: evaporation temperature, activation temperature, the pressure of argon and deposition time. The variation of these parameters allows for a certain degree of control over the resulting deposited functional layer. By changing these deposition parameters, layers with very different properties such as amine density and roughness was produced on silicon substrates (Arroyo-Hernández et al., 2014). Thus, the

functional layer deposited by AVS can be optimized according to the required application.

1.6 Chemistries involving reaction with amines

Binding a biomolecule to the functional layer requires employing an adequate cross-linker chemistry. The cross-linker should bind the amine groups of the functional layer to a group of the target biomolecule. Several strategies were proposed for this goal.

1.6.1 Amine-amine cross-linking

1.6.1.1 NHS esters

N-Hydroxysuccinimide (NHS) esters are homobifunctional cross-linkers composed of two NHS ester tails connected by a spacer arm. They were introduced in the 1970s (Lomant & Fairbanks, 1976). Although they show high reactivity at physiological pH, most of the NHS ester cross-linkers are not soluble in water. N-hydroxysulfosuccinimide (sulfo-NHS) is a water-soluble derivation of NHS ester cross-linkers (Staros, 1982). Cross-linkers containing NHS esters react with amine groups and create an acylated product which is reactive toward sulfhydryl, hydroxyl and amine groups. However, the only stable product is created by the reaction with primary and secondary amines through the formation of an amide bond (Figure 1.9) (Cuatrecasas & Parikh, 1972). Therefore, they are used to link an amine group from one molecule to amine groups of a second molecule.

Dithiobis(succinimidylpropionate) (DSP) is an example of a water-insoluble NHS ester cross-linker with a spacer arm length of 8 atoms and 12 Å (Lomant & Fairbanks, 1976). Due to its hydrophobic nature, DSP is permeable to the cell membrane and thus, has been used for investigating interactions of the cell membrane proteins (Hamada & Tsuruo, 1987). The water-soluble counterpart of DSP is dithio bis (sulfosuccinimidyl propionate) (DTSSP). In contrast to DSP, it is impermeable to the cell membrane because of its hydrophilic nature and has been used in applications where the modification of the cell membrane without modifying intracellular components is necessary (Staros, 1982). Other NHS ester cross-linkers include: Disuccinimidyl suberate (DSS) and its counterpart Bis(sulfosuccinimidyl) suberate (BS), Disuccinimidyl tartarate (DST) and Sulfo DST, Ethylene glycol bis(succinimidylsuccinate) (EGS) and sulfo-EGS.

Figure 1.9: Reaction of NHS esters with amines (Banerjee et al., 2012).

1.6.1.2 Imidoesters

Another type of cross-linkers that bind amine groups are imidoesters. The molecule of such cross-linkers is composed of two imidoesters at both ends of the molecules connected by a spacer arm. These cross-linkers can bind two amine groups through the formation of an amidine bond between them (Figure 1.10). The created amidine bond is highly stable at acidic pH, but might experience hydrolysis at alkaline environments. Imidoester cross linkers are water-soluble but they are susceptible to hydrolysis. One of the advantages of imidoester cross-linkers is that they do not alter the charge distribution on the targeted protein. The imidoester-based crosslinkers include: Dimethyl adipimidate (DMA), Dimethyl pimelimidate (DMP) and Dimethyl suberimidate (DMS) (Wilbur, 1992, Hartman & Wold, 1966). These cross-linkers have been used in applications such as crosslinking of hemoglobin (Pennathur-Das et al., 1982) and crosslinking of lactose synthetase (Brew et al., 1975).

Figure 1.10: The reaction of imidoesters with amines (Wilbur, 1992).

1.6.1.3 Difluorobenzenes

An alternative cross-linker group for amine groups are difluorobenzenes. Difluorobenzenes possess two fluorine atoms which are capable of reacting with amine groups. The outcome of the reaction is the creation of an arylamine bond between the two amine-containing moieties. A disadvantage of these cross-linkers is that apart from the amine groups, they are also reactive towards sulfhydryl, imidazolyl and phenolate groups (Webpage: Thermofisher). 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene (DFDNB) and 4,4'-difluoro-3,3'-dinitrophenylsulfone (DFDNPS) are the most common of these cross-linkers which have been used in applications such as linking peptides to albumin carriers (Tager, 1976). The reaction of DFDNB with amines is shown in Figure 1.11.

Figure 1.11: The reaction of DFDNB with amine containing compounds (Hermanson, 2013).

1.6.1.4 Aldehydes

Aldehyde cross-linkers are reactive toward amine groups and consist of two aldehyde groups. The most frequently used aldehyde cross-linkers are formaldehyde and glutaraldehyde. Formaldehyde is the smallest non-zero length cross-linker (spacer arm ~ 2 Å). The reactions including formaldehyde proceeds in two steps. First, formaldehyde reacts with amine groups and forms a methylol intermediate. This intermediate can react

with a range of other functional groups and form stable bonds (Feldman, 1973). Since the methylol intermediate is reactive towards different functional groups, several different products of the cross-linking reaction may be obtained. Formaldehyde has been used for crosslinking proteins in cells or tissues (Hoffman et al., 2015).

Glutaraldehyde is another important aldehyde cross-linker which is capable of linking amines to other functional groups. The progression of the crosslinking reactions using glutaraldehyde is very complex and occurs through different pathways. Since glutaraldehyde in aqueous solutions polymerizes, the size and structure of the glutaraldehyde polymers are difficult to determine and vary with pH and the age of the solution. Therefore, the reproducibility of the experiments that use glutaraldehyde as cross-linker is poor (Monsan et al., 1975). In spite of these limitations, glutaraldehyde has been widely used for linking antibodies and enzymes (Avrameas & Ternynck, 1969).

1.6.2 Amine-carboxyl cross-linkers

1.6.2.1 Carbodiimides

Carbodiimides are linker species that are able to form amide bonds between carboxylates and amines, and phosphoramidate bonds between phosphates and amines (Ghosh et al., 1990). The carbodiimide molecule itself does not participate in the resulting bond but forms an intermediate moiety that favors the formation of the product from the two reactant molecules. Consequently, carbodiimides are considered a zero-length cross-linker.

Some carbodiimides are water-soluble, while others are only soluble in organic solvents. The water-soluble carbodiimides are one of the most common choice as cross-linkers in biochemistry, since the biological target molecules are mostly soluble in aqueous buffers.

1.6.2.1.1 EDC

The most-common carbodiimide used in biological reactions is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (J. Sheehan et al., 1961, J. C. Sheehan & Hlavka, 1956). The products of the reaction of EDC with carboxylates are o-acylisourea intermediates which are highly reactive. This o-acylisoureas in turn, can react with nucleophiles such as primary amines and form amide bonds (Figure 1.12) (Williams & Ibrahim, 1981). The by-product of the reactions involving water-soluble carbodiimides is isourea which is also water-soluble. A drawback of this method is the possible

hydrolysis of the intermediates, since the oxygen atoms of the water molecules can react with these intermediates and regenerate the carboxylate group in a competing reaction with the amines (Gilles et al., 1990). This problem can be avoided by the precise adjustment of pH. Thus, the amide bond formation is most-optimal at pH 4-6, while hydrolysis of EDC occurs mostly above pH 6.5 (Nakajima & Ikada, 1995). Additionally, if the target molecules possess both amine and carboxyl groups, this method can lead to self-polymerization, due to the reaction of one molecule with another molecule of the same substance.

Figure 1.12: The cross-linking reaction of EDC (Hermanson, 2013).

1.6.2.1.2 EDC and NHS

NHS is a reactive chemical that can form a stable amide bond with amines (Anjaneyulu & Staros, 1987). When EDC reacts with carboxyl groups, the reaction of the resulting activated ester intermediate with amines proceeds at a slow rate. Additionally, the produced intermediate is susceptible to hydrolyzation in water which reduces the efficiency of the reaction. The addition of NHS increases the stability and solubility of the intermediates through the reaction of its hydroxyl group with the EDC activated ester (Nakajima & Ikada, 1995). Therefore, the addition of NHS significantly increases the rate of the reaction and, consequently, the efficiency of the cross-linking.

1.6.2.1.3 CMC

1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC) is another water-soluble carbodiimide cross-linker capable of forming amide bonds between carboxyl and amine groups. The o-acylisourea intermediate ester is formed when a carboxyl group reacts with CMC. The addition of NHS can improve the efficiency of the reaction, as it occurs with EDC (J. C. Sheehan & Hlavka, 1956). Some of the uses of CMC include: linking ligands to support substrates for affinity chromatography applications (Marcus & Balbinder, 1972) and estimation of the number of carboxyl groups in proteins (Hoare & Koshland, 1967).

1.6.2.1.4 DCC

Dicyclohexyl carbodiimide (DCC) is the most-commonly used water-insoluble cross-linker in organic synthesis applications. In contrast to EDC, since the reactions including DCC are performed in organic solvents, the hydrolysis of the intermediates is not relevant. For this chemistry, the disadvantage associated with the use of DCC is its toxic nature, which complicates its use (J. C. Sheehan & Hess, 1955). DCC has been used for bonding molecules to carboxyl-functionalized substrates (Lowe et al., 1973).

1.6.2.1.5 DIC

Diisopropyl carbodiimide (DIC) is another carbodiimide which is insoluble in water. Its advantage over DCC is that it is liquid at ambient temperature and thus, easier to manipulate.

1.6.2.2 CDI

N,N'-carbonyldiimidazole (CDI) is a highly reactive cross-linker that can bind carboxyl or hydroxyl groups to amines via either an amide or one-carbon-length N-alkyl carbamate linkage, respectively. Since CDI is highly susceptible to hydrolysis, it cannot be normally used in aqueous solvents. However, if a tight control is exerted on the pH of the solution, CDI may be used in aqueous solutions, although the efficiency of the reaction is low. An advantage of CDI is that it is possible to be used in both organic and aqueous solvents (G. W. Anderson & Paul, 1958).

1.6.2.3 Reagent K

Another zero-length cross-linker capable of coupling amines to carboxyl groups is N-ethyl-3-phenylisoxazolium-3'-sulfonate also known as Woodward reagent K. In alkaline

solutions the reagent reacts with carboxyl groups and forms an enol ester. The enol ester, in turn, reacts with amine groups which results in the formation of an amide bond (Woodward et al., 1961). This reagent is susceptible to rapid hydrolysis in aqueous media, which reduces the efficiency of the crosslinking reaction (Dunn & Anfinsen, 1974). In spite of this drawback, Woodward reagent K has been used successfully for crosslinking proteins (Boyer, 1986).

1.7 Target biomolecules and cell response

After establishing the surface functionalization method and the proper crosslinking chemistry, the next step is the choice of a target biomolecule capable of inducing the desired cell response. The important factor that should be kept in mind is that for the immobilization of the biomolecule to be successful, it is necessary that the surface functional group, cross-linker and biomolecule must be compatible. Since there are various biomolecules, proteins and peptides that are capable of inducing specific responses in different cell lines, it is worth reviewing some of the main candidates.

Bone tissue contains different types of cells such as osteoblasts, osteocytes and osteoclasts. In addition, MSCs and osteoprogenitor cells present in bone marrow, can differentiate into osteoblasts. Differentiation and proliferation of these cells is regulated by signals from the extracellular environment, that can appear as adhesion molecules or as soluble factors.

The adhesion of cells to surfaces and to other cells are regulated by adhesion receptor proteins in the cell membrane which can be grouped into four families: integrins, cadherins, immunoglobulins and selectins. Cadherins, immunoglobulins and selectins are responsible for cell – cell adhesion (Hynes & Lander, 1992), while integrins are transmembrane proteins that are responsible for cell – extracellular matrix (ECM) interactions. Integrins consist of two subunits α and β which are linked together by a non-covalent bond (Huhtala et al., 2005). Different cell types possess specialized integrins. For instance, mesenchymal cell lines possess $\alpha 1\beta 1$ integrins while, $\alpha 2\beta 1$ integrins are dominant in epithelial cells (Heino, 2007). Each combination of α and β integrins shows different affinity for various target proteins. The function of integrins serves as signaling mechanism that controls the subsequent cell survival, proliferation, migration and differentiation of the cells (Giancotti & Ruoslahti, 1999). Hence, bone implants capable

of mimicking the ECM, might modulate the integrin response of the cells and lead to an enhanced osseointegration.

Consequently, ECM molecules are obvious candidates as the biological moiety of biofunctional surfaces. ECM is made up of carbohydrates and proteins such as collagen, elastin, hyaluronic acid, proteoglycans and glycoproteins. As indicated above, the interaction between ECM and cells may lead to migration or differentiation of the latter (Ruoslahti & Pierschbacher, 1987). Consequently, there have been several attempts to control the cellular response to the implants by grafting ECM components such as collagen (Morra et al., 2005, d'Aquino et al., 2009), hyaluronic acid (Solchaga et al., 1999), bone sialoprotein (Graf et al., 2008) and fibrin (Perka et al., 2000). Some of these potential target proteins are discussed in the following section.

1.7.1 Collagen type I

Collagen is the most abundant protein in the body of all vertebrates. It accounts for 30% of the total proteins in the human body and is the major proteinaceous component of the ECM, bone, skin and cartilage. Collagen plays different roles in several parts of the body and is responsible for toughness of various kinds of tissue (Fratzl, 2008). The functional collagen structure (tropocollagen) is composed of three polypeptide chains that form a right-handed triple helix (Figure 1.13). Each chain presents the characteristic amino acid motif of $(-X-Y\text{-glycine})_n$. X and Y positions may be occupied with different amino acids, but usually correspond to proline (Pro) and hydroxylproline (Hyp), making the –Pro – Hyp – Gly motif. Tropocollagen molecules assemble into fibrils, which imparts toughness to the tissue (Shoulders & Raines, 2009). Currently 28 different type of collagen have been recognized (Kadler et al., 2007).

Type I collagen is the most abundant collagen and is present in fibrous tissue, skin, ligament, cornea and blood vessels, and constitutes 80% of the total protein present in bone (Viguet-Carrin et al., 2006). Type I collagen found in human body is composed of 338 uninterrupted –X-Y-Gly motifs in each one of the chains. Approximately 1/3 of the X positions are occupied by Pro, 1/3 of the Y positions by Hyp and 1/8 of the Y positions by arginine (Arg) (Makareeva & Leikin, 2014), although other amino acids can also participate in the structure.

Figure 1.13: Collagen triple helical structure (Hulmes, 2008).

Due to its major role in the ECM, collagen has been used extensively as coating in bone-contacting biomaterials. Cells can identify several features of collagen with their surface receptors. Thus, some receptors can identify the triple helical domains in collagen, while others identify certain amino acid motifs such as GFOGER (Glycine – Phenylalanine – Hydroxyproline – Glycine – Glutamic acid – Arginine) which shows high affinity for some integrins (Knight et al., 2000). Not only complete collagen molecules but also collagen-like peptides were used to control the adhesion of cells and their differentiation (Reyes & Garcia, 2003). In this regard, the motif RGD (Arginine – Glycine – Aspartic acid) which, is exposed after denaturation of the triple helix has been extensively used (Heino, 2007). KGDE (Lysine – Glycine – Aspartic acid – Glutamic acid) is also another recognition site for cells in collagen (Cooper et al., 1993) Due to the presence of these cell receptor binding sites, collagen can be considered as a viable target biomolecule for covalent attachment onto the surface in order to control the reaction of the body to the implant.

1.7.2 Fibronectin

Fibronectin is a protein found in blood plasma in its soluble form and in the ECM in its insoluble form. It is produced in the liver by hepatocytes as a soluble dimer. ECM fibronectin however, is produced by several cells as insoluble fibrils (Pankov & Yamada, 2002).

Fibronectin is a dimeric protein formed from two peptide subunits. Each subunit has a molecular weight of approximately 250 kDa and both subunits are linked together near the C-termini through disulfide bonds. Each subunit is composed of three types of repeating units (FN-I, FN-II, and FN-III): in a proportion of 12:2:15-17 that makes up almost 90% of the total sequence. The type I unit comprises of roughly 40 amino acids,

Type II units about 60 and type III approximately 90 amino acids. The fibronectin molecule as a whole contains binding sites towards integrins (i.e. cells), heparin, fibrin and collagen (Vogel et al., 2001, Pankov & Yamada, 2002). A schematic representation of fibronectin structure and binding sites can be seen in Figure 1.14.

The main cell binding site in fibronectin is the Arginine – Glycine – Aspartic acid (RGD) site in the FN-III10 repeating unit (Ruoslahti & Öbrink, 1996). Additionally, there are other peptide sequences in the structure of fibronectin that are also reported as binding site for cells, including Proline – Histidine – Serine – Arginine – Asparagine (PHSRN) (Aota et al., 1994), Arginine – Glutamic acid – Aspartic acid – Valine (REDV) (Humphries et al., 1986) and Leucine – Aspartic acid – Valine (LDV) (Komoriya et al., 1991). It is stated that the presence of PHSRN alongside RGD sites can increase affinity for integrins significantly (Aota et al., 1994). Synthetic peptides containing both an RGD site and a PHSRN site attached to polyethylene glycol were observed to increase adhesion and lead to the differentiation of osteoblasts, when compared to an RGD coating (Benoit & Anseth, 2005). Recombinant fragments of fibronectin were also used as coating. The peptide sequence FNIII7-10, which refers to the 7th to 10th type III units of fibronectin, was shown to increase osteoblast differentiation and cell adhesion strength (Petrie et al., 2008).

Figure 1.14: Fibronectin dimeric structure (Muazzez et al., 2014).

Thanks to its properties mentioned above, fibronectin can be a potential target protein for immobilization, capable of improving cell response to the implant.

1.7.3 Albumin

Since not only ECM proteins might improve the response of the organism to the implant, it is worth considering some other candidates as well. Albumin is a globular, water-soluble protein found in the blood plasma. It accounts for 50% of the total plasma proteins in humans (Farrugia, 2010). Albumin is responsible for the transport and metabolism of hormones, fatty acids, amino acids and regulates the osmotic pressure inside the blood vessels. Serum albumin has been the subject of extensive study due to its commercial availability and ease of purification. Human serum albumin is a protein with a molecular weight of 65 kDa and consists of 585 amino acids. The molecule of the human serum albumin is formed by six helical subdomains assembled in a heart-shaped figure (Figure 1.15) (He & Carter, 1992).

Figure 1.15: Human serum albumin molecule containing six helical subdomains (He & Carter, 1992).

Since albumin possesses anti-thrombogenic properties and decreases platelet adhesion, it can be used to minimize blood-biomaterial interactions. Consequently, one of its main applications in biomaterial science is coating of polymeric implants that are in contact

with blood (Kottke-Marchant et al., 1989). Moreover, it was found that an albumin coating on metallic implants can reduce bacterial adhesion and reduce the possibility of infections (An et al., 1996). Additionally, albumin-based scaffolds have been used in bone tissue engineering and have been shown to support human mesenchymal stem cell attachment and growth (P. S. Li et al., 2014). Hence, albumin could be another potential biomolecule of interest for immobilization on the surface of biomaterials.

1.7.4 Fibrinogen

Fibrinogen is a blood plasma protein found in high concentrations in all vertebrates. Fibrinogen plays a main role in the wound healing process through the formation of fibrin clots. The fibrinogen-fibrin system also participates in homeostasis, inflammation and angiogenesis (Doolittle, 2010). Fibrinogen is a large and complex protein made up of two sets of three non-identical polypeptide chains referred to as A α , B β , γ which in their conventional form possess 610, 461, 411 amino acids, respectively. The whole molecule is about 45 nm in size and has a molecular weight of 340 kDa (Doolittle, 1984). Figure 1.16 shows the structure of the fibrinogen molecule.

Figure 1.16: The structure of fibrinogen (Medved et al., 2009).

Platelets, endothelial cells, lymphocytes, neutrophils, monocytes and fibroblasts are able to identify and bind to fibrinogen via their integrins. One of the most important adhesion sites on fibrinogen is the Lysine – Glutamine – Alanine – Glycine – Aspartic acid – Valine (KQAGDV) motif located at the end of the γ chain which is recognized by platelets. (Hawiger et al., 1982). Additionally, RGD motifs are found close to the C-terminal and N-terminal domains of the A α chain (Savage & Ruggeri, 1991). These RGD motifs also bind to platelets. It is also reported that the RGD sites in fibrinogen can interact with osteoblasts (Horasawa et al., 2015).

Due to these properties, fibrinogen was used as coating on blood-contacting biomaterials, in an attempt to control the subsequent inflammatory responses (Tang & Eaton, 1993) and to study platelet adhesion (Tsai et al., 1999). Additionally, the effect of a fibrinogen coating on the adhesion and proliferation of osteoblasts was also addressed (Horasawa et al., 2015). Thus, fibrinogen is another attractive biomolecule as target for the immobilization process.

1.8 State of the art

There have been numerous attempts to improve biocompatibility of Ti implants through the covalent immobilization of biomolecules. A summary of the published work covering this topic is presented in Table 1.2 in chronological order.

As mentioned earlier, biofunctionalization requires selecting a surface functionalization technique, a cross-linker chemistry and a target biomolecule. In some approaches however, there is no need for the utilization of a cross-linker as the surface functional groups are readily reactive towards the functional groups of the target biomolecule. Consequently, the presentation of the works will follow this basic scheme.

Most of the techniques reviewed here employ the silanization technique for functionalization of the Ti substrates. A very commonly used method is the silanization in liquid phase that proceeds by dissolving the organosilane in an organic solvent and exposing the surface of the Ti substrates to this solution, which is accompanied with several disadvantages as mentioned previously in section 1.5.

Table 1.2: Summary of the published papers concerning covalent attachment of biomolecules on Ti surface.

Substrate	Surface functionalization method	Cross-linker	Target biomolecule	Results	Year
Ti	Immersion in APTMS/ methanol	SMCC	FHRRIKA and RFHARIK peptides	Improved ROC cell attachment (Rezania et al., 1999)	1999
Ti	Immersion in APTS/ toluene	CDI	BMP-2	Improved bone-implant contact (Voggenreiter et al., 2001)/bone density/direct bone apposition (J. Becker et al., 2006) in vivo	2001
Ti-6Al-4V	Plasma polymerization of allyl amine	EDC/NHS	BMP-4	Improved ALP activity (Puleo et al., 2002)	2002
Ti	Plasma deposition- acrylic acid grafting	EDC/NHS	Collagen	Lower growth of SaOS-2 cells No difference in ALP activity Better in-vivo bone growth (Morra et al., 2003)	2003

Ti	Immersion in APTS/ acetone	EDC	Fibronectin/RGD peptide	Improved SaOS-2 adhesion/spreading (Pham et al., 2003)	2003
Ti	Tresyl chloride	N/A	Fibronectin/Collagen	Improved fibroblast attachment (Hayakawa et al., 2004)	2004
Ti-6Al-4V	Immersion in APTS/ hexane	SMP	RGD peptide/cysteine	Improved osteoprogenitor cell adhesion (Porté-Durrieu et al., 2004)/ALP activity (Pallu et al., 2005)	2005
Ti	Hydrogen peroxide treatment	CDI	RGD peptide	Enhanced gingival fibroblast and epithelial cells adhesion and proliferation (B. Zhao et al., 2005)	2005
Ti-6Al-4V	Immersion in APTS/ toluene	EDC/NHS	Collagen type-I	Improved adhesion/proliferation of MG63 cells (Müller et al., 2006)	2006
Ti	Immersion in APTS/ hexane solution	SMCC	Synthetic BMP-2	Improved cell growth rate/ALP activity Enhanced bone growth in-vivo (Seol et al., 2006)	2006

Ti	Immersion in APTS/epoxysilane/toluene solution	H2N-PEG-COOH/DMF, DCC/NHS	LL37 peptide	Enhanced bactericidal effect towards <i>E. Coli</i> . (Gabriel et al., 2006)	2006
Ti	Immersion in APTS /toluene solution	Glutaraldehyde	(GRGDSP) ₄ K, (351–359) HVP peptides	Improved osteoblast adhesion (Bagno et al., 2007)	2007
Ti	Immersion in tresyl chloride	N/A	Fibronectin	Improved MC3T3-E1 cell adhesion/gene expression (Pugdee et al., 2007)	2007
Porous Ti	Immersion in p-nitrophenyl chloroformate	N/A	Collagen type I	Enhanced differentiation of rat bone marrow cells (van den Dolder & Jansen, 2007)	2007
Ti-6Al-4V	Immersion in APTS/acetone	Glutaraldehyde	Fibronectin	Improved fibroblast attachment (Middleton et al., 2007)	2007
Ti	Immersion in APTS/toluene	EDC	RGDS peptide	Enhanced MC3T3-E1 adhesion/resistance to apoptosis (Secchi et al., 2007)	2007

Ti	Tresyl chloride	N/A	GRGDSP	Accelerated MC3T3-E1 gene expression (Yamamichi et al., 2008)	2008
Ti	Plasma discharge of allyl amine	Glutaraldehyde	Collagen type I	Enhanced MG63 cell adhesion (Chang et al., 2008)	2008
Ti	Poly (methacrylic acid)/trichlorosilane	EDC/NHS	Silk sericin	Reduced <i>S. aureus</i> and <i>S. epidermidis</i> adhesion/improved MC3T3-E1 cell attachment, proliferation and ALP activity (F. Zhang et al., 2008)	2008
Porous Ti	Immersion in APTS/xylene	Glutaraldehyde	Fibrinogen/Bisphosphonate	Improved fixation of Ti screws in vivo (Wermelin et al., 2008)	2008
Ti-6Al-4V	Immersion in dopamine/water	Glutaraldehyde and EDC/NHS	Chitosan/RGD peptide	Decreased adhesion of <i>S. aureus</i> and <i>S. epidermidis</i> Increased adhesion/proliferation/ALP activity of MC3T3-E1 (Shi et al., 2008)	2008

Ti-6Al-4V	Immersion in dopamine/ water	EDC/NHS	Chitosan/BMP-2	Reduced <i>S. aureus</i> and <i>S. epidermidis</i> adhesion Increased adhesion/ALP activity/calcium phosphate deposition of hMSCs (Shi et al., 2009)	2009
Ti	Immersion in APTS/ toluene	Glutaric anhydride	(GRGDSP) ₄ K peptide	Improved osteoblast adhesion/proliferation/differentiation (Dettin et al., 2009)	2009
Ti	Immersion in dopamine/ water	N/A	Dextran/BMP-2	Reduced adhesion of <i>S. aureus</i> and <i>S. epidermidis</i> /enhanced MC3T3- E1 cell spreading/ALP activity (Shi et al., 2009)	2009
Ti	Immersion in APTS/ ethanol	EDC/NHS	Heparin/fibronectin	Improved anti-coagulation properties (G. Li et al., 2010)	2010
Ti	Plasma polymerization of allyl amine	EDC/NHS	Hyaluronic acid	Enhanced MC3T3-E1 attachment/proliferation (Seo et al., 2010)	2010

Ti-6Al-4V	Immersion in dopamine/ Tris	N/A	Vascular endothelial growth factor (VEGF)	Enhanced survival/proliferation of endothelial cells (EC) Induction of differentiation of hMSCs into ECs (Poh et al., 2010)	2010
Ti	Immersion in APTS/ acetone	EDC/NHS	JH8194 peptide	Reduced <i>P. gingivalis</i> colonization/Increase ALP activity mf MC3T3-E1 cells (Makihira et al., 2010)	2010
Ti	Immersion in APTS/ acetone	EDC/NHS	Osteoprotegerin	Reduced RAW264.7 osteoclasts differentiation (Makihira et al., 2010)	2010
Ti	Immersion in APTS/ toluene	EDC/NHS	Heparin/BMP-2	Higher ALP activity/calcium content (S. E. Kim et al., 2011)	2011
Ti	Immersion in APTS/ ethanol	EDC/NHS	Heparin/fibronectin	Improved endothelial cell adhesion/proliferation (G. Li et al., 2011)	2011

Ti	Plasma deposition of allyl amine	EDC/NHS	Hyaluronic acid /carboxymethyl chitosan	Enhanced MG63 cell proliferation (B. H. Kim, 2011)	2011
Ti	Immersion in dopamine/ Tris-HCl	EDC	Recombinant human platelet-derived growth factor (rhPDGF-BB)/heparin	Increased ALP activity of MG63 cells (Huh et al., 2011)	2011
Ti-6Al-4V	Immersion in APTS/ ethanol	Glutaraldehyde	Chitosan/vancomycin Chitosan/semaphorin 3A	Inhibited <i>S. aureus</i> growth (Swanson et al., 2011) Enhanced osteogenic differentiation (Fang et al., 2014)	2011
Ti	Immersion in tresyl chloride	N/A	Fibronectin	Improved MC3T3-E1 cell morphology, no effect on ALP activity and cell/adhesion proliferation (Yoshida et al., 2012)	2012
Ti	Immersion in APTS/ ethanol	Glutaraldehyde	Silk sericin	Improved MG63 cell adhesion and spreading (Nayak et al., 2013)	2013

Ti	Immersion in dopamine/Tris-HCL	EDC/NHS	Collagen	Improved MCT3T-E1 cell adhesion/proliferation/differentiation (Yu et al., 2013)	2013
Ti	Polydopamine	N/A	RGD peptide/BMP-2	Improved cell adhesion/ proliferation/ differentiation of hBMSC 3A6 (Chien & Tsai, 2013)	2013
Ti	Immersion in dopamine	EDC/NHS	BMP-2	Enhanced ALP activity of C2C12 cells (Kang et al., 2013)	2013
TiO ₂ /Ti	Immersion in APTS and glutamic acid γ -benzyl ester/water	EDC/NHS	Pamidronic acid	Enhanced adhesion and proliferation of MC3T3-E1 (Koo et al., 2013)	2013
Ti	Immersion in CPTS/ pentane	Diisopropyl ethylamine	RGD/PHSRN peptide	Improved MC3T3-E1 proliferation (X. Chen et al., 2013)	2013
Ti-6Al-4V	Immersion in APTMS/ethanol	EDC/NHS and Ethylenediamine	Oligo(ethylene glycol) bis(carboxymethyl)/ methacryloyloxyethyl phosphorylcholine	Improved anti-coagulation/reduced platelet adhesion (Pan et al., 2013)	2013

Ti-6Al-4V	Immersion in APTMS/ ethanol	EDC/NHS	Poly (ethylene glycol) Collagen type I	Reduced platelet adhesion/improved endothelial cell proliferation (Pan et al., 2013)	2013
Ti	Immersion in APTS/ toluene and CPTES/ toluene	N/A	hLf1-11 peptide	Inhibiting attachment of <i>S. sanguinis</i> and <i>L. salivarius</i> (anti-microbial activity) (Godoy-Gallardo et al., 2014)	2014
Ti	Immersion in APTMS/ ethanol	EDC/NHS	Polyethylene glycol/heparin	Reduced platelet adhesion/improved endothelial cell proliferation/adhesion/spreading (Pan et al., 2014)	2014
Ti	Immersion in dopamine hydrochloride/Tris-HCl	EDC/NHS	Heparin/BMP-2	Improved ALP activity/calcium deposition/gene expression of MG-63 cells (S. E. Kim et al., 2014)	2014
Ti	Poly-L-lysine	EDC/NHS	Heparin/laminine	Enhanced proliferation of endothelial progenitor cells (J. Wang et al., 2014)	2014

Ti	Immersion in APTS/ toluene	EDC/NHS	Hyaluronic acid	Improved hMSC adhesion, proliferation/differentiation in vitro Enhanced osseointegration in vitro (Ao et al., 2014)	2014
Ti	Immersion in dopamine/ Tris	N/A	Chitosan/lauric acids	Improved osteoblast adhesion/proliferation/ALP activity Reduced <i>S. aureus</i> and <i>P. aeruginosa</i> growth (L. Zhao et al., 2014)	2014
Ti	Immersion in trisyl chloride	N/A	TGF- β 2	Improved bone integration in rats (Suzuki et al., 2014)	2014
Ti	Immersion in APTS/ toluene	N/A	Silk fibroin	Enhanced osteoblast adhesion/spreading/differentiation (Naskar et al., 2014)	2014
Ti porous scaffold	Immersion in trisyl chloride	N/A	Collagen or fibronectin	Improved bone formation and augmentation in vivo (Amemiya et al., 2014)	2014

Ti	Immersion in 3-chloropropyltriethoxy-silane (CPTES) or 3-glycidyloxypropyl-triethoxy-silane (GPTES)/pentane	N/A	Collagen type I	Improved fibroblast attachment/spreading (Marin-Pareja et al., 2015)	2015
Ti	Plasma polymerization of allyl amine	Di-NHS	Fibronectin, collagen, laminin, osteopontin	Improved osteoblast response to fibronectin and collagen-immobilized surface (Heller et al., 2015)	2015
Ti	Immersion in APTS	Hexamethylene diisocyanate/heptane	Vancomycin and caspofungin	Reduced colonization of <i>S. aureus</i> and <i>C. albicans</i> (Kucharikova et al., 2016)	2016
Ti-6Al-4V	Immersion in Dopamine/Tris	EDC/NHS	Bacitracin	Enhanced hMSC differentiation/ Reduced <i>S. aureus</i> colonization and macrophage inflammation (Nie et al., 2016)	2016

Ti-Nb-Hf	Immersion in APTS/ toluene	SMP	FNIII 8 th -10 th /FNIII 12 th -14 th peptides	Improved cell proliferation/spreading (Herranz-Diez et al., 2016)	2016
Ti	Plasma deposition of allylamine	EDC/NHS	Collagen	Better cell proliferation/ALP activity/ECM mineralization (Costa et al., 2017)	2017

1.9 Objectives

As it was stated previously in detail, Ti-based biomaterials possess a range of properties that makes them the most suitable choice for use in bone tissue engineering among metals. However, the lack of complete integration with bone is a severe drawback to long-time applications of this material. An approach to overcome this limitation is to modify the surface of the Ti with bioactive molecules.

In this regard, the aim of this work is to enhance the biological response to the Ti-6Al-4V alloy through utilization of the AVS functionalization technique for the deposition of amine groups and to use the deposited functional amine groups to covalently bind different proteins to the surface of the alloy. In this regard, several partial objectives are also defined:

- 1- Implementation of the AVS technique for the functionalization of the surface of Ti-6Al-4V alloys.
- 2- Characterization of the functional amine layer deposited by AVS on Ti-6Al-4V samples and optimization of the deposition parameters accordingly.
- 3- Assessment of the effect of the functional amine layer on the adhesion, survival and proliferation of cells.
- 4- Analysis of the adsorption process of different proteins on Ti-6Al-4V substrates.
- 5- Determination of the effect of the adsorbed proteins on the adhesion, survival and proliferation of cells on Ti-6Al-4V substrates.
- 6- Development of a procedure for the covalent immobilization of proteins on Ti-6Al-4V surface using an appropriate cross-linking chemistry.
- 7- Designing a procedure to distinguish between the physically adsorbed proteins and the covalently immobilized ones.
- 8- Investigating the possible cytotoxic effects of the cross-linkers used in the process of covalent immobilization.
- 9- Development of an effective and robust cleaning procedure for the removal of the excess of cross-linkers from the samples, in order to limit their possible toxic effects.

10- Address the adhesion, survival and proliferation of cells on Ti-6Al-4V with covalently immobilized proteins.

Ultimately, the development of a reproducible and robust protocol for the covalent attachment of proteins on AVS-functionalized Ti-6Al-4V, which leads to an enhanced adhesion and spreading of bone marrow mesenchymal stem cells over the Ti-6Al-4V alloy, will be the main objective of this work.

2 MATERIALS AND METHODS

2.1 Substrate preparation

2.1.1 Materials

Table 2.1: Materials used for the preparation of Ti-6Al-4V substrates.

Material	Chemical Formula	Purity	Manufacturer
Isopropanol	C_3H_8O	99.5%	Scharlau
Acetone	C_3H_6O	99.5%	Scharlau

2.1.2 Preparation of the substrates

Ti-6Al-4V substrates were cut from an ingot of the commercial alloy in dimensions of 10 mm $\times$ 10 mm $\times$ 1 mm. The substrates were polished with an automated grinding machine (Struers Abramin) using the following series of sandpapers: grit No. 80, grit No 400, grit No.1200 and grit No. 4000. In some samples, polishing was stopped before completing the series and samples were named accordingly using the grit of the finishing sandpaper: grit No. 400 – 400 samples, grit No. 1200 – 1200 samples and grit No. 4000 – 4000 samples. 4000 samples showed a mirror-like finish. After the polishing process was finished, the substrates were rinsed with distilled water, sonicated in acetone for 15 min followed by sonication for 15 min in isopropanol and dried by an argon flow to remove any remaining organic contaminating particles.

2.1.3 Characterization of Ti-6Al-4V substrates

2.1.3.1 Surface characterization

2.1.3.1.1 Profilometry

After cleaning, the surface topography of the samples was characterized using an Optimet CT-2000 profilometer. Three profile data sets were recorded for each substrate and 10 samples were scanned corresponding to each sample type (400, 1200 and 4000). Root mean square (RMS) roughness of the samples was calculated according to the following expression:

$$RMS = \sqrt{\frac{1}{n} \sum_{i=1}^n (z_i - \bar{z})^2}$$

Where:

z_i : is the height of the i th point and

$\bar{z}$: is the average height

2.1.3.1.2 Atomic force microscopy

The topography and surface roughness of the samples polished with different grinding papers were also characterized by means of atomic force microscopy (AFM, Cervantes, Nanotec S.L.). Profile data and roughness were analyzed using WSxM 5.0 software. AFM measurements were performed in the dynamic mode using a pyramidal cantilever (Olympus OMCL RC800, semi-angle 39°, nominal resonance frequency 69 KHz). 10 substrates were scanned corresponding to each sample type. Roughness parameters RMS, average roughness, maximum value and average height were reported where maximum value is the maximum value of the heights z_{max} , average height $\bar{z}$ and average roughness is:

$$R_a = \sum_{i=1}^n \frac{|z_i - \bar{z}|}{n}$$

2.1.3.2 In-vitro cell cultures

2.1.3.2.1 Materials

Table 2.2: Materials used for culturing MC3T3-E1 cells on bare Ti-6Al-4V substrates with different surface roughness.

Material	Manufacturer
Dulbecco's Modified Eagle Medium (DMEM)	HyClone
Fetal Bovine Serum (FBS)	HyClone
Penicillin/Streptomycin	Sigma
Trypsin-Ethylenediamine Tetracetic Acid (EDTA)	HyClone
Calcein Acetoxymethyl (AM)	Life Technologies
Propidium Iodide	Sigma-Aldrich
2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)	Applichem

2.1.3.2.2 Cell maintenance and cultures

The in-vitro cytocompatibility and the effect of surface roughness of Ti-6Al-4V samples on cell adhesion, cell proliferation and survival were determined by performing cell culture experiments. Osteoblast-like murine MC3T3-E1 cell line was used as cell model and acquired from American Type Culture Collection (ATCC). Cells were cultured on polystyrene P100 dishes (Corning) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin in a humidified atmosphere at 37 °C and at 5% CO₂ until they reached confluence. Medium was changed twice a week. The cells were detached from the dishes using trypsin-EDTA (0.05% in Hank's balanced salt solution (HBSS)) when needed. Passages 5 through 15 of the cells were used to perform the experiments.

2.1.3.2.3 Sample sterilization

Prior to cell culturing, all the substrates were autoclaved (Sturdy, SA252F) and placed into 24-well plates. Autoclaving was performed for 50 min of sterilization time at 121 °C and 50 min of drying time.

2.1.3.2.4 Cell viability assessment

For cell viability experiments, MC3T3-E1 cells suspended in the culture medium were seeded on the substrates in a concentration of 5×10^4 cells/ml and 0.5 ml of medium per well. After either 4 or 48 hours of incubation at 37 °C in a humidified atmosphere of 5% CO₂ (incubator-Thermo scientific), the cells were dyed with a combined solution of 1 µl/ml of calcein AM (0.5 µg/µl in dimethyl sulfoxide (DMSO)) and 1 µl/ml propidium iodide (750 µM in phosphate-buffered saline (PBS)) in DMEM and incubated for another 30 mins. Calcein AM is a non-fluorescent cell-permeable dye that inside the living cell is converted to green fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases and is used to stain the viable cells (Johnson et al., 2001). Propidium iodide is a red-fluorescent nuclear marker impermeable to the cellular membrane. Therefore, only the nuclei of cells with damaged membranes, i.e. dead cells, will be stained with this dye (Arndt-Jovin & Jovin, 1989). Finally, the samples were observed by fluorescence microscopy using an inverted Leica DMIRB microscope equipped with a digital camera, Leica DC100 at emission wavelengths of 520 nm and 620 nm for calcein AM and propidium iodide, respectively. Data were obtained from three independent experiments running in duplicate. At least three representative images were taken from the surface of each substrate. ImageJ software was used to determine the number of viable and dead cells in each image as described in section 2.4.2. The number of viable cells was normalized by surface area of each sample and reported for each incubation time. Cellular viability percentage was defined as:

$$\text{Cellular viability \%} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

2.1.3.2.5 Cell proliferation

MC3T3-E1 cells suspended in medium (1×10^4 cells/ml) were seeded on the substrates (0.5 ml per well) and the substrates were incubated for either 1 or 7 days at 37 °C in a humidified atmosphere of 5% CO₂. Cell proliferation was determined using the Cell Proliferation Assay kit (XTT) following the manufacturer's instructions. In living cells, mitochondria have the capability to reduce XTT to form an orange-colored, water-soluble dye (Berridge et al., 2005). Therefore, the concentration of this dye is proportional to the number of metabolically active cells. Briefly, for each well of a 24-well plate, the XTT working solution was prepared consisting of 334:162:2.85 µl DMEM: XTT reagent: activation reagent. The medium in each well was replaced with the prepared XTT working solution and incubated for 4 h. Subsequently, 100 µl of the XTT solution from each well was pipetted into a 96-well plate and the absorbance of each well was measured spectrophotometrically at 450 nm using a microplate reader (BioTeK ELX808). For comparative reasons the absorbance values were normalized according to the surface area of each sample. Data were obtained from experiments running in triplicate.

2.2 Functionalization of Ti-6Al-4V samples

2.2.1 Materials

Table 2.3: Materials used for amino-functionalization of Ti-6Al-4V samples by activated vapor silanization.

Material	Chemical Formula	Purity	Manufacturer
3-Aminopropyltriethoxysilane	C ₉ H ₂₃ NO ₃ Si	99%	Acros Organics
Isopropanol	C ₃ H ₈ O	99.5%	Scharlau
Acetone	C ₃ H ₆ O	99.5%	Scharlau
Argon	Ar	≥ 99, 9997 %	BIP

2.2.2 The AVS process

Aminofunctionalization of the substrates was performed with a home-made Activated Vapor Silanization (AVS) reactor. The reactor (Figure 2.1) consists of two chambers and connecting elements. The evaporation chamber contains the organometallic 3-aminopropyltriethoxysilane (APTS) and is heated by a furnace up to a temperature in the range of 100-200 °C. APTS vapor is carried via an argon flux through the activation tube which is heated by another furnace up to a temperature in the range of 700-800 °C. In this tube, the APTS molecules are activated and enter the activation chamber. The substrates are located in the activation chamber where activated APTS molecules impinge on their surface.

Subsequently, the APTS vapor is evacuated through a trap cooled down to -80 °C by a mixture of acetone and dry ice. The APTS vapor is condensed inside the trap and the argon exits the system through another tube connected to a rotary pump (Pfeiffer-Duo5) which maintains the vacuum in the system. A pressure sensor (Inficon PSG) is used to measure the pressure inside the system. Three valves control the flow of the gas along the system: one needle-valve before the evaporation chamber control the flow of Ar, one valve after the trap that isolates the pump from the rest of the equipment when necessary and one valve that isolates the pressure sensor from the rest of the system, and is closed when the APTS flow is established to prevent the contamination of the sensor.

The process is controlled by four parameters: temperature of the evaporation furnace (T_{evap}), temperature of the activation furnace (T_{act}), working pressure (P_{ar}) and deposition time (t).

A detailed protocol of the functionalization process using AVS equipment is presented in Appendix I.

Figure 2.1: Activated vapor silanization (AVS) equipment. Different parts of the system are shown.

In this study, in order to determine the optimal conditions for AVS-functionalization of Ti-6Al-4V substrates, three of the processing parameters (T_{evap} , T_{act} , P_{Ar}) were varied, while the deposition time (t) was kept constant at 20 min (Table 2.4).

Table 2.4: AVS functionalization parameters used in the different processes.

T_{Evap} (°C)	T_{Act} (°C)	P_{Ar} (mbar)
130	750	1
150	750	1
170	750	1
150	700	1
150	800	1
150	750	2
150	750	0.5
150	800	2

2.2.3 Characterization of the functionalized samples

The resulting amine functional layers deposited on Ti-6Al-4V substrates were characterized by means of fluorescence microscopy, atomic force microscopy (AFM), contact angle measurements and nanoscratch tests.

2.2.3.1 Fluorescence microscopy

Table 2.5: Materials used for characterization of the amine functional layer by fluorescence microscopy.

Materials	Chemical Formula	Purity	Manufacturer
Fluorescein 5(6)-isothiocyanate (FITC)	C ₂₁ H ₁₁ NO ₅ S	>90%	Fluka
Phosphate Buffer Saline (PBS)	0.137M NaCl 0.003M KCl 0.010M Na ₂ HPO ₄ 0.002M KH ₂ PO ₄	N/A	Home-made

In order to visualize the amine functional layer, and to compare the density of amine groups deposited on Ti-6Al-4V substrates obtained with different processing conditions, FITC was used as a fluorescent dye capable of binding to the amine groups.

Samples were incubated with a solution of 0.5 mg/ml of FITC in phosphate buffer saline (PBS, pH = 7.4) for 20 min. To remove any FITC molecules not covalently linked to the surface amines, samples were cleaned in PBS for 5 min (3 times), rinsed with distilled water (3 times) and subsequently, dried with an argon flow. Non-functionalized Ti-6Al-4V samples were also used as control.

The presence of FITC was checked by fluorescence microscopy (Leica DMI 3000B) at emission wavelength of 520 nm. The same observation conditions: exposure time 1.3 sec, gain 2.1X and gamma 0.68 were used for all observations to allow comparison of the different samples in terms of surface density of the amine groups. Two samples corresponding to each functionalization condition were analyzed and three images were recorded from each sample.

Fluorescence intensities were quantified in each captured image using ImageJ software as described in section 2.4.1

FITC is a fluorescein molecule functionalized with an isothiocyanate group ($-N=C=S$). Isothiocyanates are reactive toward nucleophiles such as amines or sulfhydryls however, the only stable product is with primary amino groups (Hermanson, 2013). The reaction of FITC with primary amino groups involves an electron shift between the carbons of the isothiocyanate groups and those of the amine groups which results in the formation of a thiourea bond. The schematics of the reaction can be seen in Figure 2.2.

Figure 2.2: Schematic representation of the reaction of fluorescein isothiocyanate with a functionalized surface of Ti-6Al-4V.

2.2.3.2 Atomic force microscopy

The topography and surface roughness of the functionalized samples were characterized by means of atomic force microscopy (AFM, Cervantes, Nanotec S.L.). AFM measurements were performed in the dynamic mode using a pyramidal cantilever (Olympus OMCL RC800, semi-angle 39°, nominal resonance frequency 69 KHz). Two substrates were scanned corresponding to each functionalization condition. Profile data and roughness were analyzed using WSxM 5.0 software.

2.2.3.3 Thickness measurement

4000 samples were covered with a resin mask in some parts of the surface prior to functionalization. The resin mask was prepared by drop casting with photoresist (microchemical AZ5214E) and spun at 5000 rpm during 45 sec in order to get a uniform thickness of 1.4 μm . Optical lithography was carried out using a Karl Süss MJB mask aligner. The resist development was made in AZ400K + H₂O (1:5) for 1 min and final rinsing in H₂O for 10 sec. After subsequent functionalization the samples were sonicated in acetone in order to remove the resin. The step created between the regions previously covered by the resin and those covered by the functional layer was measured by AFM in contact mode. The method is shown schematically in Figure 2.3.

Figure 2.3: Schematic representation of the method used for the measurement of the thickness of the amine layer.

Optical images were captured by an optical camera (Union-DZ2) mounted on top of the AFM. Additionally, the samples were analyzed using field emission scanning electron microscopy (Zeiss, Oberkochen) for observation of the functionalized and non-functionalized segments. Images were captured using the detector for backscattered electrons.

2.2.3.4 In-vitro cell cultures

Cell cultures, cell viability assessment and cell proliferation studies were performed following the same protocols used for bare Ti-6Al-4V samples described in section 2.1.3.2

2.2.3.5 Stability under physiological conditions

The stability of the functionalized layer under cell culture conditions was checked by assessing the presence of amine groups on the surface after finishing the 7-day incubation period of the cell culture experiments. The samples were removed from the medium and rinsed with distilled water. The cells were removed from the samples using trypsin-EDTA and rinsed with distilled water several times. Subsequently the samples were dyed with FITC following the protocol described in section 2.2.3.1.

2.2.3.6 Water contact angle measurements

The water contact angle of the bare Ti-6Al-4V 4000 samples (used as reference) and of the F1 and F2 samples was measured according to the ASTM D7334-08 standard, by depositing a small volume of water ($V = 5 \mu\text{l}$) on each surface and capturing a micrograph of the droplet using a precision camera (EO-1312C, Edmund Optics) equipped with a micro inspection lens system (Optem zoom 125). The contact angle was measured in the micrograph using ImageJ software. The measurement was performed on six samples of each condition and four times on each sample.

2.2.3.7 Nanoscratch tests

In order to determine the strength of the amine functional layer deposited on Ti-6Al-4V samples, nanoscratch tests were employed. For this purpose, a commercial nanoindenter instrument (Hysitron TI 950 Triboindenter) equipped with a sphero-conical diamond tip with a radius of 10 μm was used in the nanoscratching mode. The maximum normal loads applied during ramping load tests were 50 mN, 100 mN and 150 mN. The loading rate of the indenter was 0.16667 mN/ μm and 3.333 $\mu\text{m/s}$. Five nanoscratches were made to check reproducibility. Subsequently, the samples were dyed with FITC and observed as described in 2.2.3.1, in order to visualize the scratches on the samples.

2.3 Adsorption and covalent immobilization of proteins on bare and functionalized Ti-6Al-4V substrates

In this study two different methods for protein attachment to the surface of the samples were used. Proteins were either adsorbed physically or immobilized covalently on Ti-6Al-4V samples. Protein adsorption proceeded on both functionalized and non-functionalized (bare) samples. Covalent immobilization was possible only on functionalized samples. Figure 2.4 shows the flowchart followed for characterizing the attachment of the proteins to the samples.

Figure 2.4: Flowchart of the characterization of adsorbed/immobilized proteins on bare and functionalized Ti-6Al-4V substrates.

Four different proteins were used in this study: rat tail collagen type I, bovine serum albumin, fibrinogen and fibronectin from human plasma. Each protein was first adsorbed onto the surface of non-functionalized (bare) Ti-6Al-4V samples (see section 2.3.1). The effect of the presence of the adsorbed protein on the viability, adhesion and proliferation of cells on the samples was assessed by cell culturing. Two different cell lines were used for these experiments: pre-osteoblastic MC3T3-E1 and murine bone marrow mesenchymal stem cells (BM-MSC). If any enhancement in the cell adhesion or proliferation was observed, experiments were repeated with the same cell line, but with samples with covalently immobilized protein.

In parallel, the presence of covalently bound proteins on the samples was assessed by atomic force microscopy and fluorescence microscopy. Usage of fluorescence microscopy required the labeling of the proteins with FITC (see section 2.3.2.2.1.1). The stability of the attached protein (either adsorbed or covalently immobilized) was checked by incubating the samples with a detergent. After the treatment with detergent, the samples were analyzed by atomic force microscopy and fluorescence microscopy to assess the stability of the proteins on the material.

2.3.1 Physical adsorption of proteins on Ti-6Al-4V substrates

2.3.1.1 Materials

Table 2.6: Materials used for the adsorption experiments of different proteins onto non-functionalized Ti-6Al-4V samples.

Material	Purity	Manufacturer
Bovine Serum Albumin (BSA)	$\geq 96\%$	Sigma-Aldrich
Fibrinogen	$\geq 70\%$	Sigma-Aldrich
Fibronectin	98%	Home-made
Rat Tail Collagen Type I	$\geq 90\%$	Corning
Phosphate Buffer Saline (PBS)	1X (see Table 2.5)	Home-made
Sodium Hydroxide	98%	Panreac
Sodium Dodecyl Sulfate (SDS)	99%	Fisher Scientific

2.3.1.2 Collagen

Collagen was diluted in PBS to obtain a suspension of 2.5 mg/ml. A solution of 1 M sodium hydroxide was added dropwise to the solution to adjust the pH in the range of 6.0-8.0. The pH was measured continuously using a pH paper. When the desired pH was reached, the solution was pipetted on the surface of the substrates. Subsequently, the samples were incubated at 37 °C for 2 h in a wet chamber followed by 1 h incubation at 37 °C in a dry chamber. Finally, the samples were immersed in distilled water for 1 h to rehydrate the collagen and air dried. This procedure leads to the formation of an adsorbed collagen film on the Ti-6Al-4V samples.

2.3.1.3 Bovine serum albumin (BSA)

BSA was dissolved in PBS to obtain a solution of 2.5 mg/ml. Bare Ti-6Al-4V samples were incubated with this solution for 5 h. Finally, the samples were removed from the BSA solution and gently rinsed with distilled water.

2.3.1.4 Fibrinogen

In order to adsorb fibrinogen on bare Ti-6Al-4V samples, a solution of 2.5 mg/ml fibrinogen in PBS was prepared. Bare Ti-6Al-4V samples were incubated with the said solution for 5 h. After the incubation period was over, the sample were removed from the solution and gently rinsed with distilled water.

2.3.1.5 Fibronectin

The extraction of fibronectin was done from cryoprecipitated human plasma as described by Poulouin et al. (Poulouin et al., 1999) using gelatin-heparin chromatography affinity. The fibronectin stock was received as a 500 µg/ml solution in carbonate-bicarbonate buffer. The stock solution was diluted in PBS to obtain a fibronectin solution of 200 µg/ml. Ti-6Al-4V samples were incubated with this solution for 5 h. Finally, the samples were taken out of the fibronectin solution and gently rinsed with distilled water.

2.3.1.6 Characterization of samples with adsorbed proteins

2.3.1.6.1 *In-vitro Cell cultures*

2.3.1.6.1.1 *Materials*

Table 2.7: Materials used for the extraction, expansion and staining of BM-MSCs.

Material	Manufacturer
Dulbecco's Modified Eagle Medium (DMEM)	HyClone
Fetal Bovine Serum	HyClone
Penicillin/Streptomycin	Sigma
Trypsin-EDTA	HyClone
Iscove's Modified Dulbecco's Medium (IMDM)	HyClone
Mesenchymal stem cell stimulatory supplements	Stem Cell Technologies
2-Mercaptoethanol	Sigma
Penicillin	Sigma
Streptomycin	GIBCO
L-glutamine	GIBCO
Human PDGF-BB	Peprtech
Recombinant Mouse Epidermal Growth Factor (rm-EGF)	Peprtech
XTT	Applichem
Calcein acetoxymethyl (AM)	Life Technologies
Propidium Iodide	Sigma-Aldrich
Trypsin-EDTA	HyClone

2.3.1.6.1.2 Cell maintenance and extraction

In-vitro cytocompatibility and the effect on cell behavior of adsorbed proteins on Ti-6Al-4V samples was assessed by cell culture experiments. Two cell types were used in this part of the study: osteoblast-like murine cells MC3T3-E1 and bone marrow murine mesenchymal stem cells (BM-MSC).

MC3T3-E1 cells were acquired from the American Type Culture Collection (ATCC) and were maintained in polystyrene P100 dishes (Corning) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin in a humidified atmosphere at 37 °C and 5% CO₂ (incubator, Thermo Scientific) until they reached confluence. Medium was changed twice a week. The cells were detached from the dishes using Trypsin-EDTA when needed. MC3T3-E1 passages 5 through 15 were used for performing the experiments.

Isolation and expansion of BM-MSCs were performed on fibronectin-coated wells (Corning) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% of MSC stimulatory supplements, 100 µmol/l 2-mercaptoethanol, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l L-glutamine, 10 ng/ml human PDGF-BB, and 10 ng/ml rm-EGF. Adherent cell clusters were grown for a minimum of 5 passages. After this step, the cells were regularly maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were detached from the cultures dishes when necessary using Trypsin-EDTA. All experiments were performed on MSC in passages 5 through 15.

2.3.1.6.1.3 Sample sterilization

The samples of Ti-6Al-4V with adsorbed proteins were sterilized prior to cell culturing by UV irradiation. For this purpose, the samples were exposed to ultraviolet light for 20 min on each side.

2.3.1.6.1.4 Cell viability

For cell viability experiments, the cells suspended in medium were seeded on the substrates containing adsorbed proteins in a concentration of 5×10^4 cells/ml and 0.5 ml of medium per well. After either 4 or 48 h of incubation at 37 °C in a humidified atmosphere of 5% CO₂, the cells were dyed with a combined solution of 1 µl/ml of calcein AM (0.5 µg/µl in DMSO) and 1 µl/ml propidium iodide (750 µM in PBS) in DMEM and

incubated for another 30 min. Finally, the samples were observed by fluorescence microscopy at emission wavelength of 520 nm and 620 nm for detecting calcein and propidium iodide, respectively, using an inverted Leica DMIRB microscope equipped with a digital camera, Leica DC100. Data were obtained from two independent experiments running in duplicate. At least three representative images were taken from the surface of each substrate. ImageJ software was used to determine the number of cells in each image as explained in section 2.4.2. Cells cultured in blank wells under the same conditions were used as controls. Data were reported as number of viable cells/mm² and cellular viability was calculated as described in 2.1.3.2.4

2.3.1.6.1.5 Cell proliferation

MC3T3-E1 and BM-MSCs were seeded on the samples separately in concentration of 5×10^4 cells/ml and 0.5 ml/well and incubated for 48 h. The proliferation of cells on non-functionalized Ti-6Al-4V samples with adsorbed proteins was assessed with the cell proliferation assay kit (XTT). The XTT solution was prepared by mixing 334:162:2.85 μ l of DMEM: XTT reagent: Activation reagent for each well. Samples were incubated with XTT solution for 4 h. Subsequently, 100 μ l of the XTT solution was pipetted into the wells of a 96-well plate and the absorbance of the solutions was measured spectrophotometrically at 450 nm using a microplate reader (BioTeK ELX808). For comparative reasons the absorbance values were normalized according to the surface area of each sample. Data were obtained from experiments running in triplicate.

2.3.2 Covalent immobilization of proteins on functionalized Ti-6Al-4V substrates

2.3.2.1 Materials

Table 2.8: Materials used for the covalent immobilization of proteins on functionalized Ti-6Al-4V samples and their characterization.

Material	Chemical Formula	Purity	Manufacturer
N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)	$C_8H_{17}N_3 \cdot HCl$	N/A	Sigma-Aldrich
N-Hydroxysuccinimide (NHS)	$C_4H_5NO_3$	98%	Sigma-Aldrich
4-Morpholineethanesulfonic acid (MES)	$C_6H_{13}NO_4S \cdot xH_2O$	$\geq 99.5\%$	Sigma
Fluorescein 5(6)-isothiocyanate (FITC)	$C_{21}H_{11}NO_5S$	$> 90\%$	Fluka
t-Octylphenoxypolyethoxyethanol (Triton X-100)	$(C_2H_4O)_n C_{14}H_{22}O$	$\geq 99\%$	Sigma
Polyoxyethylene sorbitan monolaurate (Tween-20)	$C_{58}H_{114}O_{26}$	$\geq 99\%$	VWR
Sodium dodecyl sulfate (SDS)	$C_{12}H_{25}NaO_4S$	$\geq 99\%$	Fisher Scientific

2.3.2.2 Preparation of samples with covalently immobilized protein on Ti-6Al-4V for atomic force microscopy and fluorescence microscopy examination

2.3.2.2.1 Immobilization of collagen

2.3.2.2.1.1 Preparation of FITC-labeled collagen suspension in MES buffer

In order to achieve a fluorescent protein observable by fluorescence microscope, collagen type I molecules were reacted with FITC dye.

Collagen was diluted in PBS to a final concentration of 2 mg/ml. Afterwards, FITC was added to the suspension to a concentration of 0.5 mg/ml in order to tag the proteins. The

labeling reaction proceeded in the absence of light to protect the FITC molecules from photobleaching.

A process of dialysis was employed to eliminate the excess FITC molecules that did not react with the collagen molecules. Snakeskin dialysis membranes (Thermo Scientific, molecular weight cut off = 3500 Da) were used for the dialysis. Dialyses were performed against 1 l of PBS. The dialysis medium was changed every 8 h up to three times in total.

The absorbance/excitation maximum of FITC was determined by performing a wavelength scan on a solution of 0.025 mg/ml FITC in PBS. The resulting spectrum is shown in Figure 2.5. It can be seen that maximum absorbance of the solution occurs at 495 nm.

The concentration of FITC in the solution was measured from the dialysis medium at the end of each 8-hour time period, based on the assumption that equilibrium was established at this moment between the collagen solution and dialysis medium. The absorbance of FITC was measured with a spectrophotometer (HALO Rb-10) at 490 nm. A standard absorbance-concentration curve for FITC in PBS was prepared by measuring the absorbance of serial dilutions of FITC in PBS with known concentrations. The concentration of free FITC in the dialysis medium was measured by comparing the absorbance of the unknown samples with the standard curve. Once the concentration of FITC was below the resolution of the spectrophotometer, the dialysis was stopped. The standard absorbance-concentration curve is shown in Figure 2.6. It was seen that the absorbance signal becomes saturated for a concentration higher than 0.0625 mg/ml. The linear part of the calibration curve was used to determine the concentration of FITC in the dialysis medium using the Beer-Lambert equation (Ingle & Crouch, 1988). The absorbance of the dialysis medium was read after each change and the concentration of FITC was calculated based on the fitted line (Table 2.9).

After the dialysis was over, the collagen suspension was taken out of the membrane and an equal volume of MES 0.2 M was added to the suspension.

Figure 2.5: Wavelength scan of FITC in PBS solution. FITC absorbance maximum occurs at 495 nm.

Figure 2.6: Standard absorbance-concentration curve for FITC in PBS solutions.

Table 2.9: Absorbance of the dialysis medium after each change at 495 nm with the corresponding FITC concentrations.

	Absorbance @ 495 nm (a.u.)	Free FITC Concentration (mg/ml)
1 st change of the dialysis medium	0.126	0.002
2 nd change of the dialysis medium	0.077	0.001
3 rd change of the dialysis medium	0.005	≈ 0.000

2.3.2.2.1.2 Covalent attachment of FITC-labeled collagen to functionalized Ti-6Al-4V samples

Functionalized F2 (see section 3.2.1) Ti-6Al-4V samples were incubated with the FITC-collagen solution for 1 h. Subsequently, a combined solution of EDC and NHS in MES was added to the samples and the incubation continued for 4 h. Finally, the samples were removed from the solution, gently rinsed with distilled water, dried by an argon flow and stored in air. The final concentration of the used reagents is shown in Table 2.10. A schematic representation of the protein immobilization process can be seen in Figure 2.7

The effect of substrate functionalization and EDC/NHS cross-linkers on the process was determined by repeating the process using bare and functionalized Ti-6Al-4V samples with and without using the EDC/NHS cross-linkers.

Additionally, an attempt was made to increase the amount of immobilized collagen on the samples by performing the incubation of the functionalized (F2) samples with collagen and EDC/NHS twice.

Table 2.10: Final concentration of the reagents used for covalent immobilization of collagen on Ti-6Al-4V substrates

Reagent	Final concentration
Collagen	1 mg/ml
MES	0.1 M
EDC	2.5, 0.25, 0.125 mg/ml
NHS	0.63, 0.063, 0.0315, 0 mg/ml

Figure 2.7: Schematic representation of the covalent immobilization of proteins on AVS-functionalized Ti-6Al-4V samples.

2.3.2.2.1.3 Designing a protocol for the removal of adsorbed proteins from the samples

In order to evaluate the stability of the covalently immobilized proteins versus the physically adsorbed ones, it was necessary to develop a procedure to eliminate the physically adsorbed proteins from the surface of Ti-6Al-4V samples. FITC-labeled collagen type I was chosen as the model protein for these experiments and Ti-6Al-4V samples with adsorbed collagen films were prepared as stated previously in section 2.3.1.2. Samples were immersed in three different solutions: 10 % v/v triton X-100, 10 % v/v tween 20 and 10 % w/v sodium dodecyl sulfate (SDS) in PBS for 24 h. Later, the treatment with SDS was expanded to include concentrations of 10% and 30 % w/v of SDS in PBS and, in some cases, sonication in the said solutions for 30 min. Using a fluorescence microscope, images were captured from the surface of the samples before and after the treatment. The intensity of fluorescence was compared in the images obtained before and after the treatment in order to estimate the stability of adsorbed collagen on the material.

2.3.2.2.2 Immobilization of BSA

2.3.2.2.2.1 Preparation of FITC-labeled BSA solution in MES buffer

A solution of 5 mg/ml albumin in PBS was prepared. FITC was added to obtain a concentration of 0.5 mg/ml and the solution was mixed thoroughly. In order to eliminate the excess of FITC not reacted with BSA, a dialysis process was employed as described in section 2.3.2.2.1.1

Finally, the FITC-labeled BSA solution was mixed with an equal volume of MES 0.2 M buffer.

2.3.2.2.2.2 Covalent attachment of FITC-labeled BSA to functionalized Ti-6Al-4V samples

In order to achieve a covalent bond between the FITC-BSA and the Ti-6Al-4V samples, functionalized (F2) samples were incubated with the FITC-BSA solution for 1 h. Subsequently, a combined solution of EDC and NHS in MES was added to the samples and the incubation period was maintained for 4 h more. The final concentration of the used reagents is shown in Table 2.11. Eventually, the samples were removed from the solution, gently rinsed with distilled water in order to remove any non-adherent albumin from the sample, dried using an argon flow and stored in air.

For comparison purposes, the process was repeated with and without the addition of EDC/NHS cross-linkers with bare and functionalized Ti-6Al-4V samples.

Table 2.11: Final concentration of the reagents used for covalent immobilization of BSA, fibrinogen and fibronectin on Ti-6Al-4V substrates

Reagent	Concentration
BSA	2.5 mg/ml
Fibrinogen	2.5 mg/ml
Fibronectin	200 µg/ml
MES	0.1 M
EDC	2.5 mg/ml
NHS	0.63 mg/ml

2.3.2.2.3 Immobilization of fibrinogen

2.3.2.2.3.1 Preparation of FITC-labeled fibrinogen solution in MES buffer

Initially, a 5 mg/ml fibrinogen solution in PBS was prepared. FITC labeling of fibrinogen proceeded by addition of FITC to the original fibrinogen solution up to a final concentration of 0.5 mg/ml.

In order to eliminate the excess of FITC not reacted with fibrinogen, the dialysis process described in section 2.3.2.2.1.1 was used. Finally, the FITC-labeled fibrinogen solution was mixed with an equal amount of MES 0.2 M buffer.

2.3.2.2.3.2 Covalent attachment of FITC-labeled fibrinogen to functionalized Ti-6Al-4V samples

Initially, FITC-labeled fibrinogen solution was incubated with functionalized (F2) Ti-6Al-4V samples for 1 h. Then, a combined solution of EDC and NHS in MES was added to the samples and the incubation was maintained for 4 h more. The final concentration of the used solutions is shown in Table 2.11. Finally, the samples were extracted from the solution, gently rinsed with distilled water, dried using an argon flow and stored in air.

For comparison and to determine the effects of surface functionalization and the use of cross-linkers, the process was repeated with and without the use of EDC/NHS on bare and functionalized Ti-6Al-4V samples.

2.3.2.2.4 Immobilization of fibronectin

Fibronectin molecules were labeled with FITC by a method described by Hoffmann et al. (Hoffmann et al., 2008). The stock solution of FITC-labeled fibronectin had a concentration of 416.4 µg/ml fibronectin-FITC in carbonate-bicarbonate buffer. The stock solution was diluted in MES buffer to achieve a concentration of 200 µg/ml of fibronectin. Functionalized samples were incubated with this solution for 1 h. Subsequently, a combined solution of EDC and NHS in MES was added to the samples and the incubation continued for 4 h. The final concentration of the solutions is shown in Table 2.11. Finally, the samples were removed from the solution, rinsed gently with distilled water, dried using an argon flow and stored in air.

2.3.2.3 Characterization of samples containing covalently immobilized FITC-labeled proteins

2.3.2.3.1 Removal of non-covalently immobilized protein

In order to remove the proteins simply adsorbed on the samples (not covalently bound) and to confirm the covalent immobilization and stability of the covalently bound proteins, the samples were sonicated in an SDS 10% w/v solution in PBS and stored in a fresh SDS solution for 24 h.

2.3.2.3.2 Fluorescence microscopy

In order to check the presence of the FITC labeled proteins on the substrates, the samples were observed using a fluorescence microscope (Leica DMI 3000B) at emission wavelength of 520 nm. Images of the samples were captured before and after the treatment with SDS detergent.

2.3.2.3.3 Atomic force microscopy

The topography of the samples subjected to the protein immobilization protocol before and after the treatment with SDS was checked by atomic force microscopy (AFM, Cervantes, Nanotec S.L.). Data were analyzed using the WSxM 5.0 software. AFM measurements were performed in the dynamic mode using a pyramidal tip (Olympus OMCL RC800, semi-angle 39°, nominal resonance frequency 69 KHz).

2.3.2.3.4 Protein quantification

The amount of immobilized protein was measured on some collagen-immobilized samples. For this goal, the amount of proteins immobilized on 1X, 1/10X and 1/20X samples after completing the cleaning protocol VIII (see section 2.3.2.4.2) was measured using Micro-BCA protein assay kit (Thermo Scientific). Bicinchoninic acid (BCA) assay was used for protein quantification. The BCA test is based on a color change from greenish to purple when Cu^{2+} are reduced by proteins in an alkaline environment. The resulting solution shows strong absorbance at 562 nm (Smith et al., 1985). The samples were incubated with the working solution of the kit (working solution was prepared by mixing A:B:C:PBS = 25:24:1:50) for 1 h at 60 °C followed by equilibration at room temperature for 30 min. The absorbance values were obtained at 562 nm and compared with a standard curve obtained from serial dilutions of BSA in PBS. Assays were done in duplicates.

2.3.2.4 Preparation of samples containing covalently immobilized protein on Ti-6Al-4V samples for cell cultures

Following the results obtained from the cell culture experiments performed on Ti-6Al-4V samples with adsorbed proteins, collagen type I and fibronectin were chosen as the proteins that could improve the adhesion of BM-MSCs. Therefore, the response of BM-MSCs to covalently immobilized collagen type I and fibronectin on functionalized Ti-6Al-4V samples was assessed.

2.3.2.4.1 Preparation of covalently immobilized collagen on Ti-6Al-4V for cell cultures

A collagen solution in MES was prepared to a final concentration of 2.5 mg/ml collagen and a final concentration of 0.1 M MES. This solution was pipetted on each functionalized (F2) sample and incubated for 1 h. A solution of EDC/NHS in MES was prepared separately and was added to the samples and the incubation continued for 4 h. The final concentration of reagents is shown in Table 2.12.

After the incubation period was over, the samples were rinsed with distilled water.

2.3.2.4.2 Establishing an effective cleaning procedure

In the preliminary results obtained from cell cultures with samples subjected to the protocol of covalent immobilization of collagen, massive cell death was observed after 48 h of incubation. It was hypothesized that cytotoxicity might be related to the presence of free cross-linkers in the culture medium.

To check the possible cytotoxic effect of the cross-linkers, BM-MSCs in a concentration of 5×10^4 cell/ml and 0.5 ml per well were seeded in a 24-well plate and different concentrations of EDC/NHS were added to the wells. The cells were incubated for either 4 or 48 h. Subsequently, the cells were stained with a combined solution of 1 μ l/ml of calcein AM (0.5 μ g/ μ l in DMSO) and 1 μ l/ml propidium iodide (750 μ M in PBS) in DMEM and incubated for another 30 min. Finally, the samples were observed by fluorescence microscopy at emission wavelength of 520 nm and 620 nm for calcein AM and propidium iodide, respectively. An inverted Leica DMIRB microscope equipped with a digital camera (Leica DC100) was used to observe the cells.

Table 2.12: Final concentration of the reagents used for the covalent immobilization of collagen on Ti-6Al-4V substrates for cell culture experiments.

Reagent	Final concentration
Collagen	2.5 mg/ml
MES	0.1 M
EDC	2.5, 0.25, 0.125, 0 mg/ml
NHS	0.63, 0.063, 0.0315, 0 mg/ml

In order to eliminate the possible cytotoxicity of the cross-linkers, a series of cleaning processes were developed and their effectiveness was assessed by performing cell cultures on the resulting samples. All cleaning processes started with a basic rinsing step with distilled water. Several additional steps were added subsequently up to a total of eight complete cleaning procedures. These procedures are summarized in Table 2.13.

Table 2.13: Summary of the cleaning processes for the removal of free EDC/NHS cross-linkers from samples with immobilized collagen.

Protocol	Steps	EDC/NHS concentrations (mg/ml)
I	i) Rinse with distilled water	2.5/0.63 (1X)
II	i) Rinse with distilled water ii) Immersion in PBS for 24 h	2.5/0.63 (1X)
III	i) Rinse with distilled water ii) Immersion in MES for 24 h	2.5/0.63 (1X)
IV	i) Rinse with distilled water ii) Immersion in serum-free DMEM for 24 h	2.5/0.63 (1X)
V	i) Rinse with distilled water ii) Immersion in MES for 2 h iii) Immersion in MES for 2 h iv) Immersion in MES overnight	1.25/0.315 (1/2X) 0.5/0.126 (1/5X) 0.25/0.063 (1/10X)
VI	i) Rinse with distilled water ii) Immersion in PBS for 2 h iii) Immersion in PBS for 2 h iv) Immersion in serum-free DMEM overnight	0.25/0.063 (1/10X) 0.125/0.0315 (1/20X)
VII	i) Rinse with distilled water ii) Immersion in PBS for 2 h iii) Immersion in PBS for 2 h iv) Immersion in MES for 48 h v) Immersion in serum-free DMEM for 24 h	0.25/0.063 (1/10X) 0.125/0.0315 (1/20X)
VIII	i) Rinse with distilled water ii) Immersion in PBS for 2 h iii) Immersion in PBS for 2 h iv) Immersion in MES for 72 h v) Immersion in serum-free DMEM for 12 h vi) Immersion in serum-free DMEM for 12 h	2.5/0.63 (1X) 0.25/0.063 (1/10X) 0.125/0.0315 (1/20X)

2.3.2.4.3 Preparation of covalently immobilized fibronectin on Ti-6Al-4V for cell cultures

Cellular viability and adhesion on Ti-6Al-4V samples with covalently immobilized fibronectin was also addressed. For this aim, a stock solution of 500 µg/ml fibronectin in carbonate-bicarbonate buffer was used. This solution was diluted in MES buffer and functionalized (F2) Ti-6Al-4V samples were incubated with this solution for 1 h. Subsequently, a solution of EDC/NHS in MES was added to the samples and the incubation continued for 4 h. The final concentration of reagents is shown in Table 2.14. Subsequently, the samples were removed from the fibronectin solution and cleaned following the protocol VIII of Table 2.13.

Table 2.14: Final concentration of reagents for covalent immobilization of fibronectin on Ti-6Al-4V substrates for cell culture experiments.

Reagent	Final concentration
Fibronectin	200 µg/ml
MES	0.1 M
EDC	0.125 mg/ml
NHS	0.0315 mg/ml

2.3.2.5 In-vitro cell cultures

In order to evaluate the effect of covalently immobilized collagen type I and fibronectin on cell adhesion, survival and proliferation and to assess the efficacy of the cleaning processes, cell culture experiments were performed using BM-MSCs. These experiments were carried out following the protocol used for cell culture on samples with adsorbed proteins as described in section 2.3.1.6.1.

2.3.2.5.1 Phalloidin/Hoechst staining

2.3.2.5.1.1 Materials

Table 2.15: Materials used for staining and measuring the cell area.

Material	Manufacturer
Phalloidin tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC)	Sigma
Hoechst-33258	Molecular Probes
Paraformaldehyde	Acros Organics

2.3.2.5.1.2 Staining

In addition to the staining protocols mentioned above, phalloidin/hoechst dyes were used to stain actin filaments of the cells. Prior to staining, the samples were washed with PBS and the cells were fixed in a solution of paraformaldehyde 4%. Afterwards, the fixed cells were rinsed with PBS once more, permeabilized using a solution of 0.1 % triton X-100 and stained with a combined solution of 2 µg/ml phalloidin and 0.2 mg/ml hoechst in PBS. Finally, the samples were observed in a fluorescence microscope (Leica DMIRB) equipped with a digital camera (Leica DC100) at emission wavelengths 570 and 461 nm for phalloidin and hoechst, respectively.

2.4 Image analysis

2.4.1 Fluorescence intensity quantification

The intensity of fluorescence was quantified from fluorescence microscopy images using the ImageJ software. Raw images in RGB format were recorded from the fluorescence microscope. In the RGB format, every color is constructed by a combination of red, green and blue colors, and each color is defined by 8-bits of data, which corresponds to an integer between 0 and 255. Thus, a matrix of 1×3 can be used to describe each pixel in which each array depicts the intensity for each primary color. The average of these three values is known as the *gray value*. The average of *gray values* for all of the pixels building

the image can be calculated. This average defines the *mean gray value* for each image. Consequently, for an image comprising of n pixels:

$$\text{Mean gray value} = \frac{1}{3n} \sum_{i=1}^n (\text{Red}_i + \text{Green}_i + \text{Blue}_i)$$

Mean gray values of the images were measured by ImageJ directly by selecting *Analyze/Set Measurements* and selecting the *mean gray value* and *standard deviation* followed by executing *Analyze/Measure*. The software reports the mean gray value and its standard deviation.

2.4.2 Cell counting with ImageJ

The number of viable and dead cells in images obtained by fluorescence microscopy was counted using ImageJ software. Figure 2.8 shows a typical unprocessed image captured at 520 nm using the 2.5X objective. The images have a .TIFF format and possess an RGB color profile. In a first step, the image was split into three different channels that correspond to the primary colors (red, green and blue). This was done by executing the *Image/Color/Split Channels* command in ImageJ, which converts the primary RGB image into three different images, one for each primary color, with a color depth of 8-bit grayscale (Figure 2.9) in which each pixel has a value of intensity of light, zero being total absence of light (black) and 255 being total presence of light (white). Different shades of gray have values in between.

Figure 2.8: A typical image of viable cells captured using fluorescence microscopy at 520 nm with a 2.5X objective.

Figure 2.9: Three grayscale images obtained by splitting the original RGB image into three different channels corresponding to the primary colors: a) Blue b) Red and C) Green.

For the 520 nm wavelength the most relevant information is contained in the green channel. In the green channel image, cells appear white on a black background. The contrast between the white cells and black background in the green channel, allows the automatization of the counting process. In order to separate the background from the foreground (cells) an intensity threshold can be imposed by selecting *Image/Adjust/Threshold* in the menu of the ImageJ software. Application of this command classifies the pixels with an intensity outside the threshold range as background, while the pixels with intensities inside the range will be classified as foreground. In this example (Figure 2.10), the threshold range is selected as 100-255 for separating the foreground from the background. For each image the threshold range was manually adjusted to achieve the most accurate separation between cells and the background for that particular image.

Figure 2.10: An example of using the threshold window to distinguish the cells from the background.

The outcome of this process is an image in which cells appear as black dots on a white background as illustrated in Figure 2.11.

Figure 2.11: An example of the image produced after applying the threshold command to the green channel of a fluorescence image. Cells appear as black dots on a white background.

The number of particles (cells) in the image was counted automatically by executing *Analyze/Analyze Particles* command of the ImageJ software. This command requires defining the range of the sizes of the particles, so that the particles that exceed the maximum size or are below the minimum size are not counted. In this study, the minimum value of the size of the cells was considered to be $10 \mu\text{m}^2$. The particles below that size were considered as background noise and discarded.

The final outcome of this procedure is shown in Figure 2.12 which corresponds to the *Summary* window. The number of cells is obtained, and detailed information on each cell can be seen in the *results* window. Additionally, the software creates a new image with all the counted particles outlined and numbered (Figure 2.13).

Figure 2.12: Summary and Results windows of the ImageJ software. The number of particles is obtained in the *Count* parameter. The *Results* window provides information on the individual particles.

Figure 2.13: An illustration of the outline image generated by the software. Each individual particle is marked and numbered.

The number of dead cells was counted following the same procedure explained in the previous section except that propidium iodide is observed at a wavelength of 620 nm. Consequently, the observation channel in this case corresponds to the red channel. An example of the fluorescence image of dead cells is shown in Figure 2.14. Additionally, since propidium iodide stains the nuclei of the dead cells, the range of sizes of the particles used for cell counting was adjusted accordingly.

The number of viable or dead cells was divided by the area of each image and reported as cells/mm².

Figure 2.14: A typical image of dead cells captured using fluorescence microscopy at 620nm.

2.4.3 Measurement of cell surface area

In addition to the number of viable cells on different samples, the surface area of BM-MSCs adhered on samples with covalently immobilized collagen was measured and compared to that of cells adhered on bare Ti-6Al-4V and polystyrene controls. For this goal, the phalloidin staining images obtained after 4 h of seeding were captured with 20X and 40X objectives and analyzed. Figure 2.15 shows a representative image obtained using the 40X objective. For each sample, 10 individual and isolated cells were selected at random, and the surface area of each cell was measured using the ImageJ software. Calibration grids of the microscope were used to determine the image size. It was found that the images obtained with 20X and 40X objectives correspond to dimensions of $500 \times 375 \mu\text{m}$ and $250 \times 187.5 \mu\text{m}$, respectively. The images of calibrations grids in 20X and 40X objectives can be seen in Figure 2.16. All images were obtained with a resolution of 1600×1200 pixels. These data were used to establish the ratio of pixels/ μm which was defined for each image by using the *Analyze/Set Scale* command.

Figure 2.15: A representative image of phalloidin stained BM-MSC cells obtained by fluorescence microscopy using 40X objective.

Figure 2.16: Images of calibration grids of the microscope. Each side of the squares is 100μm. a) 20X and b) 40X objectives.

The original RGB image was split into three different channels by executing *Image/Color/Split Channels* command and the red channel was used for further processing. Next, by using the *Freehand selections* tool, a separate cell was selected and was copied into a blank 8-bit image file. The outcome can be seen in Figure 2.17. At this point, by executing the *Image/Adjust/Threshold* command, the image was split into foreground and background. In the example presented in Figure 2.17, the threshold was set between 118 and 198 as indicated in Figure 2.18. Consequently, pixels with intensity values between 118 and 198 are considered as foreground while the other pixels are classified as background. The threshold window and the product of thresholding are shown in Figure 2.18 and Figure 2.19.

Figure 2.17: A selected isolated cell copied in a new 8-bit image.

Figure 2.18: The threshold window allows distinguishing the foreground (cells) from the background.

Finally, the surface area of the selected cell was measured by running *Analyze/Analyze Particles*. The measured cell surface area is then shown in the Results window (Figure 2.20).

Figure 2.19: The outcome of applying the threshold command to the image presented in Figure 2.17. The area of the cells appears in black color.

	Area	Mean
1	3977.661	158.470

Figure 2.20: The area of the cells is obtained in the Results window.

2.5 Statistical analysis

The statistical analysis of the data was performed in three complementary steps:

Firstly, the normality of the data was checked with the Shapiro-Wilk test. Secondly, the equality of variances was assessed by Levene's test. Thirdly, Games-Howell test was selected as a suitable post-hoc test and performed on the data in order to detect any significant difference between the means of the groups. The applicability of the Games-Howell test to a dataset was determined by the results obtained from Shapiro-Wilk and Levene's tests on that dataset.

2.5.1 Normality Test

Normality of the data is a prerequisite for most statistical tests and is an underlying assumption in parametric tests. In this work, Shapiro-Wilk test was used in order to test for normality of the data.

Shapiro-Wilk test was chosen since it provides better power compared to the other tests (Steinskog et al., 2007) and is recommended as the best choice for testing the normality of the data when the sample size is relatively small ($n \leq 50$) (Thode, 2002). Shapiro-Wilk test examines the null hypothesis that a sample of $x_1, x_2, \dots, x_n$ originated from a normal population. The test statistic is defined as:

$$W = \frac{(\sum_{i=1}^n a_i x_{(i)})^2}{\sum_{i=1}^n (x - \bar{x})^2}, \text{ For } i = 1, \dots, n$$

Where:

$x_{(i)}$: are the ordered sample values ($x_{(1)}$ being the smallest)

a_i : are the constants generated from the means, variances and covariances of the order statistics of a sample of size n from a normal distribution.

$\bar{x}$: is the sample mean.

The calculated W value is then transformed into p-value by referring to a standard table. α level of the Shapiro-Wilk test was taken as 0.05. If significance $< \alpha$, the null hypothesis is rejected and the hypothesis of normal distribution discarded. Table 2.16 shows an example of the results of Shapiro-Wilk test performed by SPSS Statistics software where the test statistic, (W), df (degree of freedom) and Sig. (significance, P-value) are calculated.

Table 2.16: An example of Shapiro-Wilk test performed by SPSS Statistics software. The calculated P-value is 0.038 which indicates a non-normally distributed data.

		Shapiro-Wilk		
Sample		Statistic	df	Sig.
Cell_No_4H	Control	0.851	12	0.038

Based on the obtained results from both Shapiro-Wilk test, it was determined that the assumption of normality was not met (Shapiro-Wilk P-value <0.05) for most of the data. Thus, the comparison of the means required a statistical test that does not require the data to be normally distributed.

2.5.2 Homogeneity of variances

Another prerequisite for most of statistical tests, such as ANOVA and t-test is homogeneity of variances, so that this hypothesis should be checked a priori. There are a large number of statistical tests that allow detecting differences between variances. However, an assumption for most of these tests is that the data are normally distributed. As it was mentioned before, most of the data analyzed in this work showed significant deviations from a normal distribution. Thus, to check for the equality of variances, Levene's test was chosen, since it is less sensitive to deviations from normality of the data. Levene's test is used to examine if N samples have equal variances. This test checks the null hypothesis that the variances across all groups are all equal. The alternate hypothesis assumes that there is at least one variance that differs from those of the other groups:

$$H_0: \sigma_1^2 = \sigma_2^2 = \dots = \sigma_n^2$$

$$H_a: \sigma_i^2 \neq \sigma_j^2 \text{ for at least one pair}$$

The Levene's test statistic, W , is defined as:

$$W = \frac{(N - k) \sum_{i=1}^k N_i (Z_{i.} - Z_{..})^2}{(k - 1) \sum_{i=1}^k \sum_{j=1}^{N_i} (Z_{ij} - Z_{i.})^2}$$

Where:

$$Z_{ij} = |Y_{ij} - Y_{i.}|$$

k : Number of different groups.

N_i : Number of cases in the i th group.

N : Total number of cases in all groups.

Y_{ij} : Measured variable for the j th case from the i th group.

$$Z_{i.} = \frac{1}{N_i} \sum_{j=1}^{N_i} Z_{ij}: \text{ is the mean of } Z_{ij} \text{ for group } i.$$

$Z_{..} = \frac{1}{N} \sum_{i=1}^k \sum_{j=1}^{N_i} Z_{ij}$ is the mean of all Z_{ij} .

The statistic of the test, W , is approximately F-distributed. If $W > F_{\alpha, k-1, N-k}$, Levene's test leads to the rejection of the null hypothesis. In this case, the parameters are defined as:

α : Is the significance level which was considered 0.05.

$k - 1, N - k$ are degrees of freedom.

Levene's test was performed by SPSS Statistics software. The output of the test generated by SPSS on a dataset as an example is shown in Table 2.17 where the values of Levene's statistic (W), df1 ($k - 1$), df2, ($N - k$) and Sig. (significance, P-value) are calculated.

Table 2.17: An example of Levene's test performed by SPSS Statistics software. The calculated P-value is 0.041 which indicates a dataset with at least one pair of unequal variances across groups.

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
4.631	2	9	0.041

The results of the Shapiro-Wilk and Levene's tests performed in this work for the datasets of each section are provided in Appendix II.

2.5.3 Comparison between means

After performing Levene's test on the obtained data, it was concluded that in most cases, especially the results obtained from cell culture studies, the variances of groups were not homogenous. Therefore, after doing a short review on the statistical tests available in the handbooks, it was decided that Games-Howell pairwise comparison was the most adequate test for comparing the means of different datasets. Games-Howell test is a correction of the Tukey-Kramer test, which accounts for unequal variances and unequal sample sizes and is robust with respect to deviations from normality. Null and alternate hypotheses of the test are:

$H_0: \mu_i = \mu_j \text{ for } \forall i \neq j = 1, \dots, k$

$H_a: \mu_i \neq \mu_j \text{ for at least one pair}$

Where μ_i, μ_j are the population means for the i th and j th group.

The test statistic W is defined:

$$W = \frac{\bar{x}_i - \bar{x}_j}{\sqrt{\frac{s_i^2}{n_i} + \frac{s_j^2}{n_j}}}$$

Where:

$\bar{x}_i$ and $\bar{x}_j$ are the two sample means.

s_i^2 and s_j^2 are the two sample variances.

n_i and n_j are the respective sample sizes from population i and j .

The test statistic is compared to the critical value $\frac{q_{\alpha,k,v}}{\sqrt{2}}$.

In this case, α is the significance level, and $v = \frac{(\frac{s_i^2}{n_i} + \frac{s_j^2}{n_j})^2}{\frac{(\frac{s_i^2}{n_i})^2}{n_i-1} + \frac{(\frac{s_j^2}{n_j})^2}{n_j-1}}$

If the calculated $W > \frac{q_{\alpha,k,v}}{\sqrt{2}}$ the null hypothesis is rejected in favor of the alternate hypothesis (Games & Howell, 1976).

The Games-Howell test was carried out by SPSS Statistics software. The output of the software for an example dataset with three groups can be seen in Table 2.18 where Sig. (significance, P-value) < 0.05 denotes statistical significance between the two compared groups.

Table 2.18: An example of Games-Howell test performed by SPSS Statistics software. The P-value is calculated for each pairwise comparison.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Fibronectin-immobilized Ti-6Al-4V	24.703	0.225
		Bare Ti-6Al-4V	66.659*	0.000
	Fibronectin-immobilized Ti-6Al-4V	Control	-24.703	0.225
		Bare Ti-6Al-4V	41.956*	0.009
	Bare Ti-6Al-4V	Control	-66.659*	0.000
		Fibronectin-immobilized Ti-6Al-4V	-41.956*	0.009

2.5.4 Use of statistical software

All the statistical analyses and calculations were performed by IBM SPSS Statistics 20. The graphs were created by Kaleida Graph 4.5.2 and the statistical tables by IBM SPSS Statistics 20.

All the results are presented as mean value $\pm$ SE:

SE is the standard error of the mean defined as: $SE = \frac{s}{\sqrt{n}}$

Where:

s : is the sample standard deviation.

n : is the sample size.

3 RESULTS AND DISCUSSION

3.1 Characterization of as-polished Ti-6Al-4V substrates

3.1.1 Profilometry

The Ti-6Al-4V samples were polished using different grit No. sandpapers: 400, 1200 and 4000. The surface roughness of the samples was analyzed using profilometry. Figure 3.1 shows typical profiles for 400, 1200 and 4000 samples.

Figure 3.1: Characteristic Surface profiles of a) 400 b) 1200 and c) 4000 samples measured by profilometry.

Overall, for every finishing, the measurements were performed on 10 samples and three measurements were made on each sample (30 measurements in total). The calculated RMS roughness of the 400, 1200 and 4000 samples were compared and are shown in Figure 3.2. It can be seen that after polishing with a higher grit number (i.e. finer) sandpaper, the RMS roughness of the substrates only decreases slightly. In this regard, the results of the G-H pairwise comparisons (Table 3.1) demonstrated that these

differences in RMS roughness between the samples were not statistically significant ($P>0.05$). A limiting factor in these measurements was supposedly the accuracy of the profilometer, which was in the range of 1 μm . Consequently, differences in roughness between the samples below this limit could not be detected.

Figure 3.2: Comparison of the measured RMS roughness for 400, 1200 and 4000 Ti-6Al-4V samples obtained by profilometry.

Table 3.1: Results of Games-Howell test performed on the RMS roughness data of as-polished 400, 1200 and 4000 samples obtained by profilometry.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
400	1200	0.2	0.998
	4000	2.9	0.669
1200	400	-0.2	0.998
	4000	2.7	0.700
4000	400	-2.9	0.669
	1200	-2.7	0.700

In a similar study Osathanon et. al. (Osathanon et al., 2011) measured the average roughness values, R_a , of Ti-6Al-7Nb samples polished with 180, 400 and 1200 grit SiC sandpapers by profilometry. They found that the R_a values of polished 180 and 400

samples were statistically different from those of the 1200 samples, in contrast with our findings. However, the lateral scanning size in their work, was over 0.5 mm length of the samples, much smaller than the scan length of 10 mm used in this work. Difference in roughness between 180, 600 and 1200 grit polished Ti-6Al-4V samples measured by profilometry were also reported by Deligianni et. al. (Deligianni et al., 2001). Although, the scale in which the measurements were performed is not mentioned in that study.

Consequently, it seems that the difference between the results obtained in this study and other published papers in terms of the surface topography of polished samples might originate from the differences in the scan size of the measurements and possibly, from the different resolution of the profilometers.

Thus, in order to draw more robust conclusions, atomic force microscopy was used to characterize the effect of polishing on surface roughness. These results were used to choose the most suitable sample finishing for further studies.

3.1.2 Atomic force microscopy

10 samples of each surface finishing were measured in three different scan sizes: 500 nm, 5 μm and 50 μm by atomic force microscopy (AFM). Two measurements for each scan size were taken on each sample. Representative AFM micrographs for a 5 μm scan size are shown in Figure 3.3. In the topography images (Figure 3.3 a-c), the scratches produced by the polishing step are apparent and their size can be quantified from the profiles, such as those presented in Figure 3.3 d-e. It can be seen that by using a finer sandpaper, the size of the scratches is reduced, and their morphology becomes narrower and shallower. For the 400 samples, the width of the scratches is about 2 μm and their depth is about 70 nm, for 1200 sample the width is about 1.5 μm and the depth is about 40 nm. Finally, for 4000 samples, the scratches have a width of about 1 μm and a depth of about 25 nm. This reduction in the size of the scratches was related to the smaller abrasive particles of 4000 sandpaper compared to the other two sandpapers.

The RMS roughness of the samples was quantified from the surface topography images using the WSxM software. The results are shown in Figure 3.4. It can be seen that by applying a finer sandpaper, the RMS roughness of the samples has been reduced in all scan sizes, although this reduction is more apparent in 50 and 5 μm scan sizes.

Figure 3.3: Representative atomic force microscopy topography images (a-c) and surface profiles (d-f) of as-polished samples. (a,d) 400, (b,e) 1200 and (c,f) 4000 samples, respectively.

Figure 3.4: Comparison of RMS roughness values of 400, 1200 and 4000 samples measured by atomic force microscopy.

Table 3.2 shows the results of the Games-Howell test performed on the RMS roughness data. 4000 samples in all scan sizes possess the smoothest surface. In 50 μm scan size,

the difference between RMS roughness of 4000, 1200 and 400 samples is statistically significant. However, in 5 and 0.5 μm scan sizes, only 4000 and 400 samples show meaningful differences in RMS roughness values. Other topographical parameters were determined from the AFM micrographs and are shown in Table 3.3.

Since one of the objectives of this work required the identification of the proteins on the material, the smoothest surface (i.e. 4000 samples) were considered as the most suitable substrate for the rest of the experimental work. In addition, this choice allowed evaluating the possible increase in roughness due to the presence of the functional layer under the most demanding conditions.

Table 3.2: The results of Games-Howell test performed on RMS roughness data of as-polished samples obtained by atomic force microscopy.

Dependent Variable	(I) Sample2	(J) Sample2	Mean Difference (I-J)	Sig.
RMS_Scansize_50	400	1200	77.2*	0.000
		4000	105.7*	0.000
	1200	400	-77.2*	0.000
		4000	28.5*	0.000
	4000	400	-105.7*	0.000
		1200	-28.5*	0.000
RMS_Scansize_5	400	1200	14.1*	0.004
		4000	19.0*	0.000
	1200	400	-14.1*	0.004
		4000	4.9	0.215
	4000	400	-19.0*	0.000
		1200	-4.9	0.215
RMS_Scansize_0.5	400	1200	1.2	0.091
		4000	1.6*	0.006
	1200	400	-1.2	0.091
		4000	0.4	0.658
	4000	400	-1.6*	0.006
		1200	-0.4	0.658

*. The mean difference is significant at the 0.05 level.

In a similar study, P. Cacciafesta et al. (Cacciafesta et al., 2001) measured the profiles and roughness of commercially pure titanium by atomic force microscopy. Their

measurements revealed a RMS value of 160 ± 110 nm for grit-600 polished samples over a $20 \mu\text{m} \times 20 \mu\text{m}$ scan area. This result is consistent with the values presented in Table 3.3.

The analysis of the surface roughness highlights two main points in order to compare the data presented in different studies. First, roughness measurement should be performed on similar scan sizes. Second, the geometry of the sensor (i.e. the AFM tip) influences the resolution of the measurements and, consequently, the values of roughness (Jandt, 2001).

Table 3.3: Topographical parameters for 400, 1200 and 4000 samples in the three different scan sizes.

Quantity Sample	Root Mean Square Roughness (RMS) (nm)	Maximum Roughness (nm)	Average Height (nm)	Average Roughness (R _a) (nm)	
400	140±10	1140±70	580±40	110±10	50 µm Scan Size
1200	62±2	800±100	290±10	47±1	
4000	34±3	460±40	190±20	25±2	
400	35±3	270±20	130±10	27±3	5 µm Scan Size
1200	21±2	190±20	80±7	16±2	
4000	16±2	140±10	70±8	13±2	
400	3.9±0.4	36±3	17±2	3.0±0.3	0.5 µm Scan Size
1200	2.7±0.4	24±3	11±2	2.0±0.3	
4000	2.3±0.3	20±1	9.8±0.7	1.8±0.2	

3.1.3 In-vitro cell cultures

3.1.3.1 Cell adhesion/viability

In order to evaluate cell adhesion, proliferation and survival on bare Ti-6Al-4V, MC3T3-E1 cells were cultured on as-polished 400,1200 and 4000 samples. The morphology of the Calcein/PI stained cells at 4 and 48 h after seeding is shown in Figure 3.5. At 4 h after seeding the cells are adhered on all the samples but are not completely spread. After 48 h, the cells are more spread and the number of cells is larger on all samples

The number of cells was quantified from the obtained fluorescence images. The results of cell counting are shown in Figure 3.6 and Table 3.4. As it can be seen, the number of viable MC3T3-E1 cells on all Ti-6Al-4V samples (400, 1200 and 4000) is roughly equal at each time period, and a marginal increase from 4 to 48 h after seeding is apparent. In contrast, the controls (cells seeded on blank tissue culture plates) show a higher number of cells compared to the Ti-6Al-4V samples, and the increase in the number of cells after 48 h of seeding is substantial. Statistical analysis supports the initial assessment of the data and no statistical differences were observed between the number of cells adhered on 400, 1200 and 4000 samples (Table 3.4) at each time period.

Figure 3.5: Fluorescence microscopy images of calcein/PI stained MC3T3-E1 cells cultured on 400, 1200, 4000 as-polished Ti-6Al-4V and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.6: The results of a) cell counting and b) cellular viability at 4 and 48 h of seeding for MC3T3-E1 cells seeded on 400, 1200, 4000 as-polished Ti-6Al-4V and polystyrene control samples.

Table 3.4: The results of Games-Howell test performed on cell counting data of Ti-6Al-4V as-polished samples.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_4h_normalized	Control	400	28.939*	0.031
		1200	41.173*	0.001
		4000	42.947*	0.006
	400	Control	-28.939*	0.031
		1200	12.234	0.314
		4000	14.008	0.492
	1200	Control	-41.173*	0.001
		400	-12.234	0.314
		4000	1.774	0.998
	4000	Control	-42.947*	0.006
		400	-14.008	0.492
		1200	-1.774	0.998
Cell_48h_normalized	Control	400	107.463*	0.000
		1200	93.687*	0.000
		4000	113.101*	0.000
	400	Control	-107.463*	0.000
		1200	-13.776	0.667
		4000	5.638	0.942
	1200	Control	-93.687*	0.000
		400	13.776	0.667
		4000	19.414	0.554
	4000	Control	-113.101*	0.000
		400	-5.638	0.942
		1200	-19.414	0.554

*. The mean difference is significant at the 0.05 level.

3.1.3.2 Cell proliferation

Proliferation of MC3T3-E1 cells on the as-polished samples was also investigated. Figure 3.7 shows the XTT absorbance values after 1 and 7 days of incubation for the different samples. The results of the statistical analysis can be seen in Table 3.5. The increase in absorbance values demonstrates the increase in the number of cells during the incubation period. As shown previously from the calcein/PI studies, the cells adhered on all samples, although, the control samples show a higher number of cells. After 7 days, there is a

substantial increase in the absorbance values of the controls, in contrast with a much more modest increase of the number of cells on Ti-6Al-4V samples.

No clear difference in the number of cells could be established between 400, 1200 and 4000 samples at any of the incubation times.

Figure 3.7: Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on 400, 1200, 4000 as-polished Ti-6Al-4V and polystyrene control samples at 1 and 7 days after seeding.

Based on these results, it was established that in this scale, the roughness of the samples did not have any effect on the adhesion, viability and proliferation of MC3T3-E1 cells on the material. Consequently, 4000 samples were selected for the rest of the experimental work in order to facilitate the analysis of roughness on the functionalized samples and of the proteins adsorbed or immobilized on the surface.

The analysis of the previously published data leads to contradictory results. Cell cultures on titanium samples polished with 180, 600 and 1200 grit papers using human bone marrow stromal cells yielded a higher number of cells on the rougher 180-grit polished samples from the earliest (30 min) to the longest (16 days) incubation times (Deligianni et al., 2001).

Table 3.5: The results of Games-Howell test performed on cell proliferation data of as-polished samples.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Abs_Day1	Control	400	.028111*	.002
		1200	.038266	.140
		4000	.039875*	.022
	400	Control	-.028111*	.002
		1200	.010154	.895
		4000	.011764	.656
	1200	Control	-.038266	.140
		400	-.010154	.895
		4000	.001610	1.000
	4000	Control	-.039875*	.022
		400	-.011764	.656
		1200	-.001610	1.000
Abs_Day7	Control	400	.266333*	.000
		1200	.248362*	.000
		4000	.269140*	.000
	400	Control	-.266333*	.000
		1200	-.017971	.947
		4000	.002807	1.000
	1200	Control	-.248362*	.000
		400	.017971	.947
		4000	.020778	.905
	4000	Control	-.269140*	.000
		400	-.002807	1.000
		1200	-.020778	.905

*. The mean difference is significant at the 0.05 level.

Similar results were found with SaSO-2 human osteoblast-like cell line on Ti-6Al-4Nb alloy polished with 180, 400 and 1200 grit SiC sandpapers (Osathanon et al., 2011). In this case no differences were observed between the number of cells at 20 min after seeding, but a significantly higher number of cells were present on the 180-grit polished samples 72 h after seeding. There is an abundance of studies in support of these results which state that rough surfaces favor cell attachment and proliferation (Nishimoto et al., 2008, Degasne et al., 1999).

In contrast, a similar experiment using MC3T3-E1 cells on 100, 320, 600, 1000 and 2000 grit polished Ti-6Al-4V samples yielded a different result (Wu et al., 2015). After 5 and 7 days of culture, a significantly higher number of cells was found on the smooth 600 and 1000 samples, compared with rougher ones. This result was supported by other studies (Anselme et al., 2000, Linez-Bataillon et al., 2002) in which the survival and proliferation of MC3T3-E1 cells on grit 80, 1200 and 4000 polished Ti-6Al-4V was assessed. After 3 days of culture, the highest number of cells was found in the smooth grit 4000 polished samples.

Finally, another study (Mante et al., 2003) on the proliferation on human bone marrow stromal cells on grit 240 and 600 Ti-6Al-4V polished samples did not find significant differences at 48 h after seeding. The results obtained in this work are consistent with this later data, since no influence of surface roughness on cell survival and proliferation is found.

The contradictory findings discussed above may be the results of several factors. First, results might depend on the cell line used in each study. For example, one study showed an increased proliferation of SaOS2 osteoblast-like cells on rougher titanium surfaces after 48 h of culture (Degasne et al., 1999) while other studies show a reduced proliferation of MG-63 (Martin et al., 1995, Lincks et al., 1998) and U2OS (H. Huang et al., 2004) cell lines on rougher titanium surfaces. However, some contradictory results are found even if the same cell line (MC3T3-E1) is considered. In this case, there are studies that suggest that MC3T3-E1 cells proliferate better on smoother surfaces (Anselme et al., 2000, Linez-Bataillon et al., 2002) , while others indicate that rougher surfaces favor MC3T3-E1 proliferation (Nishimura & Kawai, 1998, Keller et al., 1994, Bowers et al., 1992).

Apart from the cell lineage, cell culture conditions (such as serum concentration) could also affect cell attachment. A study performed by Lee et al. stated that neonatal rat calvarial osteoblast attachment on Ti-6Al-4V samples was not affected by surface roughness of the samples when the cells were cultured in culture medium containing 10% FBS. However, at culture conditions containing 4% FBS, the cells' attachment was significantly higher on smoother surfaces (T. M. Lee et al., 2002).

Other researchers have stated that the behavior of cells on different surfaces is dependent on the kinetics of protein adsorption on that surface and not on the cells themselves. In

this regard, Deligianni et al. (Deligianni et al., 2001) stated that a higher amount of total protein was adsorbed on rougher Ti-6Al-4V samples from a culture medium containing 10% fetal calf serum. It should be stated that protein adsorption on smooth and rough surfaces is not identical for all proteins, as it has been shown that smooth surfaces favor the adsorption of bovine serum albumin, while higher amounts of fibronectin are adsorbed on rougher surfaces (Deligianni et al., 2001).

Finally, an additional factor that might affect the outcome of the studies is the use of different methods, scales and units for the characterization of surface roughness. In the studies mentioned above, different profilometry techniques and scan sizes for the measurement of surface roughness are used. This lack of standardization in the characterization of surface roughness could also contribute to the contradictory results found in the literature.

3.2 Preparation and characterization of AVS-functionalized samples

In this study, eight functionalization conditions were assessed and the following processing parameters: evaporation temperature, T_{Evap} , activation temperature, T_{Act} , and argon pressure, P_{Ar} were varied, and the resulting samples were characterized in terms of surface amine density and roughness.

3.2.1 Fluorescent labeling of amine groups

The presence of amine groups on samples functionalized under different functionalization conditions was assessed. The functional surface amines were labeled with FITC and observed with a fluorescence microscope. Since the observation conditions were the same for all samples, the surface concentration of amine groups could be compared between functionalized samples by comparing the intensity of fluorescence. In this regard, a higher number of amine bonding sites on the samples results in more FITC molecules being immobilized and thus, a higher fluorescence intensity. Large scale labeling and imaging of FITC on amino-functionalized substrates has been employed successfully by various researches for labeling amino groups (Baumgärtel et al., 2013, Ara et al., 2007, Gartmann & Brühwiler, 2009).

Figure 3.8 shows illustrative fluorescence microscopy images of samples functionalized under all conditions. In these images, the distribution and surface density of amine

functional groups can be observed. A non-functionalized bare Ti-6Al-4V sample also incubated with FITC is shown and used as control for comparison. (Figure 3.8 c). These results allow establishing some correlations between the functionalization conditions and the surface density of amines. Thus, the effect of varying T_{evap} from 130 °C to 170 °C can be seen in the middle column (Figure 3.8 b,e and h). It can be seen that the distribution of amino groups produced at $T_{\text{Evap}}=150$ °C is more homogeneous compared with the other two conditions. The effect of varying T_{Act} between 700 °C and 800 °C can be seen in the middle row (Figure 3.8 d, e and f). Their comparison indicates that a $T_{\text{Act}}=750$ °C leads to a higher concentration of amino groups. Lastly, the effect of varying P_{Ar} is shown in Figure 3.8 a,e and i. It can be seen that with increasing Ar pressure, the concentration of amines deposited on the substrates increases. Figure 3.8 g ($T_{\text{Act}}=800$ °C, $P_{\text{Ar}}=2$ mbar) exhibits the effect of increasing the activation temperature and argon pressure simultaneously. In this case, the fluorescence image shows a homogeneous functionalized surface with a high density of amino groups.

Figure 3.8: Fluorescence microscopy images of AVS-functionalized samples under different processing conditions. (Scale bar = 1 mm).

The intensity of the fluorescence was quantified in all the obtained images and compared (Figure 3.9). The highest fluorescence intensity corresponds to the samples functionalized with the following combination of processing parameters: T_{Evap} : 150°C, T_{Act} : 750 °C, P_{Ar} : 1 mbar (from now on samples F1) and T_{Evap} : 150 °C, T_{Act} : 750 °C, P_{Ar} : 2 mbar (from now on samples F2). The small error bar in these samples reveals that the differences in the intensity of fluorescence in various points was minimal and, consequently, that these processing conditions lead to a homogenous distribution of amine groups on the surface of Ti-6Al-4V substrates. In contrast, the large error bars found in samples processed under other conditions (for instance, T_{Evap} : 170°C, T_{Act} : 750°C, P_{Ar} : 1mbar) reflect a non-homogeneous distribution of the surface amino groups. The inhomogeneity in the fluorescence of some samples is also appreciated Figure 3.8.

Figure 3.9: Comparison of fluorescent intensity of functionalized samples with different functionalization conditions.

3.2.2 Atomic force microscopy

The surface topography of Ti-6Al-4V samples functionalized under different processing conditions was assessed with atomic force microscopy. Figure 3.10 shows representative AFM micrographs of the Ti-6Al-4V samples functionalized under different conditions. A micrograph of a non-functionalized Ti-6Al-4V sample is also shown for comparison. It can be seen that after functionalization, the grooves and scratches remaining from the polishing step are no longer visible in any of the samples. In most of the functionalized samples, the formation of a largely homogeneous film on the Ti-6Al-4V surface is apparent. The comparison of RMS roughness of the samples functionalized under different conditions can be seen in Figure 3.11 where it is also compared with a bare Ti-6Al-4V sample. In most samples, functionalization leads to a reduction in the RMS roughness although some samples show an increase in this value that is associated with the formation of an inhomogeneous film.

Figure 3.10: Atomic force microscopy topography images of samples functionalized under different conditions. Scan area 5 μm × 5 μm.

Figure 3.11: Comparison of the RMS roughness of samples functionalized under different processing conditions.

A summary of the RMS roughness and other parameters that define the topography of the samples can be seen in Table 3.6.

According to the obtained results, it was determined that F1 and F2 samples showed a suitable combination of surface amine density and low RMS roughness (<5nm).

The topography observed in AVS-functionalized samples compare well with previous studies done by other researchers. Matinlinna et al. (Matinlinna et al., 2004) examined the surface topography of amino-functionalized commercially pure titanium by atomic force microscopy. 1200-grit polished samples were functionalized by immersion in liquid APTS. Their results also demonstrated a reduction in surface roughness over a scan area of $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$ and a reduction from an RMS value of 150 nm in bare titanium to 88

nm in the functionalized samples was found. This trend was also observed in another study (Pop-Georgievski et al., 2015) in which the titanium samples were functionalized by immersion in a APTS/toluene solution. In this case a scan area of $0.5\ \mu\text{m} \times 0.5\ \mu\text{m}$ was used and a value of $\text{RMS} = 1.1 \pm 0.2\ \text{nm}$ was measured. This value is comparable to those of the F1 and F2 samples (Table 3.6). In comparison with the results presented here, the functionalized Ti-6Al-4V produced by AVS with F1 condition led to the formation of a smoother layer with a RMS roughness value of $0.3 \pm 0.1\ \text{nm}$.

Since the ultimate objective of this work is the development of a system that allows the covalent immobilization of biomolecules on Ti-6Al-4V, the conditions which produced the highest amine density and lowest surface roughness were selected as the optimal functionalization conditions to continue with the rest of the experimental work. As shown above, these conditions correspond to the F1 and F2 samples. Consequently, these samples were used for subsequent characterization in terms of:

- Reproducibility of the AVS process
- Measurement of the film thickness
- Contact angle measurement
- Nanoscratch test
- Cell viability and proliferation studies and,
- Stability of the film upon incubation in a liquid medium

Table 3.6: Roughness parameters for functionalized samples in different scan sizes.

Quantity Sample	RMS Roughness (RMS) (nm)	Maximum Value (nm)	Average Height (nm)	Average Roughness (R _a) (nm)	
130°C-750°C-1mbar-20'	2.1±0.8	18±5	7±1	1.5±0.5	5 µm Scan Size
170°C-750°C-1mbar-20'	1.3±0.4	20±3	4.7±0.4	1.0±0.4	
150°C-700°C-1mbar-20'	4.8±0.1	90±10	30±10	3.6±0.1	
150°C-800°C-1mbar-20'	16±7	120±40	70±20	13±6	
150°C-750°C-0.5mbar-20'	26±4	257.1±0.2	140±30	18±5	
150°C-750°C-1mbar-20' (F1)	1.5±0.2	20±2	7±2	1.1±0.2	
150°C-750°C-2mbar-20' (F2)	0.4±0.7	5±30	1.8±0.5	0.3±0.8	
150°C-800°C-2mbar-20'	10.0±0.4	79±6	33±3	7.9±0.1	
130°C-750°C-1mbar-20'	0.439±0.004	3.3±0.2	1.6±0.1	0.347±0.005	0.5 µm Scan Size
170°C-750°C-1mbar-20'	0.42±0.03	3.31±0.08	1.77±0.08	0.33±0.02	
150°C-700°C-1mbar-20'	1.0±0.6	20±10	10±8	0.5±0.2	
150°C-800°C-1mbar-20'	0.7±0.1	10±4	2.8±0.1	0.56±0.02	
150°C-750°C-0.5mbar-20'	2.98±0.04	24±4	10.7±0.7	2.4±0.1	
150°C-750°C-1mbar-20' (F1)	0.339±0.005	3.4±0.2	1.35±0.02	0.266±0.004	
150°C-750°C-2mbar-20' (F2)	0.427±0.009	5.1±0.9	1.765±0.004	0.340±0.006	
150°C-800°C-2mbar-20'	1.3±0.3	10±3	4.7±0.6	1.0±0.2	

3.2.3 `Reproducibility of the AVS process

In order to assess the reproducibility of F1 and F2 conditions, six different samples were produced under F1 and F2 functionalization parameters and characterized by fluorescence microscopy and AFM.

3.2.3.1 Fluorescence study

Figure 3.12 shows the fluorescence intensity measured on six samples produced under F1 and F2 sets of parameters. The reproducibility of the process is indicated by the small error bars that were determined for each set.

Figure 3.12: Comparison of fluorescence intensity of AVS functional layers produced under the F1 and F2 processing conditions. Six samples were deposited under each set of conditions.

3.2.3.2 Atomic force microscopy

The topography of six samples functionalized under F1 and F2 processing conditions was determined by AFM. The topography micrographs of a typical F1 and F2 sample in 50, 5 and 0.5 μm scan sizes can be seen in Figure 3.13.

The RMS roughness of these samples was quantified and is shown in Figure 3.14. Both F1 and F2 functionalization conditions lead to the deposition of layers with low values of RMS roughness (RMS roughness < 10 nm in 5 μm scan size) however, the F1 samples possess slightly smoother surfaces in all the scan sizes.

Figure 3.13: Representative atomic force microscopy images of the surface of a,b,c) F1 and d,e,f) F2 functionalized samples. Scan area a,d) $50\ \mu\text{m} \times 50\ \mu\text{m}$, b,e) $5\ \mu\text{m} \times 5\ \mu\text{m}$ and c,f) $0.5\ \mu\text{m} \times 0.5\ \mu\text{m}$.

The reproducibility of the process in terms of the topography of the functionalized layer is indicated again by the small error bars. The values of the RMS roughness and other parameters that describe the topography of the samples are shown in Table 3.7.

In summary, these results show the robustness of the AVS technique reflected in the high reproducibility of the F1 and F2 samples in terms of the surface density of amine groups and topography.

Figure 3.14: Comparison of the RMS roughness of F1 and F2 reproduced samples in 50, 5 and 0.5 μm scan sizes.

Table 3.7: Roughness parameters of F1 and F2 samples in different scan sizes.

Quantity Sample	RMS Roughness (RMS) (nm)	Maximum Value (nm)	Average Height (nm)	Average Roughness (R_a) (nm)	
F1	13±2	220±30	76±9	10±1	50 μm Scan Size
F2	27±8	380±60	145±7	21.4±0.6	
F1	4±1	45±5	15±3	3.8±0.8	5 μm Scan Size
F2	7.4±0.1	68±4	32±3	5.7±0.1	
F1	0.8±0.1	10±1	3.2±0.3	0.57±0.04	0.5 μm Scan Size
F2	1.2±0.2	12±1	4.6±0.5	0.9±0.1	

3.2.4 Thickness measurement

The thickness of any thin film is an important parameter than can influence its stability under different conditions. In order to measure the thickness of the functional layers, a resin mask was applied on the surface of bare Ti-6Al-4V samples. Subsequently, the samples were functionalized with either F1 or F2 processing conditions. Finally, the mask was removed by immersion in acetone. The step between the functionalized and the non-functionalized regions of the samples, previously covered by the resin, was measured by atomic force microscopy and corresponds to the thickness of the layer.

Figure 3.15 b and e show optical images in which the position of the cantilever with respect to the geometry defined by the resin mask in the F1 and F2 samples can be observed. Illustrative AFM micrographs of that regions are presented in Figure 3.15 a and d, and representative profiles across the step are shown in Figure 3.15 c and f.

From these profiles the thickness of the functional layer is found to be ~100 nm for the F1 condition and ~200 nm for the F2 condition. As it can be seen in the profiles (Figure 3.15 c and f), there seems to be a peak at the edge between the functionalized and non-functionalized regions. The presence of this peak was confirmed by scanning electron microscopy (Figure 3.16). Although, the origin of the peak is not clear, it might be related with the deposition and accumulation of the functional layer on the lateral surface of the resin mask.

Figure 3.15: Atomic force microscopy a,d), optical images b,e) and surface profiles c,f) of the step created between the functionalized and non-functionalized regions of the surface, a,b,c) F1 and d,e,f) F2 samples.

Figure 3.16: Scanning electron microscopy images of the step created between the functionalized and non-functionalized regions of the surface in a) F1 and b) F2 samples. The accumulation of material between both regions is apparent from these micrographs.

3.2.5 Contact angle measurements

Wettability is an important factor in the characterization of biomaterials. The importance of this property is related with the interaction established between the material and physiological fluids upon implantation in the body.

Contact angle measurements are usually employed to determine the hydrophilicity of a material. Consequently, the water contact angle of F1, F2 and bare Ti-6Al-4V samples was measured using the sessile drop method. Figure 3.17 shows illustrative images used for contact angle measurements. The obtained data are summarized in Figure 3.18. As it can be seen, the water contact angle was found to be $66.2 \pm 0.5^\circ$, $58.9 \pm 0.7^\circ$, $57.8 \pm 0.6^\circ$ for F1, F2 and non-functionalized samples respectively.

Figure 3.17: Lateral optical images of water drops resting on the surface of a) F1, b) F2 and c) bare Ti-6Al-4V samples.

The Games-Howell test performed on these data (Table 3.8), revealed that the water contact angle for F1 samples was significantly higher than that of F2 or bare Ti-6Al-4V samples. No statistically significant difference was found between the contact angle of F2 and non-functionalized Ti-6Al-4V samples.

On the whole, the AVS-functionalized Ti-6Al-4V samples demonstrated moderate water contact angles. It is suggested that highly hydrophobic or highly hydrophilic surfaces are not optimal for cell growth while surfaces with moderate wettability are most supportive for cell growth and proliferation (Cai et al., 2006).

When a biomaterial is put inside the body, it comes in contact with the body fluids and a thin layer of the fluid containing plasma proteins, is formed on the surface of the biomaterial. The successful formation of this layer controls the subsequent adhesion and spreading of different cell types and ultimately, the success or failure of the biomaterial in establishing direct contact with the host body. The surface wettability of the biomaterial therefore, is of crucial importance in its final acceptance by the host.

Comparison of the contact angle measurements on bare and amino-functionalized Ti materials in the available literature lead to some contradictory results. For instance, Mussano et al. used APTS as precursor in a polymer plasma deposition system to functionalize commercially pure titanium. Their water contact angle measurements led to values of $86 \pm 2^\circ$ and $62 \pm 3^\circ$ for titanium and amino-functionalized titanium, respectively (Mussano et al., 2017). In another study, Cai et al. measured the water contact angles for titanium substrates functionalized by immersion in a solution of 7-octenyltrichlorosilane and APTS in pentane. They also observed a decrease of the water contact angle from $80.7 \pm 2.4^\circ$ for titanium to $70.3 \pm 1.5^\circ$ for the functionalized material (Cai et al., 2006). Dubruel et al. (Dubruel et al., 2006) examined the effect of the concentration of amino groups on the surface of functionalized titanium on its water contact angle. These authors obtained a value of $67 \pm 5^\circ$ for the contact angle of commercially pure titanium. Subsequently, the material was functionalized by immersion in APTS in pentane solutions with increasing concentrations of APTS ranging from 0.001 to 1 vol%. It was observed that an increase in the concentration of APTS led to a reduction of the contact angle from $64 \pm 13^\circ$ to $57 \pm 1^\circ$ for 0.001 to 1 vol% solution, respectively. An alternative study (Nebe et al., 2007) used allylamine as precursor in a low-pressure plasma reactor

for amino-functionalization of titanium. In this case, the contact angle for untreated titanium was $78 \pm 3^\circ$ which was reduced to $48 \pm 3^\circ$ after functionalization.

All these studies suggest that amino-functionalization of Ti leads to a decrease in the contact angle, which implies an increase in the hydrophilicity of the surface. However, there are a number of studies that report the opposite trend. For instance, it was reported that the contact angle of titanium disks was initially $49.6 \pm 1.5^\circ$. The contact angle however, increased to $58.5 \pm 1.5^\circ$ upon functionalization by immersion in a 10 %w/v aqueous solution of APTS (Yoon et al., 2014). Studies on a different titanium alloy, Ti-16Hf-25Nb also reported an increase from the initial value of 62.1° for the bare material up to 75.2° after functionalization by immersion in a pentane solution containing diisopropylethylamine and APTS (Paredes et al., 2015). Finally, an increase of the contact angle from a value of 50° for the pristine titanium to a value of $72 \pm 0.1^\circ$ was reported after functionalization in a solution of 0.1% v/v APTS in dry toluene (Pop-Georgievski et al., 2015). These latter values are comparable with those presented in this work.

There are some factors which can contribute to the apparent contradictions among different studies. First of all, different procedures for amino-functionalization of the titanium samples might lead to different contact angles. Thus, although a process may lead to the successful formation of an aminated surface, the overall surface density of amines depends on the processing conditions. Besides, the ratio between primary and secondary amines on the surface may also depend on the details of the functionalization process. In this regard, secondary amines possess a less hydrophobic character compared to primary amines (Pawlak et al., 2013, Burke & Barrett, 2003) so that, the differences in the ratio of secondary and primary surface amines could play a role in the wettability of the surface.

In addition to the functionalization process, another important factor that influences the contact angle of the amino-functionalized titanium samples, is the titanium surface pre-treatment (cleaning, etc.), which could also contribute to the contradictions found in the cited studies. In this regard, the presence of organic contaminants could give a rise to the hydrophobicity of the titanium substrates while the presence of a hydrophilic oxide layer might lead to an increased hydrophilicity (Yuan & Lee, 2013, Watanabe, 2009).

Figure 3.18: Water contact angles measured from F1, F2 and bare Ti-6Al-4V samples.

Table 3.8: Results of Games-Howell test performed on the water contact angle data.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
F1	F2	7.3*	.000
	Non-functionalized Ti-6Al-4V	8.5*	.000
F2	F1	-7.3*	.000
	Non-functionalized Ti-6Al-4V	1.2	.428
Non-functionalized Ti-6Al-4V	F1	-8.5*	.000
	F2	-1.2	.428

*. The mean difference is significant at the 0.05 level.

3.2.6 Nanoscratch test

Critical failure load of the amine functional film on the Ti-6Al-4V substrates was determined by performing nanoscratch tests. Figure 3.19 shows the loading curve of the nanoscratch test performed with a final load of 50 mN in conjunction with fluorescence image of the scratch produced over the surface by the indenter. As it can be seen, by increasing the normal force to 50 mN, no failure nor delamination of the amine layer was observed. No massive fluctuations in the lateral force were detected and the slope of the curve was roughly the same over the whole range of the applied normal force.

Figure 3.19: a) Loading curve of the nanoscratch test performed by applying a final load of 50 mN. b) fluorescence microscopy image of the resulting scratch in the amine layer.

The next test was performed up to a 100 mN of final load (Figure 3.20). After approximately 440 μm of scratching, signs of failure in the amine layer were seen. At this point, high fluctuations in the lateral force were detected and the slope of the normal force-lateral force suddenly increased. Thus, the critical normal force which caused the failure of the amine layer was determined to be roughly 73 mN.

The final test was performed up to a final load of 150 mN over a 900 μm range (Figure 3.21). Signs of failure in the structure of the functional layer were apparent at approximately 430 μm of scratching which were coupled with the oscillations in the lateral force and variations of the slope of the normal force-lateral force curve. The critical failure force in this test was about 68 mN.

Figure 3.20: a) Loading curve of the nanoscratch test performed up to a final load of 100 mN. b) fluorescence microscopy image of the resulting scratch in the amine layer.

Two different failure modes can be defined from nanoscratch tests. First, the cohesive failure, in which, the damage or failure occurs in one part of the coating or the substrate. Second, the adhesive failure, in which a separation takes place between the coating and the substrate. Depending on the coating, one or both failures modes can occur in the system (ASTM C1624-05(2015)). However, when the coating is noticeably softer than the substrate, the applied load by the indenter causes considerable plastic deformation in the coating until the coating is scraped off the substrate. In this case, the failure load is defined as the load in which the coating has failed. Fluctuation in the lateral force and sudden changes in the lateral force-normal force curves are considered as signs for the occurrence of this failure (ASTM C1624-05(2015), Benjamin & Weaver, 1960, Bull, 1997). According to these criteria, the failure point of the amine functional layer on Ti-6Al-4V substrates was determined to be approximately 70 mN and the mechanism of the failure was plastic deformation within the amine layer that led to its cohesive failure. No significant decohesion was observed between the Ti substrate and the functionalized layer.

Figure 3.21: a) Loading curve of the nanoscratch test performed by applying a final load of 150 mN. b) fluorescence microscopy image of the resulting scratch in the amine layer.

3.2.7 In-vitro cell cultures

3.2.7.1 Cell viability/adhesion

In order to assess the in-vitro biological properties of the functionalized amine layer deposited by AVS on Ti-6Al-4V samples, cell culture experiments were performed using MC3T3-E1 cells as cell model. Fluorescence microscopy images of the calcein/PI stained cells adhered on F1, F2, non-functionalized Ti-6Al-4V and polystyrene control samples after 4 and 48 h of seeding can be seen in Figure 3.22. At 4 h, the cells are adhered to the surface of all samples and possess a prominent circular shape. In contrast, 48 h after seeding, the cells are completely spread, and the typical polygonal shape of this cell line can be observed. The morphology of the cells appears to be similar in all substrates. This result indicates that cells can adhere to and spread over the amine layer, as they do on the non-functionalized bare Ti-6Al-4V samples.

Figure 3.22: Fluorescence microscopy images of calcein/PI stained MC3T3-E1 cells cultured on F1 and F2 functionalized, bare Ti-6Al-4V and polystyrene control samples. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μm)

The number of viable cells attached to the substrates 4 and 48 h after seeding was determined from the images taken from the samples and is shown in Figure 3.23. The statistical analysis of these data is summarized in Table 3.9. The statistical analysis showed a significantly larger number of cells attached to the functionalized samples compared with the non-functionalized ones after 4 h of incubation. In contrast, after 48 h of incubation, the difference between the number of cells on functionalized and non-functionalized samples was not statistically significant. It should be noted that at both times, all the Ti-6Al-4V samples showed a significantly lower number of cells than the controls.

These results indicated that the cytocompatibility of the F1 and F2 functionalized samples was comparable to that observed for bare Ti-6Al-4V substrates. Consequently, AVS functionalization is not observed to induce any toxic effect on the survival and proliferation of the cells.

Figure 3.23: a) Number of cells and b) cellular viability after 4 and 48 h of seeding for MC3T3-E1 cells seeded on F1 and F2 functionalized, bare Ti-6Al-4V and polystyrene control samples.

Table 3.9: The results of Games-Howell test performed on the number of cells found on F1 and F2 functionalized, bare Ti-6Al-4V and polystyrene control samples after 4 h and 48 h of seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Bare Ti-6Al-4V	F1	-24.404*	.002
		F2	-30.056*	.000
		Control	-74.663*	.000
	F1	F2	-5.652	.867
		Control	-50.260*	.000
		Bare Ti-6Al-4V	24.404*	.002
	F2	F1	5.652	.867
		Control	-44.607*	.001
		Bare Ti-6Al-4V	30.056*	.000
	Control	F1	50.260*	.000
		F2	44.607*	.001
		Bare Ti-6Al-4V	74.663*	.000
Cell_No_48H	Bare Ti-6Al-4V	F1	-4.336	.969
		F2	-22.866	.220
		Control	-106.001*	.000
	F1	F2	-18.531	.332
		Control	-101.665*	.000
		Bare Ti-6Al-4V	4.336	.969
	F2	F1	18.531	.332
		Control	-83.135*	.000
		Bare Ti-6Al-4V	22.866	.220
	Control	F1	101.665*	.000
		F2	83.135*	.000
		Bare Ti-6Al-4V	106.001*	.000

*. The mean difference is significant at the 0.05 level.

3.2.7.2 Cell proliferation

In order to extend the comparison between functionalized and non-functionalized samples to longer incubation times, the proliferation of the cells on each sample after one day and seven days of incubation was compared by using the XTT assay. According to the measured absorbance levels (Figure 3.24) the number of viable cells on the functionalized and non-functionalized samples at a given incubation time, does not show any statistically

significant differences (see Table 3.10). In both types of substrates, however, the number of viable cells is considerably lower than that found in the controls.

Figure 3.24: Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on F1 and F2 functionalized, bare Ti-6Al-4V and polystyrene control samples at 1 and 7 days after seeding.

From these results it can be seen that the functional amine layer does not inhibit cell proliferation in any way, and cells behave on the functionalized layer similarly as on the non-functionalized Ti-6Al-4V substrate.

Table 3.10: Results of Games-Howell test performed on XTT absorbance obtained from MC3T3-E1 cells cultured on F1 and F2 functionalized, bare Ti-6Al-4V and polystyrene control samples.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Abs_Day1	Bare Ti-6Al-4V	F1	.043836	.093
		F2	.022555	.335
		Control	-.039875*	.022
	F1	F2	-.021282	.565
		Control	-.083712*	.004
		Bare Ti-6Al-4V	-.043836	.093
	F2	F1	.021282	.565
		Control	-.062430*	.001
		Bare Ti-6Al-4V	-.022555	.335
	Control	F1	.083712*	.004
		F2	.062430*	.001
		Bare Ti-6Al-4V	.039875*	.022
Abs_Day7	Bare Ti-6Al-4V	F1	-.025603	.729
		F2	.035378	.817
		Control	-.269140*	.000
	F1	F2	.060982	.429
		Control	-.243536*	.000
		Bare Ti-6Al-4V	.025603	.729
	F2	F1	-.060982	.429
		Control	-.304518*	.001
		Bare Ti-6Al-4V	-.035378	.817
	Control	F1	.243536*	.000
		F2	.304518*	.001
		Bare Ti-6Al-4V	.269140*	.000

*, The mean difference is significant at the 0.05 level.

Various available studies have addressed cell viability and proliferation on amino-functionalized titanium samples. Cai et al. examined the behavior of CAL-72 osteoblasts on functionalized titanium samples with amino groups. Preparation of functionalized samples was done by immersing the titanium samples in a solution of 5 vol% 7-octenyltrichlorosilane and 1 vol% APTS in anhydrous pentane for 1 h at room temperature. They observed that at 1, 4 and 7 days of culture the functionalized samples possessed considerably more viable cells compared to bare titanium (Cai et al., 2006). In another study (Mussano et al., 2017), functionalized titanium samples with amino groups

prepared by plasma polymer deposition using APTS as precursor were compared with bare titanium using the MC3T3-E1 cell line. The results indicated that after 10 min of cell seeding, a higher number of cells was found on the functionalized samples. On the contrary, after 24 and 48 h after cell seeding, the functionalized samples possessed less viable cells than bare titanium. Sharan et al. (Sharan et al., 2018) cultured primary human periodontal ligament fibroblasts on functionalized Ti-6Al-4V samples. Their functionalization process consisted of dipping the Ti-6Al-4V samples in a 1% aqueous solution of APTS for 15 min. They concluded that the functionalized Ti-6Al-4V had more viable cells at 12, 24, 48 and 72 h after seeding compared to bare Ti-6Al-4V. In another study Nebe et al. (Nebe et al., 2007) assessed the reaction of human MG-63 osteoblasts to functionalized titanium disks. Samples were functionalized with plasma polymerization technique with allylamine as precursor. They observed that initial cell adhesion on the functionalized titanium substrates after 2, 5 and 15 min after seeding in serum-free DMEM was much better than that on untreated titanium samples, and even significantly better than that on tissue culture polystyrene and collagen coated coverslips. Lu et al. (Lu et al., 2016) performed the amino-functionalization process of commercially pure titanium by plasma discharge deposition, using heptylamine as amine precursor and cultured MC3T3-E1 cells on these samples. They detected improved cell adhesion on the functionalized titanium samples after 5, 30 and 120 min after seeding however, after 6, 12, 24, 48 and 72 h after seeding no statistically significant differences were observed in cell viability on the functionalized samples compared to untreated titanium.

It seems that the general consensus in the literature is that amination of the surface of titanium or its alloys leads to better cell adhesion on the samples at short incubation times. However, depending on the studied cell line and other factors, this improved cell adhesion may or may not have been translated into an enhanced cell proliferation at longer incubation times.

It can be hypothesized that the improved cell adhesion on functionalized samples at short incubation times may be the result of at least two effects.

Firstly, by taking into account the presence of amino groups on the surface of the materials, at biological pH (~7.4) the surface is expected to be covered by positively charged NH_3^+ functional groups. On the other hand, there is a negative charge density in the glycocalyx at the surface of most mammalian cell lines. This negative charge is due

to the presence of glycosaminoglycans and sialic acid residues (Vandamme et al., 1994, Chung et al., 2007, L. Chen et al., 2011). This would result in an electrostatic attractive interaction. This interaction, in turn, would lead to an enhanced adhesion of the cells on the surface.

Besides, different adsorption kinetics of proteins on various surfaces might also play a role in cell adhesion. As soon as the biomaterial comes into contact with the biological fluids, adsorption of proteins such as fibronectin, laminin and collagen may adsorb on the surface of the biomaterial (Castner & Ratner, 2002). It is also assumed that a surface capable of adsorbing higher amounts of these ECM proteins, could exhibit improved cell adhesion. In this regard, Cai et al. demonstrated that higher amounts of fibrinogen are adsorbed on amino-functionalized titanium compared to bare titanium (Cai et al., 2006). Mussano et al also concluded that a higher total amount of protein from bovine fetal serum is adsorbed onto amino-functionalized titanium compared to a non-functionalized sample (Mussano et al., 2017). This greater affinity for adsorption of proteins in aminated titanium samples could also play a role in enhanced cell adhesion at short incubation times.

However, it is also found that the difference in the number of cells adhered on functionalized and bare Ti substrates decrease at longer incubation times. Both phenomena, (1) enhanced adhesion at short incubation times and (2) comparable behavior at longer incubation times, were also observed in this work.

3.2.8 Stability under physiological conditions

In order to complete the assessment of the interaction between cells and the functionalized material, the stability of the deposited amine layer under physiological conditions was addressed. Samples used for cell culture for seven days were used, after removal of the cells adhered on these samples. The samples were dyed with calcein/PI to check for the removal of the cells and with FITC to identify the amine layer. Figure 3.25 a and b shows a calcein/PI stained F1 sample after trypsinization, sonication with distilled water and autoclaving in the green (calcein) and red (PI) channels. The lack of any fluorescence is an indication of the removal of all the cells previously adhered to the samples. Figure 3.26 shows fluorescence microscopy images of FITC dyed F1 and F2 samples before and after seven days of cell culture and subsequent cell removal, respectively.

Figure 3.25: Fluorescence microscopy images of a F1 sample after cell removal process stained with calcein/PI. a) green channel (calcein) b) red channel (PI). (Scale bar 1 mm).

Figure 3.26: Fluorescence microscopy images of F1 and F2 functionalized Ti-6Al-4V samples stained with FITC before and after seven days of cell culture. (Scale bar = 1 mm).

Although some degradation is observed, the amine layer is shown to essentially keep its integrity and a high density of active amines at the surface is preserved even after seven days of exposure to culture medium, and the harsh treatment used to remove the cells.

3.3 Adsorption of proteins on bare and functionalized Ti-6Al-4V substrates

The assessment of the biocompatibility of the functional layer is just a first step to improve the response of the organism to the implant. Since this improvement should be the result of the reaction of the body to the molecules present on the surface, it is necessary

to study the interaction of these molecules, basically proteins, with the functionalized materials. The following proteins were considered: albumin, collagen, fibrinogen and fibronectin.

Initially, the effect of adsorbed proteins on the survival and adhesion of cells was addressed. Subsequently, protocols for the covalent immobilization of the proteins were developed and the effect of covalently immobilized proteins on the survival and adhesion of cells was assessed.

3.3.1 Collagen type I

3.3.1.1 MC3T3-E1 cells

The effect of adsorbed collagen type I on Ti-6Al-4V samples, on the adhesion and viability of MC3T3-E1 cells was assessed. Figure 3.27 shows the morphology of MC3T3-E1 cells after 4 and 48 h of seeding as revealed by the calcein/PI staining. After 4 h of seeding, the morphology of the cells on bare Ti-6Al-4V and collagen-coated Ti-6Al-4V showed no apparent differences. The cells in control samples possessed a more noticeable polygonal morphology with highly developed processes in contrast with the circular morphology of the cells seeded on the Ti-6Al-4V substrates.

After 48 h of seeding, the cells on Ti-6Al-4V and control samples had the polygonal cell shape with a spread cytoskeleton, characteristic of this cell line.

The cell numbers on the different substrates at 4 and 48 h after seeding can be seen in Figure 3.28 and Table 3.11. These data confirm that a roughly similar number of cells is found on bare and on collagen-coated Ti-6Al-4V samples at both incubation times.

Figure 3.27: Fluorescence microscopy images of calcein/PI stained MCT3T-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.28: a) Number of cells and b) cellular viability at 4 and 48 h of seeding for MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples.

Table 3.11: Results of the Games-Howell test performed on the data obtained from cell counting for MC3T3-E1 cells seeded on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Ti-6Al-4V with Adsorbed Collagen	Bare Ti-6Al-4V	-4.170	0.726
		Control	-27.789*	0.000
	Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed Collagen	4.170	0.726
		Control	-23.619*	0.000
	Control	Ti-6Al-4V with Adsorbed Collagen	27.789*	0.000
		Bare Ti-6Al-4V	23.619*	0.000
Cell_No_48H	Ti-6Al-4V with Adsorbed Collagen	Bare Ti-6Al-4V	-5.240	0.909
		Control	-76.705*	0.001
	Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed Collagen	5.240	0.909
		Control	-71.464*	0.001
	Control	Ti-6Al-4V with Adsorbed Collagen	76.705*	0.001
		Bare Ti-6Al-4V	71.464*	0.001

*, The mean difference is significant at the 0.05 level.

The results of XTT assays after 48 h of seeding for bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen and polystyrene control samples are shown in Figure 3.29 and Table 3.12. These results are in agreement with those obtained by direct cell counting and demonstrate that bare and collagen-coated Ti-6Al-4V samples support approximately the same number of viable cells, while a considerably larger number of cells is found in the controls.

These data indicate that the adsorption of collagen type I does not have any influence on the survival and proliferation of MC3T3-E1 cells on Ti-6Al-4V substrates.

Figure 3.29: Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 48 h of seeding.

Table 3.12: Results of the Games-Howell test performed on XTT absorbance data for MC3T3-E1 cells seeded on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 48 h of seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed Collagen	-0.000333	0.624
	Control	-0.004056*	0.002
Ti-6Al-4V with Adsorbed Collagen	Bare Ti-6Al-4V	0.000333	0.624
	Control	-0.003724*	0.000
Control	Bare Ti-6Al-4V	0.004056*	0.002
	Ti-6Al-4V with Adsorbed Collagen	0.003724*	0.000

*. The mean difference is significant at the 0.05 level.

3.3.1.2 BM-MSCs

The effect of collagen adsorption on bare Ti-6Al-4V samples, on the adhesion and viability of BM-MSCs was also assessed. The morphology of MSCs at 4 and 48 h of seeding can be seen in Figure 3.30 as revealed by calcein/PI staining. After 4 h of seeding, the MSCs on bare Ti-6Al-4V possessed an approximate circular morphology. In contrast,

the cells adhered on Ti-6Al-4V samples with adsorbed collagen and polystyrene controls showed a more pronounced polygonal morphology with more developed cell processes. After 48 h of seeding, no noticeable difference in cells morphology between the samples was observed and, cells showed a mostly polygonal morphology, characteristic of this cell line.

Figure 3.30: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

The number of cells on each material and the statistical analysis of the data are shown in Figure 3.31 and Table 3.13. A significantly higher number of viable cells is observed on Ti-6Al-4V samples with adsorbed collagen when compared to the bare Ti-6Al-4V material. It is worth mentioning, that at both incubation times the number of cells on bare and Ti-6Al-4V samples with adsorbed collagen was smaller than that on the controls.

Figure 3.31: a) Number of cells and b) cellular viability at 4 and 48 h of seeding for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples.

Table 3.13: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Ti-6Al-4V with Adsorbed Collagen	20.360*	0.008
		Bare Ti-6Al-4V	41.818*	0.000
	Ti-6Al-4V with Adsorbed Collagen	Control	-20.360*	0.008
		Bare Ti-6Al-4V	21.458*	0.006
	Bare Ti-6Al-4V	Control	-41.818*	0.000
		Ti-6Al-4V with Adsorbed Collagen	-21.458*	0.006
Cell_No_48H	Control	Ti-6Al-4V with Adsorbed Collagen	44.235*	0.028
		Bare Ti-6Al-4V	67.971*	0.001
	Ti-6Al-4V with Adsorbed Collagen	Control	-44.235*	0.028
		Bare Ti-6Al-4V	23.736*	0.003
	Bare Ti-6Al-4V	Control	-67.971*	0.001
		Ti-6Al-4V with Adsorbed Collagen	-23.736*	0.003

*. The mean difference is significant at the 0.05 level.

The results obtained by direct cell counting were also confirmed by XTT assays. Figure 3.32 shows the XTT absorbance values obtained for bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen and control samples after 48 h of seeding. The statistical analysis of the data is summarized in Table 3.14. These results are in good agreement with those of cell counting and confirm the presence of a larger number of viable cells on the collagen-coated samples after 48 h of seeding.

Consequently, and in contrast with the results obtained for the MC3T3-E1 cell line, adsorption of collagen type I on Ti-6Al-4V substrates led to an enhanced behavior of BM-MSCs in terms of cell adhesion and viability.

Figure 3.32: Absorbance values of XTT assay obtained from BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples at 48 h of seeding.

Table 3.14: Results of the Games-Howell test performed on XTT absorbance data for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed collagen type I and polystyrene control samples after 48 h of seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Control	Ti-6Al-4V with Adsorbed Collagen	0.001921*	0.000
	Bare Ti-6Al-4V	0.002637*	0.000
Ti-6Al-4V with Adsorbed Collagen	Control	-0.001921*	0.000
	Bare Ti-6Al-4V	0.000716*	0.002
Bare Ti-6Al-4V	Control	-0.002637*	0.000
	Ti-6Al-4V with Adsorbed Collagen	-0.000716*	0.002

*. The mean difference is significant at the 0.05 level.

At this point, it is worth comparing these results with the numerous studies available that attempt to improve the biological response of metallic implants by incorporating different type of collagen onto their surface.

Thus, van den Dolder et al. (van den Dolder et al., 2003) adsorbed collagen type I on porous titanium substrates. Rat bone marrow (RMB) cells were used as the cell model and no significant effects on cellular proliferation after 1, 4 and 8 days of culture were observed between bare and collagen-coated samples (van den Dolder et al., 2003). Becker et al. (D. Becker et al., 2002) assessed the effect of collagen coating on Ti-6Al-4V substrates using fibrillar calf skin collagen type I and rat calvarial osteoblasts as the cell model. No significant differences between the proliferation rate of cells on bare and collagen-coated Ti-6Al-4V samples was found throughout 25 days of culture (D. Becker et al., 2002). In another experiment, Nagai et al. (Nagai et al., 2002) evaluated the effect of porcine collagen type I adsorption onto commercially pure titanium disks using human gingival fibroblasts as cell model. After 90 min of seeding, the authors found a considerably higher number of cells on the collagen-coated samples than on bare Ti (Nagai et al., 2002). Moreover, Geißler et al. (Geißler et al., 2000) demonstrated the improved biological properties of collagen-coated Ti-6Al-4V samples at short incubation times. In particular, it was found that collagen-coated Ti-6Al-4V samples induced a higher initial cell attachment compared to uncoated Ti-6Al-4V samples (Geißler et al., 2000). The possible differences between short and long-incubation time were highlighted by Wang et al. (Q. Q. Wang et al., 2011). These authors reported an enhanced adhesion

of MG63 cells on collagen-coated samples after 24 h of seeding. However, after 2 days of seeding and until day 6, bare and collagen-coated samples possessed roughly the same number of cells (Q. Q. Wang et al., 2011). Finally, Cooper et al. (Cooper et al., 1993) assessed the effect of adsorption of rat tail collagen type I on commercially pure titanium using MC3T3-E1 cells as cell model. No significant difference in cell adhesion was found between bare and collagen-coated samples after 4 h of culture (Cooper et al., 1993).

The latter study is in agreement with the results obtained for the MC3T3-E1 cells in this work. In this regard, the positive influence exerted by the adsorbed collagen on the adhesion of BM-MSCs on Ti-6Al-4V suggests that the effect might depend on the cell line.

The RGD (Arginine – Glycine – Aspartic acid) (Brighton & Albelda, 1992, Pierschbacher & Ruoslahti, 1984, Ruoslahti & Pierschbacher, 1987), KGDE (Lysine – Glycine – Aspartic acid – Glutamate) (Cooper et al., 1993) and GFOGER (Knight et al., 2000) peptide sequences, present in the structure of collagen are believed to be able to directly interact with bone cells through integrins. Since collagen is a major part of the ECM, it is not surprising that cells might interact with this protein either directly, or indirectly through other proteins such as fibronectin or vitronectin associated to collagen (Schneider & Burridge, 1994, Tjia et al., 1999). In this regard, it should not be surprising that the specific interaction of the cells with those proteins might depend on the cell lineage.

The enhanced behavior of BM-MSCs on Ti-6Al-4V substrates with adsorbed collagen promoted the selection of collagen as a candidate for subsequent covalent immobilization on functionalized Ti-6Al-4V.

3.3.2 Bovine serum albumin

The possible effects of the presence of bovine serum albumin (BSA) physically adsorbed on the surface of Ti-6Al-4V on the adhesion and viability of MC3T3-E1 and BM-MSCs were assessed.

3.3.2.1 MC3T3-E1 cells

Figure 3.33 shows the morphology of MC3T3-E1 cells adhered on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 4 and 48 h after seeding. At 4 h after seeding, the cells are adhered on all the samples, but are not completely spread on the surface. After 48 h however, the cells possessed a polygonal shape with extended

processes. Higher confluence of cells can be seen in the control samples at both times, but there are no significant differences between the morphology of the cells on the different samples.

Figure 3.33: Fluorescence microscopy images of calcein/PI stained MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 4 and 48 h of seeding. Viable cells are stained green, whereas the dead cells appear as red red dots. (Scale bar = 100 μ m).

The results of cell quantification are shown in Figure 3.34 and Table 3.15. As it can be seen after 4 h of seeding, Ti-6Al-4V samples with adsorbed BSA showed better cell adhesion compared to bare Ti-6Al-4V. However, after 48 h of seeding no significant difference was observed between both types of samples. At either 4 or 48 h after seeding, the highest number of cells was observed in the control samples.

Figure 3.34: a) Number of viable cells and b) cellular viability at 4 and 48 h of seeding for MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples.

Table 3.15: Results of the Games-Howell test performed on the data obtained from cell counting for MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 4 and 48 h of seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Ti-6Al-4V with adsorbed BSA	31.614*	0.000
		Bare Ti-6Al-4V	48.819*	0.000
	Ti-6Al-4V with adsorbed BSA	Control	-31.614*	0.000
		Bare Ti-6Al-4V	17.205*	0.006
	Bare Ti-6Al-4V	Control	-48.819*	0.000
		Ti-6Al-4V with adsorbed BSA	-17.205*	0.006
Cell_No_48H	Control	Ti-6Al-4V with adsorbed BSA	75.351*	0.000
		Bare Ti-6Al-4V	67.978*	0.000
	Ti-6Al-4V with adsorbed BSA	Control	-75.351*	0.000
		Bare Ti-6Al-4V	-7.374	0.778
	Bare Ti-6Al-4V	Control	-67.978*	0.000
		Ti-6Al-4V with adsorbed BSA	7.374	0.778

*. The mean difference is significant at the 0.05 level.

The results of independent XTT experiment performed on these samples after 48 h of seeding can be seen in Figure 3.35 and Table 3.16. These data confirmed the results of cell counting and demonstrated that after 48 h of seeding, there was no statistically significant difference between the number of cells on bare and BSA-adsorbed Ti-6Al-4V.

Figure 3.35: Absorbance values of XTT assays obtained from MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples after 48 h of seeding.

Table 3.16: Results of the Games-Howell test performed on the XTT absorbance data for MC3T3-E1 cells seeded on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 48 h of seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed BSA	0.000408	0.177
	Control	-0.002267*	0.000
Ti-6Al-4V with Adsorbed BSA	Bare Ti-6Al-4V	-0.000408	0.177
	Control	-0.002675*	0.001
Control	Bare Ti-6Al-4V	0.002267*	0.000
	Ti-6Al-4V with Adsorbed BSA	0.002675*	0.001

*. The mean difference is significant at the 0.05 level.

3.3.2.2 BM-MSCs

The adhesion and survival of BM-MSCs on Ti-6Al-4V with adsorbed BSA was also assessed. The morphology of the BM-MSCs on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and control samples after 4 and 48 h of seeding is shown in Figure 3.36. It can be seen that after 4 h of seeding, the cells possessed a prominent circular morphology in all samples. After 48 h of seeding however, most of the cells show polygonal morphology. Although still some cells with a circular morphology can also be identified. The various morphologies of cells observed after 48 h of seeding can be attributed to the heterogeneity of the MSC culture used in this study.

The results of cell quantification and survival are shown in Figure 3.37 and Table 3.17. At 4 h after seeding, a larger number of cells is found on BSA adsorbed samples compared to the bare Ti-6Al-4V. However, the number of cells at 48 h is roughly the same in both samples as previously observed for MC3T3-E1 cells.

Figure 3.36: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

The comparison of the cell cultures performed with MC3T3-E1 and BM-MSCs show that the number of the latter is smaller in all cases. This observation might imply that the BM-MSCs had a slower proliferation rate than the MC3T3-E1 cells.

This slower proliferation rate might be related to the contact inhibition exhibited by the BM-MSCs. This behavior results in the inhibition of some of the cell functions when contacting another cell. One of the main consequences of the contact inhibition is to prevent the three-dimensional growth of the cell culture (Abercrombie, 1970). The limitation in cell spreading caused by contact inhibition may lead to reduced cell surface area and subsequently, reduced uptake from medium which can cause inhibited proliferation (Castor, 1968).

Figure 3.37: a) Number of viable cells and b) cellular viability after 4 and 48 h of seeding for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples.

The result of the independent XTT tests performed on BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene controls after 48 h of seeding is shown in Figure 3.38 and Table 3.18. These data supported the results obtained from cell

counting and demonstrated that BSA adsorbed on Ti-6Al-4V did not have any statistically significant effects on the behavior of BM-MSCs 48 h after seeding.

Table 3.17: Results of the Games-Howell test performed on the data obtained from cell counting of BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 4 and 48 h of seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Ti-6-Al-4V with Adsorbed BSA	21.769*	0.009
		Bare Ti-6Al-4V	42.819*	0.000
	Ti-6-Al-4V with Adsorbed BSA	Control	-21.769*	0.009
		Bare Ti-6Al-4V	21.051*	0.032
	Bare Ti-6Al-4V	Control	-42.819*	0.000
		Ti-6-Al-4V with Adsorbed BSA	-21.051*	0.032
Cell_No_48H	Control	Ti-6-Al-4V with Adsorbed BSA	49.005*	0.000
		Bare Ti-6Al-4V	49.910*	0.000
	Ti-6-Al-4V with Adsorbed BSA	Control	-49.005*	0.000
		Bare Ti-6Al-4V	0.904	0.992
	Bare Ti-6Al-4V	Control	-49.910*	0.000
		Ti-6-Al-4V with Adsorbed BSA	-0.904	0.992

*. The mean difference is significant at the 0.05 level.

Figure 3.38: Absorbance values of XTT assays obtained from BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 48 h of seeding.

Table 3.18: Results of the Games-Howell test performed on XTT absorbance data for BM-MSCs seeded on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed BSA and polystyrene control samples at 48 h of seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed BSA	0.000011	0.999
	Control	-0.003736*	0.001
Ti-6Al-4V with Adsorbed BSA	Bare Ti-6Al-4V	-0.000011	0.999
	Control	-0.003747*	0.000
Control	Bare Ti-6Al-4V	0.003736*	0.001
	Ti-6Al-4V with Adsorbed BSA	0.003747*	0.000

*. The mean difference is significant at the 0.05 level.

The results above allow addressing the response of two different cell lines, MC3T3-E1 and BM-MSCs, to the presence of bovine serum albumin adsorbed on Ti-6Al-4V samples. Albumin is a major protein constituent of the serum and acts as a carrier for some smaller molecules and lipids (Deligianni et al., 2001). It is generally believed that albumin is a non-adhesive protein for osteoblasts (Puleo & Bizios, 1992). Our results are in agreement with those obtained by Yang et al. (Yang et al., 2003) which in their study, cultured CRL 1486 human embryonic palatal mesenchyme (HEPM) cells, an osteoblast

precursor cell line, on pure titanium samples upon adsorption of BSA. 15 and 180 min after seeding, no statistically significant differences were found between the number of cells on pure titanium and titanium with BSA samples (Yang et al., 2003).

In another study, the effect of adsorption of human serum albumin on commercially pure TiO₂ was assessed using MC3T3-E1 cells. It was concluded that after 4 h of seeding there were less cells on albumin-coated samples compared to bare TiO₂. However, after 24 h after seeding, TiO₂ samples with albumin and without albumin demonstrated roughly the same number of viable cells (Sousa et al., 2008).

Some researches hypothesized that although albumin is a non-adhesive protein for osteoblasts, it can increase the uptake of other proteins from the biological media which, in turn, can lead to improved cell adhesion on the samples in question. Hayashi et al. (Hayashi et al., 2013) observed that at 1 and 3 h after incubation, hydroxyapatite and α -Al₂O₃ coated with BSA, adsorbed considerably higher amounts of protein from DMEM supplemented with 20% FBS compared to bare hydroxyapatite and α -Al₂O₃ samples. After 6 h of incubation however, the amounts of protein adsorbed on all samples were roughly equal. This study concluded that the presence of adsorbed BSA can increase the kinetics of protein adsorption from the medium onto the samples (Hayashi et al., 2013). In this regard, it is also reported that adsorbed BSA can increase the adsorption of fibronectin to tissue culture plates (Grinnell & Feld, 1982). In addition, Koblinksi et al. (Koblinksi et al., 2005) demonstrated that some ECM proteins can be activated by BSA. In particular, the adhesion of human MDA-MB-231 breast carcinoma cells on culture plates coated simultaneously by BSA and fibronectin, BSA and laminin, BSA and collagen type I and BSA and vitronectin were compared to coatings formed by fibronectin, laminin, collagen type I and vitronectin without BSA. An enhanced adhesion was observed in the samples that contained BSA and it was concluded that BSA can positively influence the conformation of the adsorbed adhesion proteins and keep them active at low concentrations (Koblinksi et al., 2005).

In this study we observed an enhanced cells adhesion at 4 h after seeding for BSA-coated Ti-6Al-4V samples compared to bare Ti-6Al-4V for both MC3T3-E1 and BM-MSCs. This positive effect however, was limited to the initial cell adhesion stage and after 48 h of seeding, no statistically significant differences were observed between BSA-coated Ti-6Al-4V and bare Ti-6Al-4V samples.

3.3.3 Fibrinogen

3.3.3.1 MC3T3-E1 cells

Fibrinogen derived from human plasma was the third protein that was assessed in terms of its ability to regulate cell adhesion and proliferation. Fibrinogen was adsorbed on Ti-6Al-4V and MC3T3-E1 cells were cultured onto it. The morphology of MC3T3-E1 cells adhered on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen and tissue culture polystyrene control samples after 4 and 48 h of seeding can be seen in Figure 3.39, as revealed by calcein/PI staining.

As it can be seen in Figure 3.39, the cells are completely adhered on all samples after 4 h of seeding although not completely spread yet.

Figure 3.39: Fluorescence microscopy images of calcein/PI stained MCT3T-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen and polystyrene control samples at 4 and 48 h of seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

The number of cells on each material and the statistical analysis of the data are shown in Figure 3.40 and Table 3.19. At 4 h after seeding, Ti-6Al-4V samples with adsorbed fibrinogen possessed roughly the same number of viable cells as bare Ti-6Al-4V samples. Likewise, after 48 h of seeding the same number of viable cells was observed for these

two samples. The number of viable cells at both incubation times was significantly higher in the controls than that observed on Ti-6Al-4V substrates.

Figure 3.40: a) Number of viable cells and b) cellular viability after 4 and 48 h of seeding for MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen and polystyrene control samples.

The results of the XTT assays after 48 h of seeding for these samples can be seen in Figure 3.41 and Table 3.20. In agreement with the results obtained from the cell counting experiments no significant differences in terms of the number of cells is found between bare Ti-6Al-4V and Ti-6Al-4V with adsorbed fibrinogen samples.

Table 3.19: Results of the Games-Howell test performed on the data obtained from cell counting for MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen and polystyrene control samples at 4 and 48 h of seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Ti-6Al-4V with Adsorbed Fibrinogen	28.383*	0.000
		Bare Ti-6Al-4V	33.250*	0.000
	Ti-6Al-4V with Adsorbed Fibrinogen	Control	-28.383*	0.000
		Bare Ti-6Al-4V	4.867	0.506
	Bare Ti-6Al-4V	Control	-33.250*	0.000
		Ti-6Al-4V with Adsorbed Fibrinogen	-4.867	0.506
Cell_No_48H	Control	Ti-6Al-4V with Adsorbed Fibrinogen	52.402*	0.000
		Bare Ti-6Al-4V	56.344*	0.000
	Ti-6Al-4V with Adsorbed Fibrinogen	Control	-52.402*	0.000
		Bare Ti-6Al-4V	3.942	0.706
	Bare Ti-6Al-4V	Control	-56.344*	0.000
		Ti-6Al-4V with Adsorbed Fibrinogen	-3.942	0.706

*. The mean difference is significant at the 0.05 level.

Figure 3.41: Absorbance values of XTT assays obtained from MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen and polystyrene control samples after 48 h of seeding.

Table 3.20: Results of the Games-Howell test performed on XTT absorbance data for MC3T3-E1 cells cultured on bare Ti-6Al-4V, Ti-6Al-4V with fibrinogen and polystyrene control samples after 48 h of seeding.

(I) sample	(J) sample	Mean Difference (I-J)	Sig.
Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed Fibrinogen	-0.000257	0.272
	Control	-0.003696*	0.000
Ti-6Al-4V with Adsorbed Fibrinogen	Bare Ti-6Al-4V	0.000257	0.272
	Control	-0.003439*	0.000
Control	Bare Ti-6Al-4V	0.003696*	0.000
	Ti-6Al-4V with Adsorbed Fibrinogen	0.003439*	0.000

*. The mean difference is significant at the 0.05 level.

3.3.3.2 BM-MSCs

The effect of adsorption of fibrinogen on Ti-6Al-4V was also assessed using BM-MSCs. In this experiment, fibrinogen was also adsorbed on F2 functionalized samples. Figure 3.42 shows the morphology of BM-MSCs on each material as revealed by the calcein/PI staining.

Figure 3.42: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen, AVS-functionalized Ti-6Al-4V (F2) with adsorbed fibrinogen and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μm).

As it can be seen, after 4 h of seeding the cells adhered on polystyrene controls and fibrinogen-coated samples show a more spread cytoskeleton compared to those adhered on bare Ti-6Al-4V. After 48 h of seeding however, there were no distinguishable differences between the morphology of cells on bare Ti-6Al-4V and fibrinogen-coated materials. Cells grown on control samples showed a larger number of processes and extended filopodia.

Figure 3.43: a) Number of viable cells and b) cellular viability at 4 and 48 h of seeding for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen, AVS-functionalized Ti-6Al-4V (F2) with adsorbed fibrinogen and polystyrene control samples.

The number of viable cells on different samples is presented in Figure 3.43 and the statistical analysis of the data in Table 3.21. The difference observed in the initial number of cells between bare and fibrinogen-coated Ti-6Al-4V samples was not statistically significant, indicating that functionalization has no effect on the number of adhered cells. In both temporal points, the number of viable cells on the control samples was considerably higher than that on any of the Ti-6Al-4V samples.

The results of the XTT assays performed on these samples can be seen in Figure 3.44 and Table 3.22. These results are in good agreement with the cell counting results and

demonstrate that adsorption of fibrinogen on Ti-6Al-4V samples, either functionalized or not, does not have any effect on the adhesion or survival of BM-MSCs.

Table 3.21: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen, AVS-functionalized Ti-6Al-4V (F2) with adsorbed fibrinogen and polystyrene control samples at 4 and 48 h of seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	F2 with Adsorbed Fibrinogen	25.159*	0.001
		Ti-6Al-4V with Adsorbed Fibrinogen	34.755*	0.000
		Bare Ti-6Al-4V	35.059*	0.000
	F2 with Adsorbed Fibrinogen	Control	-25.159*	0.001
		Ti-6Al-4V with Adsorbed Fibrinogen	9.597	0.397
		Bare Ti-6Al-4V	9.900	0.253
	Ti-6Al-4V with Adsorbed Fibrinogen	Control	-34.755*	0.000
		F2 with Adsorbed Fibrinogen	-9.597	0.397
		Bare Ti-6Al-4V	0.304	1.000
	Bare Ti-6Al-4V	Control	-35.059*	0.000
		F2 with Adsorbed Fibrinogen	-9.900	0.253
		Ti-6Al-4V with Adsorbed Fibrinogen	-0.304	1.000
Cell_No_48H	Control	F2 with Adsorbed Fibrinogen	57.304*	0.000
		Ti-6Al-4V with Adsorbed Fibrinogen	55.426*	0.000
		Bare Ti-6Al-4V	47.424*	0.000
	F2 with Adsorbed Fibrinogen	Control	-57.304*	0.000
		Ti-6Al-4V with Adsorbed Fibrinogen	-1.878	0.993
		Bare Ti-6Al-4V	-9.880	0.461
	Ti-6Al-4V with Adsorbed Fibrinogen	Control	-55.426*	0.000
		F2 with Adsorbed Fibrinogen	1.878	0.993
		Bare Ti-6Al-4V	-8.002	0.715
	Bare Ti-6Al-4V	Control	-47.424*	0.000
		F2 with Adsorbed Fibrinogen	9.880	0.461
		Ti-6Al-4V with Adsorbed Fibrinogen	8.002	0.715

*. The mean difference is significant at the 0.05 level.

Figure 3.44: Absorbance values of XTT assays obtained from BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen, AVS-functionalized Ti-6Al-4V (F2) with adsorbed fibrinogen and polystyrene control samples after 48 h of seeding.

Table 3.22: Results of the Games-Howell test performed on XTT absorbance data for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibrinogen, AVS-functionalized Ti-6Al-4V (F2) with adsorbed fibrinogen and polystyrene control samples after 48 h of seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Control	Bare Ti-6Al-4V	0.005053*	0.000
	Ti-6Al-4V with Adsorbed Fibrinogen	0.005114*	0.000
	F2 with Adsorbed Fibrinogen	0.004728*	0.001
Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed Fibrinogen	0.000061	0.975
	F2 with Adsorbed Fibrinogen	-0.000325	0.773
	Control	-0.005053*	0.000
Ti-6Al-4V with Adsorbed Fibrinogen	Bare Ti-6Al-4V	-0.000061	0.975
	F2 with Adsorbed Fibrinogen	-0.000386	0.640
	Control	-0.005114*	0.000
F2 with Adsorbed Fibrinogen	Bare Ti-6Al-4V	0.000325	0.773
	Ti-6Al-4V with Adsorbed Fibrinogen	0.000386	0.640
	Control	-0.004728*	0.001

*. The mean difference is significant at the 0.05 level.

Fibrinogen is a protein found in blood plasma (Doolittle et al., 1997). The conversion of fibrinogen to fibrin is responsible for the coagulation of blood (Hantgan et al., 1990, Endenburg et al., 1995). Fibrinogen normally, does not form part of the extracellular matrix, although it is reported to bind to different kinds of cells such as platelets (Savage et al., 1996) endothelial cells, neutrophils and monocytes (Doolittle, 2010, Hawiger et al., 1982).

In this regard, Kornu et al. (Kornu et al., 1996) investigated the effect of fibrinogen adsorption on MC3T3-E2 mouse osteoblasts adhesion on Ti-Al-V and Co-Cr-Mo alloys. It was reported that adsorption of fibrinogen did not have any effect on cell adhesion at 2, 12 and 24 h after seeding (Kornu et al., 1996). Similar results were observed in this work. It is suggested that although fibrinogen contains RGD cell attachments sites in its structure, these sites might not be accessible at the surface of the molecule or their conformation may not be suitable for the attachment of cell receptors (Ruoslahti & Pierschbacher, 1987).

In contrast, another study found out that adsorption of low concentration of fibrinogen (1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$) on titanium improves the proliferation of mouse calvarial osteoblasts after 7 days of seeding, while a higher concentration of fibrinogen (100 $\mu\text{g/ml}$) does not have any effect on cell proliferation. It was proposed that different conformations of the RGD motif in low and high concentrations of adsorbed fibrinogen, might be responsible for this effect. It is noteworthy that, at low concentrations of fibrinogen the cell proliferation enhancement occurred after 7 days of seeding and after 1 and 4 days, no improvement was observed (Horasawa et al., 2015).

3.3.4 Fibronectin

3.3.4.1 BM-MSCs

Due to the limitations in the stock of fibronectin, the experiment concerning the adsorption of fibronectin onto Ti-6Al-4V samples was only performed using the BM-MSCs.

The morphology of calcein/PI stained BM-MSCs adhered on bare Ti-6Al-4V, Ti-6-Al-4V with adsorbed fibronectin and polystyrene controls after 4 and 48 h of seeding can be seen in Figure 3.45. After 4 h of seeding, the cells adhered on bare Ti-6Al-4V samples show a marked circular morphology, compared with the cells on Ti-6Al-4V with

adsorbed fibronectin and control samples. After 48 h of seeding, cells with highly extended processes can be seen on the fibronectin-coated samples, while cells on bare Ti-6Al-4V possess rectangular morphology and less developed processes.

Figure 3.45: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibronectin and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

The results of cell quantification are shown in Figure 3.46 and the statistical analysis of the data in Table 3.23. It can be seen that both at 4 and 48 h after seeding more cells attached to the Ti-6Al-4V with adsorbed fibronectin samples compared to the bare substrates, although the number of cells is smaller than on the control samples.

The results of the XTT assays performed on these samples at 48 h after seeding are shown in Figure 3.47 and the statistical analysis of the data in Table 3.24. These results are in agreement with the previous results of cell counting as higher absorbance values are found for Ti-6Al-4V with adsorbed fibronectin compared with the bare Ti-6Al-4V substrates.

Figure 3.46: a) Number of viable cells and b) cellular viability at 4 and 48 h of seeding for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibronectin and polystyrene control samples.

Table 3.23: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibronectin and polystyrene control samples at 4 and 48 h of seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Ti-6Al-4V with Adsorbed Fibronectin	20.008*	0.015
		Bare Ti-6Al-4V	48.218*	0.000
	Ti-6Al-4V with Adsorbed Fibronectin	Control	-20.008*	0.015
		Bare Ti-6Al-4V	28.210*	0.000
	Bare Ti-6Al-4V	Control	-48.218*	0.000
		Ti-6Al-4V with Adsorbed Fibronectin	-28.210*	0.000
Cell_No_48H	Control	Ti-6Al-4V with Adsorbed Fibronectin	36.240*	0.000
		Bare Ti-6Al-4V	62.441*	0.000
	Ti-6Al-4V with Adsorbed Fibronectin	Control	-36.240*	0.000
		Bare Ti-6Al-4V	26.201*	0.000
	Bare Ti-6Al-4V	Control	-62.441*	0.000
		Ti-6Al-4V with Adsorbed Fibronectin	-26.201*	0.000

*. The mean difference is significant at the 0.05 level.

Figure 3.47: Absorbance values of XTT assays obtained from BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with adsorbed fibronectin and polystyrene control samples at 48 h of seeding.

Table 3.24: Results of the Games-Howell test performed on XTT absorbance data for BM-MSCs cultured on bare Ti-6Al-4V, Ti-6Al-4V with fibronectin and polystyrene control samples at 48 h of seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Control	Bare Ti-6Al-4V	0.004078*	0.000
	Ti-6Al-4V with Adsorbed Fibronectin	0.002531*	0.004
Bare Ti-6Al-4V	Ti-6Al-4V with Adsorbed Fibronectin	-0.001547*	0.016
	Control	-0.004078*	0.000
Ti-6Al-4V with Adsorbed Fibronectin	Bare Ti-6Al-4V	0.001547*	0.016
	Control	-0.002531*	0.004

*. The mean difference is significant at the 0.05 level.

Fibronectin is one of the main ECM proteins and contribution to cell adhesion is among its functions (Hsiao et al., 2017, Ruoslahti, 1984). The mechanisms involved in the interaction of the adsorbed fibronectin with cells are not completely understood yet. However, some researchers propose that fibronectin interacts with bone cells through the RGD (Brighton & Albelda, 1992, Miyamoto et al., 1998, Hersel et al., 2003), PHSRN (Proline – Histidine – Serine – Arginine – Asparagine) (Aota et al., 1994), LDV (Leucine – Aspartic acid – Valine) (Komoriya et al., 1991) and REDV (Arginine – Glutamate –

Aspartic acid – Valine) (Humphries et al., 1986) motifs which are recognized by the integrins.

Almost all of the studies concerning adsorption of fibronectin on titanium samples, report some kind of improvement in terms of cell response for the fibronectin-adsorbed samples. In one study, Ku et al. (Ku et al., 2005) assessed the adhesion and proliferation of MC3T3-E1 cells on fibronectin-coated commercially pure titanium. After 1 h of seeding no significant differences in the number of adhered cells on fibronectin-coated and bare titanium was observed, but a considerably larger number of cell were observed on fibronectin-coated samples after 6 h. The measured number of cells after 1 and 3 days of seeding indicated that at day 1 no significant differences were observed, but at day 3 higher number of viable cells were present on the fibronectin-coated samples compared to the titanium samples without coating (Ku et al., 2005). Another study (Rapuano & MacDonald, 2011) used three different cell lines: MG63, MC3T3-E1 and C3H10T1/2 mesenchymal stem cells to assess the influence of fibronectin on the adherence of cells on Ti-6Al-4V disks. It was observed that incubation with fibronectin solution with concentrations as low as 0.5 nM, led to a significant enhancement of cell attachment on Ti-6Al-4V samples (Rapuano & MacDonald, 2011). Human gingival fibroblasts were also used as cell model and their proliferation on fibronectin-coated Ti-6Al-4V samples over a 16-day period was studied (Säuberlich et al., 1999). It was found that the number of cells on the coated substrates was larger than that on the bare material after day 3 (Säuberlich et al., 1999). Enhanced cell adhesion on fibronectin-coated Ti was also reported for baby hamster kidney 21C/13 fibroblasts, 4 h after seeding (Cannas et al., 1988). Finally, it is worth mentioning that fibronectin did not have any effect on adhesion of human gingival fibroblasts, MG63 and HOS osteoblast-like cells, 1 h after seeding. However, fibronectin seemed to have a positive effect on the spreading of human gingival fibroblasts grown on Ti, but not on MG63 and HOS cell lines (B. S. Park et al., 2005).

The results obtained in this work are consistent with a positive effect of fibronectin on the adhesion of BM-MSCs on Ti-6Al-4V substrates. This improved cell adhesion translates into larger number of cells at longer incubation times.

3.4 Covalent immobilization of proteins on AVS-functionalized Ti-6Al-4V substrates: Development of an immobilization protocol and characterization of the immobilized biomolecules

Since the main objective of this work is the development of a robust functionalization procedure to modulate the response of the organism to the implant, it is necessary to develop protocols that allow the covalent binding of the selected proteins to the material. The selection of the proteins was based on the previous results on the effect of different adsorbed moieties on the survival and proliferation of the cells. The presentation of the different proteins used in this section will follow the same order than in the previous section.

3.4.1 Collagen

3.4.1.1 Elution of adsorbed proteins using detergents

3.4.1.1.1 Development of a protocol for the removal of non-covalently immobilized collagen

In order to distinguish between adsorbed and covalently bound proteins on the surface, samples with adsorbed fluorescent collagen type I were prepared according to the protocol described in section 2.3.2.2.1.1. Fluorescence microscopy images of the samples were captured before and after the treatment (Figure 3.48) and the removal of collagen was monitored by the variation in fluorescence intensity before and after the treatment with each detergent. SDS was chosen as an anionic, and triton X-100 and tween 20 as nonionic detergents for the treatment.

Figure 3.49 shows the fluorescence intensity for samples with adsorbed fluorescent collagen type I, before and after treatment with triton X-100, tween 20, SDS. A sample incubated with PBS was used as control.

Figure 3.48: Fluorescence microscopy images of FITC-collagen coated samples before and after treatment with Triton X-100 (a, e), Tween-20 (b, f), SDS (c, g) and PBS (d, h). (Scale bar =100 μm).

Figure 3.49: Intensity of fluorescence of FITC-collagen coated samples before and after the treatment with Triton X-100, Tween-20, SDS. Samples immersed in PBS were used as controls.

Since the highest removal of collagen, as determined by the reduction of fluorescence intensity, was observed for samples treated with SDS, this detergent was chosen for further experimentation.

3.4.1.1.2 Optimization of the protocol for the removal of non-covalently immobilized collagen

Different concentration and incubation protocols were tested to optimize the removal of adsorbed collagen type I from the Ti-6Al-4V substrates. The duration of the incubation with SDS was fixed to 24 h and in some cases a step of 30 min of sonication was also added. Two concentrations of SDS in PBS were examined: 10% and 30% (w/v). The quantification of the intensity of fluorescence after each treatment is shown in Figure 3.50.

In order to detect any possible adverse effects of the treatment with SDS on AVS-functionalized samples, F2 samples were also dyed with FITC and exposed to the same treatments. The intensity of fluorescence for these samples before and after the treatments with SDS solutions can be seen in Figure 3.51.

It was observed that samples with adsorbed collagen type I treated with 10% or 30% SDS solution for 24 h, with or without sonication step, showed almost no fluorescence after the treatments, which is an indication of the removal of adsorbed fluorescent collagen from the samples. In contrast, samples treated only with PBS did not show any significant reduction in fluorescence after incubation (Figure 3.50).

In addition, no significant variation in fluorescence before and after each treatment was observed for F2 AVS-functionalized samples (Figure 3.51). These data confirm that the functional layer can resist relatively harsh treatments, such as incubation with SDS and sonication.

The capacity of SDS for removing adsorbed proteins may be related with its ability to induce conformational changes in the proteins. SDS might diffuse to the surface-protein interface and disrupt the interactions between the protein and the surface (Bohnert & Horbett, 1986). Other studies have used SDS to elute adsorbed proteins from different surfaces, for instance collagen from polytetrafluoroethylene (PTFE) surfaces (Bax et al., 2010), albumin and fibrinogen from silicon wafers (Vandenberg et al., 1991), albumin from fluorocarbon polymers (Bohnert et al., 1990), and even DNA from mica substrates (Shlyakhtenko et al., 1999).

Figure 3.50: Intensity of fluorescence of FITC-collagen coated samples before and after treatments with SDS solutions of varying concentrations, with and without a sonication step.

Figure 3.51: Intensity of fluorescence for functionalized (F2) samples dyed with FITC, before and after treatments with SDS solutions of varying concentrations.

3.4.1.2 Covalent immobilization of collagen

3.4.1.2.1 Fluorescence microscopy study

Collagen was covalently immobilized on the surface of F2 AVS-functionalized Ti-6Al-4V substrates using EDC/NHS chemistry. F2 functionalization conditions were selected due to their higher concentration of amino groups. Figure 3.52 shows fluorescence microscopy images of F2 functionalized samples incubated with FITC-collagen and

EDC/NHS cross-linkers (F2+C), F2 functionalized samples incubated with FITC-collagen without EDC/NHS (F2-C), bare Ti-6Al-4V incubated with FITC-collagen and EDC/NHS (Ti+C) and bare Ti-6Al-4V samples incubated with FITC-collagen without EDC/NHS (Ti-C). Before the treatment with SDS, fluorescence can be observed in all the samples. The density of collagen aggregates appears to be higher in F2+C samples compared to the others. After the SDS treatment, fluorescent collagen aggregates are stable only in F2+C samples which is an indication of the presence of a covalent bond between the collagen and the surface of the substrates. In contrast, most of the collagen aggregates are removed in the other samples after the treatment.

Figure 3.52: Fluorescence microscopy images of functionalized Ti-6Al-4V samples incubated with FITC-collagen and EDC/NHS (F2+C) (a, e, i, m), functionalized Ti-6Al-4V samples incubated with FITC-collagen without EDC/NHS (F2-C) (b, f, j, n), bare Ti-6Al-4V samples incubated with FITC-collagen and EDC/NHS (Ti+C) (c, g, k, o) and bare Ti-6Al-4V samples incubated with FITC-collagen without EDC/NHS (Ti-C) (d, h, l, p). Images captured with 2.5X, scale bar = 1 mm (a-h) and 10X, scale bar = 100 μ m (i-p) objectives before (a-d, i-l) and after (e-h, m-p) treatment with SDS.

3.4.1.2.2 Atomic force microscopy study

Atomic force microscopy was used to complement the results obtained by fluorescence microscopy in order to characterize the covalently immobilized collagen. Figure 3.53 shows atomic force microscopy images of F2+C, F2-C, Ti+C and Ti-C samples at two different scan sizes before and after SDS treatment. Collagen aggregates are present on all the samples before the treatment. After the SDS treatment, collagen aggregates are only observed on the F2+C samples.

Consequently, AFM results are compatible with those previously obtained by fluorescence microscopy. These results indicate that the collagen immobilized on F2+C samples is resistant to elution by SDS.

Figure 3.53: Atomic Force microscopy topography images of functionalized Ti-6Al-4V sample incubated with FITC-collagen and EDC/NHS (F2+C) (a, e, i, m), functionalized Ti-6Al-4V samples incubated with FITC-collagen without EDC/NHS (F2-C) (b, f, j, n), bare Ti-6Al-4V samples incubated with FITC-collagen and EDC/NHS (Ti+C) (c, g, k, o) and bare Ti-6Al-4V samples incubated with FITC-collagen without EDC/NHS (d, h, l, p) in two different scan sizes. Micrographs captured before (a-d,i-l) and after (e-g, m-p) treatment with SDS.

3.4.1.2.3 Increasing the amount of immobilized collagen

In order to increase the amount of collagen immobilized on the samples and to saturate the amine bonding sites from collagen, the incubation procedure with collagen, with or without EDC/NHS cross-linkers was repeated twice for all the samples. Figure 3.54 shows fluorescence microscopy images of functionalized samples incubated with collagen and EDC/NHS (F2+C), functionalized samples incubated with collagen without EDC/NHS (F2-C), bare Ti-6Al-4V samples incubated with collagen and EDC/NHS

(Ti+C) and bare Ti-6Al-4V samples incubated with collagen without EDC/NHS (Ti-C), when the incubation procedures is repeated twice.

Figure 3.54: Fluorescence microscopy images of functionalized samples incubated with collagen and EDC/NHS (F2+C), functionalized samples incubated with collagen without EDC/NHS (F2-C), bare Ti-6Al-4V samples incubated with collagen and EDC/NHS (Ti+C) and bare Ti-6Al-4V samples incubated with collagen without EDC/NHS (Ti-C). Incubation steps were performed twice for each sample. Images captured with 2.5X, scale bar = 1 mm (a-d) and 10X, scale bar = 100 μ m (e-h) objectives.

It can be seen that by repeating the incubation procedures, the amount of immobilized collagen on F2+C samples increases considerably, whereas in the other samples no significant change is observed (comparison between Figure 3.52 and Figure 3.54)

This result further demonstrates the capacity of the process for adjusting the amount of immobilized protein on the samples by performing several incubation steps.

3.4.1.2.4 Effect of EDC/NHS cross-linker concentration

After the assessment that collagen can be covalently linked to the functional layer, a series of experiments were performed to optimize the process. In particular, the effect of the concentration of EDC/NHS on the process was evaluated.

The concentrations of EDC/NHS used for subsequent immobilization of the collagen on functionalized Ti-6Al-4V were: 2.5/0.63 (1X, since this was the original concentration used in previous experiments), 0.25/0.063 (1/10X) and 0.125/0.0315 (1/20X) mg/ml. The

resulting immobilized proteins were again characterized by fluorescence and atomic force microscopies.

3.4.1.2.4.1 Fluorescence microscopy study

Figure 3.55 shows fluorescence microscopy images of AVS-functionalized (F2) Ti-6Al-4V samples incubated with collagen type I and varying concentration EDC/NHS. Collagen aggregates are apparent in all the samples although, the density of aggregates appears to be higher for the highest concentration of EDC/NHS (concentration 1X).

Figure 3.55: Fluorescence microscopy images of F2 AVS-functionalized samples incubated with FITC-collagen and varying concentrations of EDC/NHS: 2.5/0.63 mg/ml (1X) (a, d), 0.25/0.063 mg/ml (1/10X) (b, e) and 0.125/0.0315 mg/ml (1/20X) (c, f). Images captured with 2.5X, scale bar = 1 mm (a, b, c) and 10X, scale bar = 100 μ m (d, e, f) objectives.

3.4.1.2.4.2 Atomic force microscopy study

Atomic force microscopy was used to complement the results obtained from fluorescence microscopy. AFM micrographs of the functionalized samples incubated with collagen and varying concentrations of EDC/NHS can be seen in Figure 3.56. Collagen aggregates are detected on all the samples, even at the lowest concentration of EDC/NHS (1/20X).

Figure 3.56: : Atomic force microscopy topography images of F2 AVS-functionalized samples incubated with FITC-collagen and varying concentrations of EDC/NHS: 2.5/0.63 mg/ml (1X) (a, d), 0.25/0.063 mg/ml (1/10X) (b, e) and 0.125/0.0315 mg/ml (1/20X) (c, f) in two different scan sizes.

3.4.1.2.4.3 Quantification of immobilized collagen

The qualitative results on the density of immobilized collagen obtained by fluorescence microscopy and AFM were complemented with a quantitative measurement of the amount of the protein on the surface of the samples. Samples for these quantitative measurements were cleaned according to the protocol VIII described in section 2.3.2.4.2, after the protein immobilization process. Figure 3.57 shows the standard concentration-absorbance curve obtained with the BCA kit using serial dilutions of BSA and fitting of a polynomial curve to the data. The absorbance values obtained from incubating the samples with immobilized collagen with BCA kit working solution, were converted to mass of protein using this calibration curve. The values are presented in Table 3.25. Subsequently, the amount of protein obtained for each sample was normalized by the surface area of each sample in order to determine the surface density of collagen. The obtained values were: $0.20 \pm 0.04 \mu\text{g}/\text{mm}^2$, $0.114 \pm 0.009 \mu\text{g}/\text{mm}^2$, $0.117 \pm 0.009 \mu\text{g}/\text{mm}^2$ for 1X, 1/10X, and 1/20X EDC/NHS concentrations, respectively.

Figure 3.57: Standard absorbance-concentration curve for BCA working solution obtained by incubating the working solution with serial dilutions of BSA in PBS.

Table 3.25: Absorbance values of BCA solution for 1X, 1/10X and 1/20X samples and corresponding amounts of collagen.

Quantity Sample	Absorbance @ 562nm (a.u.)	Amount of collagen (µg)	µg of collagen/mm ²
1X	0.191	11.275	0.158
	0.284	16.217	0.232
1/10X	0.119	7.222	0.106
	0.140	8.427	0.123
1/20X	0.135	8.142	0.108
	0.150	8.994	0.125

3.4.2 Bovine serum albumin

3.4.2.1 Fluorescence microscopy study

BSA was covalently immobilized on F2 AVS-functionalized Ti-6Al-4V samples. BSA proteins were labeled with FITC and the EDC/NHS chemistry was used to bind the proteins to the amine groups of the functional layer (see the experimental section for details). Figure 3.58 shows fluorescence microscopy images of the following samples: functionalized Ti-6Al-4V sample incubated with FITC-BSA and EDC/NHS (F2+C), functionalized Ti-6Al-4V samples incubated with FITC-BSA, but not with EDC/NHS (F2-C), bare Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (Ti+C), and bare Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (Ti-C). Micrographs were taken before and after the treatment with SDS and at two different magnifications. Immersion in SDS was intended to remove the adsorbed protein, so that only covalently bound proteins are assumed to stay after the SDS treatment.

These micrographs show that the highest amount of BSA-FITC is found on the F2+C sample, and no significant variation in fluorescence is observed after SDS treatment. Some fluorescence is observed on Ti+C samples, although it decreases after the SDS treatment. Finally, no significant fluorescence is observed on F2-C and Ti-C samples. Consequently, it can be seen that the samples to which EDC/NHS cross-linkers were added (F2+C and Ti+C), show higher amounts of BSA compared to the samples without the cross-linkers (F2-C and Ti-C).

In order to justify these results, it is worth considering the possible interactions established between the BSA and the different substrates. The protein immobilization experiments were performed in MES buffer at pH = 6.0. Since the isoelectric point of BSA is pI = 4.7 (Ge et al., 1998), the protein will have a net negative charge under these conditions.

Functionalized layers with their high surface density of amine groups, are supposed to be positively charged at pH = 6.0. In contrast, TiO₂, which is assumed to be the major component of the surface of bare Ti-6Al-4V samples (Rinner et al., 2000), is negatively charged at pH = 6.0 (Rodríguez-Sánchez et al., 2016, MacDonald et al., 2010).

Assuming that the electrostatic forces are a significant contribution to the interaction between BSA and the surface, BSA should appear preferentially on the functionalized samples and much more scarcely on bare substrates.

As indicated above, the experimental data fits to this scheme. The lowest amount of BSA is found on Ti-C samples, whereas a greater amount of BSA is present on F2-C samples. The amount of BSA on the substrates increases with the addition of cross-linkers in Ti+C samples and reaches its maximum in F2+C samples.

In the samples containing the cross-linkers, the formation of aggregates in solution and their interaction with the surface seems to play a major role in determining the properties of the protein-surface system. It can be hypothesized that BSA aggregates are formed in the presence of EDC/NHS cross-linkers via cross-linking of BSA molecules with each other. Aggregates seem to interact more strongly with the material than the non-crosslinked proteins.

In the case of F2+C samples, the functional amino groups on the F2 samples participate in the chain cross-linking reactions. This leads to the formation of a stable covalent bond between the surface amino groups on the functionalized Ti-6Al-4V substrates and the carboxyl groups of the protein. By comparing F2+C and Ti+C samples, the highest amount and largest aggregate size of BSA is apparent in F2+C samples, which could be related to the participation of functional surface amino groups of F2 samples in the cross-linking reactions. Covalent binding justifies the persistence of fluorescence in F2+C samples even after immersion in SDS solution.

Figure 3.58: Fluorescence microscopy images of functionalized Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (F2+C) (a, e, i, m), functionalized Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (F2-C) (b, f, j, n), bare Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (Ti+C) (c, g, k, o) and bare Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (Ti-C) (d, h, l, p). Images captured with 2.5X, scale bar = 1 mm (a-h) and 10X, scale bar = 100 μ m (i-p) objectives before (a-d, i-l) and after (e-h, m-p) treatment with SDS.

3.4.2.2 Atomic force microscopy study

Atomic force microscopy micrographs of functionalized Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS solution (F2+C), functionalized Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (F2-C), bare Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS solution (Ti+C), and bare Ti-6Al-4V samples incubated with FITC-BSA solution without EDC/NHS (Ti-C) before and after treatment with SDS and in different scan sizes are shown in Figure 3.59. F2+C samples seem to possess the highest amount of BSA on their surface. The larger BSA aggregate size is also

distinguishable for F2+C samples. Ti+C samples have lesser amounts of BSA compared with F2+C. Finally, minor amounts of BSA can be seen on F2-C and almost no BSA Ti-C samples. These results are consistent with the fluorescence microscopy observations and show that BSA can be covalently linked to the functionalized surface using the EDC/NHS as cross-linker.

Figure 3.59: Atomic force microscopy topography images of functionalized Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (F2+C) (a, e, i, m), functionalized Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (F2-C) (b, f, j, n), bare Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (Ti+C) (c, g, k, o) and bare Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (d, h, l, p) in two different scan sizes. Micrographs captured before (a-d, i-l) and after (e-g, m-p) treatment with SDS.

3.4.3 Fibrinogen

3.4.3.1 Fluorescence microscopy study

Figure 3.60 shows fluorescence microscopy images of the following samples: functionalized Ti-6Al-4V sample incubated with FITC-fibrinogen and EDC/NHS (F2+C), functionalized Ti-6Al-4V incubated with FITC-fibrinogen, but not with EDC/NHS (F2-C), bare Ti-6Al-4V samples incubated with FITC-fibrinogen and EDC/NHS (Ti+C), and bare Ti-6Al-4V samples incubated with FITC-fibrinogen, but not with EDC/NHS (Ti-C). Micrographs were captured before and after the treatment with SDS at two different magnifications.

In the samples where no cross-linkers are used (Ti-C and F2-C), there seems to be more amounts of fibrinogen adsorbed on the functionalized (F2-C) samples compared with bare (Ti-C) samples. This phenomenon can be justified by considering the electrostatic interactions between the protein and the surface. As it was stated above, at pH = 6.0 in which the experiments were performed, the surface of bare Ti-6Al-4V samples is negatively charged which is due the presence of TiO₂ surface oxide layer (Rapuano & MacDonald, 2011, MacDonald et al., 2010). On the other hand, the isoelectric point of the fibrinogen is found at pH = 5.5 (Tsapikouni et al., 2008, Salim et al., 2007). Therefore, fibrinogen also has a slight negative net charge. In contrast, the surface of AVS-functionalized samples is positively charged at pH = 6.0, which makes them more favorable for adsorption of a negatively charged protein (i.e. fibrinogen) than a negatively charged surface.

The effect of addition of EDC/NHS cross-linkers can be seen in Figure 3.60. The aggregation of fibrinogen occurs due to the cross-linking of amino and carboxyl groups of the protein. This aggregation can overcome the weak electrostatic forces and leads to adsorption of higher amounts of fibrinogen on bare Ti-6Al-4V samples (Ti+C).

In the case of F2+C samples, apart from the cross-linking of the protein, the surface functional amine groups present on the functionalized samples also participate in the cross-linking reactions. This leads to the formation of a stable covalent bond between the fibrinogen and the surface of the substrates. Consequently, the highest amount of fibrinogen is immobilized on these samples.

After the treatment with SDS, it seems that in Ti+C (Figure 3.60 g, o) and Ti-C (Figure 3.60 h, p) samples almost all the previously adsorbed fibrinogen is removed, as indicated

by the lack of fluorescence in these images. In F2-C samples, although not all of the fluorescent fibrinogen is removed, there is a marked decrease in fluorescence after the SDS treatment (Figure 3.60 f, n).

Finally, in F2+C samples, no loss of fluorescence is observed after the SDS treatment which indicates a high stability of the immobilized protein on these samples, and confirms the formation of a stable covalent bond between the fibrinogen and the surface of the samples that is able to resist being eluted by SDS.

Figure 3.60: Fluorescence microscopy images of functionalized Ti-6Al-4V samples incubated with FITC-fibrinogen and EDC/NHS (F2+C) (a, e, i, m), functionalized Ti-6Al-4V samples incubated with FITC-fibrinogen without EDC/NHS (F2-C) (b, f, j, n), bare Ti-6Al-4V samples incubated with FITC-fibrinogen and EDC/NHS (Ti+C) (c, g, k, o) and bare Ti-6Al-4V samples incubated with FITC-fibrinogen without EDC/NHS (Ti-C) (d, h, l, p). Images captured with 2.5X, scale bar = 1 mm (a-h) and 10X, scale bar = 100 μ m (i-p) objectives before (a-d, i-l) and after (e-h, m-p) treatment with SDS.

3.4.3.2 Atomic force microscopy study

Atomic force microscopy micrographs of functionalized Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (F2+C), functionalized Ti-6Al-4V samples incubated with FITC-BSA without EDC/NHS (F2-C), bare Ti-6Al-4V samples incubated with FITC-BSA and EDC/NHS (Ti+C) and bare Ti-6Al-4V samples incubated with FITC-BSA solution without EDC/NHS (Ti-C) before and after treatment with SDS and in different scan sizes are shown in Figure 3.61. F2+C samples seem to possess the greatest amount of fibrinogen and Ti-C samples the smallest amount. The stability of covalently immobilized fibrinogen on F2+C samples can be appreciated from these micrographs, since the covalently immobilized protein is stable after the SDS treatment.

These results are coherent with those obtained by fluorescence microscopy and validate the stability of the covalently immobilized fibrinogen on AVS-functionalized Ti-6Al-4V substrates.

Figure 3.61: Atomic force microscopy topography images of functionalized Ti-6Al-4V sample incubated with FITC-fibrinogen and EDC/NHS (F2+C) (a, e, i, m), functionalized Ti-6Al-4V samples incubated with FITC-fibrinogen without EDC/NHS (F2-C) (b, f, j, n), bare Ti-6Al-4V samples incubated with FITC-fibrinogen and EDC/NHS (Ti+C) (c, g, k, o) and bare Ti-6Al-4V samples incubated with FITC-fibrinogen without EDC/NHS (Ti-C) (d, h, l, p) in two different scan sizes. Micrographs captured before (a-d, i-l) and after (e-g, m-p) treatment with SDS.

3.4.4 Fibronectin

3.4.4.1 Fluorescence microscopy study

Figure 3.62 shows fluorescence microscopy images of AVS-functionalized Ti-6Al-4V samples incubated with FITC-fibronectin and EDC/NHS (F2+C), functionalized Ti-6Al-4V incubated with FITC-fibronectin without EDC/NHS (F2-C), bare Ti-6Al-4V incubated with FITC-fibronectin and EDC/NHS (Ti+C) and bare Ti-6Al-4V incubated

with FITC-fibronectin without EDC/NHS (Ti-C) in different magnifications. In contrast to previous results, no fluorescence related to the protein aggregates was observable in any of the samples. It was hypothesized that this lack of fluorescence might be related to the low concentration of the protein solution employed for the study (200 $\mu\text{g/ml}$) compared to the concentration of 2.5 mg/ml used for collagen. The low concentration of the fluorescent fibronectin would, in turn, lead to a smaller number of protein aggregates that cannot be detected by fluorescence microscopy.

Figure 3.62: Fluorescence microscopy images of functionalized Ti-6Al-4V samples incubated with FITC-fibronectin and EDC/NHS (F2+C) (a, e), functionalized Ti-6Al-4V samples incubated with FITC-fibronectin without EDC/NHS (F2-C) (b, f), bare Ti-6Al-4V samples incubated with FITC-fibronectin and EDC/NHS (Ti+C) (c, g) and bare Ti-6Al-4V samples incubated with FITC-fibronectin without EDC/NHS (Ti-C) (d, h). Images captured with 2.5X, scale bar = 1 mm (a-d) and 10X, scale bar = 100 μm (e-h) objectives.

3.4.4.2 Atomic force microscopy study

The absence of a positive identification of fibronectin on the surface by fluorescence microscopy increased the importance of identifying the protein on the surface by atomic force microscopy. Atomic force microscopy is able to detect the presence of proteins on the surface at densities below the resolution of fluorescence microscopy. The AFM micrographs of functionalized Ti-6Al-4V samples incubated with FITC-fibronectin and EDC/NHS (F2+C), functionalized Ti-6Al-4V incubated with FITC-fibronectin without EDC/NHS (F2-C), bare Ti-6Al-4V incubated with FITC-fibronectin and EDC/NHS

(Ti+C) and bare Ti-6Al-4V incubated with FITC-fibronectin without EDC/NHS (Ti-C) can be seen in Figure 3.63. The presence of fibronectin is apparent in F2+C and F2-C samples. In contrast, no distinctive structures that might be associated with the presence of fibronectin on the surface are found either in Ti+C and Ti-C samples.

Figure 3.63: Atomic force microscopy topography images of functionalized Ti-6Al-4V samples incubated with FITC-fibronectin and EDC/NHS (F2+C) (a,e), functionalized Ti-6Al-4V incubated with FITC-fibronectin without EDC/NHS (F2-C) (b,f), bare Ti-6Al-4V incubated with FITC-fibronectin and EDC/NHS (Ti+C) (c,g) and bare Ti-6Al-4V incubated with FITC-fibronectin without EDC/NHS (Ti-C) (d,h) in two different scan sizes.

Figure 3.64 shows the AFM topography images of F2+C and F2-C samples after the treatment with SDS in different scan sizes. It can be seen that while fibronectin is still present on F2+C samples after the treatment, almost all of the initially present fibronectin on F2-C samples is removed. This is an indication of the higher stability of the covalently immobilized versus the physically adsorbed fibronectin. The relatively high amount of fibronectin on F2-C samples before the treatment with SDS can be justified considering the possible electrostatic interactions between the substrate and fibronectin. The isoelectric point of plasma fibronectin is reported to be at $pI = 5.2$ (Tooney et al., 1983). Consequently, at $pH = 6.0$, which is the value of the buffer used during the incubation, fibronectin is slightly negatively charged. It can be hypothesized that attraction forces are generated between the positively-charged surface amines and the negatively-charged

fibronectin. However, adsorbed fibronectin was not able to resist the SDS treatment and was removed, while the covalently bound fibronectin (F2+C samples) was stable before and after the treatment.

Figure 3.64: Atomic force microscopy topography images of functionalized Ti-6Al-4V samples incubated with FITC-fibronectin and EDC/NHS (F2+C) (a,c) and functionalized Ti-6Al-4V incubated with FITC-fibronectin without EDC/NHS (F2-C) (b,d) after treatment with SDS.

3.5 Cell cultures on covalently immobilized proteins

In the experiments performed with adsorbed proteins on Ti-6Al-4V substrates, it was demonstrated that among the evaluated proteins and cells lines, collagen type I and fibronectin were able to enhance the response of BM-MSCs to the material in terms of initial cell adhesion. Thus, these two proteins were selected to proceed with the in-vitro cell culture experiments on protein-immobilized Ti-6Al-4V substrates.

As a first step, the possible toxic effect of EDC/NHS cross-linkers on BM-MSCs was evaluated.

3.5.1 Cytotoxicity of EDC/NHS cross-linkers

In order to assess the effect of EDC/NHS cross-linkers on BM-MSCs, BM-MSCs were exposed to serial dilutions of EDC/NHS in the medium. Cells were seeded in a P24 dish

and at the time of seeding, EDC/NHS were mixed with the cell culture medium. Fluorescence microscopy images of calcein/PI stained cells after 4 and 48 h of seeding can be seen in Figure 3.65. 1X refers to a concentration of 2.5/0.63 mg/ml EDC/NHS in the medium and control sample denotes the samples without EDC/NHS.

As it can be seen in Figure 3.65 massive cell death has occurred in all the samples that contained EDC/NHS. In the highest concentration of EDC/NHS (1X) almost all the cells are PI positive. Even at the lowest concentration (1/100X) in the 4 h images, and although some viable cells are observable, their number is much smaller than that on the control culture wells. Besides, the morphology of cells incubated with EDC/NHS is very different from those grown in the controls. 48 h after seeding no viable cells could be seen in any of the EDC/NHS containing culture wells.

Thus, in this concentration range and from its early effect on the cells, it can be assumed that the cytotoxic effects of EDC/NHS appear almost instantly when it comes in contact with the cells.

Although several available studies report no cytotoxic effect of carbodiimide cross-linkers (S. Park et al., 2002, Osborne et al., 1997, Pieper et al., 1999), the results obtained in this study demonstrate that EDC/NHS cross-linkers can be very cytotoxic. In this regard, our study supports previous reports on the negative effect of EDC or EDC/NHS on the survival and proliferation of cells. EDC/NHS cytotoxicity was also found by Müller et al. which stated that medium concentration of 5 mg/ml EDC significantly reduced human MSC cell attachment and proliferation (Müller et al., 2005). The cytotoxic effect of EDC on different cell lines was assessed by Moshnikova et al. through exposure of the cells to 2-200 μ M (0.38×10^{-3} - 38.34×10^{-3} mg/ml) of EDC in the medium. It was concluded that EDC shows cytotoxicity in-vitro through apoptosis at low concentrations and thorough necrosis at high concentrations (Moshnikova et al., 2006). The mechanism involved in the cytotoxicity of EDC is believed to be the interchain cross-linking within DNA of cells caused by EDC, which leads to disturbances in the cell cycle and eventual cell death (Moshnikova et al., 2006). Since the EDC/NHS concentrations in this study are higher than those reported in the literature in order to match the concentrations required for protein immobilization, these results highlight the importance of removing any traces of free cross-linkers to minimize their cytotoxic effects.

Figure 3.65: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on polystyrene dish in the presence of varying concentrations of EDC/NHS at 4 and 48 h of seeding. (Scale bar = 100 μm).

3.5.2 Cell cultures on Ti-6Al-4V samples with covalently immobilized collagen type I

This section addresses the results of cell cultures performed on Ti-6Al-4V samples with immobilized collagen and the use of different cleaning procedures for the removal of the excess cross-linkers. The experiments were performed in a trial-and-error basis until the desirable outcome was achieved.

Collagen type I was covalently immobilized on functionalized (F2) Ti-6Al-4V samples according to the protocol described in section 2.3.2.4.1. After the protein immobilization step, different cleaning procedures were employed in order to remove the excess of non-reacted EDC/NHS cross-linkers from the samples.

3.5.2.1 Protocol I: Rinsing with distilled water

In protocol I, the immobilization process was performed with 1X EDC/NHS (1X: 2.5/0.63 mg/ml) and after completing the process the samples were thoroughly rinsed with distilled water. Cell culture experiments proceeded immediately.

Figure 3.66 shows the morphology of the BM-MSCs adhered on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V and control samples after 4 and 48 h of seeding. It can be seen that after 4 h, the cells are adhered on all the samples. The cells adhered on immobilized-collagen samples possessed higher extension of the cytoskeleton compared to those adhered on bare Ti-6Al-4V samples. After 48 h of seeding however, the cells on bare Ti-6Al-4V and control samples showed a more prominent polygonal morphology typical for this cell line. In contrast, cells adhered on collagen-immobilized Ti-6Al-4V possessed a more pronounced circular morphology. The results of cell quantification are shown in Figure 3.67 and the statistical analysis of the data in Table 3.26.

Figure 3.66: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol I and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.67: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol I and polystyrene control samples.

Table 3.26: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol I and polystyrene control samples at 4 and 48 after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Collagen-immobilized Ti-6Al-4V	16.404*	0.009
		Bare Ti-6Al-4V	33.568*	0.000
	Collagen-immobilized Ti-6Al-4V	Control	-16.404*	0.009
		Bare Ti-6Al-4V	17.164*	0.007
	Bare Ti-6Al-4V	Control	-33.568*	0.000
		Collagen-immobilized Ti-6Al-4V	-17.164*	0.007
Cell_No_48H	Control	Collagen-immobilized Ti-6Al-4V	101.325*	0.000
		Bare Ti-6Al-4V	51.325*	0.015
	Collagen-immobilized Ti-6Al-4V	Control	-101.325*	0.000
		Bare Ti-6Al-4V	-50.000*	0.000
	Bare Ti-6Al-4V	Control	-51.325*	0.015
		Collagen-immobilized Ti-6Al-4V	50.000*	0.000

*. The mean difference is significant at the 0.05 level.

These results demonstrate that although at 4 h after seeding collagen-immobilized Ti-6Al-4V samples showed improved cell adhesion, after 48 h some evidence of cytotoxicity is observed as indicated by an increased cell death and lower cellular viability. It was hypothesized that an excess of non-removed EDC might cause this cytotoxic effect (Moshnikova et al., 2006). Therefore, it was concluded that a more thorough cleaning process was needed for the complete removal of the residual EDC from the samples.

Our initial assumption was that since EDC and urea, the only by-product of the cross-linking reaction, are water soluble (Ahmad et al., 2015), a simple rinsing process could eliminate any unwanted residues. On the other hand, EDC/NHS is a zero-length cross-linker system. In other word, EDC does not get integrated inside the material structure (Olde Damink et al., 1996). However, we observed that such a rudimentary cleaning process may not be thorough enough to eliminate residual EDC and any possible reaction by-products. Thus, the cleaning process was greatly expanded and revised until desirable results were obtained.

3.5.2.2 Protocol II: Cleaning in PBS for 24 h

The next experiment was performed by using 1X concentration of the cross-linkers for the immobilization process, followed by rinsing with distilled water and immersion in PBS buffer for 24 h.

Figure 3.68 shows the morphology of the BM-MSCs adhered to bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V and polystyrene control samples at 4 and 48 h after seeding. At 4 h after seeding, the cells adhered on the collagen-immobilized Ti-6Al-4V samples showed a more marked expansion of the cytoskeleton and filipodia, as well as higher confluency compared to the cells cultured on bare Ti-6Al-4V. After 48 h of seeding however, the confluency seemed to be the same in the bare and collagen-immobilized Ti-6Al-4V samples. It is apparent that cells on collagen-immobilized substrates are more prone to show a circular morphology. The results of cell quantification are shown in Figure 3.69 and the statistical analysis of the data in Table 3.27. Since there was no increase in the number of cells attached to the collagen-immobilized samples between 4 and 48 h after seeding, it can be deduced that the proliferation of the cells on these samples was rather inhibited.

Figure 3.68: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol II and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.69: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol II and polystyrene control samples.

Table 3.27: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol II and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Collagen-immobilized Ti-6Al-4V	17.951*	0.015
		Bare Ti-6Al-4V	57.056*	0.000
	Collagen-immobilized Ti-6Al-4V	Control	-17.951*	0.015
		Bare Ti-6Al-4V	39.105*	0.000
	Bare Ti-6Al-4V	Control	-57.056*	0.000
		Collagen-immobilized Ti-6Al-4V	-39.105*	0.000
Cell_No_48H	Control	Collagen-immobilized Ti-6Al-4V	67.550*	0.001
		Bare Ti-6Al-4V	68.530*	0.001
	Collagen-immobilized Ti-6Al-4V	Control	-67.550*	0.001
		Bare Ti-6Al-4V	0.980	0.991
	Bare Ti-6Al-4V	Control	-68.530*	0.001
		Collagen-immobilized Ti-6Al-4V	-0.980	0.991

*. The mean difference is significant at the 0.05 level.

The inhibited proliferation of the BM-MSCs in collagen-immobilized samples was believed to be due to the presence of traces of EDC. In this case, although the concentration of free EDC does not seem to be high enough to cause massive cell death, it might be sufficient to disturb the cell cycle and to prevent proliferation (Moshnikova et al., 2006). At low concentrations, the cytotoxic effects of EDC are reported to be connected to a cytostatic activity, which prevents cell proliferation and that eventually results in cell death by apoptosis (Moshnikova et al., 2006).

Although in this experiment some improvements are observed with respect to protocol I, the cleaning protocol II was not sufficient to eliminate the traces of EDC. Thus, a new cleaning protocol was deemed necessary.

3.5.2.3 Protocol III: Cleaning in MES buffer for 24 h

As the next attempt, the collagen immobilization on the functionalized samples was performed with 1X concentrations of the cross-linkers and the samples were rinsed with distilled water followed by immersion in MES buffer for 24 h. The BM-MSCs were subsequently seeded on these samples.

Figure 3.70 shows the morphology of the BM-MSCs adhered on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V and control samples after 4 and 48 h of seeding. After 4 h of seeding, the cells adhered on collagen-immobilized samples appeared to possess higher extension of the cytoskeleton compared to those adhered on bare Ti-6Al-4V.

After 48 h of seeding, the cells attached on polystyrene controls and bare Ti-6-Al-4V samples possessed a prominent triangular cell shape, in contrast with the abundant circular morphology of the cells on collagen-immobilized samples. The quantification of these data is presented in Figure 3.71 and Table 3.28. At 4 h after seeding a considerably higher number of cells were attached to collagen-immobilized Ti-6Al-4V compared to the bare Ti-6Al-4V samples. In contrast, at 48 h after seeding, there were no meaningful differences between the number of viable cells on both types of samples. An approximately constant number of cells on collagen-immobilized samples between 4 and 48 h, reflects the hampered proliferation of the cells in these samples.

Figure 3.70: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol III and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.71: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol III and polystyrene control samples.

Table 3.28: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol III and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Collagen-immobilized Ti-6Al-4V	19.939*	0.003
		Bare Ti-6Al-4V	43.040*	0.000
	Collagen-immobilized Ti-6Al-4V	Control	-19.939*	0.003
		Bare Ti-6Al-4V	23.101*	0.001
	Bare Ti-6Al-4V	Control	-43.040*	0.000
		Collagen-immobilized Ti-6Al-4V	-23.101*	0.001
Cell_No_48H	Control	Collagen-immobilized Ti-6Al-4V	49.502*	0.000
		Bare Ti-6Al-4V	51.298*	0.001
	Collagen-immobilized Ti-6Al-4V	Control	-49.502*	0.000
		Bare Ti-6Al-4V	1.795	0.967
	Bare Ti-6Al-4V	Control	-51.298*	0.001
		Collagen-immobilized Ti-6Al-4V	-1.795	0.967

*, The mean difference is significant at the 0.05 level.

By considering the obtained results, it can be concluded that the cytotoxic effects of EDC are still prevalent in protocol III.

3.5.2.4 Protocol IV: Cleaning in DMEM for 24 h

For the next attempt, 1X concentration of the cross-linkers was used and the samples were rinsed with distilled water and immersed in serum-free DMEM for 24 h.

The morphology of BM-MSCs adhered on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V and control samples subjected to cleaning protocol IV after 4 and 48 h of seeding is shown in Figure 3.72. Higher extension of the cytoskeleton can be seen for the cells adhered on collagen-immobilized samples compared to bare Ti-6Al-4V at 4 h after seeding. After 48 h of seeding however, the cells on collagen-immobilized Ti-6Al-4V appear smaller in size compared to those on bare Ti-6Al-4V samples. The quantification of the results is presented in Figure 3.73 and Table 3.29. As observed in previous experiments, improved cell adhesion was observed for collagen-immobilized samples compared to bare Ti-6Al-4V, at 4 h after seeding. At 48 h however, bare and collagen-immobilized Ti-6Al-4V samples had roughly the same number of attached viable cells.

By comparing the number of cells on collagen-immobilized samples at 4 and 48 h after seeding, it can be concluded that the proliferation of cells is hampered.

Figure 3.72: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol IV and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μm).

Figure 3.73: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol IV and polystyrene control samples.

Table 3.29: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V cleaned with protocol IV and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Collagen-immobilized Ti-6Al-4V	29.619*	0.000
		Bare Ti-6Al-4V	50.193*	0.000
	Collagen-immobilized Ti-6Al-4V	Control	-29.619*	0.000
		Bare Ti-6Al-4V	20.574*	0.005
	Bare Ti-6Al-4V	Control	-50.193*	0.000
		Collagen-immobilized Ti-6Al-4V	-20.574*	0.005
Cell_No_48H	Control	Collagen-immobilized Ti-6Al-4V	57.511*	0.000
		Bare Ti-6Al-4V	58.726*	0.000
	Collagen-immobilized Ti-6Al-4V	Control	-57.511*	0.000
		Bare Ti-6Al-4V	1.215	0.969
	Bare Ti-6Al-4V	Control	-58.726*	0.000
		Collagen-immobilized Ti-6Al-4V	-1.215	0.969

*, The mean difference is significant at the 0.05 level.

For the development of the next cleaning protocol, it was decided to employ a combination of the previous cleaning procedures. In addition, the concentration of the EDC/NHS cross-linkers was reduced.

3.5.2.5 Protocol V: Continuous cleaning in MES buffer

In this experiment the collagen immobilization procedure was performed using one half (1/2X), one fifth (1/5X) and one tenth (1/10X) of the original EDC/NHS cross-linkers concentration. The samples were rinsed with distilled water and cleaned in MES buffer while the buffer was renewed as explained in-detail in section 2.3.2.4.2.

Figure 3.74 shows the morphology of calcein/PI stained BM-MSCs adhered on these samples after 4 and 48 h of seeding. Similar to the previous experiments, the cells adhered on collagen-immobilized samples appear to have higher degrees of cell extension compared to bare Ti-6Al-4V. By comparing the 1/2X, 1/5X and 1/10X samples after 48 h seeding, it can be seen that a reduction in the concentration of the cross-linkers leads to a decrease in the number of cell with circular shape. It can be noticed that the morphology

of the BM-MSCs adhered on 1/10X samples is the most similar to that of cells cultured on bare Ti-6Al-4V.

Figure 3.74: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/2X, 1/5X and 1/10X concentrations of EDC/NHS cleaned with protocol V and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

The quantification of these results is presented in Figure 3.75 and Table 3.30. Collagen-immobilized samples (1/2X, 1/5X and 1/10X) show improved cell adhesion at 4 h after seeding. However, at 48 h after seeding, no cell proliferation was observed in these samples even at the lowest concentration of the cross-linkers (1/10X). In all cases, the cellular viability was lower for collagen-immobilized samples compared to control and bare Ti-6Al-4V samples at both incubation times.

Figure 3.75: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/2X, 1/5X and 1/10X concentrations of EDC/NHS cleaned with protocol V and polystyrene control samples.

The obtained results pointed out that even a reduction in the concentration of the cross-linkers of one order of magnitude is not sufficient to prevent the inhibitory effects of EDC on the proliferation of the cells.

Table 3.30: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/2X, 1/5X and 1/10X concentrations of EDC/NHS cleaned with protocol V and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	1/2X	26.291*	0.008
		1/5X	22.507*	0.041
		1/10X	26.981*	0.007
		Bare Ti-6Al-4V	42.736*	0.001
	1/2X	Control	-26.291*	0.008
		1/5X	-3.783	0.968
		1/10X	0.690	1.000
		Bare Ti-6Al-4V	16.446*	0.031
	1/5X	Control	-22.507*	0.041
		1/2X	3.783	0.968
		1/10X	4.474	0.940
		Bare Ti-6Al-4V	20.229*	0.039
	1/10X	Control	-26.981*	0.007
		1/2X	-0.690	1.000
		1/5X	-4.474	0.940
		Bare Ti-6Al-4V	15.755*	0.031
	Bare Ti-6Al-4V	Control	-42.736*	0.001
		1/2X	-16.446*	0.031
		1/5X	-20.229*	0.039
		1/10X	-15.755*	0.031

*. The mean difference is significant at the 0.05 level.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_48H	Control	1/2X	83.623*	0.000
		1/5X	61.226*	0.000
		1/10X	78.016*	0.000
		Bare Ti-6Al-4V	56.144*	0.000
	1/2X	Control	-83.623*	0.000
		1/5X	-22.397	0.059
		1/10X	-5.606	0.684
		Bare Ti-6Al-4V	-27.478*	0.026
	1/5X	Control	-61.226*	0.000
		1/2X	22.397	0.059
		1/10X	16.791	0.206
		Bare Ti-6Al-4V	-5.081	0.972
	1/10X	Control	-78.016*	0.000
		1/2X	5.606	0.684
		1/5X	-16.791	0.206
		Bare Ti-6Al-4V	-21.872	0.079
	Bare Ti-6Al-4V	Control	-56.144*	0.000
		1/2X	27.478*	0.026
		1/5X	5.081	0.972
		1/10X	21.872	0.079

*. The mean difference is significant at the 0.05 level.

3.5.2.6 Protocol VI: Cleaning in PBS and DMEM

In this new attempt, the immobilization of collagen on functionalized Ti-6Al-4V samples was performed by using 1/10X and 1/20X concentrations of EDC/NHS. Subsequently, the samples were rinsed with distilled water and cleaned in PBS and serum-free DMEM while the buffers were renewed several times as previously described in section 2.3.2.4.2.

Figure 3.76: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VI and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μm).

Figure 3.76 shows the morphology of BM-MSCs adhered on samples cleaned using protocol VI. At 4 h after seeding, the cells attached to the collagen-immobilized samples present a better extended morphology compared to those attached to bare Ti-6Al-4V. At

48 h, a healthy cell morphology was observed in all the samples. In particular, the morphology of the cells on 1/20X samples were comparable to that on the bare Ti-6Al-4V samples.

The quantification of the results is presented in Figure 3.77 and Table 3.31. As it can be seen, the number of cells attached to 1/10X and 1/20X samples at 4 h after seeding was significantly greater than that of bare Ti-6Al-4V denoting improved cell adhesion for these samples at short incubation time. At 48 h after cell seeding however, there was no increase in the number of viable cells on 1/10X and 1/20X samples, in contrast to the proliferation observed in the cells on bare Ti-6Al-4V and controls. The cellular viability was more than 90% for all the samples and no abnormal cell death was observed in any of the samples.

Figure 3.77: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VI and polystyrene control samples.

Table 3.31: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VI and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	1/10X	22.687*	0.018
		1/20X	22.949*	0.018
		Bare Ti-6Al-4V	37.462*	0.001
	1/10X	Control	-22.687*	0.018
		1/20X	0.262	1.000
		Bare Ti-6Al-4V	14.775*	0.023
	1/20X	Control	-22.949*	0.018
		1/10X	-0.262	1.000
		Bare Ti-6Al-4V	14.512*	0.039
	Bare Ti-6Al-4V	Control	-37.462*	0.001
		1/10X	-14.775*	0.023
		1/20X	-14.512*	0.039
Cell_No_48H	Control	1/10X	63.394*	0.000
		1/20X	66.638*	0.002
		Bare Ti-6Al-4V	58.588*	0.000
	1/10X	Control	-63.394*	0.000
		1/20X	3.245	0.990
		Bare Ti-6Al-4V	-4.805	0.913
	1/20X	Control	-66.638*	0.002
		1/10X	-3.245	0.990
		Bare Ti-6Al-4V	-8.050	0.881
	Bare Ti-6Al-4V	Control	-58.588*	0.000
		1/10X	4.805	0.913
		1/20X	8.050	0.881

*. The mean difference is significant at the 0.05 level.

The results obtained in this trial indicated that the cleaning protocol VI was able to diminish the cytotoxic effect of the EDC to the point that no abnormal cell death was detected in 1/10X and 1/20X samples. However, there still appears to be some traces of EDC in these samples that hinders cell proliferation.

3.5.2.7 Cleaning protocol VII: PBS, MES and DMEM

Cleaning protocol VII started from 1/10X and 1/20X concentrations of EDC/NHS for the immobilization of collagen on functionalized Ti-6Al-4V samples. Afterwards, the samples were cleaned in PBS, MES and DMEM, respectively as described in section 2.3.2.4.2.

The morphology of BM-MSCs cultured on these samples can be seen in Figure 3.78. At 4 h after seeding, the cells on bare Ti-6Al-4V showed a more prominent circular morphology while the cells on 1/10X and 1/20X samples possessed a more prominent polygonal shape. The highest confluency of cells was observed in the polystyrene control samples. After 48 h, the cells on all the samples demonstrated elongated cell shape with developed processes. No signs of cytotoxicity were observed either in the 1/10X or 1/20X samples.

The results of cell quantification are shown in Figure 3.79 and Table 3.32. It can be seen that at 4 h, 1/10X samples possessed significantly higher number of viable cells compared to bare Ti-6Al-4V. At this time, the difference in the number of viable cells between the 1/20X and bare Ti-6Al-4V samples is not statistically significant ($p>0.05$). After 48 h of seeding, the number of viable BM-MSCs in all the samples increased, although for 1/10X and 1/20X samples, the increase was smaller. At this temporal point bare Ti-6Al-4V, 1/10X and 1/20X samples had roughly the same number of viable cells. Cell viability for all the samples was high and no abnormal cell death was observed in any of the samples.

Figure 3.78: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VII and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μm).

In this trial, the results demonstrated a slight increase in the number of viable cells for 1/10X and 1/20X samples, which indicates moderate cell proliferation. However, cell proliferation in collagen-immobilized samples was slower than that in bare Ti-6Al-4V samples. Based on these results, and although the cleaning protocol VII was not effective enough to completely diminish the cytotoxic effects of residual EDC, it was thought to be a step in the right direction. Thus, this protocol was used as the basis of a new protocol in which the duration and the number of changes in the buffers was increased.

Figure 3.79: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VII and polystyrene control samples.

Table 3.32: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VII and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) samples	(J) samples	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	1/10X	22.162*	0.001
		1/20X	28.031*	0.029
		Bare Ti-6Al-4V	41.508*	0.000
	1/10X	Control	-22.162*	0.001
		1/20X	5.868	0.840
		Bare Ti-6Al-4V	19.345*	0.000
	1/20X	Control	-28.031*	0.029
		1/10X	-5.868	0.840
		Bare Ti-6Al-4V	13.477	0.321
	Bare Ti-6Al-4V	Control	-41.508*	0.000
		1/10X	-19.345*	0.000
		1/20X	-13.477	0.321
Cell_No_48H	Control	1/10X	45.457*	0.003
		1/20X	48.771*	0.000
		Bare Ti-6Al-4V	51.670*	0.033
	1/10X	Control	-45.457*	0.003
		1/20X	3.314	0.979
		Bare Ti-6Al-4V	6.214	0.921
	1/20X	Control	-48.771*	0.000
		1/10X	-3.314	0.979
		Bare Ti-6Al-4V	2.900	0.986
	Bare Ti-6Al-4V	Control	-51.670*	0.033
		1/10X	-6.214	0.921
		1/20X	-2.900	0.986

*. The mean difference is significant at the 0.05 level.

3.5.2.8 Cleaning protocol VIII: PBS, MES and DMEM

In the cleaning protocol VIII, 1X, 1/10X and 1/20X concentration of the cross-linkers were used for immobilization of collagen on functionalized Ti-6Al-4V samples. The cleaning consisted of rinsing the samples with distilled water, immersion of the samples in PBS for 2 h + 2 h, MES for 72 h and serum-free DMEM for 12 h + 12 h, as previously indicated in section 2.3.2.4.2.

The morphology of the BM-MSCs adhered on these samples is shown in Figure 3.80. At 4 h after seeding, the cells are adhered on all the samples. The highest confluency of the cells can be observed in the polystyrene control samples. After 48 h of seeding, there is an apparent difference in cell morphology between 1X, 1/10X and 1/20X samples. It can be seen that by reducing the concentration of the cross-linkers, the morphology of the cells becomes more similar to that of the control samples. In this regard, a reduced number of cells with circular morphology and an increase in cell spreading in a large number of cells can be seen in 1/20X samples at this temporal point.

The results of cell quantification are shown in Figure 3.81 and Table 3.33. At 4 h after seeding, a significantly higher number of viable cells were adhered to 1/20X samples compared to bare Ti-6Al-4V. In contrast, the number of viable cells adhered on 1X and 1/10X samples was not statistically different from that of bare Ti-6Al-4V. After 48 h after seeding, the signs of cytotoxicity were observed in 1X samples, as indicated by the reduction in the number of viable cells, lack of cell proliferation and lower cellular viability. In 1/10X and 1/20X samples however, no cytotoxicity was detected and an increase in the number of viable cells was measured. While there was no significant difference between the number of viable cells for 1/10X and bare Ti-6Al-4V after 48 h, 1/20X samples possessed statistically significant more viable cells than bare Ti-6Al-4V.

Figure 3.80: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.81: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples.

In order to confirm the positive results from the quantification of the cell numbers, independent XTT experiments were also performed on these samples. The results are shown in Figure 3.82 and Table 3.34 and are in agreement with those previously obtained by cell counting and demonstrated that 48 h after seeding, a significantly larger number of cells are found on 1/20X samples compared with bare Ti-6Al-4V substrates.

The results obtained from 1/20X samples and cleaning protocol VIII fulfil the main objective of this work since they show the possibility of enhancing the biological response to Ti-6Al-4V substrates in vitro, through covalent immobilization of a protein on the surface of the material.

Table 3.33: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Samples	(J) Samples	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	1/20X	32.159*	0.000
		1/10X	48.398*	0.000
		1X	46.154*	0.000
		Bare Ti-6Al-4V	51.173*	0.000
	1/20X	Control	-32.159*	0.000
		1/10X	16.238	0.073
		1X	13.995	0.055
		Bare Ti-6Al-4V	19.014*	0.014
	1/10X	Control	-48.398*	0.000
		1/20X	-16.238	0.073
		1X	-2.244	0.994
		Bare Ti-6Al-4V	2.775	0.991
	1X	Control	-46.154*	0.000
		1/20X	-13.995	0.055
		1/10X	2.244	0.994
		Bare Ti-6Al-4V	5.019	0.863
	Bare Ti-6Al-4V	Control	-51.173*	0.000
		1/20X	-19.014*	0.014
		1/10X	-2.775	0.991
		1X	-5.019	0.863

*. The mean difference is significant at the 0.05 level.

Dependent Variable	(I) Samples	(J) Samples	Mean Difference (I-J)	Sig.
Cell_No_48H	Control	1/20X	32.905*	0.044
		1/10X	63.421*	0.001
		1X	115.478*	0.000
		Bare Ti-6Al-4V	76.353*	0.000
	1/20X	Control	-32.905*	0.044
		1/10X	30.516	0.099
		1X	82.573*	0.000
		Bare Ti-6Al-4V	43.448*	0.000
	1/10X	Control	-63.421*	0.001
		1/20X	-30.516	0.099
		1X	52.057*	0.002
		Bare Ti-6Al-4V	12.931	0.819
	1X	Control	-115.478*	0.000
		1/20X	-82.573*	0.000
		1/10X	-52.057*	0.002
		Bare Ti-6Al-4V	-39.126*	0.001
	Bare Ti-6Al-4V	Control	-76.353*	0.000
		1/20X	-43.448*	0.000
		1/10X	-12.931	0.819
		1X	39.126*	0.001

*. The mean difference is significant at the 0.05 level.

Figure 3.82: Absorbance values of XTT assays obtained from BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 48 h after seeding.

Table 3.34: Results of the Games-Howell test performed on the data obtained from XTT assays on BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 48 h after seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Control	1X	0.006573*	0.000
	1/10X	0.006899*	0.010
	1/20X	0.005043*	0.001
	Bare Ti-6Al-4V	0.006737*	0.000
1/20X	Control	-0.005043*	0.001
	1X	0.001530	0.103
	1/10X	0.001856	0.325
	Bare Ti-6Al-4V	0.001694*	0.045
1/10X	Control	-0.006899*	0.010
	1X	-0.000326	0.993
	1/20X	-0.001856	0.325
	Bare Ti-6Al-4V	-0.000161	0.999
1X	Control	-0.006573*	0.000
	1/10X	0.000326	0.993
	1/20X	-0.001530	0.103
	Bare Ti-6Al-4V	0.000164	0.993
Bare Ti-6Al-4V	Control	-0.006737*	0.000
	1X	-0.000164	0.993
	1/10X	0.000161	0.999
	1/20X	-0.001694*	0.045

*, The mean difference is significant at the 0.05 level.

In this regard, an enhancement associated with the presence of collagen on the surface of the materials had been reported by other authors. Ao et al. (Ao et al., 2014) used collagen immobilized on Ti-6Al-4V alloy and human bone marrow mesenchymal stem cells in their study. Immobilization of collagen was done through EDC/NHS chemistry and the sample amino-functionalization by immersing the Ti-6Al-4V substrates in a solution of APTS in toluene. The concentration of the EDC/NHS cross-linkers used was 2.5/0.63 mg/ml. They concluded that better cell adhesion was achieved for samples with immobilized collagen compared to the bare alloy at all temporal points (4, 8, 12 and 24 h) and a higher cell proliferation was observed after 3, 5 and 7 days of culture (Ao et al.,

2014). Müller et al. addressed the biological response of human osteoblast-like MG-63 cell line on commercially pure titanium, Ti-6Al-4V and Co-35Ni-20Cr-10Mo with immobilized collagen. A larger number of cells was observed on collagen-immobilized samples after 1 day of seeding compared with the bare substrates. However, at 7 days after seeding, no statistically significant differences were observed between the collagen-immobilized and bare substrates (Müller et al., 2006). In the study mentioned above EDC/NHS chemistry coupled with surface functionalization through amino groups was used for covalent attachment of collagen onto the substrates. The concentrations of EDC/NHS used corresponded to the 1X concentration (1X - 2.5/0.63 mg/ml), but no cytotoxic effects for EDC/NHS nor any distinct sample cleaning processes for removal of excess EDC/NHS was reported.

Another study performed by the same group, evaluated the effect of collagen binding on stainless steel substrates on the response of human bone marrow mesenchymal stem cells. Collagen was bound to stainless steel through functionalizing the stainless steel substrates by immersion in a solution of APTS in toluene and through n,n'-disulphosuccinimidyl-suberate (DS3) as the cross-linking system. They stated that collagen bound stainless steel substrates held considerably higher number of cells compared to untreated stainless steel after 1 day and 7 days of seeding (Müller et al., 2005). One study investigated the effect of collagen immobilization on titanium samples on the proliferation of MC3T3-E1 cells. Functionalization of the Ti samples was performed through immersion in a solution of dopamine in Tris-HCl and EDC/NHS were used as cross-linker. The concentrations of EDC/NHS used were: 1.9/2.9 mg/ml. Their results indicated that after 3 days of culture, no differences were observed between bare Ti and collagen-immobilized Ti. However, at day 7 and 14 of culture, they observed higher number of cells on collagen immobilized samples. No cytotoxic effects nor any cleaning procedure for the samples were reported (Yu et al., 2013).

In contrast to these results, other studies reported cytotoxic effects associated with EDC. Thus, Powell et al. (Powell & Boyce, 2006) used increasing concentrations of EDC/NHS for cross-linking collagen scaffolds. They reported low cell density and poor cellular organization of the human fibroblast and keratinocyte cultures when elevated concentrations of EDC were used in the process. A cleaning process was developed that consisted of several washes with ethanol, HEPES buffered saline and cell culture media

(Powell & Boyce, 2006). In another similar study a comprehensive cleaning process comprising of Earle's balanced salt solution and cell culture medium was proposed (Hanthamrongwit et al., 1996).

In another approach, collagen was covalently bound to titanium using p-nitrophenylchloroformate. No improvement in adhesion and proliferation of rat bone marrow cells seeded on collagen-immobilized samples over bare Ti samples was observed throughout the 16-day study period (van den Dolder & Jansen, 2007).

Based on the results obtained in this work, it was concluded that the cleaning process VIII coupled with reducing the concentration of the cross-linkers to 1/20 of the primary concentration was sufficient for the elimination of the cytotoxic effects of the residual EDC. It was demonstrated that by covalent immobilization of collagen to AVS-functionalized Ti-6Al-4V and employing the indicated cleaning process and cross-linker concentration, an improved cell response can be achieved.

3.5.2.8.1 Assessment of cell morphology

The results of the cell adhesion and viability as assessed from the number of cells were complemented with a more detailed study of cell morphology. The surface area of the cells after 4 h of seeding were characterized and compared using the Phalloidin/Hoechst staining. Figure 3.83 shows the fluorescence microscopy images of Phalloidin/Hoechst stained BM-MSCs adhered on bare Ti-6Al-4V, 1X, 1/10X, 1/20X and control samples. The surface area of the adhered cells was measured from these micrographs. As is shown in Figure 3.84, it was found that cells grown on the polystyrene controls possess the largest cell surface area. In contrast, the smallest cell surface area was found in cells adhered to bare Ti-6Al-4V samples. The surface area of cells adhered on samples with immobilized collagen (1X, 1/10X and 1/20X) ranked somewhere between the control and bare Ti-6Al-4V. The results of the statistical analysis of these data can be seen in Table 3.35.

Figure 3.83: Fluorescence microscopy images of phalloidin/hoechst stained BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 4 h after seeding. (Scale bar = 10 µm).

Figure 3.84: Surface area of BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 4 h after seeding.

Table 3.35: Results of the Games-Howell test performed on the data obtained from measurement of surface area of BM-MSCs cultured on bare Ti-6Al-4V, collagen-immobilized Ti-6Al-4V with 1X, 1/10X and 1/20X concentrations of EDC/NHS cleaned with protocol VIII and polystyrene control samples at 4 h after seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Control	1X	1434.5	0.109
	1/10X	1186.6	0.266
	1/20X	1077.9	0.342
	Bare Ti-6Al-4V	1728.3*	0.041
1X	Control	-1434.5	0.109
	1/10X	-247.9	0.944
	1/20X	-356.5	0.808
	Bare Ti-6Al-4V	293.8	0.742
1/10X	Control	-1186.6	0.266
	1X	247.9	0.944
	1/20X	-108.7	0.998
	Bare Ti-6Al-4V	541.7	0.436
1/20X	Control	-1077.9	0.342
	1X	356.5	0.808
	1/10X	108.7	0.998
	Bare Ti-6Al-4V	650.3	0.244
Bare Ti-6Al-4V	Control	-1728.3*	0.041
	1X	-293.8	0.742
	1/10X	-541.7	0.436
	1/20X	-650.3	0.244

*, The mean difference is significant at the 0.05 level.

3.5.3 Cell culture on covalently immobilized fibronectin

The promising results obtained from collagen-immobilized samples substantiated the possibility of enhancing the biological response to Ti-6Al-4V samples in vitro through the covalent immobilization of proteins to the material. Following this rationale, the studies were expanded to include the effect of covalently immobilizing fibronectin to the material in its biological response. As presented in section 3.3.4, the presence of adsorbed fibronectin had been observed to improve the adhesion of the cells on the material.

Fibronectin was covalently immobilized on amino-functionalized Ti-6Al-4V using an EDC/NHS concentration of 1/20X and cleaning protocol VIII.

Figure 3.85 shows the morphology of the MSCs adhered to bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and control samples after 4 and 48 h of seeding. At 4 h of seeding, the cells adhered on fibronectin-immobilized samples showed a higher expansion of the cytoskeleton compared to those on bare Ti-6Al-4V. At 48 h of seeding, the cells on fibronectin-immobilized Ti-6Al-4V showed a better cell arrangement, more developed processes and extended filopodia compared to those on bare Ti-6Al-4V.

The results of cell quantification are shown in Figure 3.86 and Table 3.36. A considerably higher number of viable cells are observed on the fibronectin-immobilized samples compared with the bare Ti-6Al-4V substrates both at 4 and 48 h after seeding.

Figure 3.85: Fluorescence microscopy images of calcein/PI stained BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 4 and 48 h after seeding. Viable cells are stained green, whereas the dead cells appear as red dots. (Scale bar = 100 μ m).

Figure 3.86: a) Number of viable cells and b) cellular viability at 4 and 48 h after seeding for BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples.

The results of the independent XTT assays at 48 h of seeding are shown in Figure 3.87 and Table 3.37. The obtained results from cell counting are in agreement with those obtained from XTT experiments, which confirm that fibronectin-immobilized Ti-6Al-4V samples possessed significantly more metabolically active cells compared to bare Ti-6Al-4V.

Table 3.36: Results of the Games-Howell test performed on the data obtained from cell counting for BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 4 and 48 h after seeding.

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Cell_No_4H	Control	Fibronectin-immobilized Ti-6Al-4V	24.703	0.225
		Bare Ti-6Al-4V	66.659*	0.000
	Fibronectin-immobilized Ti-6Al-4V	Control	-24.703	0.225
		Bare Ti-6Al-4V	41.956*	0.009
	Bare Ti-6Al-4V	Control	-66.659*	0.000
		Fibronectin-immobilized Ti-6Al-4V	-41.956*	0.009
Cell_No_48H	Control	Fibronectin-immobilized Ti-6Al-4V	44.214*	0.046
		Bare Ti-6Al-4V	99.585*	0.000
	Fibronectin-immobilized Ti-6Al-4V	Control	-44.214*	0.046
		Bare Ti-6Al-4V	55.371*	0.002
	Bare Ti-6Al-4V	Control	-99.585*	0.000
		Fibronectin-immobilized Ti-6Al-4V	-55.371*	0.002

*. The mean difference is significant at the 0.05 level.

Figure 3.87: Absorbance values of XTT assays obtained from BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 48 h after seeding.

Table 3.37: Results of the Games-Howell test performed on the data obtained from XTT assays on BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 48 h after seeding.

(I) Sample	(J) Sample	Mean Difference (I-J)	Sig.
Control	Fibronectin-immobilized Ti-6Al-4V	0.003761*	0.003
	Bare Ti-6Al-4V	0.005892*	0.000
Fibronectin-immobilized Ti-6Al-4V	Control	-0.003761*	0.003
	Bare Ti-6Al-4V	0.002131*	0.013
Bare Ti-6Al-4V	Control	-0.005892*	0.000
	Fibronectin-immobilized Ti-6Al-4V	-0.002131*	0.013

*. The mean difference is significant at the 0.05 level.

Fibronectin is an ECM protein that favors cell adhesion and spreading (Seitz et al., 1982, Dessau et al., 1978). A few studies can be found that report the effect of covalently bound fibronectin on the biological response of Ti samples. In this regard, an improved initial adhesion of MC3T3-E1 cells 30 min after seeding was reported on fibronectin-immobilized Ti. Fibronectin was covalently bound using a tresyl chloride chemistry (Pugdee et al., 2007). In a similar study, fibronectin was covalently immobilized on a Ti surface using EDC as cross-linker and immersion in APTS solution as the functionalization method. It was reported that osteoblast-like SaOS2 cells showed a better cell adhesion and spreading on fibronectin-immobilized samples after 4 h of seeding (Pham et al., 2003). In another study, fibronectin was covalently immobilized on Ti using the same tresyl chloride chemistry and the proliferation of MC3T3-E1 cells was studied throughout a 52-day period by measuring the DNA content. In this case, the experiment concluded that fibronectin immobilization on titanium did not have any effect on the proliferation of the cells (Yoshida et al., 2012). In contrast, an independent experiment performed by the same group using the MTT assay revealed a higher number of cells for sandblasted titanium with immobilized fibronectin compared to bare sandblasted titanium. The presence of fibronectin also seemed to influence the arrangement and morphology of the cells (Hayakawa et al., 2012). Another study investigated the immobilization of fibronectin over polyethylene terephthalate (PET) surface using glutaraldehyde as cross-linker. It was reported that this immobilization strategy led to an inhibited adhesion of BHK21 cells after 2 h of seeding which might be related to the

undesired conformational changes of glutaraldehyde-immobilized fibronectin (Y. Zhang et al., 2007).

Our results demonstrated that covalent immobilization of fibronectin on AVS amino-functionalized Ti-6Al-4V samples is possible using the EDC/NHS chemistry. Fibronectin immobilization improves the biological response of the material in terms of enhanced initial cell adhesion which leads to higher number of adhered cells for these samples at longer incubation times.

3.5.3.1 Assessment of cell morphology

In parallel with the experimental procedure used for collagen-immobilized Ti-6Al-4V, the morphology of the cells cultured on fibronectin-immobilized samples was assessed using phalloidin/hoechst system.

The fluorescence images of BM-MSCs adhered on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples after 4 h of seeding can be seen in Figure 3.88. The results of cell surface area measurement are presented in Figure 3.89 and the statistical analysis of the data in Table 3.38. It can be seen that the cells adhered on the polystyrene control samples possess the highest cell surface area with highly developed processes. The cells adhered on fibronectin-immobilized Ti-6Al-4V samples show slightly less surface area than the controls, but a much larger surface area than the cells cultured on bare Ti-6Al-4V substrates.

Figure 3.88: Fluorescence microscopy images of phalloidin/hoechst stained BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 4 h after seeding. (Scale bar = 10 μ m).

The statistical analysis of the data shows that there is not a statistical difference between the cell surface area of fibronectin-immobilized Ti-6Al-4V and polystyrene control samples. Thus, the covalent immobilization of fibronectin on Ti-6Al-4V samples not only improves cell adhesion, but also enhances initial cell spreading. In a similar study Middleton et al. reported the positive effect of fibronectin on cell spreading. They covalently attached fibronectin to the surface of Ti-6Al-4V by functionalizing the surface of Ti-6Al-4V through immersion in APTS solution using glutaraldehyde as cross-linker. They reported higher surface area for dermal fibroblasts cultured on the fibronectin-immobilized samples compared to the polystyrene controls at 1, 4, 24 and 96 h after seeding (Middleton et al., 2007).

Figure 3.89: Surface area of BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 4 h after seeding.

It is acknowledged that proliferation of substrate-dependent cells is highly reliant on the extension of the cells on the substrate. So that the lack of spreading over a substrate can even cause apoptosis in the cells (Straface et al., 1999). Consequently, various researchers have stated that initial cell spreading magnitude has a positive influence on subsequent proliferation (Thomas et al., 1999, S. Huang et al., 1998, C. S. Chen et al., 1997). It is assumed that two mechanisms might be involved in this process. The first mechanism

establishes that cells with a higher surface area are able to uptake higher amount of proteins from the biological fluids. This higher protein uptake induces the transition of the cells from G0 to G1 and from G1 to S phase of the cell cycle and favors cell proliferation (Avraham et al., 2003, Dwivedi et al., 2008, Zamir & Geiger, 2001). The second mechanism is based on mechanical cues, as sensed by the actin cytoskeleton. As the cells expand on the substrate, the stress in the actin fibers increases. The increase in stress in the actin fibers would result in the stimulation of cell proliferation through DNA synthesis and nuclear expansion (Assoian & Klein, 2008, Bacakova et al., 2011, Ingber et al., 1995).

Table 3.38: Results of the Games-Howell test performed on the data obtained from measurement of surface area of BM-MSCs cultured on bare Ti-6Al-4V, fibronectin-immobilized Ti-6Al-4V and polystyrene control samples at 4 h after seeding.

(I) Samples	(J) Sample	Mean Difference (I-J)	Sig.
Control	Fibronectin-immobilized Ti-6Al-4V	1531.5	0.595
	Bare Ti-6Al-4V	8449.5*	0.000
Fibronectin-immobilized Ti-6Al-4V	Control	-1531.5	0.595
	Bare Ti-6Al-4V	6918.0*	0.000
Bare Ti-6Al-4V	Control	-8449.5*	0.000
	Fibronectin-immobilized Ti-6Al-4V	-6918.0*	0.000

*. The mean difference is significant at the 0.05 level.

4 CONCLUSIONS

1. In this work, a novel method for the deposition of functional amine groups was employed to successfully functionalize Ti-6Al-4V alloy. This technique allows for a certain degree of control over the deposited amine layer.
2. By varying the deposition parameters, different functional amine films were deposited on Ti-6Al-4V substrates and the resulting layers were characterized in terms of amine concentration and surface topography.
3. By considering the highest amine concentration and lowest surface roughness, two sets of optimal deposition conditions were determined: F1: T_{Evap} : 150 °C, T_{Act} : 750 °C, P_{Ar} : 1 mbar, t =20 min and F2: T_{Evap} : 150 °C, T_{Act} : 750 °C, P_{Ar} : 2 mbar, t =20.
4. It was determined that the AVS functionalization was a highly reproducible method for the functionalization of Ti-6Al-4V.
5. AVS functionalization led to the formation of an amine layer that was roughly 100-200 nm thick.
6. F2 samples showed roughly the same contact angle as bare Ti-6Al-4V samples however, the contact angle for F1 samples was slightly higher.
7. The attachment and proliferation of MC3T3-E1 cells on functionalized samples was comparable to that on bare Ti-6Al-4V and no inhibition in cell proliferation nor any form of cytotoxicity was observed for MC3T3-E1 cell attached on functionalized samples after 7 days of incubation.
8. After 7 days of exposure to cells and cell culture conditions, the amine layer was still stable and essentially kept its integrity.
9. Physical adsorption of collagen type I on bare Ti-6Al-4V did not have any effect on the adhesion and proliferation of MC3T3-E1 cells during the first 48 h.

10. Physical adsorption of collagen type I on bare Ti-6Al-4V improved the initial adhesion of BM-MSCs.
11. Physical adsorption of BSA on bare Ti-6Al-4V did not have any effect on the adhesion and proliferation of MC3T3-E1 cells during first 48 h.
12. Physical adsorption of BSA on bare Ti-6Al-4V did not have any effect on the adhesion and proliferation of BM-MSCs during the first 48 h.
13. Physical adsorption of fibrinogen on bare Ti-6Al-4V did not have any effect on the adhesion and proliferation of MC3T3-E1 cells during the first 48 h.
14. Physical adsorption of fibrinogen on bare Ti-6Al-4V did not have any effect on the adhesion and proliferation of BM-MSCs during the first 48 h.
15. Physical adsorption of fibronectin on bare Ti-6Al-4V improved the initial adhesion of BM-MSCs.
16. Collagen type I, albumin, fibrinogen and fibronectin were successfully immobilized on the surface of AVS-functionalized Ti-6Al-4V through covalent bonds using EDC/NHS as cross-linker. The immobilized proteins were characterized by atomic force microscopy and fluorescence microscopy.
17. The effect of concentration of cross-linker on the immobilization of collagen was determined and the amount of collagen present on the samples was quantified.
18. A protocol was developed for the elution of adsorbed collagen from the Ti-6Al-4V samples using sodium dodecyl sulfate (SDS) detergent.
19. Usage of this protocol allowed distinguishing between adsorbed and covalently bound proteins on the surface.
20. EDC/NHS residues caused cytotoxicity and inhibited proliferation in BM-MSCs.
21. A robust protocol for the removal of excess EDC/NHS from the samples was developed based on sequential cleaning step by PBS, MES and DMEM.
22. Covalent attachment of collagen type I on Ti-6Al-4V led to an enhanced adhesion of BM-MSCs on the material.
23. Covalent attachment of fibronectin on Ti-6Al-4V led to an enhanced cell adhesion and spreading on the material.

In summary, this work presents a robust and reproducible method for the covalent attachment of different proteins on the surface of Ti-6Al-4V that leads to an enhanced in-vitro biological response of the material. The applications of this method are not

limited to the studied proteins and potentially could include any biomolecule which possess the appropriate functional groups.

The results provided in this work pave the way for the development of novel biomaterials with enhanced properties that are able to trigger desired responses in the host organism through the biomolecules immobilized on their surface.

5 FUTURE WORK

1. Assessment of the differentiation of BM-MSCs on the collagen/fibronectin immobilized Ti-6Al-4V substrates.
2. Addressing the effect of simultaneous immobilization of collagen and fibronectin on adhesion, proliferation and differentiation of cells.
3. Assessment of the effect of covalent immobilization of collagen/fibronectin on Ti-6Al-4V implants on the bone healing process in vivo, using animal models such as mice or rabbits.
4. Immobilization of several other proteins such as laminin and bone morphogenic proteins (BMP) on Ti-6Al-4V and evaluation of cell adhesion, proliferation and differentiation on such surfaces.
5. Study of different cross-linking chemistries for the covalent attachment of biomolecules to the AVS-functionalized Ti-6Al-4V.

APPENDIX I: PROTOCOL OF THE AVS PROCESS

- Introduce clean Ti-6Al-4V in the deposition chamber and close the chamber.
- Pipette 3 ml of APTS into the evaporation chamber and close the chamber.
- Turn on the rotary pump and open the valve connecting the pump to the system.
- Open the valve of the pressure sensor. The system should reach a base pressure of 10^{-1} mbar.
- Fill the trap with dry ice and acetone, the base pressure should reach 10^{-2} mbar.
- Turn on the activation furnace.
- Turn on the evaporation furnace. The evaporation chamber remains outside the evaporation furnace at this stage.
- When both furnaces reach their temperature set points, the needle-valve is open and Ar is allowed into the system.
- The pressure of the system is controlled by the needle-valve.
- When pressure reaches its set point, the valve of the pressure sensor is closed.
- Evaporation furnace is displaced to enclose the evaporation chamber in order to initiate the evaporation of APTS. Deposition time starts at this moment.
- After the desired deposition time is over, the needle-valve is closed and the evaporation furnace is removed from the evaporation chamber.
- The activation and evaporation furnaces are turned off.
- After the system cools down, the rotary pump is turned off and the samples are taken out of the deposition chamber.
- Samples are sonicated in acetone (2min), isopropanol (2min), rinsed with distilled water and dried with Ar.

- Samples are stored in air.

APPENDIX II: SUPPLEMENTARY STATISTICAL INFORMATION

3.1.1 Profilometry

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
RMS_μm	400	0.889	30	0.004
	1200	0.719	30	0.000
	4000	0.903	30	0.010

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
0.572	2	87	0.566

3.1.2 Atomic force microscopy

	Sample	Shapiro-Wilk		
		Statistic	df	Sig.
RMS_scansize_50	50	0.878	20	0.016
	5	0.947	20	0.323
	0.5	0.859	20	0.008
RMS_scansize_5	50	0.913	20	0.073
	5	0.935	20	0.191
	0.5	0.791	20	0.001
RMS_scansize_0.5	50	0.935	20	0.194
	5	0.798	20	0.001
	0.5	0.778	20	0.000

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
RMS_scansize_50	20.834	2	57	0.000
RMS_scansize_5	3.632	2	57	0.033
RMS_scansize_0.5	1.863	2	57	0.165

3.1.3.1 Cell adhesion/viability

	Sample	Shapiro-Wilk		
		Statistic	df	Sig.
Cell_4h	Control	0.826	24	0.001
	400	0.927	12	0.346
	1200	0.837	9	0.053
	4000	0.966	9	0.856
Cell_48h	Control	0.951	24	0.284
	400	0.977	12	0.970
	1200	0.934	9	0.520
	4000	0.849	9	0.072

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_4h	3.780	3	56	0.015
Cell_48h	8.867	3	50	0.000

3.1.3.2 Cell proliferation

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Abs_1day	Control	0.755	6	0.022
	400	0.932	6	0.592
	1200	0.954	6	0.773
	4000	0.988	6	0.983
Abs_7day	Control	0.895	6	0.343
	400	0.954	6	0.775
	1200	0.833	6	0.114
	4000	0.882	6	0.279

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Abs_1day	4.517	3	20	0.014
Abs_7day	3.389	3	20	0.038

3.2.5 Contact angle measurements

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Contact_angle	F1	0.951	32	0.150
	F2	0.957	32	0.233
	Non-functionalized Ti-6Al-4V	0.969	32	0.476

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2.227	2	93	0.114

3.2.7.1 Cell viability/adhesion

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
cell_4h_normalized	F1	0.932	18	0.211
	F2	0.897	18	0.051
	Control	0.793	36	0.000
	Non-functionalized Ti-6Al-4V	0.914	18	0.102
cell_48h_normalized	F1	0.931	18	0.204
	F2	0.953	18	0.482
	Control	0.783	36	0.000
	Non-functionalized Ti-6Al-4V	0.924	18	0.151

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
cell_4h	8.797	3	86	0.000
cell_48h	7.611	3	86	0.000

3.2.7.2 Cell proliferation

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Abs_1day	F1	0.920	6	0.509
	F2	0.986	6	0.977
	Control	0.755	6	0.022
	Non-functionalized Ti-6Al-4V	0.988	6	0.983
Abs_7day	F1	0.783	6	0.041
	F2	0.868	6	0.217
	Control	0.895	6	0.343
	Non-functionalized Ti-6Al-4V	0.882	6	0.279

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Abs_1day	2.680	3	20	0.075
Abs_7day	6.035	3	20	0.004

3.3.1.1 MC3T3-E1 cells

Cell counting

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Ti-6Al-4V with Adsorbed Collagen	0.896	12	0.141
	Bare Ti-6Al-4V	0.973	12	0.941
	Control	0.963	12	0.823
Cell_No_48H	Ti-6Al-4V with Adsorbed Collagen	0.897	12	0.146
	Bare Ti-6Al-4V	0.931	12	0.391
	Control	0.897	12	0.145

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No4H	1.398	2	33	0.261
Cell_No_48H	2.360	2	33	0.110

XTT

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
XTT_Absorbance	Bare Ti-6Al-4V	0.943	4	0.675
	Ti-6Al-4V with Adsorbed Collagen	0.892	4	0.392
	Control	0.972	4	0.851

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
16.960	2	9	0.001

3.3.1.2 BM-MSCs

Cell counting

		Shapiro-Wilk		
Sample		Statistic	df	Sig.
Cell_No_4H	Control	0.909	12	0.205
	Ti-6Al-4V with Adsorbed Collagen	0.928	12	0.362
	Bare Ti-6Al-4V	0.873	12	0.071
Cell_No_48H	Control	0.809	12	0.012
	Ti-6Al-4V with Adsorbed Collagen	0.964	12	0.839
	Bare Ti-6Al-4V	0.914	12	0.241

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	2.170	2	33	0.130
Cell_No_48H	6.596	2	33	0.004

XTT

		Shapiro-Wilk		
Sample		Statistic	df	Sig.
XTT_Absorbance	Control	0.949	4	0.709
	Ti-6Al-4V with Adsorbed Collagen	0.972	4	0.855
	Bare Ti-6Al-4V	0.672	4	0.005

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
1.157	2	9	0.357

3.3.2.1 MC3T3-E1 cells

Cell counting

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Control	0.936	12	0.450
	Ti-6Al-4V with adsorbed BSA	0.926	12	0.342
	Bare Ti-6Al-4V	0.940	12	0.499
Cell_No_48H	Control	0.918	12	0.266
	Ti-6Al-4V with adsorbed BSA	0.861	12	0.050
	Bare Ti-6Al-4V	0.906	12	0.188

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	2.849	2	33	0.072
Cell_No_48H	1.362	2	33	0.270

XTT

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
XTT_ Absorbance	Bare Ti-6Al-4V	0.970	4	0.844
	Ti-6Al-4V with Adsorbed BSA	0.879	4	0.332
	Control	0.926	4	0.572

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
28.602	2	9	0.000

3.3.2.2 BM-MSCs

Cell counting

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Control	0.959	12	0.773
	Ti-6-Al-4V with Adsorbed BSA	0.896	12	0.142
	Bare Ti-6Al-4V	0.915	12	0.245
Cell_No_48H	Control	0.837	12	0.026
	Ti-6-Al-4V with Adsorbed BSA	0.936	12	0.449
	Bare Ti-6Al-4V	0.909	12	0.208

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	1.508	2	33	0.236
Cell_No_48H	0.844	2	33	0.439

XTT

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
XTT_Absorbance	Bare Ti-6Al-4V	0.876	4	0.320
	Ti-6Al-4V with Adsorbed BSA	0.793	4	0.090
	Control	0.931	4	0.602

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
17.578	2	9	0.001

3.3.3.1 MC3T3-E1 cells

Cell counting

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4h	Control	0.868	12	0.061
	Ti-6Al-4V with Adsorbed Fibrinogen	0.938	12	0.467
	Bare Ti-6Al-4V	0.943	12	0.544
Cell_No_48h	Control	0.748	12	0.003
	Ti-6Al-4V with Adsorbed Fibrinogen	0.874	12	0.073
	Bare Ti-6Al-4V	0.941	12	0.516

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4h	3.859	2	33	0.031
Cell_No_48h	1.664	2	33	0.205

XTT

		Shapiro-Wilk		
	sample	Statistic	df	Sig.
XTT_Absorbance	Bare Ti-6Al-4V	0.851	4	0.229
	Ti-6Al-4V with Adsorbed Fibrinogen	0.918	4	0.524
	Control	0.835	4	0.182

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
4.631	2	9	0.041

3.3.3.2 BM-MSCs

Cell counting

Sample		Shapiro-Wilk		
		Statistic	df	Sig.
Cell_No_4H	Control	0.939	12	0.484
	F2 with Adsorbed Fibrinogen	0.972	12	0.931
	Bare Ti-6Al-4V with Adsorbed Fibrinogen	0.930	12	0.379
	Bare Ti-6Al-4V	0.900	12	0.159
Cell_No_48H	Control	0.978	12	0.974
	F2 with Adsorbed Fibrinogen	0.897	12	0.143
	Bare Ti-6Al-4V with Adsorbed Fibrinogen	0.941	12	0.506
	Bare Ti-6Al-4V	0.873	12	0.071

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	1.141	3	44	0.343
Cell_No_48H	0.989	3	44	0.407

XTT

Sample		Shapiro-Wilk		
		Statistic	df	Sig.
Absorbance_48H	Bare Ti-6Al-4V	0.792	4	0.089
	Ti-6Al-4V with Adsorbed Fibrinogen	0.857	4	0.248
	F2 with Adsorbed Fibrinogen	0.809	4	0.118
	Control	0.912	4	0.495

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
68.115	3	12	0.000

3.3.4.1 BM-MSCs

Cell counting

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Control	0.981	12	0.988
	Ti-6Al-4V with Adsorbed Fibronectin	0.861	12	0.051
	Bare Ti-6Al-4V	0.969	12	0.895
Cell_No_48H	Control	0.831	12	0.022
	Ti-6Al-4V with Adsorbed Fibronectin	0.971	12	0.919
	Bare Ti-6Al-4V	0.918	12	0.271

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	1.514	2	33	0.235
Cell_No_48H	2.131	2	33	0.135

XTT

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
XTT_Absorbance	Bare Ti-6Al-4V	0.916	4	0.514
	Ti-6Al-4V with Adsorbed Fibronectin	0.799	4	0.101
	Control	0.805	4	0.111

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
12.109	2	9	0.003

3.5.2.1 Protocol I: Rinsing with distilled water

	Sample	Shapiro-Wilk		
		Statistic	df	Sig.
Cell_No_4H	Control	0.926	6	0.552
	Ti-6Al-4V with Immobilized Collagen	0.849	6	0.153
	Bare Ti-6Al-4V	0.924	6	0.538
Cell_No_48H	Control	0.861	6	0.193
	Ti-6Al-4V with Immobilized Collagen	0.915	6	0.471
	Bare Ti-6Al-4V	0.845	6	0.142

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	0.974	2	15	0.400
Cell_No_48H	8.497	2	15	0.003

3.5.2.2 Protocol II: Cleaning in PBS for 24 h

	Sample	Shapiro-Wilk		
		Statistic	df	Sig.
Cell_No_4h	Control	0.903	6	0.394
	Ti-6Al-4V with Immobilized Collagen	0.875	6	0.247
	Bare Ti-6Al-4V	0.835	6	0.118
Cell_No_48h	Control	0.903	6	0.395
	Ti-6Al-4V with Immobilized Collagen	0.943	6	0.682
	Bare Ti-6Al-4V	0.892	6	0.328

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4h	1.007	2	15	0.389
Cell_No_48h	3.280	2	15	0.066

3.5.2.3 Protocol III: Cleaning in MES buffer for 24 h

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Control	0.900	6	0.373
	Ti-6Al-4V with Immobilized Collagen	0.903	6	0.392
	Bare Ti-6Al-4V	0.960	6	0.824
Cell_No_48H	Control	0.907	6	0.420
	Ti-6Al-4V with Immobilized Collagen	0.951	6	0.750
	Bare Ti-6Al-4V	0.994	6	0.996

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	0.013	2	15	0.987
Cell_No_48H	0.800	2	15	0.468

3.5.2.4 Protocol IV: Cleaning in DMEM for 24 h

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Control	0.791	6	0.049
	Ti-6Al-4V with Immobilized Collagen	0.987	6	0.980
	Bare Ti-6Al-4V	0.920	6	0.504
Cell_No_48H	Control	0.827	6	0.102
	Ti-6Al-4V with Immobilized Collagen	0.927	6	0.560
	Bare Ti-6Al-4V	0.891	6	0.325

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	0.246	2	15	0.785
Cell_No_48H	1.044	2	15	0.376

3.5.2.5 Protocol V: Continuous cleaning in MES buffer

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4H	Control	0.851	6	0.161
	1/2X	0.899	6	0.370
	1/5X	0.974	6	0.915
	1/10X	0.887	6	0.302
	Bare Ti-6Al-4V	0.933	6	0.606
Cell_No_48H	Control	0.962	6	0.834
	1/2X	0.701	6	0.006
	1/5X	0.927	6	0.556
	1/10X	0.962	6	0.834
	Bare Ti-6Al-4V	0.768	6	0.030

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4h	1.765	4	25	0.168
Cell_No_48h	0.667	4	25	0.621

3.5.2.6 Protocol VI: Cleaning in PBS and DMEM

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_No_4h	Control	0.944	6	0.691
	1/10X	0.918	6	0.489
	1/20X	0.878	6	0.260
	Bare Ti-6Al-4V	0.949	6	0.734
Cell_No_48h	Control	0.901	6	0.380
	1/10X	0.941	6	0.669
	1/20X	0.933	6	0.600
	Bare Ti-6Al-4V	0.850	6	0.158

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4h	2.688	3	20	0.074
Cell_No_48h	0.994	3	20	0.416

3.5.2.7 Cleaning protocol VII: PBS, MES and DMEM

		Shapiro-Wilk		
	samples	Statistic	df	Sig.
Cell_No_4h	Control	0.889	6	0.312
	1/10X	0.885	6	0.292
	1/20X	0.836	6	0.120
	Bare Ti-6Al-4V	0.825	3	0.175
Cell_No_48h	Control	0.973	6	0.911
	1/10X	0.913	6	0.457
	1/20X	0.909	6	0.433
	Bare Ti-6Al-4V	0.968	3	0.656

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4h	5.894	3	20	0.005
Cell_No_48h	2.557	3	17	0.089

3.5.2.8 Cleaning protocol VIII: PBS, MES and DMEM

Cell counting

		Shapiro-Wilk		
	Samples	Statistic	df	Sig.
Cell_No_4H	Control	0.940	12	0.503
	1/20X	0.984	12	0.995
	1/10X	0.986	12	0.998
	1X	0.923	12	0.309
	Bare Ti-6Al-4V	0.942	12	0.518
Cell_No_48H	Control	0.879	12	0.085
	1/20X	0.970	12	0.912
	1/10X	0.807	12	0.011
	1X	0.967	12	0.881
	Bare Ti-6Al-4V	0.940	12	0.497

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	3.190	4	55	0.020
Cell_No_48H	4.938	4	55	0.002

XTT

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
XTT_Absorbance	Control	0.961	4	0.783
	1X	0.816	4	0.134
	1/10X	0.791	4	0.088
	1/20X	0.770	4	0.058
	Bare Ti-6Al-4V	0.899	4	0.426

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
66.101	4	15	0.000

3.5.2.8.1 Assessment of cell morphology

		Shapiro-Wilk		
	Sample	Statistic	df	Sig.
Cell_area	Control	0.914	10	0.310
	1X	0.835	10	0.039
	1/10X	0.776	10	0.007
	1/20X	0.864	10	0.086
	Bare Ti-6Al-4V	0.856	10	0.069

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
3.494	4	45	0.014

3.5.3 Cell culture on covalently immobilized fibronectin

Cell counting

		Shapiro-Wilk		
Sample		Statistic	df	Sig.
Cell_No_4H	Control	0.851	12	0.038
	Ti-6Al-4V with Immobilized Fibronectin	0.846	12	0.033
	Bare Ti-6Al-4V	0.937	12	0.465
Cell_No_48H	Control	0.953	12	0.677
	Ti-6Al-4V with Immobilized Fibronectin	0.774	12	0.005
	Bare Ti-6Al-4V	0.891	12	0.120

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Cell_No_4H	5.633	2	33	0.008
Cell_No_48H	11.729	2	33	0.000

XTT

		Shapiro-Wilk		
Sample		Statistic	df	Sig.
XTT_Absorbance	Control	0.722	8	0.004
	Ti-6Al-4V with Immobilized Fibronectin	0.813	8	0.039
	Bare Ti-6Al-4V	0.893	8	0.247

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
12.622	2	21	0.000

3.5.3.1 Assessment of cell morphology

		Shapiro-Wilk		
Sample		Statistic	df	Sig.
Cell_area	Control	0.897	10	0.203
	Ti-6Al-4V with Immobilized Fibronectin	0.931	10	0.459
	Bare Ti-6Al-4V	0.882	10	0.138

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
3.455	2	27	0.046

APPENDIX III: REPRINTS OF THE PUBLISHED PAPERS

Development of a versatile procedure for the biofunctionalization of Ti-6Al-4V implants

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ARTICLE INFO

Article history:

Received 7 April 2016

Received in revised form 10 June 2016

Accepted 23 June 2016

Available online 25 June 2016

Keywords:

Biofunctionalization

Silanization

Titanium

Fluorescence

Atomic force microscopy

ABSTRACT

Titanium (Ti) and titanium alloys are among the most commonly used metallic materials for implantation in the human body for the purpose of replacing hard tissue. Although Ti and its alloys are widely used for such an aim, in implants of a long duration they exhibit some shortcomings due to the loosening of the very implant. This phenomenon is highly dependent on the interaction between the organic tissues and the surface of the implant. In this study, the authors introduce a surface treatment technique for functionalization of the surface of Ti-6Al-4V alloy with amino groups that could help to control this interaction. The functionalized layer was deposited by activated vapor silanization (AVS), which has been proven as a reliable and robust technique with other materials. The resulting biofunctional layers were characterized by atomic force microscopy and fluorescence microscopy, with the optimal conditions for the deposition of a homogeneous film with a high density of amino groups being determined. Additionally, the non-toxic nature and stability of the biofunctional layer were confirmed by cell culturing. The results show the formation of a homogeneous biofunctional amine layer on Ti-6Al-4V alloy that may be used as a platform for the subsequent covalent immobilization of proteins or other biomolecules.

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1. Introduction

The ever-increasing need for implants to replace damaged parts of the human body calls for biomaterials with improved properties able to mimic the functions of failed organs. Among these biomaterials, metallic biomaterials are of significant interest for hard tissue replacement due to their advantageous mechanical properties. The biomaterials used for bone replacement are required to be chemically stable, be resistant to fracture and fatigue and, possibly, able to possess an elastic modulus close to that of bone [1]. In this regard, titanium and titanium alloys offer a combination of properties that make them suitable as load-bearing implants for hard tissue replacement. Their combined properties of high tensile strength, low density, high corrosion resistance and low elastic modulus have made them the ultimate choice for load-bearing implants for such replacement [2]. In particular, their low elastic modulus compared with other metallic biomaterials (stainless steel and Co-Cr alloys) improves stress transfer to the adjacent bone. Consequently, titanium implants diminish bone resorption and reduce the risk of

a loosening of the implant [3], since its elastic modulus is closer to the 30 GPa of cortical bone [4]. Ti-6Al-4V is the most common titanium alloy used in biomedical applications, given that the biphasic structure of Ti-6Al-4V causes the alloy to have superior corrosion resistance and tensile strength compared with pure titanium [1].

However, and despite its extensive use as an implant for bone replacement, metals in general and titanium alloys in particular show a significant drawback as prostheses, since they are unable to integrate directly into the adjacent bone tissue. Thus, metallic prostheses constantly appear to be encapsulated by a layer of connective tissue [5]. Healthy functional tissue can grow on top of the connective tissue, though this non-specialized tissue remains as an arguably weak interface between the metal and the bone [6], and tend to cause problems in implants of long duration.

In order to improve the performance of metallic implants, different methods of chemical or physical surface modification have been employed. Application of these methods is based on the assumption that the reaction of the organism to the implant depends on the events that occur at the surface of the material. In particular, the proteins adsorbed on the surface and their recognition by distinct cell lineages are presumed to determine the response of the organism to the material [7,8]. Consequently, various surface modification procedures such as deposition of calcium phosphates [9], acid etching [10], plasma spraying [11]

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and alkaline treatment [12] were proposed to monitor interaction between the material and the organism and to improve the osseointegration of Ti alloys. An increased interest has recently emerged in functionalization routes that allow the covalent immobilization of biomolecules on the surface of biomaterials in an attempt to trigger specific responses in the host tissue and thus improve cell adhesion and osseointegration [13].

Silanization is a commonly used technique for functionalization which results in an amine-terminated functional surface. Aminosilanes are used as precursors in this procedure because they are able to bond with hydroxyl groups which may be induced on the oxidized surface of metals [14,15] and, in particular, may be used to functionalize titanium and its alloys [16–19]. In turn, the resulting amino groups of the functionalized surface are capable of binding themselves to a wide range of biomolecules [20]. In general, this process proceeds by immersing the substrate in a solution of 3-aminopropyltriethoxysilane (APTS) with toluene or pentane [21] (immersion silanization, IS), although it has been found that this process does not necessarily lead to reproducible functionalized layers [22,23]. The difficulties associated with IS are firstly related with the need for surface activation to induce the appearance of –OH groups at the surface. Activation requires a pre-processing of the material by either immersion in acid [19,24] or with an oxygen plasma treatment [16]. Secondly, IS tends to result in a relatively low concentration of amine functional groups on the surface which, in addition, may be non-homogeneously distributed. These undesired features of IS seem to be highly dependent on the sensitivity of the process to the ambient conditions, since reactions are highly susceptible to hydrolyzation and oligomerization [22,23].

In order to address these obstacles, an alternative silanization technique referred to as activated vapor silanization (AVS) has been developed by the authors and is used in this work to functionalize Ti-6Al-4V substrates. This method has been previously employed to functionalize various materials, such as silicon, [25,26] successfully. In this procedure, the need for surface activation is eliminated, since the APTS molecules are activated in the vapor phase at high temperatures (700–800 °C) before impinging on the substrate. Additionally, as activation and deposition reactions take place under low vacuum ($P \approx 10^{-2}$ mbar), the reactions generally do not depend on ambient conditions, which then lead to higher reproducibility. As a result, AVS functionalized samples show a high concentration of amines which are homogeneously distributed. Lastly, the control exerted on the process through the deposition parameters allows a certain degree of variation in the properties of the functionalized layers.

This work demonstrates the ability of AVS for the effective bio-functionalization of Ti-6Al-4V alloy. Various biofunctional layers were prepared and characterized in terms of amine reactivity and surface topography. Optimal functionalization conditions that lead to the deposition of a homogeneous functional layer on Ti-6Al-4V were selected in terms of density of surface amine groups. Biocompatibility of the biofunctionalized layer was assessed through *in-vitro* cell culture experiments. In particular, the proliferation of MC3T3-E1 cells on the functionalized Ti-6Al-4V samples was studied. Biofunctionalized layers were also shown to remain stable even after being used as substrates for cell culturing for a period of at least one week.

2. Materials and methods

2.1. Preparation of Ti-6Al-4V substrates

The substrates were cut from a block of commercial Ti-6Al-4V alloy into samples with dimensions of approximately $10 \times 10 \times 1$ mm. All the samples were subjected to a grinding process with SiC grinding papers grit no. 400. The samples that

Table 1

Parameters used in the AVS functionalization processes featured in this work. Deposition time was fixed at 20 min for all processes.

T_{Evap} (°C)	T_{Act} (°C)	P_{Ar} (mbar)
130	750	1
150	750	1
170	750	1
150	700	1
150	800	1
150	750	2
150	750	0.5
150	800	2

underwent this process only were labeled as 400 samples. The rest of the samples were subjected to a grinding process with SiC grinding paper grit no. 1200. Those that were not additionally polished were labeled 1200 samples. Lastly, the rest of the samples were polished with SiC grinding paper grit no. 4000 and labeled 4000 samples. After grinding, all the substrates were cleaned by sonication in acetone, isopropanol and distilled water.

2.2. Functionalization of substrates

The substrates were functionalized by activated vapor silanization (AVS) [25] by using APTS ($\text{NH}_2(\text{CH}_2)_3\text{Si}(\text{OC}_2\text{H}_5)_3$, (Fluka, Germany) as a precursor. Briefly, APTS is evaporated in an evaporation chamber and the vapor is then transported via an Argon flux (BIP, Purity $\geq 99.9997\%$) to the activation chamber in which the APTS molecules impinge on the substrate after being activated at 700–800 °C. The substrates were cleaned after deposition by sonication with acetone and isopropanol, rinsed with distilled water, dried by using the argon flux and stored in air.

The AVS process is defined by four parameters: evaporation temperature of APTS (T_{Evap}), activation temperature (T_{Act}), argon pressure (P_{Ar}) and deposition time (t). In order to determine the optimal conditions that lead to the deposition of a homogeneous amine layer on Ti-6Al-4V substrates, the first three parameters (T_{Evap} , T_{Act} , P_{Ar}) were varied while the deposition time (t) was kept constant, and the resulting amine layers characterized. The deposition parameters used in this study are presented in Table 1.

2.3. Characterization

2.3.1. Topography characterization

The topography of the bare Ti-6Al-4V and functionalized samples was studied by atomic force microscopy (Cervantes AFM, Nanotec S.L.). Profile data and roughness were analyzed by use of WSxM 5.0 [27] software. AFM measurements were performed in dynamic mode by using a pyramidal cantilever (Olympus OMCL RC800, semi-angle 39°, nominal resonance frequency 69 kHz). 10 substrates were scanned corresponding to each type of non-functionalized samples (i.e. 400, 1200 and 4000 samples) and two substrates for each functionalization condition.

In order to measure the thickness of the functionalized layers, 4000 samples were covered with a resin mask in some parts of the surface prior to functionalization. The resin mask was prepared by drop casting with photoresist (microchemical AZ5214E), and spun at 5000 rpm for 45 s in order to obtain a uniform thickness of 1.4 μm . The optical lithography was carried out with a Karl Süss MJB mask aligner. The resist development was made at AZ400K + H_2O (1:5) for one minute and final rinsing in H_2O for 10 s. After the subsequent functionalization, the samples were sonicated in acetone to remove the resin. The step created between the regions previously covered by the resin and those covered by the functional layer was measured by AFM in contact mode.

2.3.2. Fluorescence labeling of amines

Fluorescein isothiocyanate (FITC) was used as a fluorescent marker to measure the density of amines on the surface. FITC is a fluorescent molecule which can react with the amino groups through the isothiocyanate group. After functionalization, relative amine concentrations were compared by examining the intensity of fluorescent light emitted from each sample. The measurement of the intensity of fluorescence under the same observation conditions represents a semi-quantitative procedure to determine the density of amine surface groups.

For this purpose, the samples were incubated in 0.5 mg/ml solution of FITC in phosphate buffer saline (PBS 10 mM, pH = 7.4) for 20 min. With the aim of removing any FITC non-covalently linked to the surface, the samples were cleaned in the same buffer for five minutes (three times) and subsequently rinsed with distilled water (three times). The presence of FITC was checked by fluorescence microscopy (Leica DMI 3000B) at an emission wavelength of 520 nm. The observation conditions were: exposure time: 1.3 ms, gain: 2.1X and gamma: 0.68.

2.3.3. Contact angle measurements

The contact angle of the bare Ti-6Al-4V 4000 samples (used as reference) and the F1 and F2 biofunctional layers (defined below) was measured according to the ASTM D7334-08 standard, by depositing a small volume of water ($V = 5 \mu\text{l}$) on each surface and taking a micrograph of the droplet using a precision camera (EO-1312C, Edmund Optics) equipped with a micro inspection lens system (Optem zoom 125). The measurement was performed on six samples of each condition and four times on each sample.

2.3.4. Reproducibility

In order to check the reproducibility of the F1 and F2 functionalization conditions, six samples of each condition were produced and characterized in terms of roughness (AFM) and amine concentration (FITC labeling).

2.3.5. Cell culture

The *in-vitro* cytocompatibility of bare Ti-6Al-4V and functionalized samples was compared by culturing osteoblast-like murine MC3T3-E1 cells on the materials. The cells were cultured in polystyrene Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere at 37 °C and at 5% CO₂ until they reached confluence. The medium was changed twice a week. The cells were detached from the dishes by using Trypsin-EDTA (0.05% in HBSS, HyClone) when needed. Prior to cell culturing, the substrates were autoclaved and placed in 24-well plates. Cells cultured in blank wells under the same conditions were used as controls.

2.3.5.1. Cell viability. For cell viability experiments, MC3T3-E1 cells suspended in medium were seeded on the substrates in a concentration of 50,000 cells/ml and 0.5 ml of medium per well. After either four or 48 h of incubation at 37 °C in a humidified atmosphere of 5% CO₂, the cells were dyed with a combined solution of 1 μl /ml of calcein acetoxymethyl (Calcein AM, Life Technologies, 0.5 $\mu\text{g}/\mu\text{l}$ in DMSO) and 1 μl /ml propidium iodide (Sigma-Aldrich, 750 μM in PBS) in DMEM and incubated for another 30 min. Calcein AM is a non-fluorescent cell-permeable dye that inside the living cell is converted to green fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases, and is used to stain the viable cells [28]. Propidium iodide is a red-fluorescent nuclear marker impermeable to the cellular membrane. Therefore, the nuclei of cells with damaged membranes, i.e. dead cells, will be stained with this dye [29]. Lastly, the samples were observed by fluorescence microscopy at an emission wavelength of 520 nm and 620 nm for

calcein and propidium iodide, respectively, by using an inverted Leica DMIRB microscope equipped with a digital camera, Leica DC100 (Leica, Nussloch, Germany). Data were obtained from three independent experiments run in duplicate. At least three representative images were taken from the surface of each substrate. ImageJ software was used to determine the number of cells in each such image.

2.3.5.2. Cell proliferation. MC3T3-E1 cells suspended in medium (10,000 cells/ml) were seeded on the substrates (0.5 ml per well) and the substrates were incubated for either one or seven days at 37 °C in a humidified atmosphere of 5% CO₂. Cell proliferation was determined by using the Cell Proliferation Assay kit (AppliChem) following manufacturer instructions. This employs 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT). In living cells, mitochondria have the capability to reduce XTT to form an orange-colored, water-soluble dye [31]. Therefore, the concentration of this dye is proportional to the number of metabolically active cells. The absorbance of each well was measured spectrophotometrically at 450 nm by using an ELX808 microplate reader (BioTeK). For comparative reasons, the absorbance values were normalized according to the surface area of each sample. Data were obtained from experiments running in triplicate.

2.3.6. Determination of stability of the functional layer

In order to examine the stability of the functional layer exposed to cells and culture medium, after the cell culturing experiment was over (seven days of incubation) the samples were checked for the presence of the amine groups on the surface. The samples were removed from the medium, rinsed with distilled water and, subsequently, dyed with FITC following the protocol described above.

2.3.7. Statistical methods

Statistical analyses were performed with IBM SPSS Statistics 20 software by using one-way analysis of variance (ANOVA) followed by the Games-Howell post-hoc test [30]. $p < 0.05$ was considered significant. All data are presented as a mean value $\pm$ standard error.

3. Results and discussion

3.1. Characterization of non-functionalized samples

3.1.1. Roughness

Fig. 1 shows the root mean square (RMS) roughness for 400, 1200 and 4000 Ti-6Al-4V samples at three scan sizes (50 μm , 5 μm and 0.5 μm) measured by AFM. It can be seen that after each grinding step, the RMS roughness at 50 μm and 5 μm scan sizes decreases considerably. In contrast, roughness only shows a marginal decrease when the 0.5 μm scan size is considered. In all cases, all the samples are smooth at this scale with an average roughness of 16 ± 2 nm for the 4000 samples in 5 μm scan size.

3.1.2. Cell cultures on non-functionalized samples

In order to examine the possible effect of surface roughness of the substrates on the adhesion and viability of MC3T3-E1 cells, samples with distinct degrees of surface roughness were used as substrates for the cell culture. Cells seeded on polystyrene wells were used as controls. After seeding the cells on the substrates, the samples were incubated for four or 48 h. The number of viable cells attached to each substrate was then determined from the images of calcein AM/propidium iodide assay.

As can be seen in Fig. 2, after four hours of incubation, the cells adhered to the surface of the bare Ti-6Al-4V substrates, although they are not completely spread, and most have a circular shape. After 48 h of incubation, the cells are completely spread

Fig. 1. Comparison of RMS roughness values of Ti-6Al-4V substrates after each grinding step at different AFM scan sizes. Data are presented as a mean value $\pm$ standard error.

on the surface and exhibit a well-structured polygonal shape, typical for this cell line in its adhered state with many cytoplasmic protrusions extending along the surfaces. There is no obvious difference between the morphology of the cells that were seeded on polystyrene blank wells used as control, and of those on the substrates, although cell density seems to be higher in the former.

In order to compare the adhesion and viability rates of cells on each substrate after four hours of incubation, the number of cells present in each image taken from the surface of the samples was determined. The results showed (see the supplementary material) that after four hours of incubation time there is no sta-

Fig. 3. Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on Ti-6Al-4V substrates with different degrees of surface roughness for one and seven days. Data are presented as mean value $\pm$ standard error. * denotes statistically significant difference versus the corresponding control sample.

tistically significant difference between the number of viable cells on the substrates with distinct surface degrees of roughness (i.e. 400, 1200 and 4000). Moreover, the number of dead cells on each substrate was appreciably low and similar to that obtained when culturing the cells on polystyrene plates (control samples). In contrast, the number of viable attached cells on the control samples was significantly higher (Fig. 1 in the Supplementary material).

In order to examine the proliferation rate of cells on the substrates with different degrees of roughness at longer incubation times, an XTT assay was used. Fig. 3 shows the absorbance values after one and seven days of incubation time. It may be seen that as there is no significant difference between the absorbance values, the numbers of viable cells on all substrates are similar. In contrast, the number of cells is significantly higher on the controls at these

Fig. 2. Fluorescence microscopy images of MC3T3-E1 cells on substrates with different degrees of surface roughness after four and 48 h of incubation. Viable cells are dyed green; dead cells appear as red dots. Cells seeded on polystyrene wells are shown as control. Scale bar 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Fluorescence microscopy images of Ti-6Al-4V substrates functionalized by using different deposition conditions after incubation with FITC. A non-functionalized 4000 sample incubated with FITC and observed under the same conditions is shown as control (c). Scale bar 1 μ m.

longer incubation times. These results show that the roughness of the substrates in the range included in this study had no significant effect on the proliferation rate of cells. In all cases, Ti-6Al-4V samples were not as favorable for the proliferation of the cells as polystyrene controls.

Since no difference was observed between the substrates with a different surface roughness in terms of cell adhesion, viability and proliferation, those with the lowest surface roughness (4000 samples) were chosen as the substrates for functionalization. This choice was influenced by an interest in evaluating the possible increase in roughness due to the presence of the functional layer under the most demanding of conditions.

3.2. Characterization of functionalized samples

3.2.1. Roughness, contact angle and surface density of amino groups

Biofunctional layers deposited by AVS and by using APTS as a precursor are composed of Si, O, C, H and N, with an approximate empirical formula of $\text{SiO}_{2.5}\text{C}_{2.5}\text{H}_{2.5}\text{N}_{0.5}$, as determined in a previous paper by X-ray photoelectron spectroscopy (XPS) and Rutherford backscattering spectroscopy (RBS) [32]. This elemental composition is the result of the decomposition of the organometallic moiety, which results in a thin solid film stabilized by Si—O—Si bonds, while maintaining a relatively high proportion of the other elements, most importantly nitrogen. The analysis by FTIR of layers deposited with different AVS processing parameters also showed that,

although AVS always yields the same decomposition products, their quantitative proportion is highly sensitive to processing conditions. Quantitative variations are especially important with regard to the content of amino groups in the functionalized layer [33].

Various functionalized samples were prepared by varying the deposition conditions of the process: T_{Evap} , T_{Act} and P_{Ar} . The samples were characterized in order to establish at least one set of optimal functionalization conditions in terms of the high density of amino groups and low roughness.

Fluorescence microscope images of substrates functionalized under different conditions are shown in Fig. 4. By comparing these images with the non-functionalized 4000 samples (Fig. 4c) the presence of an amine layer is evident. By varying T_{Evap} from 130 to 170 °C (Figs. 4b, e and h), it can be seen that the amine layer produced at $T_{\text{Evap}} = 150$ °C is more homogeneous compared with the other two. Figs. 4d–f show samples that differ in T_{Act} and their comparison indicates that a $T_{\text{Act}} = 750$ °C leads to a higher concentration of amino groups present. Lastly, micrographs d, e and i show samples that differ in P_{Ar} . It can be seen that with increasing Ar pressure, the concentration of amines deposited on the substrates increases. Fig. 4g ($T_{\text{Act}} = 800$ °C, $P_{\text{Ar}} = 2$ mbar) exhibits the effect of increasing the activation temperature and argon pressure simultaneously. In this case, the fluorescence image shows a homogeneous functionalized surface with a high density of amino groups.

Fig. 5 shows the AFM topography images of functionalized samples under distinct functionalization conditions (scan size: $5 \mu\text{m} \times 5 \mu\text{m}$). From comparison of the surface morphology of a

Fig. 5. Atomic-force microscopy (AFM) micrographs of Ti-6Al-4V samples functionalized under different deposition conditions. The topography of a non-functionalized 4000 sample (c) is shown for comparison purposes.

Fig. 6. Comparison of (a) fluorescence intensity and (b) RMS roughness values of layers deposited under different functionalization conditions.

Fig. 7. Comparison of (a) fluorescence intensity and (b) RMS roughness values of biofunctional layers deposited under the F1 and F2 conditions. Six samples were deposited under each condition.

non-functionalized 4000 sample (c) with those of functionalized samples, it is apparent that the amine layer buries the characteristic scratches and grooves that result from grinding. In most of the functionalization conditions, the formation of a homogeneous thin film over the surface results in a reduction of roughness values as measured by AFM.

Fig. 6 summarizes the comparison of (a) surface density of amines (as measured by fluorescence) and (b) roughness (measured as RMS) of samples functionalized under different conditions. Two sets of optimal functionalization conditions were selected (F1): T_{Evap} : 150 °C, T_{AET} : 750 °C, P_{AET} : 1 mbar, t : 20 min and (F2): T_{Evap} : 150 °C, T_{AET} : 750 °C, P_{AET} : 2 mbar, t : 20 min. Both conditions result in homogeneous and smooth (low values of RMS from AFM micrographs) layers combined with a high density of amines (high values of fluorescence). Since the F1 and F2 sets of conditions were selected to continue the biological assessment, their characterization was extended to include the thickness of the film, the contact angle and

the reproducibility of the biofunctional layers deposited. The thickness of the functional layers was measured by using AFM and values in the range of 150–200 nm were obtained. The contact angle values obtained were F1: $66.2 \pm 0.5^\circ$ and F2: $58.9 \pm 0.7^\circ$, which can be compared with the value of $57.8 \pm 0.6^\circ$ measured for the bare Ti-6Al-4V 4000 samples. The reproducibility of the deposited layers was checked by characterizing six samples of each condition (either F1 or F2). Fig. 7 compares (a) the fluorescence intensity and (b) the RMS roughness values of the samples. The comparison of the error bars with the values of the corresponding feature is an indication of the excellent reproducibility obtained for the deposition of the biofunctional layers.

3.2.2. Cell cultures on functionalized samples

Long- and short-term cell culture experiments were performed to determine the response of cells to the functionalized layers in terms of their viability and proliferation rates. After either four or

Fig. 8. Fluorescence microscopy images of MC3T3-E1 cells on non-functionalized 4000 and two functionalized samples (conditions F1 and F2) after four and 48 h of incubation. Viable cells are dyed green while dead cells appear as red dots. Cells seeded on blank polystyrene wells are shown as control. Scale bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9. Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on non functionalized and functionalized substrates (samples F1 and F2) for one and seven days. Data are presented as mean value $\pm$ standard error. * denotes statistically significant difference versus the corresponding control sample.

48 h of incubation, cells were stained with calcein AM and propidium iodide and observed with a fluorescence microscope. Fig. 8 compares the morphology of cells adhered on functionalized and non-functionalized (4000) samples with polystyrene controls after four and 48 h of incubation. At four hours, the cells are adhered to the surface of all samples and possess a circular shape. This rounded cell shape is more pronounced in cells growing on the functionalized substrates. However, 48 h after seeding, the cells are

completely spread and the typical triangular shape of this cell line can be observed. The morphology of the cells appears to be similar in all substrates. This result indicates that cells can adhere to and spread over the amine layer, as well as they do on the non-functionalized Ti-6Al-4V samples.

The number of viable cells attached to the substrates four hours after seeding was determined from the micrographs taken from different samples and a statistical analysis performed (see the Supplementary material). The statistical analysis showed a significant increase in the number of cells attached to the functionalized compared with the non-functionalized substrates after four hours of incubation (Fig. 2 in the Supplementary material). Moreover, the number of dead cells (Propidium iodide positive cells) obtained for all substrates was somewhat low and close to that obtained with cells seeded on polystyrene controls, demonstrating a cytocompatibility for the functionalized substrates comparable to that observed on bare Ti-6Al-4V substrates.

In order to extend the comparison between functionalized and non-functionalized samples to longer incubation times, the proliferation rate of the cells on each sample after one day and seven days of incubation was compared by using the XTT assay (Fig. 9). According to the measured absorbance levels, the number of viable cells on the functionalized and non-functionalized samples at a given incubation time, does not show any statistically significant differences. In both types of substrates, however, the number of viable cells is considerably lower than that found in the controls.

From these results it can be seen that the functional amine layer does not inhibit cell proliferation in any way, and cells behave on the functionalized layer similarly as on the non-functionalized Ti-6Al-4V substrate.

Lastly, the influence of cell culturing on the biofunctional layer was assessed by removing the cells after seven days of culture and measuring the density of amino groups by using FITC and fluorescence microscopy (Fig. 10). These images show that although some degradation is observed, after cell culturing the amine layer keeps essentially its integrity and a high density of active amines at the surface is preserved after this incubation time.

Fig. 10. Fluorescence images of F1 and F2 samples before and after seven days of cell culture. Scale bar 1 mm.

4. Conclusions

In this study it is shown that the AVS technique is an effective, reliable and controllable method for depositing biofunctional amine layers on Ti-6Al-4V substrates. The amine layer was characterized by using fluorescence microscopy to determine the density of amines, and atomic force microscopy to measure the topography of the layer. Two sets of optimal functionalization conditions were defined that lead to the deposition of a homogeneous and smooth biofunctional layer on Ti-6Al-4V substrates.

MC3T3-E1 osteoblastic-like murine cells were used as a cell model for *in-vitro* experiments in order to assess the response of the cells to the biofunctional layer. It was shown that the amine thin film does not inhibit cell proliferation in any way and does not produce any toxic effect on the cells. Additionally, the stability of amine layer was confirmed after seven days of cell culturing.

The results confirm the formation of a stable, biofunctional and non-toxic amine layer which could create a platform that allows the subsequent covalent immobilization of biomolecules and proteins onto the surface of Ti-6Al-4V alloy.

Acknowledgements

The authors would like to express their appreciation to Dr. Mihaela Iordăchescu for providing the Ti-6Al-4V alloy, Soledad Martínez-Montero for her assistance in cell culturing and Dr. Javier Martínez for preparing the resin-coated samples. The work was funded by the Ministerio de Economía y Competitividad in Spain through projects MAT2012-38412-CO2-01 and DPI2013-45485 and by the Comunidad de Madrid in Spain (Grant S2011/BMD-2460).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.susc.2016.05.139>.

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SCIENTIFIC REPORTS

OPEN

Enhanced Biological Response of AVS-Functionalized Ti-6Al-4V Alloy through Covalent Immobilization of Collagen

Received: 22 September 2017

Accepted: 8 February 2018

Published online: 20 February 2018

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This study presents the development of an efficient procedure for covalently immobilizing collagen molecules on AVS-functionalized Ti-6Al-4V samples, and the assessment of the survival and proliferation of cells cultured on these substrates. Activated Vapor Silanization (AVS) is a versatile functionalization technique that allows obtaining a high density of active amine groups on the surface. A procedure is presented to covalently bind collagen to the functional layer using EDC/NHS as cross-linker. The covalently bound collagen proteins are characterized by fluorescence microscopy and atomic force microscopy and their stability is tested. The effect of the cross-linker concentration on the process is assessed. The concentration of the cross-linker is optimized and a reliable cleaning protocol is developed for the removal of the excess of carbodiimide from the samples. The results demonstrate that the covalent immobilization of collagen type I on Ti-6Al-4V substrates, using the optimized protocol, increases the number of viable cells present on the material. Consequently, AVS in combination with the carbodiimide chemistry appears as a robust method for the immobilization of proteins and, for the first time, it is shown that it can be used to enhance the biological response to the material.

Titanium (Ti) and titanium alloys are among the most commonly used biomaterials for hard tissue replacement^{1,2}. This choice is due to their good corrosion resistance, good mechanical properties and excellent biocompatibility^{1,3,4}. However, the difficulty for establishing a direct contact with adjacent tissues has been a drawback for their application as implants⁵. Hence, a variety of surface engineering techniques have been proposed that, by modifying the morphological and chemical properties of the surface, intend to modulate the response of the body to the implanted titanium and to improve the interaction between the implant and bone tissue^{2,6,7}. A promising approach consists of immobilizing biomolecules, so that specific responses in the tissue in contact with the implant are induced^{8,9}. Extra cellular matrix (ECM) proteins are usual targets in this approach, since their biological function implies an intimate interaction with cells^{10–12}.

Collagen is the most abundant protein component of the ECM and was proven to support proliferation and differentiation of different cell lineages^{13,14}. Fibroblast and osteoblast adhesion onto collagen is believed to be regulated by the recognition of the protein through specific proteins, such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins^{15,16}. Consequently, collagen has been used as a coating in an attempt to improve the biological properties of metallic biomaterials^{17–19}. Although adsorption is the most simple procedure to cover a surface with any given protein, adsorbed proteins are not strongly bonded to the surface and variations in the environment can cause them to be easily desorbed^{20,21}. Moreover, adsorbed collagen is highly susceptible to biodegradation¹⁶, and adsorption may lead to conformational changes which can affect its properties²².

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Figure 1. Morphology of the MSC cells cultured on bare Ti-6Al-4V, adsorbed collagen on Ti-6Al-4V (Col-ads) and plastic control samples after 4 and 48 hours of seeding. Viable cells are stained green while the dead cells appear as red dots. Scale Bar: 100 μm.

Covalent immobilization of proteins provides an alternative approach to modulate the biological response to materials. Covalent immobilization creates a stable link between the protein and the surface that is resistant to environmental changes and increases the stability of the immobilized protein²². However, there is a need to functionalize the surface of the metallic substrate with appropriate reactive groups. Subsequently, proteins can be covalently bound to these reactive groups using a cross-linker. Different strategies were proposed for the covalent immobilization of biomolecules on metallic surfaces which include different functionalization procedures and active proteins. For instance, bone morphogenic proteins (BMP) have been immobilized on titanium after functionalizing the material through liquid phase silanization using a solution of 3-aminopropyltriethoxysilane (APTS) in toluene and carbonyl diimidazole as cross-linker²⁴. Other examples of functionalization protocols include the functionalization in liquid phase by APTS in toluene using glutaraldehyde as cross-linker to immobilize alkaline phosphatase and rat albumin on titanium²⁵, functionalization with isocyanatopropyl-triethoxysilane (IPS) in toluene in combination with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for immobilization of collagen on stainless steel¹⁶ and functionalization with polydopamine using EDC as crosslinker for the immobilization of collagen on titanium²². The selection of the crosslinker was recognized as a critical point in the whole functionalization process since, for instance, it was found that glutaraldehyde might act as a cytotoxic agent *in-vitro*²⁶. In contrast, carbodiimide cross-linkers are water-soluble and the excess molecules can be relatively easily removed^{27–29}.

In this study, we use activated vapor silanization (AVS), a method developed by the authors that allows the surface functionalization of a wide range of materials^{30–32}, in combination with the EDC/NHS chemistry to covalently immobilize collagen on Ti-6Al-4V samples. The covalently immobilized collagen is characterized and its stability on the surface demonstrated. Finally, by performing *in-vitro* cell cultures, we address the hypothesis that the covalently immobilized collagen is able to enhance the adhesion and proliferation of mesenchymal stem cells on functionalized Ti-6Al-4V samples compared with the bare material.

Results

Collagen adsorption. As a means to investigate the effect on cell behavior of a collagen type I coating on Ti-6Al-4V samples, collagen type I was adsorbed onto non-functionalized (bare) Ti-6Al-4V substrates, leading to the formation of a film (Col-ads). Figure 1 shows the calcein AM/propidium iodide stained MSC cells on bare Ti-6Al-4V, Col-ads and polystyrene control samples after 4 and 48 hours of seeding.

After 4 hours of seeding the cells are adhered to all samples, however the morphology of cells on Col-ads and control samples show a higher degree of extension of cellular processes, probably related with a dynamic cytoskeletal rearrangement, compared to the cells adhered on Ti-6Al-4V samples. The number of adhered cells on Col-ads samples is higher than that on bare Ti-6Al-4V, but lesser than on the controls (Fig. 2a). This trend in the number of cells on each sample remains consistent after 48 hours of seeding, demonstrating improved cell adhesion after 4 hours of seeding and a higher number of cells after 48 hours on Col-ads samples compared to bare Ti-6Al-4V. XTT assay performed at 48 hours after seeding also confirms the results of cell counting (Fig. 2b).

Covalent immobilization of collagen. Figure 3 shows fluorescence microscopy images of a functionalized Ti-6Al-4V sample incubated with collagen and EDC/NHS (Fig. 3c) compared to a bare (non-functionalized) Ti-6Al-4V sample, which was incubated with the same FITC-tagged collagen and EDC/NHS solution (Fig. 3a), and a functionalized Ti-6Al-4V sample incubated with FITC-tagged collagen, but without the addition of EDC/NHS cross-linker in the solution (Fig. 3b). Collagen aggregates are apparent on the surface of all three samples, but the number of aggregates is higher on Col-imm-f (Fig. 3c) samples. After the treatment with SDS, it can be

Figure 2. Number of cells obtained by (a) cell counting from micrographs after 4 and 48 hours of seeding, and (b) XTT reaction after 48 hours of seeding for MSC cells on bare Ti-6Al-4V, collagen adsorbed on Ti-6Al-4V (Col-ads) and control samples. *Denotes $p < 0.05$.

Figure 3. Fluorescence microscopy images of samples incubated with FITC-tagged collagen on (a) bare Ti-6Al-4V incubated with EDC/NHS cross-linker before SDS treatment and (d) after SDS treatment, (b) AVS-functionalized Ti-6Al-4V without EDC/NHS cross-linker before SDS treatment and (e) after SDS treatment, and (c) AVS-functionalized Ti-6Al-4V incubated with EDC/NHS cross-linker (Col-imm-f) before SDS treatment and (f) after SDS treatment. Scale Bar 100 μm.

seen that fluorescence remains practically unchanged in the Col-imm-f (Fig. 3f), while a significant reduction is observed in the other two samples (Fig. 3d,e).

Figure 4 shows AFM topography images of the aforementioned samples. Before SDS treatment, the collagen aggregates are clearly visible in all three samples. After the SDS treatment, a significant removal of non-immobilized collagen is observed in micrographs d and e, but not in the functionalized Ti-6Al-4V sample incubated with collagen and EDC/NHS (f). AFM topography images of a bare and functionalized Ti-6Al-4V are also shown in the Supplementary data Fig. 1 for comparison.

The ability of SDS to remove adsorbed protein from the bare material was assessed by forming films of FITC-tagged collagen on Ti-6Al-4V samples and, subsequently, incubating them with 10% SDS solution in PBS. The fluorescence microscopy images can be seen in Supplementary data Fig. 2. It can be seen that after the incubation with SDS almost all the FITC-tagged collagen is removed from the substrate. On the other hand, the SDS treatment did not have any adverse effects on the amine functional layer. Since the layer is observed to be completely stable after the SDS treatment (Supplementary data Fig. 2).

Figure 4. AFM topography images of samples incubated with FITC-tagged collagen on (a) bare Ti-6Al-4V incubated with EDC/NHS cross-linker before SDS treatment and (d) after SDS treatment, (b) AVS-functionalized Ti-6Al-4V without EDC/NHS cross-linker before SDS treatment and (e) after SDS treatment, and (c) AVS-functionalized Ti-6Al-4V incubated with EDC/NHS cross-linker (Col-imm-f) before SDS treatment and (f) after SDS treatment. Scale Bar 5 μ m.

Figure 5. Fluorescence microscopy images of functionalized Ti-6Al-4V samples incubated with collagen and different concentrations of EDC/NHS. (a,d) 1X concentration of EDC/NHS (b,e) 1/10X concentration of EDC/NHS (c,f) 1/20X concentration of EDC/NHS. (a,b,c) Scale Bar 1 mm and (d,e,f) Scale Bar: 100 μ m.

In order to determine the effect of EDC/NHS cross-linker concentration on the covalent binding of the protein, collagen was immobilized on functionalized Ti-6Al-4V samples, changing the concentration of the EDC/NHS cross-linker while keeping constant the concentration of collagen. Figure 5 shows fluorescence microscopy images of the FITC-tagged collagen covalently immobilized on functionalized Ti-6Al-4V samples using EDC/NHS concentrations of 2.5/0.63 mg/ml (1X), 0.25/0.063 mg/ml (1/10X) and 0.125/0.0315 mg/ml (1/20X). Although, the fluorescence content appears higher on the 1X sample, even in case of the lowest EDC/NHS concentration (1/20X), a significant fluorescence is still observable on the surface of the samples. The presence of immobilized collagen even in the 1/20X samples is confirmed by the AFM topography images as shown in Fig. 6. The amount of immobilized collagen on 1X, 1/10X and 1/20X samples was determined

Figure 6. AFM topography images of functionalized Ti-6Al-4V samples incubated with collagen and different concentration of EDC/NHS at different scan sizes (**a,d**) 1X concentration of EDC/NHS, (**b,e**) 1/10X concentration of EDC/NHS, and (**c,f**) 1/20X concentration of EDC/NHS.

Figure 7. MSC cells adhered on bare Ti-6Al-4V, functionalized Ti-6Al-4V with immobilized collagen containing 1X, 1/10X and 1/20X concentrations of EDC/NHS, and control sample at 4 and 48 hours after seeding. Viable cells are stained green, while the dead cells appear as red dots. Scale Bar 100 μm.

quantitatively by the Micro-BCA assay leading to the values: $0.20 \pm 0.04 \mu\text{g}/\text{mm}^2$ (1X), $0.114 \pm 0.009 \mu\text{g}/\text{mm}^2$ (1/10X), $0.117 \pm 0.009 \mu\text{g}/\text{mm}^2$ (1/20X).

In order to assess the cytocompatibility and the effect of covalently immobilized collagen on the surface of Ti-6Al-4V samples on cell behavior, *in-vitro* cell cultures were performed. The morphology of MSC cells on 1X, 1/10X and 1/20X compared to bare Ti-6Al-4V and polystyrene controls at 4 and 48 hours after seeding can be seen in Fig. 7. The number of cells counted on each sample (Fig. 8a) demonstrated that at either time period, 1/20X sample had significantly more attached cells compared to bare Ti-6Al-4V. The better adhesion of 1/20X group was translated into a higher number of viable cells at later time points, as confirmed by XTT assay (Fig. 8b).

Although at 4 hours after seeding the cells were adhered on all the samples, those on the samples containing immobilized collagen showed a more widespread morphology compared with bare Ti-6Al-4V samples. This observation was further supported by phalloidin/hoecchst staining (Fig. 9) and cell surface area measurements (Fig. 10).

Although the phalloidin/hoecchst analysis was not extended to longer incubation times, it is apparent from Fig. 7 that cells on 1X samples show a spherical morphology at 48 hours after seeding, in contrast to all the other samples which mostly exhibit cells with polygonal shapes.

Figure 8. Number of MSC cells obtained by (a) cell counting from micrographs after 4 and 48 hours of seeding and (b) XTT reaction at 48 hours after seeding on bare Ti-6Al-4V, functionalized Ti-6Al-4V with immobilized collagen containing 1X, 1/10X and 1/20X concentrations of EDC/NHS, and control sample. *Denotes $p < 0.05$.

Discussion

Although in a previous paper we showed the capability of AVS to effectively functionalize Ti-6Al-4V substrates, the enhancement of the biological response of biomaterials requires two additional steps: (1) using an adequate crosslinking chemistry to immobilize the biomolecule on the material, and (2) making a choice with regard to the biomolecule (or biomolecules) to be immobilized.

Collagen type I is a convenient choice due to its ubiquitous presence in the ECM and availability. Consequently, there are several studies on cell adhesion and proliferation on collagen coated Ti-alloy substrates. *As et al.* reported improved cell adhesion of human MSCs on collagen type I coated titanium after 4 hours of culture time¹⁵. *Rochlecke et al.* also demonstrated the enhanced cell adhesion and proliferation for collagen type I adsorbed onto titanium compared to bare titanium¹⁴, suggesting a possible positive effect of collagen on cell cycle evolution. It was also found that a collagen coating on titanium alloys promotes long and short term cell adhesion and proliferation of rat calvarial osteoblasts²⁰. However, some contradictory results can be also found in the literature. *Bierbaum et al.* observed no improvement on cell proliferation after 2 days of culturing rat calvarial osteoblast on collagen type I coated Ti-6Al-4V²⁵.

Therefore, we decided to perform an initial assessment of the influence of a collagen coating on the survival and proliferation of murine MSC cells using collagen type I simply adsorbed on Ti-6Al-4V samples (Col-ads samples). As it can be seen in Fig. 2, the number of viable cells present on Col-ads samples is significantly higher than on bare Ti-6Al-4V at both time periods.

The enhanced cell adhesion and higher final number of cells on Ti-6Al-4V with an adsorbed collagen layer led to the development of a protocol to covalently immobilize the protein on the functionalized samples. Among the different possible choices, the EDC/NHS chemistry was employed due to its simplicity. Covalent immobilization proceeds by the initial formation of a semi-stable reactive ester between the $-COOH$ groups of the glutamate or aspartate residues present in collagen^{36,37}, and the NHS molecule. When the semi-stable ester comes into contact with an amine located on the functionalized surface of Ti-6Al-4V, a stable amide bond is created between the protein and the amine group^{37–40}.

The stability of the covalently immobilized collagen was examined by exposing the samples to an SDS solution. It was observed that, in contrast to the results that showed the removal of adsorbed collagen on bare titanium samples upon SDS washing, no significant effect was observed on the covalently immobilized collagen samples (Col-imm-f) (Fig. 3c and f). Additionally, it was checked that the functional layer was not altered by exposure to an SDS solution and keeps its integrity even after the treatment, as shown in Supplementary data Fig. 2.

The *in-vitro* biological response of samples with immobilized collagen was assessed by cell culture of MSCs (Fig. 7). As it can be seen, at 4 hours after seeding all the functionalized samples show a relatively high number of adhered cells. However, depending on the concentration of EDC/NHS used, the response of the cells after 48 hours of seeding was quite different. In 1X samples, the number of cells declined at 48 hours after seeding, suggesting an inhibited cell proliferation and lower cell survival. Besides, the morphology of the cells on 1X samples show a dominantly circular morphology. This anomalous morphology can be explained by a possible cytotoxic effect of the EDC/NHS cross-linker. Despite the fact that there are several available studies which report no cytotoxic effect of carbodiimide cross-linker^{41–43}, EDC in high concentrations might be toxic. This effect could be the result of DNA crosslinking caused by EDC, which is membrane permeable, and would lead to the disturbance of the cell cycle⁴⁴. Concentrations between 10 mM (1.9 mg/ml)³⁷ and 1 mg/ml⁴⁵ were reported to produce cytotoxic effects.

In contrast to the 1X samples, the number of cells on 1/10X and 1/20X samples grew after 48 hours of culture. In particular, it was shown that 1/20X samples not only showed better cell adhesion after 4 hours of cell seeding ($p = 0.0143$), but also a higher number of attached cells at 48 hours after seeding compared to bare Ti-6Al-4V ($p = 0.0002$).

Figure 9. Fluorescence microscopy images of Phalloidin/Hoechst stained MSC cells adhered on bare Ti-6Al-4V, functionalized Ti-6Al-4V with immobilized collagen containing 1X, 1/10X and 1/20X concentrations of EDC/NHS, and control sample after 4 hours of seeding. Scale Bar 20µm.

Figure 10. Surface area of MSCs on bare Ti-6Al-4V, functionalized Ti-6Al-4V with immobilized collagen containing 1X, 1/10X and 1/20X concentrations of EDC/NHS, and control sample after 4 hours of seeding. * Denotes $p < 0.05$ with respect to the control sample.

The assessment of biological response of the functionalized samples was completed with the study of cell morphology at short incubation times. It was observed that after 4 hours of seeding, the cells growing on all functionalized samples showed a slightly more extended cytoskeleton compared to bare Ti-6Al-4V. In particular, the measurements of the surface area of the cells adhered to the samples (Figs 9 and 10) demonstrated that the cells adhered on the plastic control samples possess the highest surface area, while those adhered on bare Ti-6Al-4V had a significantly lower surface area. The surface area of cells adhered on samples with immobilized collagen ranked between the polystyrene control and bare Ti-6Al-4V samples. It was observed that among these samples, the cells adhered on the 1/20X samples seem to possess a slightly higher average surface area.

The observed increase in the cell surface area of the functionalized samples compared with bare Ti-6Al-4V can be interpreted as the result of a more extended cell cytoskeleton which, in turn, can contribute to an improved cell attachment to the substrates. It has been reported that the initial extent of adhesion and spreading of the cell can influence its subsequent survival and proliferation⁶⁵, so that this result is consistent with the higher number of viable cells found on 1/20X at longer incubation times.

In our case we have found an optimal concentration of EDC of 0.125 mg/ml (1/20X), when combined with the cleaning process indicated in the Materials and Methods section. The comparison of the tests on 1/20X, 1/10X and 1X samples highlights the importance of cleaning as one of the critical steps in the whole functionalization process as suggested previously^{27,66}.

Conclusion

In this work, we demonstrate the enhancement in cell adhesion and survival of MSCs on Ti-6Al-4V alloy induced by the covalent immobilization of collagen Type I on AVS-functionalized samples. An efficient protocol for the covalent binding of collagen to the amine functional layer based on the EDC/NHS chemistry is developed and complemented by a suitable cleaning process. The immobilization of collagen was characterized by fluorescence microscopy and atomic force microscopy, including its stability even after relatively harsh treatments, such as incubation with SDS.

The biological response was assessed by *in-vitro* cell culture experiments using MSCs. The results demonstrated that even relatively low concentrations of EDC/NHS could cause cytotoxicity for cells 48 hours after

seeding. Finally, an optimized protocol based on a reduced EDC/NHS concentration and an exhaustive cleaning procedure was defined and proven to lead to an increase in the cell number on the material compared with those observed on bare Ti-6Al-4V samples.

Although these results illustrate the robustness of the whole process that combines AVS-functionalization and protein covalent immobilization, they are far from exhausting all the possibilities of the technique. In this regard, it can be stressed that the AVS process is compatible not only with a wide range of biomaterials and processing methods, but also with a variety of crosslinkers and biomolecules. We believe that this versatility offers a great opportunity to improve the biological response of numerous biomaterials significantly with a minimum modification of the fabrication procedures. This improvement, in turn, should reflect in an enhanced biocompatibility of implants and devices.

Materials and Methods

Substrate preparation. Ti-6Al-4V substrates with dimensions of $10 \times 10 \times 1$ mm were cut from an ingot of commercially available alloy. Subsequently, the substrates were subjected to a polishing process using sandpapers grit No. 80, 400, 1200 and 4000 sequentially. Ultimately, the samples were cleaned by sonication in acetone, isopropanol and distilled water.

Adsorption of collagen on bare Ti-6Al-4V substrates. In order to determine the effect exerted by the presence of collagen on Ti-6Al-4V substrates on cell adhesion/proliferation, initial tests with collagen adsorbed on bare (non-functionalized) Ti-6Al-4V substrates were conducted. Samples were incubated with a suspension of 2.5 mg/ml collagen (Rat tail Collagen type I, Corning) in PBS (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 137 mM, KCl 2.7 mM, pH = 7.4). A solution of NaOH (Panreac) 1 M was added dropwise to the collagen suspension to adjust the pH in the range of 6.0–8.0. Subsequently, 100 μl of the suspension was deposited on the surface of each bare Ti-6Al-4V substrate and the substrates were incubated at 37 °C for 2 h in a wet chamber followed by 1 h incubation at 37 °C in a dry chamber. Finally, the samples were immersed in distilled water for 1 h to rehydrate the collagen films. These samples were named Col-ads.

Preparation of covalently immobilized collagen on functionalized Ti-6Al-4V substrates.

Functionalization. Functionalization of Ti-6Al-4V substrates was performed using an Activated Vapor Silanization (AVS) equipment as described elsewhere³². Briefly, 3-Aminopropyltriethoxysilane (APTS, Fluka) is put inside a closed chamber and evaporated. The vapor is carried by an argon flux (BIP, Purity ≥ 99 , 9997%) to an activation chamber where the temperature rises to 750 °C. Afterwards, the activated APTS vapor impinges on the substrates in the deposition chamber. Finally, the vapor phase is evacuated from the system by a rotary pump. After functionalization with AVS, the substrates were cleaned by sonication with acetone and isopropanol, rinsed with distilled water, dried using an argon flux and stored in air.

In the AVS process there are four controllable parameters: Evaporation temperature of the APTS (T_{evap}), activation temperature of the APTS (T_{act}), pressure of argon (P_{Ar}) and time of deposition (t). For the functionalization of the substrates used in this study, the processing parameters were chosen as: $T_{\text{evap}} = 150$ °C, $T_{\text{act}} = 750$ °C, $P_{\text{Ar}} = 2$ mbar, $t = 20$ min. This choice of parameters was based on a previous study³² that established the optimized functionalization conditions. This process results in a homogenous and dense amino-functionalized surface on Ti-6Al-4V substrates.

Labeling collagen with FITC. In order to assess the immobilization of the protein on the substrates, collagen molecules were tagged with fluorescein 5(6)-isothiocyanate (FITC, Fluka) which binds to the amine groups of the proteins. A suspension of collagen 2 mg/ml in PBS was prepared and FITC was added until a final FITC concentration of 0.5 mg/ml was reached.

Dialysis was employed in order to remove any free FITC molecules that had not reacted with the collagen molecules. The collagen-FITC solution was put inside a dialysis membrane (Snakeskin dialysis tubing, Thermo Scientific) and dialysis was performed against 1 L of PBS as medium. The medium was changed every 8 hours (three times in total). Before each change of the dialysis medium, 1 ml of the PBS medium was measured with a spectrophotometer (Halo RB-10) at a wavelength of 490 nm, and absorbance was compared to an absorbance-concentration standard curve to determine the concentration of free FITC molecules. The dialysis process was stopped when the concentration of FITC in the medium was determined to be below the resolution of the spectrophotometer (less than 0.005).

Covalent immobilization of FITC-labeled collagen. After the completion of the dialysis process, a solution of 4-Morpholine-ethanesulfonic acid (MES, Sigma-Aldrich) 0.2 M, pH = 6.0 was added to the collagen-FITC solution to a final concentration of collagen of 1 mg/ml. The functionalized samples were incubated with 1 ml of the solution of collagen in MES for 1 h. Subsequently, a solution of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma-Aldrich) and N-Hydroxysuccinimide (NHS, Aldrich) in MES 0.1 M, pH = 6.0 was added to the samples to obtain a final concentration of 2.5 mg/ml EDC, 0.63 mg/ml NHS (hereafter referred to as 1X concentration) and the incubation was continued for 4 h. Finally, the samples were removed from the solution and gently rinsed with distilled water to remove any non-adhered collagen. These samples were named Col-imm-f.

In order to determine the effect of the EDC/NHS cross-linker concentration on the process, different concentrations of EDC/NHS were used taking the 1X concentration as reference, and named 1/10X (one tenth of the reference concentration) and 1/20X (one twentieth of the reference concentration).

To eliminate the non-covalently bound collagen present on the surface, some samples were immersed in a solution of 10% sodium dodecyl sulfate (SDS, Fisher scientific) in PBS. The samples were sonicated initially for 1 h in the SDS solution, then the SDS solution was changed and the samples were incubated overnight.

Covalent immobilization of collagen on functionalized Ti-6Al-4V substrates for cell cultures (Col-imm). Functionalized Ti-6Al-4V samples were incubated with 75 μ l of a 3.26 mg/ml collagen suspension in MES buffer (0.1 M, pH = 0.6) for 1 h. Subsequently, 25 μ l of the EDC/NHS solution were added onto the substrates and the incubation continued for 4 h. The final concentrations were: Collagen 2.5 mg/ml, MES 0.1 M, (pH = 0.6), EDC 2.5 mg/ml and NHS 0.63 mg/ml. These concentrations correspond to the 1X concentration of EDC/NHS. In some samples, the initial concentration of EDC/NHS was modified correspondingly to produce the 1/10X and 1/20X samples.

Finally, in order to remove any free cross-linker molecules still present, the samples were cleaned in PBS (10 mM, pH = 7.4), MES (0.1 M, pH = 6.0) and Dulbecco's Modified Eagle Medium (DMEM, pH = 7.4) for 5 h, 72 h and 24 h, respectively.

Characterization. Fluorescence microscopy. In order to visualize the FITC-tagged collagen aggregates on the substrates, fluorescence microscopy was used. Samples were observed using an inverted microscope (Leica DMIRB) equipped with a digital camera (Leica DC100) at an emission wavelength of 520 nm for FITC. The observation conditions were: exposure time: 1.3 ms, gain: 2.1X and gamma: 0.68.

Atomic force microscopy. The topography of samples was studied by atomic force microscopy (Cervantes AFM, Nanotec S.L.). Profile data and roughness were analyzed using WSxM 5.0 software. AFM measurements were performed in air using a pyramidal cantilever (Olympus OMCL RC800, semi-angle 39°, nominal resonance frequency 69 KHz) in dynamic mode.

In vitro cell cultures. Bone marrow (BM) murine mesenchymal stem cells (MSC) were used as cell model. Isolation and expansion of BM cells were performed on fibronectin-coated wells (Corning Inc., NY) in Iscove's Modified Dulbecco's Medium (IMDM, HyClone) supplemented with 20% MSC stimulatory supplements (Stem Cell Technologies), 100 μ mol/L 2-mercaptoethanol (Sigma), 100 IU/ml penicillin (Sigma), 0.1 mg/ml streptomycin (GIBCO), 2 mmol/L L-glutamine (GIBCO), 10 ng/ml human PDGF-BB (Peprotech), and 10 ng/ml rh-EGF (Peprotech). Adherent cell clusters were grown for a minimum of 5 passages. After this step, cells were regularly maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin (Sigma). Cells were detached from the cultures dishes when necessary using Trypsin-EDTA (HyClone). All experiments were performed on MSC in passages 5 through 15. Prior to culturing, substrates were placed in p24 multiwells and were sterilized using UV irradiation for 20 min on each side. Cells were seeded on the substrates in a concentration of 50000 cells/ml (0.5 ml per well). The cells were maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO₂. Cells seeded on empty wells were used as controls and each experiment was done twice with duplicate samples.

Although one of the advantages of using MSC is their ability to differentiate to a number of cell lineages, including osteoblasts and chondrocytes⁴⁰, it was considered that a proper analysis of this process was more adequate for a future experimental campaign. Following this rationale, the present study is focused on the survival and proliferation of MSC on biofunctionalized and bare Ti-6Al-4V substrates.

Cell Viability. To perform a live/dead assay, calcein AM/propidium iodide staining was used. Staining was done either at 4 or 48 hours after seeding. Cells were incubated with a combined solution of 1 μ l/ml of calcein acetoxymethyl (Calcein AM, Life Technologies, 0.5 μ g/ μ l in DMSO) and 1 μ l/ml of propidium iodide (Sigma-Aldrich, 750 μ M in PBS) in DMEM for 30 min. Cells were observed using a fluorescence microscope (Leica DMIRB). At least three representative images were captured of each sample.

Phalloidin/Hoechst staining. To observe the morphology of cells adhered on different substrates, actin filaments of the cells were stained with phalloidin tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC, Sigma) and the nuclei counterstained with hoechst 33258 (Molecular Probes). Prior to staining, the samples were washed with PBS and cells were fixed in a solution of paraformaldehyde 4%. Afterwards, the fixed cells were rinsed with PBS once more, permeabilized using a solution of 0.1% triton X-100 and stained with a combined solution of 2 μ g/ml phalloidin and 0.2 mg/ml hoechst in PBS. Finally, the samples were observed in a fluorescence microscope (Leica DMIRB) equipped with a digital camera (Leica DC100) at emission wavelengths 570 and 461 nm for phalloidin and hoechst, respectively.

In order to compare the level of cell spreading on each sample, the phalloidin/hoechst images obtained after 4 hours of seeding were analyzed. For each sample, 10 individual and perfectly separate cells were selected at random and the surface area of each cell was measured.

Cell proliferation assay. Cell proliferation on each sample after 48 hours of seeding was assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT, AppliChem) following manufacturer's instructions. The absorbance of each well was measured spectrophotometrically at 450 nm by using an ELX808 microplate reader (BioTek). For comparative reasons, the absorbance values were normalized according to the surface area of each sample.

The maximum duration of the cell viability and proliferation assays was fixed to 48 h for two main reasons. Firstly, the results obtained at 48 h of incubation are less likely to be affected by the possible contact inhibition effect, when the cell density is high⁴¹. This effect might be important at longer incubation times when comparing

the number of cells grown on the polystyrene control samples and those on the other substrates. Secondly, the large number of trials performed in order to obtain reliable data from the cell culture experiments, especially during the development of the cleaning protocol, converted the duration of these experiments in a critical parameter for the development of an efficient experimental campaign. A duration of 48 h for cell culturing was selected as a convenient time that allows studying the survival and proliferation of cells, while preventing contact inhibition and, simultaneously, keeping the time required for the experimental work within a reasonable limit. This range of cell culture times was used by other authors that assessed the *in vitro* response of cells to different materials^{1,10,28}.

Protein quantification. The amount of proteins immobilized on 1X, 1/10X and 1/20X samples after completing the cleaning process was measured using Micro-BCA protein assay kit (Thermo Scientific). The samples were incubated with the working solution of the kit (A:B:C:PBS = 25:24:1:50) for 1 h at 60 °C followed by equilibration at room temperature for 30 min. The absorbance values were obtained at 562 nm and compared with a standard curve obtained from serial dilutions of bovine serum albumin in PBS. The assay was done with duplicate samples for each concentration.

Image analysis. Image J software was used for image analysis, counting the number of viable cells and measuring cell surface area.

Statistical analysis. To perform statistical analysis IBM SPSS Statistics 20 software was used. Statistical significance was determined using a one-way ANOVA followed by a Games-Howell post-hoc test. $p < 0.05$ was considered significant. All data are presented as mean value $\pm$ standard error.

Data availability statement. No datasets were generated or analysed during the current study.

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Acknowledgements

The work was funded by the Ministry of Economy and Competitiveness in Spain through Projects MAT2016-75544-C2-1-R and MAT2016-79832-R.

Author Contributions

P.R. performed the experimental work with the help of P.A.L., and wrote the first version of the manuscript. R.D. obtained the AFM images. M.R. and D.G.-N. obtained the cells and supervised the cell culture experiments. M.E. and G.V.G. contributed to the data analysis and reviewed the experiments. J.P.-R. proposed the experiments and coordinated the work.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-21685-3>.

Competing Interests: The authors declare no competing interests.

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