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Escuela de Doctorado de la Universitat Jaume I

Doctoral Thesis

Protein adsorption and macrophage polarization as biocompatibility markers for biomaterial design

Memoria presentada por Nuno Miguel Araújo da Cunha Gomes para optar al grado de doctor/a por la Universitat Jaume I

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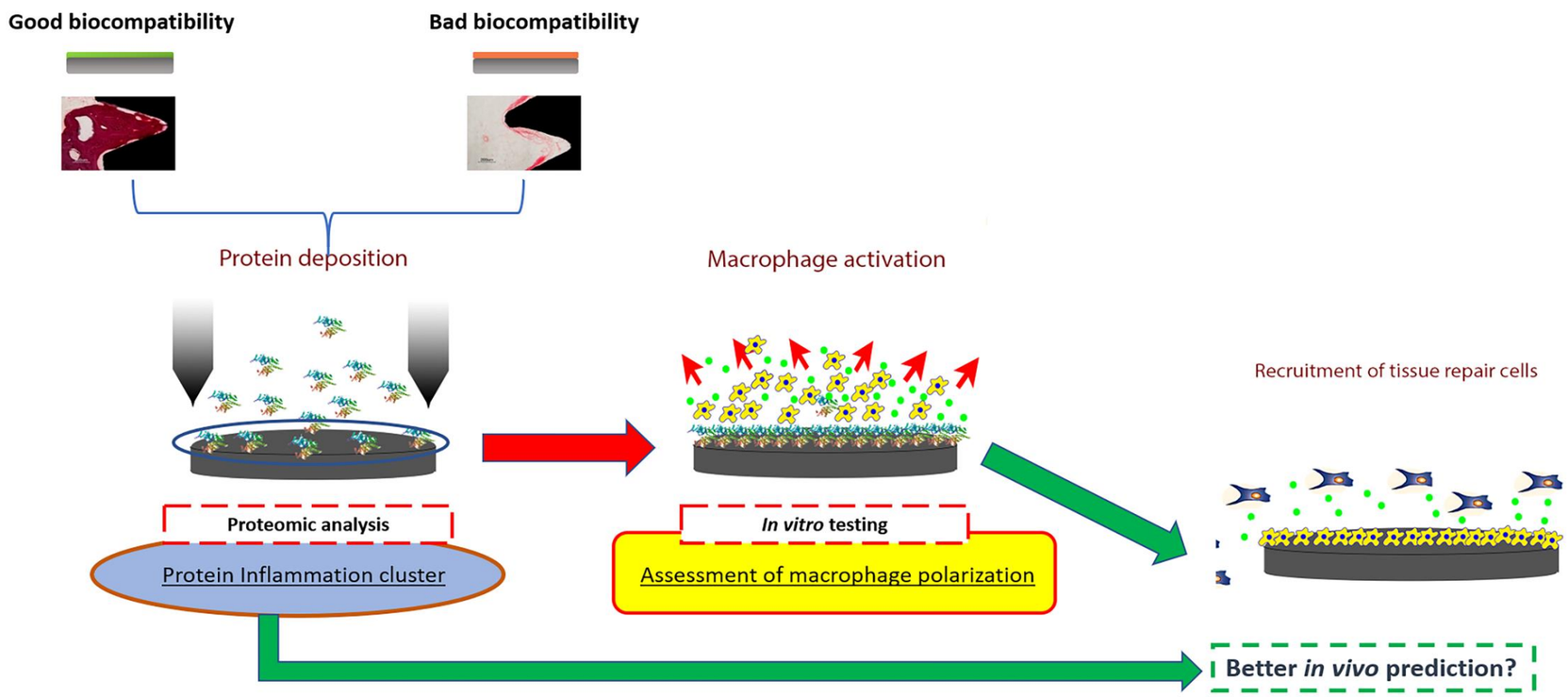
“Equipped with his five senses, man explores the universe around him and calls the adventure science.”

- Edwin Powell Hubble

Graphical Summary



Summary

When testing a new biomaterial, the standard protocol before proceeding to clinical trials is to follow a battery of determined *in vitro* assays in order to select the most successful material to undergo *in vivo* experimentation. However, the lack of correlation between *in vitro* and *in vivo* experimentation is a critical and well-documented problematic to have in account when addressing the material compatibility on the biological context.

In consequence, the search for new methodologies to approach the *in vivo* experimentation with more certainty is needed to avoid extra ethical and economic problems/costs when testing biomaterials, in specific for dental implantation purposes.

One possibility could be the study of characterisation of the layer of proteins formed post-implantation using mass spectrometry analysis (LC-MS/MS), as it comes as a possible and groundbreaking tool to assess and evaluate patterns of clusters of proteins related with biocompatibility problems, establishing a relationship with the future *in vivo* outcome. Subsequently, these clusters of proteins can be held responsible for macrophage activation and migration at the moment of implantation.

Hence, the study of inflammatory markers expressed by each phenotype of macrophages when cultured onto biomaterials gains additional interest to establish an effective tool to approach the inflammatory response to a determined material.

This thesis opens up the possibility of adopting these two methodologies as new potential tools to approach *in vitro* experimentation on the field of biomaterials for dental implantation purposes. Different biomaterials will be synthetized in a systematically way in order to obtain various biological responses. Physicochemical and biological (proteomic, *in vitro* and *in vivo*) characterisation will be done and results from different techniques correlated. Results obtained on each group of biomaterials will be presented in each chapter of this document and will successfully establish some clear correlations between the *in vitro* (cell cultures and proteomics) and *in vivo* response, proving them feasible and conceivable to take up for a near future.

Resumen

A la hora de probar un nuevo biomaterial, el protocolo estándar antes de proceder a los ensayos clínicos es seguir una batería de ensayos *in vitro* determinados con el fin de seleccionar el material más exitoso para someterse a la experimentación *in vivo*. Sin embargo, la falta de correlación entre la experimentación *in vitro* e *in vivo* es una problemática crítica y bien documentada que debe tenerse en cuenta al abordar la compatibilidad del material en el contexto biológico.

En consecuencia, la búsqueda de nuevas metodologías para abordar la experimentación *in vivo* con más certeza es necesaria para evitar problemas y costes extra, tanto éticos y económicos, cuando se prueban biomateriales, en particular para fines de implantación dental.

Una posibilidad podría ser el estudio de la caracterización de la capa de proteínas formada después de la implantación mediante análisis de espectrometría de masas (LC-MS / MS), ya que se presenta como una herramienta innovadora para evaluar patrones de grupos de proteínas relacionadas con problemas de biocompatibilidad, estableciendo una relación con el futuro resultado *in vivo*. Posteriormente, estos grupos de proteínas pueden ser responsables de la activación y migración de macrófagos en el momento de la implantación.

Por lo tanto, el estudio de los marcadores inflamatorios expresados por cada fenotipo de macrófago cuando se cultivan en biomateriales gana un interés adicional para establecer una herramienta efectiva para abordar la respuesta inflamatoria a un material determinado.

Esta tesis abre la posibilidad de adoptar estas dos metodologías como nuevas herramientas potenciales para abordar la experimentación *in vitro* en el campo de los biomateriales para fines de implantación dental. Se sintetizarán diferentes biomateriales de forma sistemática para obtener diversas respuestas biológicas. Se realizará una caracterización fisicoquímica y biológica (proteómica, *in vitro* e *in vivo*) y se correlacionarán los resultados de diferentes técnicas. Los resultados obtenidos en cada grupo de biomateriales se presentarán en cada capítulo de este documento y establecerán con éxito algunas correlaciones claras entre la respuesta *in vitro* (cultivos celulares y proteómica) y la respuesta *in vivo*, demostrando que estas correlaciones son factibles y concebibles para asumir en un futuro próximo.

Resúm

A l´hora de provar un nou biomaterial, el protocol estàndard abans de procedir amb els assajos clínics, és seguir una bateria d'assajos *in vitro* determinats per tal de seleccionar el material més exitós per a sotmetre a l'experimentació *in vivo*. Però, la falta de correlació entre l'experimentació *in vivo* i *in vivo* és una problemàtica crítica i bé documentada que ha de tenir-se en compte en abordar la compatibilitat del material en el context biològic.

En conseqüència, la recerca de noves tecnologies per abordar amb més certesa l'experimentació *in vivo* és necessària per evitar problemes i costos extra, tant ètics com econòmics, quan es proven biomaterials, en particular per a fins d'implantació dental.

Una possibilitat podria ser l'estudi de la caracterització de la capa de proteïnes formada després de la implantació mediant anàlisi d'espectrometria de masses (LC-MS / MS), ja que se presenta com una eina innovadora per avaluar patrons de grups de proteïnes relacionades amb problemes de biocompatibilitat, establint una relació amb el futur resultat *in vivo*.

Per tant, l'estudi dels marcadors inflamatoris expressats per cada fenotip dels macròfags quan es cultiven en biomaterials guanya un interès addicional per establir una eina efectiva per abordar la resposta inflamatòria a un material determinat.

Aquesta tesi obri la possibilitat d'adoptar aquestes dues metodologies com noves eines potencials per abordar l'experimentació *in vitro* en el camp dels biomaterials per a fins d'implantació dental. Es sintetitzaran diferents biomaterials de forma sistemàtica per obtenir diverses respostes biològiques. Es realitzarà una caracterització fisicoquímica i biològica (proteòmica, *in vitro* i *in vivo*) i es correlacionaran els resultats de diferents tècniques. Els resultats obtinguts en cada grup de biomaterials es presentaran en cada capítol d'aquest document i establiran amb èxit algunes correlacions clares entre la resposta *in vitro* (cultius cel·lulars i proteòmica) i la resposta *in vivo*, demostrant que aquestes correlacions són factibles i concebibles per assumir en un futur pròxim.

Content

1. Introduction

1.1. Bone Tissue biology

Bone tissue is a specialized and mineralized connective tissue with a hierarchical organization comprising several degrees of length scales that go from macro-component to nanostructured organized matrices [1] , arranged either in a compact pattern (cortical bone tissue) or in a trabecular pattern (spongy bone) .

Constituted by both mineral and organic phases, the hybrid composition of this tissue confers to it unique properties. At the same time that displays mechanical resistance and consequent flexibility, this specialized tissue is characterised by its stiffness, and it is constituted by various distinct and specialized types of cells.

These properties exert, as a whole set, structural, locomotive, protective and storage functions to the soft tissues of a living organism, representing the permanent framework of the human body [2]. The continuous dynamic of bone metabolism is a feature of this tissue, being in constant remodulation throughout an individual lifespan.

For dental implantology purposes, the bone tissue, as expected, is the fundamental object of study to follow the biological events of bone regeneration in response the implantation of a foreign body onto a living organism.

However, the process known as osteogenesis is not the only one that plays the definite sequence of events culminating in the complete regeneration of bone tissue post-implantation.

1.2. Bone healing following implant placement

The bone regenerative process is a complex system comprised of a series of events involving a great number of cells, signalling molecules and pathways, well-timed and defined on their role, leading to phenoms of inflammation, coagulation, bone induction and conduction, that interplay actions between them, optimizing the impaired tissue to complete restauration of it [3].

These are activated in response to a severe tissue trauma or defect such as it is a bone defect derived from an implantation of a foreign body onto the organism [4]. The bleeding resulting of that trauma will give the "kick-start" to a cascade of events and processes that will initiate the healing process of the affected tissue.

Depending on the type of trauma, the bone healing development encompasses three overlapping main stages that follow specific patterns, actively changing cell behaviour, subsequent to constant changes of expression of genes that lead the whole process (**Figure 1**).

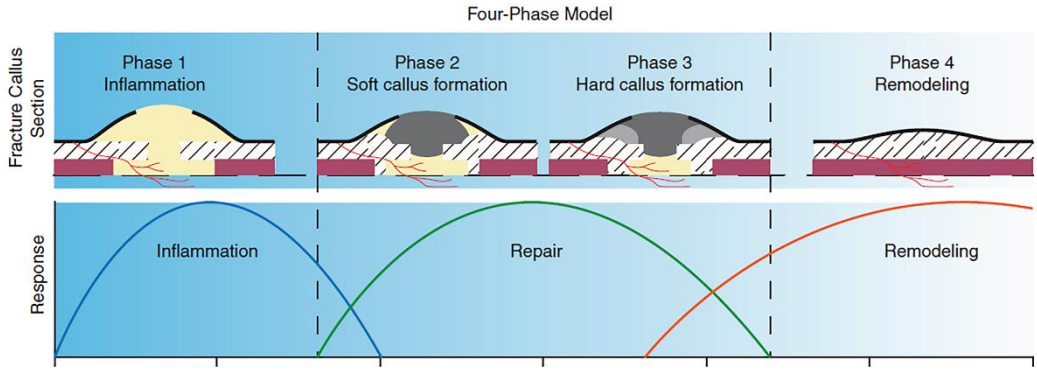


Figure 1. The three overlapping phases of bone healing: inflammatory phase, bone formation phase and bone remodelling phase. Image collected from [5].

In the specific case of dental implantation procedures, soft and hard tissue healing following implant placement leads to marginal soft tissue attachment and osseointegration [6], establishing the initial linkage to this phases. The minimal adaptation of the soft tissue to the implant surface has the function of settling a physical seal between the bone-surrounded implant surface and the oral microenvironment [7].

1.2.1. Early and acute inflammatory response

When a trauma following implantation is produced leading to, a haematoma is produced in response to the disruption of the tissue. This structure is produced in result of peripheral, intramedullary and bone marrow cell migration to the impaired tissue [8].

This haematoma eventually coagulates all around the damaged site, assembling a structural template for a posterior intermediate cartilaginous callus formation. This coagulation mainly involves the conversion of fibrinogen to fibrin in the defect gap, and its necessary to unleash cell, factor and mediator migration, removal of debris, processes ultimately required to ulterior angiogenesis of the bone tissue [9].

The activated platelets resulting from the trauma release growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) into the microenvironment surrounding the affected site, thus triggering the influx of inflammatory cells.

In parallel, during this phase, the secretion of pro-inflammatory cytokines such as TNF-α, IL1β, IL-6, IL-11 and IL-18 is activated, a commitment between immune and coagulation systems, promoting macrophage, lymphocyte and polymorphonuclear leukocyte chemotaxis to the affected tissue [10]. The secretion of these cytokines, besides the recruitment of immune response cells, is regarded too as having a secondary role on the following osteogenesis, especially on MSCs [11].

The following steps involve fibroblast migration, collagen and growth factor production and release, steadily turning the haematoma into a soft callus, overlapping the inflammatory phase, and initiating the bone formation phase [12] (**Figure 2**).

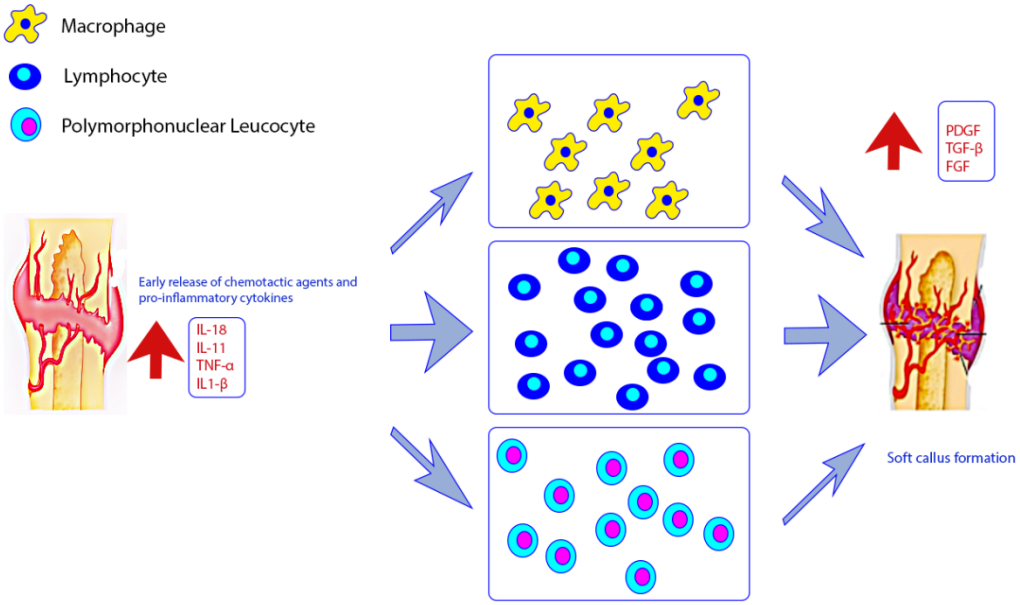


Figure 2. Development of the inflammatory processes and cytokine liberation in response to a trauma/fracture.

1.2.2. Bone formation phase

The combined release of these growth factors, namely bone morphogenetic proteins (BMPs) and the mentioned inflammatory mediators, will ultimately unleash the recruitment of MSCs to the fracture site, providing guidance to proliferative and differentiative processes, initiating the following bone formation phase [10]

The newly-formed soft callus will set up the events leading to new bone tissue formation and will be driven mainly by fibroblasts. This phase, also known by proliferative phase, is characterized by the early angiogenesis of fibrous tissue between the fragmented bone ends, in a stable and fixed position of them.

In this phase, the soft callus is progressively converted to hard callus, also known as woven bone.

The differentiation of osteoblasts and chondrocytes , consequent mineralization and bone matrix production, progressing to the fracture line, will allow formation of the new trabeculae surrounding the fractured area, in a thicker density than the previous impaired bone [13]. In this phase is observable a predominance of angiogenic factors and metalloproteinases, such as VEGF and angiopoietins 1 and 2, and a high expression of type I collagen, that will endow this new formed bone of the vascularization needed to the final bone remodelling phase.

1.2.3. Bone remodelling phase

Bone remodelling is part of the dynamic mechanism required for the maintenance of the bone structural architecture in response to mechanical needs of the organism, but also is part of a whole system that acts promptly in the repair of the impaired tissue following injury.

This process relies mainly on two types of cells of the bone tissue: the osteoblasts, that display osteogenic functions; and the osteoclasts, large and multinucleated cells responsible for bone matrix degradation.

The balance between the activities of these two types of cells define a good bone health and maintenance [14].

The impairment of this balance can have critical consequences on an individual's bone tissue health, being the osteoporosis one of the most widely known pathologies caused by this imbalance, due to excessive bone resorption by osteoclasts [15,16].

The callus is a physiological reaction to inter-fragmentary movement, requiring the presence of adequate blood flow and healthy cell viability [17]. In that sense, bone remodelling is part of the dynamic mechanism required for the maintenance of the bone structural architecture in response to mechanical needs of the organism, but also is part of a whole system that acts promptly in the repair of the impaired tissue following implantation.

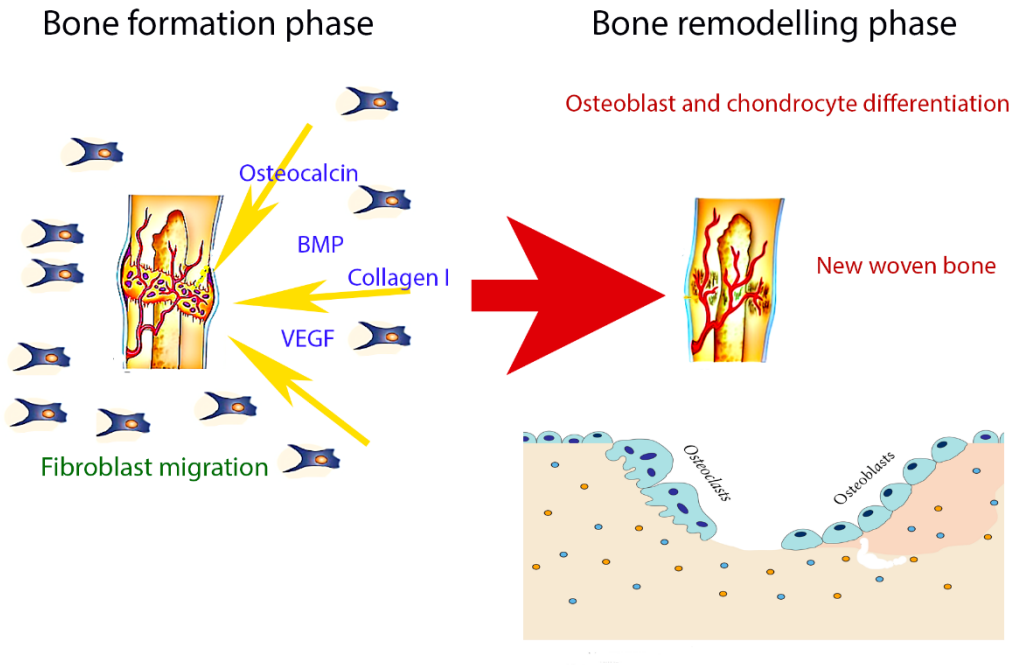


Figure 3. Development and evolution of the latter phases of bone healing – Bone formation and remodelling.

1.3. Bone healing post implantation

The necessity for the human being to live a longer and healthier life has exerted the lookout for materials with the capability of restoring lost or damaged body parts. In consequence, in the last years, the field of dental implantology research and industry has arised as one of the most technologically advanced when concerning biomedical purposes.

Tooth loss, or edentulism, is an event that might happen as a final consequence for the existence of disease or trauma [18,19]. In response to that problematic, dental implants are envisioned in order to replace the natural root of the missing tooth and posterior installation of a dental prothesis, and can even be traced back to the early civilizations, where the missing teeth were replaced by shells, bones and gold [20]. Due to the amount of time in history dental implants had been used, multiple factors have been recognized as fundamental to obtain a successful osseointegration outcome, such as implant design (including surface design), biomechanical factors, the host health and respective bone quality, and the type of biomaterial employed [21].

As defined by Williams DF [22], " A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine" , with the goal of restoring or enhance impaired tissues following trauma or disease.

In effect, the development of materials for biomedical purposes, in this case for dental implantation, has changed its focus on the last 30 years from being a simple replacement biologically inert device, to be supplemented with biologically active materials [20,23].

Among the biomaterials used, it is accountable a large selection of metals, polymers, carbons, and combinations of them to be employed for a dental implant development [21,24,25].

In that sense, Titanium (Ti) and its alloys have become the reference metal to use for dental application and biomedical purposes, as it boosts great osseointegrative properties. Its corrosive resistance to bodily fluids and its osteoconductive potential for cell proliferation and differentiation makes Ti an excellent substrate for tissue growth [26]. Although is bioinert, Ti surfaces can be modified in order to enhance cell activation and following tissue regeneration. These surface modifications can include mechanical and chemical treatments that directly affect

biocompatibility and include surface texture (roughness), ion binding sites, and even hydrophobicity alterations [27].

Moreover, many distinct types of coatings have been tested to provide the implant surface with antibacterial properties to enhance for example cell anchorage, growth and colonization all around the device surface with bactericidal compounds as gentamicin, tetracyclin and octenidin [28–30]. Other strategies include coating the implant surface with growth factors such as TGF-β and BMP, described as enhancers of bone healing, and allow their controlled dissemination on the surrounding tissue [31–34].

They act by degrading compounds gradually onto the microenvironment around the implant providing a doped environment capable of exerting some influence on the surrounding cells and biological tissues in contact with it.

In this aspect, achieving an optimal and correct bone healing after a dental implantation procedure in the lesser time possible is the prime objective when developing biomaterials for bone implantation [25]. So, it is of major importance understanding phenoms such as osteoinduction and osteoconduction, that lead to a complete osseointegration of a material on the living organism microenvironment, with a successful stability and longevity [35].

In that sense, the third generation of biomaterials, specifically for dental application, represent a hallmark on the development of biocompatible materials. This generation combines the bioactive and the resorbable characteristics of the second-generation in a synergistic formulation, allowing organism self-healing, post-implantation [36].

This group of materials seek the modulation of the cell behaviour to generate the best possible outcome of it on a living organism microenvironment, even at a molecular level, both relying on the controlled liberation of compounds, genes and growth factors capable of improving the osteogenesis process and even physico-chemical surface modifications [37,38].

1.4. Osseointegration

The extent of the integration of the biomaterial in a living organism setting is dependent on several factors, many of which involved onto the microenvironment formed shortly after the moment of implantation.

Activation of coagulation cascades, antibody production, platelet activation, adhesion and aggregation are all part of the major characteristics involved on the generic host response to biomaterial implantation onto the organism setting.

These characteristics can be led by the first layer of proteins adsorbed onto the material surface, considered major key players on the activation of all of these processes. It is generally believed that the composition, conformation and type of these proteins interplay a key role on the following host processes in response to the contact with the foreign body [39,40].

These will deposit onto its surface by competitive displacement, called “Vroman effect”, initiating the whole process of regeneration [41]. This “Vroman effect” describes the competitive nature of proteins adsorbed onto a determined surface depending on the molecular weight of the protein [42]. These proteins, at the moment of implantation, are mostly derived by blood plasma, one of the first fluids in contact with the implant surface [43].

These will unleash events like initial cell adhesion and consequent intrinsic cell processes, like cellular proliferation and differentiation [44].

The type, conformation and quantity of these proteins is dependent not only on the organism in which the material is implanted, but also of the characteristics of the material, regarding physico and chemical properties such as hydrophilic potential, surface chemistry, and micro- and/or nano-roughness, as mentioned.

The adsorption post-implantation of blood proteins will immediately cause the formation of a blood clot, composed mainly by growth factors and cytokines that will evoke cell migration to the implanted site [45].

This migration occur due to the interaction of cell adhesion receptors with the adsorbed proteins, being the first step on the activation of the mentioned immune response processes [46]. In specific, proteins adsorbed on the surface of biomaterials (such as fibrinogen, fibronectin, vitronectin, complement component C3b, among others) are crucial for the implant recognition and initiation of a foreign body reaction to an implant surface [45].

Firstly, the non-specific protein adsorption onto the implant, together with the immune and inflammatory response, will occur in order to protect the organism from the foreign body, in a determined magnitude [47,48].

The immune response process shares some traits and interactions with the bone formation processes (*e.g.* osteogenesis), by consequence forming the field of osteoimmunology [49]. In fact, bone cells are described to have a role on the maintenance, homeostasis and mobilization of the HSCs (Hematopoietic Stem Cells), being considered part of the immune processes system by some authors [50]. Such implant-adhered proteins are then recognized by macrophage integrins, like macrophage-1 antigen (CD11b/CD18) and arginine-glycine-aspartic (RGD sequence) acid-binding integrins avb3, avb5, and a5b1 [51]. In fact, it is described that when the coating containing the RGD peptide is applied to a titanium surface significantly enhance bone formation and osteoblastic activity [52,53], and at the same time is capable of modulating the immune response by macrophages [48]

Shortly, the following “sub-processes” intervein in one way or the other on the bone tissue healing process development, which are coordinated on great part by specific proteins or protein clusters.

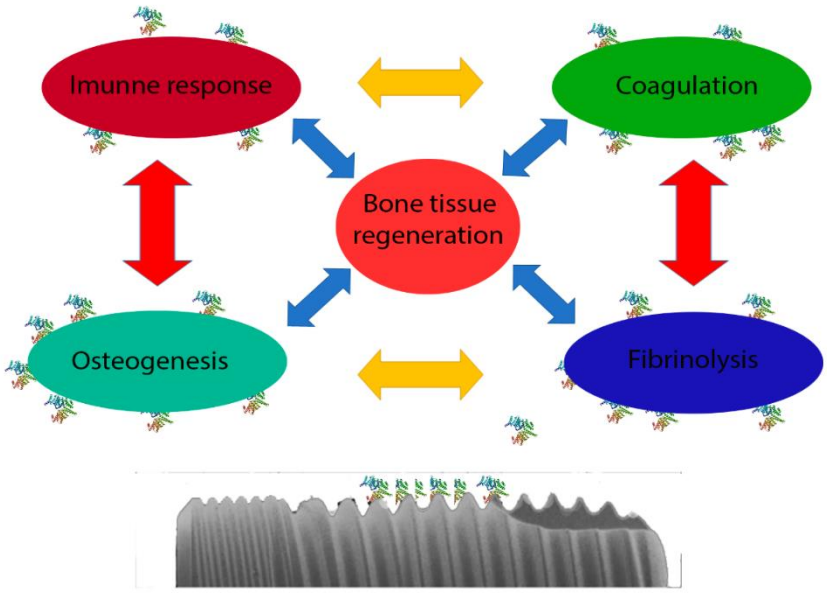


Figure 4. Processes involved on bone tissue regeneration and interaction between them.

1.4.1. Immune response

This phase is mainly driven by complement proteins that unleash mechanisms of innate and acquired immunity. The complement cluster is comprised by a series of proteins synthesised on the liver and on cell surfaces in an inactive form, also called zymogens [54]. It follows a coordinated sequence of events and interactions between these with the final goal of recognizing and opsonizing the pathogen, leading to its lysis, triggering the inflammatory response. Depending on the type and quantity of the proteins involved, as well as the type of pathogen surface recognition, the complement system can act by distinct pathways resulting in the generation of C3 convertases, the core and common component of all the complement pathway systems [55]. Each pathway acts dependent on the recognition of the distinct signalling molecules.

The classical pathway is usually initiated from the interaction of C1q with pentraxins, such as C-reactive protein, or antigen/antibody binding with IgG or IgM, leading to C1r activation and C2s cleavage. Following these events, serine proteases are activated, leading to the cleavage of C4 and C2, ultimately originating the C3 convertase C4b2a, which breaks C3 into C3a and C3b [56]. C3a is

described to have the function of recruitment of inflammatory cells to the site, while C3b interacts with C5, assembling with C6, C7, C8 and C9 the Membrane attack complex (MAC), a structure that forms transmembrane channels disrupts onto the pathogen membrane, facilitating its lysis [57]. This pathway is the most commonly activated in response to the recognition of immune complexes. The alternative pathway is mostly driven by the low continuous formation of a soluble C3 convertase, which results in the formation of a conformationally distinct C3, named C3 (H_2O), capable of binding factor B. Following this binding, the change of conformation factor B allows its cleavage by the serum protease factor D, resulting in the generation of Ba and Bb. This Bb fragment is in its turn, capable of cleaving C3 molecules, forming C3b, generating more C3 convertases [58]. This pathway is the most commonly activated in response to the recognition to pathogen surfaces. Finally, the lectin pathway relies mostly by the binding of mannose-binding lectins (MASPs) and ficolins to the C4 and later to C2, having later a similar pathway to the classical complement pathway, except on their initial activation [59]. This pathway is mostly common from the recognition of *e.g.* microbial carbohydrates.

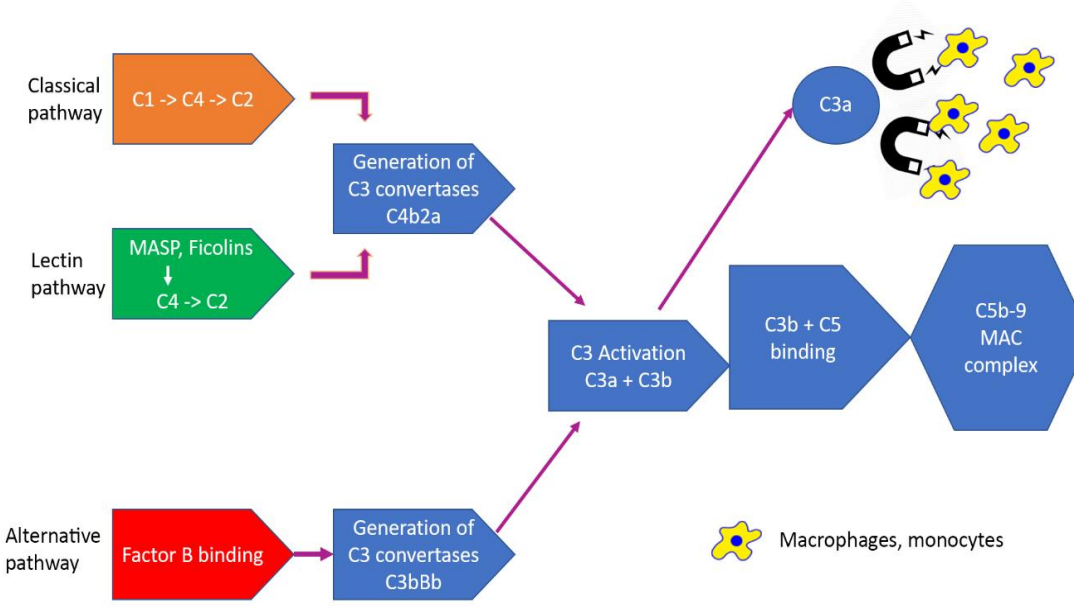


Figure 5. Pathways of complement activation. Image inspired by [54]

1.4.2. Osteogenesis

On this process intervene a great amount of proteins that will have a role on the osteoblastic cell response and consequent differentiation, topping the bone formation process in the literal sense of the event.

In response to chemical signalling and physical stress, specific signalling pathways are activated, such as the mitogen-activated kinase pathway (MAPK) that will allow the linkage between mechanical interactions with the ECM and activation of Runx2. This central control gene is considered to be defining of the osteoblast phenotype [60]. The induction of a determined osteoblastic phenotype requires interactions between osteoblastic precursors and the non-collagenous extracellular matrix secreted by this kind of cells [61]. This ECM of osteoblasts is comprised by collagenous and non-collagenous proteins, bone morphogenic proteins (BMPs) and growth factors that, depending on their expression profile, and controlled release will establish the rate of osteoblastic differentiation and consequent bone formation. Proteins like collagen I, BMP-2, BMP-7, and matrix metalloproteases (MMPs) are described to be key players on the development of this process [60,62].

1.4.3. Coagulation

In short, the main purpose of the coagulatory processes is to form a fibrin mesh made from a great amount of platelets, leading ultimately to the formation of a haemostatic plug, preventing the continued bleeding [63,64].

This complex process was initially thought to start by the exposure of the damaged/impaired tissue to a group of proteins known as Tissue Factors (TF).

It can be activated by mainly two pathways: an intrinsic and extrinsic pathway. The extrinsic pathway generally occurs in response to a trauma, where TF is produced during a trauma, and it works by activating factor VII (FVII) into FVIIa, leading to the production of extrinsic tenase complexes (TF-FVIIa) [63] . The TF can be produced by macrophages, endothelial cells, monocytes, among others following damage or impairment of the tissue, or in response to inflammatory stimuli as TNF-α and IL1α release [65,66].

The intrinsic pathway usually is activated by mixed surface contacts of proteins such as prekallikrein, kininogen and Factor XII (FXII), forming a sequential cascade of complexes zymogen-enzyme, on some steps depending on Ca^{2+} ion participation. This ultimately leads to the conversion of prothrombin to thrombin, the end product of the whole cascade, responsible for the catalysis of fibrinogen onto fibrin. On this step of bone healing, multiple growth factors of platelets are involved, such as TGF-β and IGF-1 [67].

1.4.4. Fibrinolysis

Intrinsically involved with the coagulation system, the fibrinolytic system is complex and a key part of the haemostasis processes, as it degrades the fibrin, product of the coagulation process and the substrate for the development of this process [68]. Two main events take place during the development and occurrence of this stage: the generation of plasmin, enzyme with blood plasma proteolytic activity; and subsequent degradation of fibrin by this enzyme [69]. Plasmin is generated from the zymogen plasminogen on the cell or fibrin clot surface and it is controlled by plasminogen activators, namely the tissue plasminogen activator (tPA) and the the urokinase plasminogen activator (uPA) and regulated by a series of other mechanisms and inhibitors.

Among these, the tissue plasminogen activator (tPA) is the most widely known and studied and it is regarded as being one main thrombolytic agent for acute isquemic stroke [70]. Its assembly on the fibrin surface, dependent of the lysine-binding sites, enables the formation of a complex tPA-plasminogen, in which the plasminogen is cleaved, generating plasmin. In parallel, the urokinase plasminogen activator is also described to cleave plasminogen, although with less affinity [68]. The consequent generation of fibrin by the plasmin is thus giving place to fibrin degradation products, which are described to have both immunomodulatory [71] and thromboregulatory effects [72].

1.5. Current approaches on the development of biomaterials

To this day, the present approach on biological biomaterial evaluation is banked almost entirely on the cell reaction to the exposure to a determined biomaterial with determined and protocolled *in vitro* assays, part of a filtering process that enables the material pre-selection for *in vivo* evaluation of materials and posterior clinical trial.

The exposure of the cell to the material can occur by direct or indirect contact, and the parameters evaluated contemplate not only the material cytotoxicity, but also if the proliferation and differentiation potential are affected positively or negatively by that exposure [73]. Moreover, these *in vitro* studies tend to focus on the bone tissue-forming potential of the material, mainly working with cell lines correlated with osteogenesis [74,75]. This represents a costly limitation to the studies, as they don't contemplate nor simulate completely what happens in an *in vivo* context.

This fact is well documented in Hulsart *et al*. [76], that presents the lack of correlative data between *in vivo* and *in vitro* evaluations of materials, presenting exceptionally low percentages of correlation. This lack of correlation is, of course, due to the no-contemplation of other processes associated with the whole regenerative process of bone, such as immune response to foreign body and coagulation cascades that affect equally the final outcome of the bone healing post-implantation.

This leads ultimately to costly and time-consuming consequences at the hour of evaluate whether if the material will osseointegrate perfectly onto the biological context.

Hence, and in consequence, the bone material development paradigm is shifting towards the field of osteoimmunology, envisaging the immune system process and its interactions with the other biological processes, in an effort to enhance and strengthen the possibilities of a successful outcome of a biomaterial. In fact, a recent review on dental and orthopaedic addresses this problematic, finding that almost 90% of research in this area exclusively focus on the *in vitro* behaviour of osteoblasts on surfaces, with the remaining 10% contemplating the immune cell demeanour (macrophages, monocytes, leukocytes and multinucleated giant cells) [77].

By this reason, the current standard assays only using one cell line (osteoblasts), that do not contemplate the inflammatory processes are too limited to assess the biomaterial behaviour *in vivo*, and by that fact, can lead to serious complications when testing them in the biological context. Thus, this fact arises the need to work with alternative cellular lines, like macrophages or monocytes, to appraise the potential inflammatory response to a biomaterial.

1.6. Macrophage polarization – critical role on osseointegration

The homeostasis of the immune cell state is critical for the tissue development, regeneration and repair [78]. As mentioned, the implant subsequent development of the microenvironment cues will be dependant on the biomaterial implanted, and on of the organism "foreign body reaction" (FBR) to the biomaterial. This FBR results in the formation of a granuloma fibrous tissue, which in turn can follow two paths: the desired wound/damaged site healing or the undesired fibrous tissue formation.

In that sense, macrophages are major key players on these processes, differentiating from monocytes and mediating the inflammatory events that lead wound healing processes post-implantation [79,80].

Being the most plastic cells of the hematopoietic system, macrophages are scattered through all human body tissues with variate roles, namely of tissue repairing and immunity purposes. They get activated in response to the microenvironmental cues provoked by the released cytokines. Following their activation, macrophages are both responsible for the recruitment of other immune cells to the impaired tissue by secreting a wide array of inflammatory mediators and natural pathogen removal by phagocytosis [81].

Early studies in the 80`s, suggested a role of macrophages in the bone regenerative processes as their presence was always found near the bone surface and adjacent to mature osteoblasts [82,83]. This fact opened the hypothesis of the possible and active role of macrophages on the bone formation development and biology.

The bone renewal and regenerative processes in response to an injury require the eviction of dead/damaged/old cells belonging to the impaired tissue [84]. Throughout this process, and naturally, macrophages will intervene and initiate the mentioned inflammatory processes. Although their role on these processes are widely known and studied, evidences regarding the role of these cells and their interaction with osteoblasts are still unknown.

Specifically, resident (osteal) macrophages are described to have a central role on bone metabolism. Bone tissue enclose a determined population of this type of cells and they are thought to provide pro-anabolic support for osteoblasts, the prime bone tissue formation cells [83]. Moreover, *in vitro* studies strongly suggest an critical role of these cells on the sketetal health [85].

Raggatt *et al.* have proven that the depletion of these macrophages on the post-inflammatory anabolic phase of the bone tissue repair leads to inhibition of the callus, consequent endochondral tissue formation and less bone deposition. In parallel, the same study shows that the injection of pro-macrophage stimulation factor-1 a few days after fracture enhances the formation of soft callus [86]. In another study, it has been shown that the marrow containing macrophages from juvenile mice (4-weeks old), when transplanted onto middle-aged mice, facilitates bone healing and enhances callus formation [87].

Hence, in the biomaterial implantation context, the adherent macrophages to the biomaterial become activated not only to attempt to phagocytose the material [45], but also to unleash the bone regenerative processes associated with osteoblasts.

1.6.1. Macrophage functional states and immune responses

Depending on their functional states, macrophages can display two main ways of activation leading to two distinct phenotypes.

These macrophage activation phenotypes are identifiable nowadays by reference markers, both membrane proteins and distinct cytokine expression profiles [88].

The classically activated macrophages, also known as M1 subtype, due to the dependence of Th1 lymphocytes, secrete pro-inflammatory cytokines like TNF-α, IL1-β, IL-6, IL-8, in response to TLR and CLR activation by invading organisms/foreign bodies. They typically express chemokines CXCR3 and CCR5 which promote the recruitment of leukocytes involved in tissue repair and remodelling [89,90].

The alternatively activated phenotype, also known as M2, is dependent of the Th2-type lymphocytes, and they possess the ability to suppress inflammation processes [91]. M2 cells can clear apoptotic cells and promote wound healing, besides promoting angiogenesis. M2 express different cytokines than M1, having a pattern of cytokine production of typically IL-10^{high} and IL-12^{low} [92,93]. They preferentially occur in diseased tissues where they can induce the proliferation of endothelial cells [94]. This phenotype, which displays high phenotypic heterogeneity, has at least three main subdivisions: M2a, M2b and M2c, each one capable of being activated by a different set of immune complexes [95], with distinct specific functions of immunoregulation, tissue remodelling and angiogenesis [96]. However, the differences between these sub phenotypes is not well clear, and the diversity of results makes it difficult to reach a consensus regarding this entity [95,97,98].

In the broad sense, the M1 phenotype shift naturally towards the M2 phenotype during the tissue remodelling process [99]. , M1 macrophages are predominantly found at early timepoints (1-5 days) after implantation, while M2, apart from having an initial presence, are mostly common at latter stages of the bone healing processes [100,101]. Pointedly, the early phase of the tissue repair post-implantation is dominated by the presence of the inflammatory M1 phenotype. A progressive switch to M2 anti-inflammatory shift with a consequent osteogenic cytokine release then occurs, promoting the initiation of the bone formation and bone remodelling phases. (**Figure 6**)

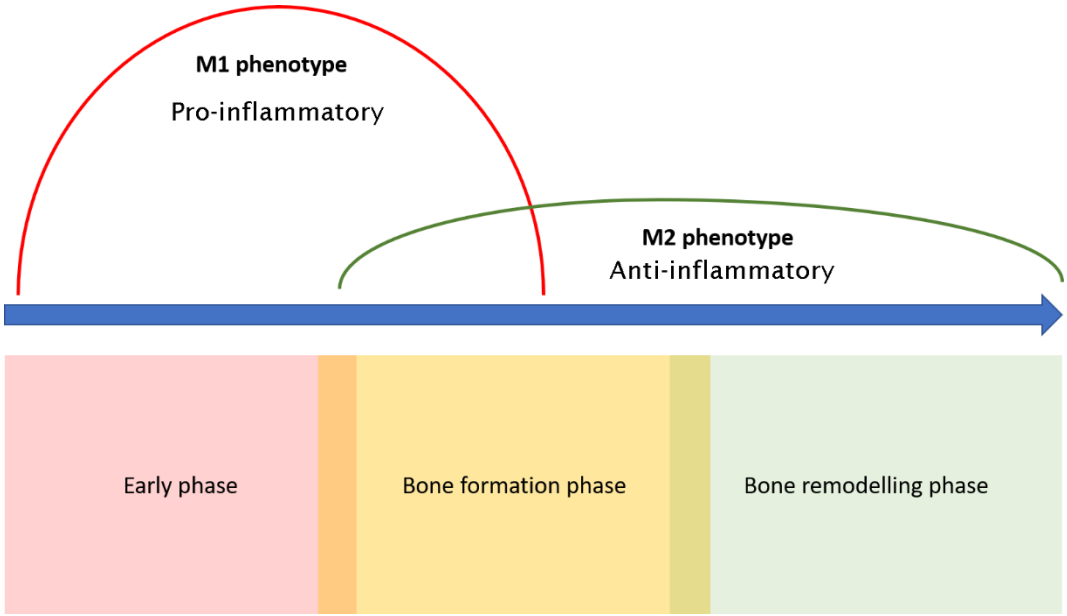


Figure 6. Normal macrophage phenotype changes during the formation of new bone. Based on [102].

The continued prevalence and unbalance of the M1 phenotype during the inflammatory stage can lead to fibrosis and consequent rejection of the implant, as macrophages are highly regarded as regulators of the fibrotic healing development of the wound [103] (**Figure 7**)

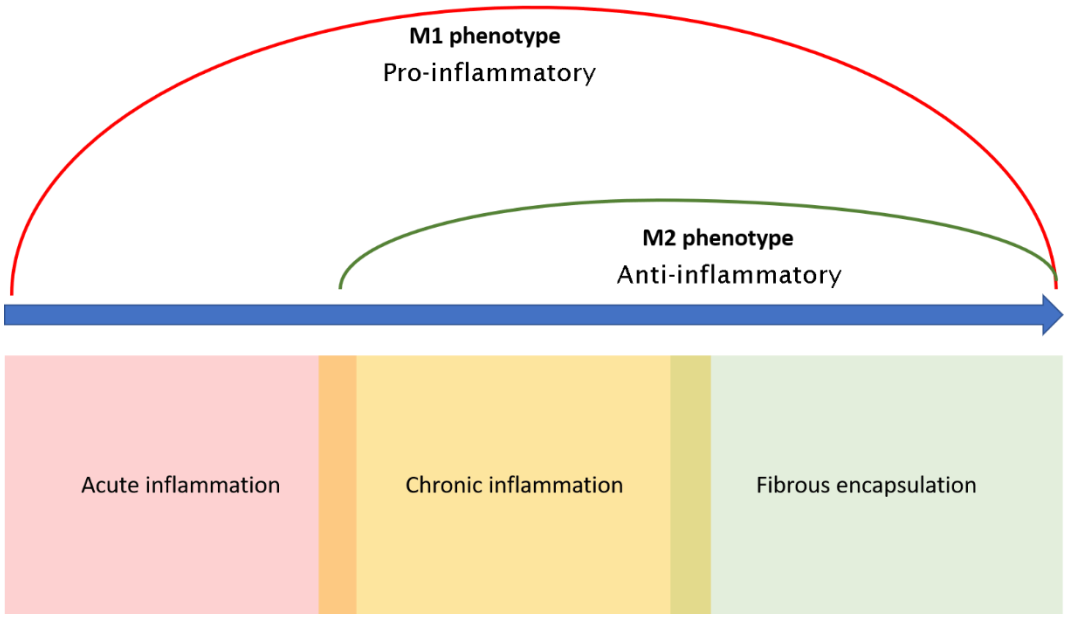


Figure 7. Macrophage phenotype change during the formation of fibrous capsule. Based on [102]

This can lead to immediate implantation-related pathologies such as chronic inflammation, acute and chronic pain, ultimately leading to implant rejection.

1.6.2. Macrophages and osteogenesis

Regarding bone healing processes, there is still a great way to go in order to establish the precise role of each phenotype on the development and renewal of the bone tissue. Schlundt *et al.* [104] have proven the influence and prevalence of each phenotype of macrophages on the evolution of a bone fracture. The predominance of M2 phenotype macrophages onto the fracture surroundings from 3 to 7 days post-trauma has led the authors to investigate the possible role of this phenotype as a therapeutic target for bone, as the induction of this phenotype through IL-4 and IL-13 has shown increased bone tissue angiogenesis by stimulating the osteogenic differentiation of MSCs [105].

Moreover, it has been shown that the depletion of this cells onto mice decreases the quantity of mineralized tissue, in particular during the stage of the endochondral ossification, when compared

to wild-type mice, reducing the healing time, which supposes a significant role for macrophages at the time in which these processes occur [106].

Some other studies refer that the prolonged predominance of M1 phenotype cells post-trauma impairs the recovery time and successfulness [107,108].

Taking this into account, to control the M1/M2 ratio following implantation of biomaterials might be an essential and relevant aspect not only to determine whether if the procedure is successful regarding immune responses, but also in regard to define their contribution to bone homeostasis [109,110].

1.6.3. Protein deposition and macrophage modulation

The protein deposition phenomenon will be the defining of this ratio, as it will unleash the mechanisms of activation of macrophages to the implanted site, and, depending on the type, amount and conformation of them, will promote phenotypical changes onto one or other phenotype, as time progresses (**Figure 8**).

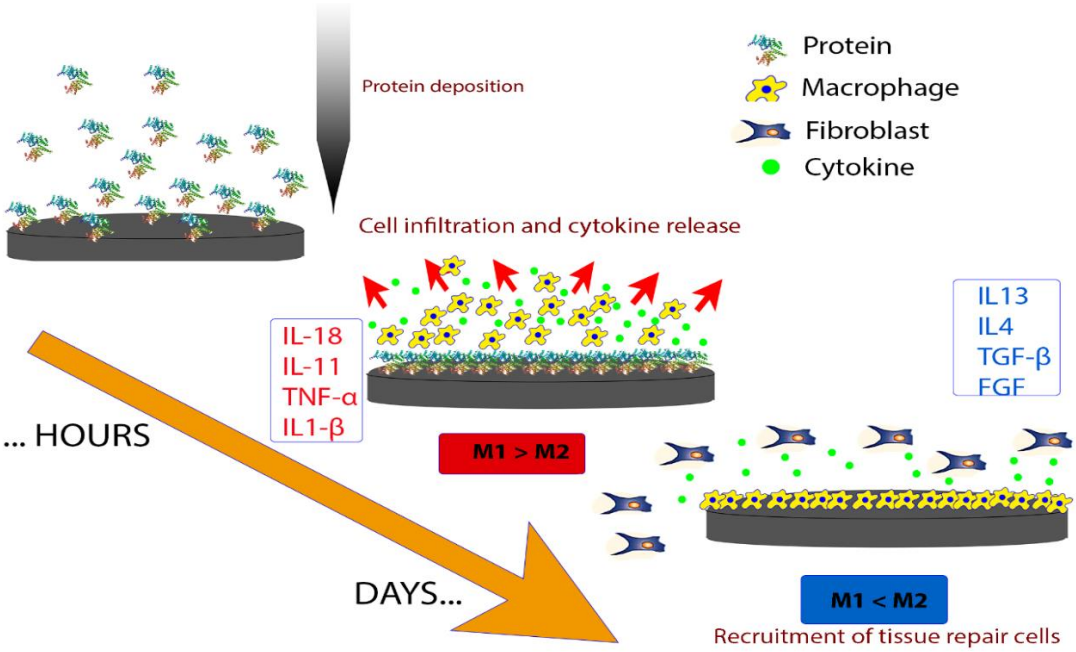


Figure 8. Evolution of protein deposition and macrophage phenotype modulation onto an implant surface.

The complexity of the bone healing in this context can depend on the gene expression profiles belonging to each phenotype and consequent interactions involved on these processes.

Some studies are starting to be made on this topic, in an attempt to modulate the immune response controlling the macrophage polarization as a strategy to improve the tissue repair outcomes in response to biomaterials.

Li *et al.* [111] evaluated changes on macrophage phenotype when cells were cultured onto titanium discs supplemented with different magnesium concentration, through the membrane markers for each phenotype (CCR7 for M1 and CD206 for M2). They have found out that magnesium induced the phenotypical change into M2. Plus, the cells were capable of liberating anti-inflammatory cytokines IL4 and IL10 and had upregulated expression profiles of BMP-2 and VEGF, confirming the anti-inflammatory and wound healing potential of the tested materials. In another study, Fernandes *et al*. [112] have tested the effects of surface properties of bone implants

coated with hydroxyapatite (HA) and *β*-tricalcium phosphate (*β*-TCP) on macrophage cultures, finding out that the coatings induced increased secretion of pro- and anti-inflammatory cytokines on these coatings when compared to control Ti. Moreover, HA was observed to induce earlier phenotypic shifts to M1 rather than the *β*-TCP.

1.6.4. Complement proteins and macrophage polarization

As described above, the activation of the complement system starts with a protein recognition step, mainly involving C1q, mannan binding lectin (MBL), and ficolins, known as innate pattern recognition receptors (PRRs), capable of recognizing the pathogen, initiating the pathway leading to its lysis.

In the dental implantology context, the deposition of these signaling proteins following the procedure and consequent contact with blood, are dependent on the amount, type and conformation onto the implant surface and may signal whether if a material can be more “harmful” to the organism and activate the implant cascades in a specific magnitude. This magnitude can ultimately lead to the predominant activation of each one of the phenotypes. Therefore, the cellular response might be dependable on the type of proteins adsorbed to a material surface.

Dependent on the recognition protein that become attached to a biomaterial, the complement pathway is triggered as described above, having as a final product the release of C3a and C3b by C3 convertases.

Major players on the complement-derived response, macrophages express various complement receptors as CR1 (CD35), CR3 (CD11b/CD18), CR4 (CD11c/CD18), besides binding complement opsonins C1q, C3b and C5b, which can be adsorbed onto surfaces or cell membranes [113] (**Figure 9**). In this regard, it has been shown and proven that C1q binds to macrophages directly modulating the cell response [114].

The complement components C3, C5 and the C5b-9 are described as capable of modulating the macrophage functional M1 phenotype, by the binding of C3a and C5a anaphylatoxins, resultant of C3 and C5 convertases, to undifferentiated macrophages. At the same time, the opsonization of the implant surface by C1q and C3b shift the cytokine production towards the differentiation onto an M2 phenotype [115]. On the specific case of C1q, this shift is said to occur towards apoptotic cells and during clearance of modified lipoproteins in atherosclerosis, as a measure to prevent autoimmunity and preserve tissue homeostasis [114,116,117]. Moreover, it weakens TLR signaling suggesting reverse inflammatory polarization, in which MBL are still described to be involved due to their similarity [118]. However, there is still a long way to go to unravel the specific role of these proteins on the macrophage activation context.

It remains to be seen the specific influence of these family of proteins on the biocompatibility outcome of a material.

Hence, the strict relationship between complement adsorption on material surfaces and consequent immune response mechanisms associated with predominance of a certain type of macrophage phenotype is interesting to take up as a possible and approachable subject concerning biocompatible material evaluation. It might be interesting to predict *in vivo* outcomes adopting the study of the protein content adsorbed onto a biomaterial, relating it with cellular responses.

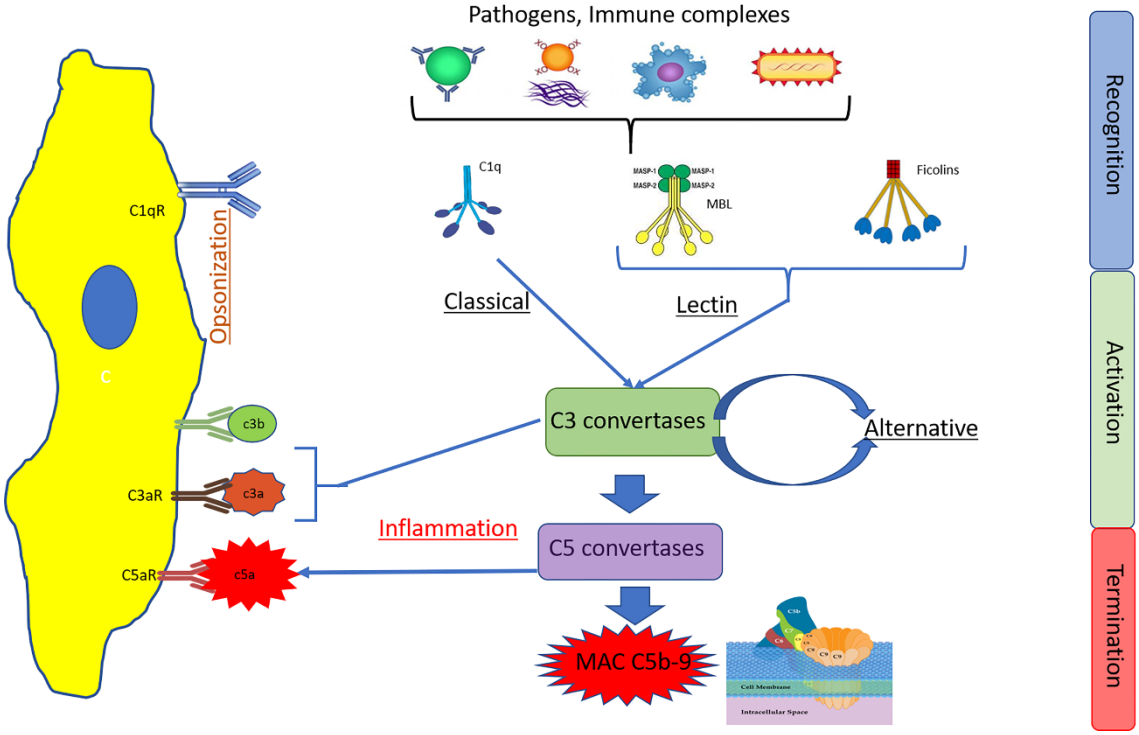


Figure 9. The interaction of macrophages with complement. Image based on [114].

In that sense, liquid-chromatography mass-spectrometry (LC-MS/MS) comes as a promising method to undertake this characterisation, due to its sensitivity, processing ability and high-throughput analysis capability [119,120].

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2. Objectives

2. Objectives

In order to evaluate the outcome of biomaterials for clinical applications, such as for dental implementation, the *in vivo* testing is still a demanded step for the following up clinical trial testing.

As of this day, there is no effective approach to evaluate a biomaterial, independent of systemic immune-suppressors or inhibitors, that can guarantee the implant outcome success. Assessing, characterizing and understanding the sequence of events taking place after implantation is decisive to develop materials capable of modulating the immune response by one side, and the osteoinductive and osteoconductive potential of the material by other. One of the solutions could be by characterizing the layer of proteins adsorbed onto the surface of the implanted material, relating it with the predominance of a determined macrophage phenotype presence on it.

In this context, the objective of this thesis is mainly focused on the discussion and development of a new possible methodology to assess *in vitro* experimentation for dental implantation purposes, regarding both the characterisation of the adsorbed layer of proteins formed post-implantation using liquid chromatography–mass spectrometry (LC-MS/MS) and the assessment of macrophage phenotype when in contact with a biomaterial. The correlation between these and the *in vivo* outcome are assessed.

Pointedly, this research work is centred on the assessment of the following questions:

1) Is there a relationship between adsorbed proteins and biological response?

2) Which proteins are associated with biocompatibility problems?

3) Can the macrophage polarization be correlated with these protein adsorption patterns and possible emergence of biocompatibility problems?

4) Can it be possible to predict the *in vivo* response with the study of macrophage polarization?

2. Objetivos

Con el objetivo de evaluar el resultado de los biomateriales para aplicaciones clínicas, como la implementación dental, la experimentación *in vivo* sigue siendo un paso necesario para proceder a las siguientes pruebas de ensayos clínicos.

Hoy en día, no existe un enfoque efectivo para evaluar un biomaterial, independientemente del uso de supresores inmunes o inhibidores sistémicos en contexto biológico, que pueda garantizar el éxito de una implantación.

Evaluar, caracterizar y comprender la secuencia de eventos que tiene lugar después de la implantación es decisivo para desarrollar materiales capaces de modular, por un lado, su respuesta inmune y, por otro, maximizar su potencial osteoinductivo y osteoconductivo. Una de las soluciones podría ser caracterizar la capa de proteínas adsorbidas en la superficie del material implantado, relacionándolo con la mayor presencia de un determinado fenotipo de macrófago en él.

En este contexto, el objetivo de esta tesis se centra principalmente en la discusión y desarrollo de una nueva metodología para evaluar la experimentación *in vitro* para la implantación dental, tanto en la caracterización de la capa de proteínas adsorbida después de la implantación mediante espectrometría de masas (LC-MS / MS) y la evaluación del fenotipo de macrófagos en contacto con un determinado biomaterial. La correlación entre estos y el resultado *in vivo* serán evaluadas.

Concretamente, este trabajo de investigación se centra en la evaluación de las siguientes preguntas:

1) ¿Existe una relación entre las proteínas adsorbidas y la respuesta biológica?

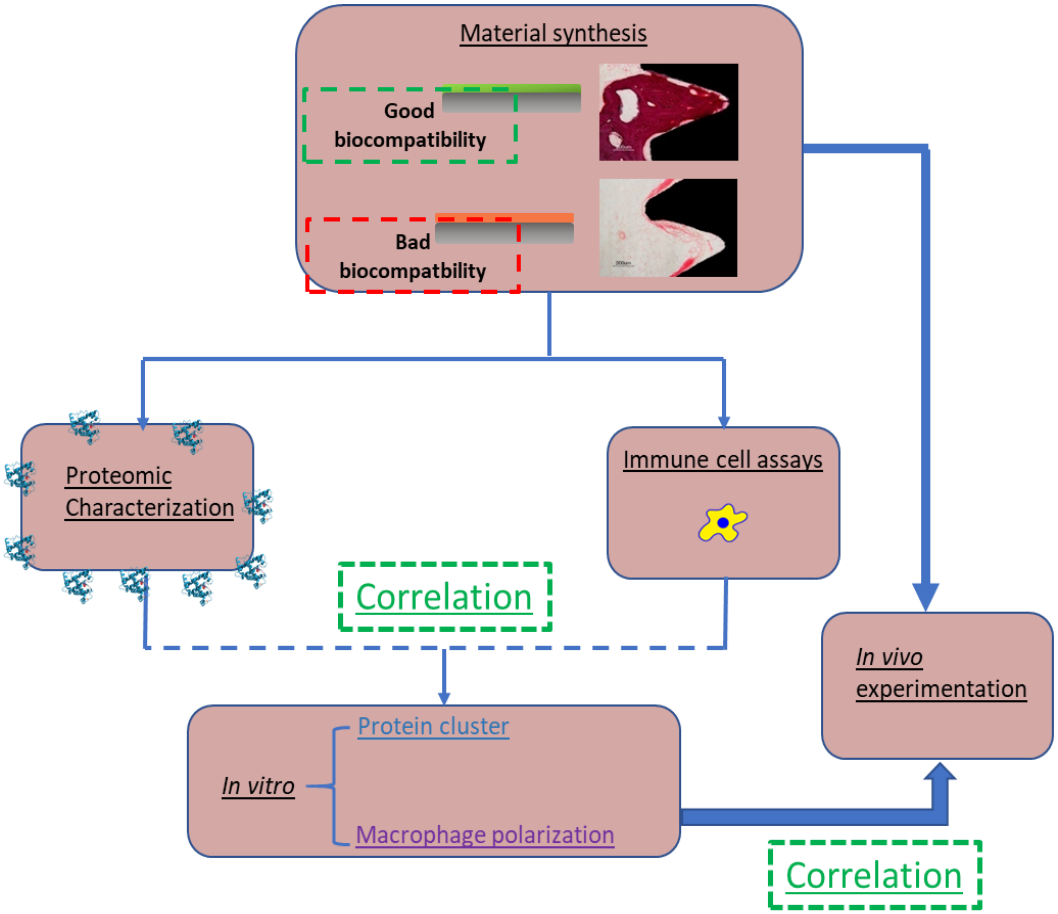
2) ¿Qué proteínas están asociadas con problemas de biocompatibilidad?

3) ¿Puede la polarización de los macrófagos correlacionarse con estos patrones de adsorción de proteínas y la posible aparición de problemas de biocompatibilidad?

4) ¿Se puede predecir la respuesta *in vivo* con el estudio de la polarización de los macrófagos?

3. Experimental Design

3. Experimental design



4. Chapter 1

CHAPTER 1

Characterisation of serum proteins attached to distinct sol-gel hybrid surfaces.

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ABSTRACT

The success of a dental implant depends on its osseointegration, an important feature of the implant biocompatibility. In this study, two distinct sol-gel hybrid coating formulations (50% methyltrimethoxysilane: 50% 3-glycidoxypropyl-trimethoxysilane (50M50G) and 70% methyltrimethoxysilane with 30 % tetraethyl orthosilicate (70M30T)) were applied onto titanium implants. To evaluate their osseointegration, *in vitro* and *in vivo* assays were performed. Cell proliferation and differentiation *in vitro* did not show any differences between the coatings. However, four and eight weeks after *in vivo* implantation, the fibrous capsule area surrounding 50M50G-implant was 10 and 4 times, respectively, bigger than the area of connective tissue surrounding the 70M30T treated implant. Thus, the *in vitro* results gave no prediction or explanation for the 50M50G-implant failure *in vivo*. We hypothesized that the first protein layer adhered to the surface may have direct implication in implant osseointegration, and perhaps correlate with the *in vivo* outcome**.** Human serum was used for adsorption analysis on the biomaterials, the first layer of serum proteins adhered to the implant surface was analysed by proteomic analysis, using mass spectrometry (LC/MS/MS). From the 171 proteins identified; 30 proteins were significantly enriched on the 50M50G implant surface. This group comprised numerous proteins of the immune complement system, including several subcomponents of the C1 complement, complement factor H, C4b-binding protein alpha chain, complement C5 and C-reactive protein. This result suggests that these proteins enriched in 50M50G surface might trigger the cascade leading to the formation of the fibrous capsule observed. The implications of these results could open up future possibilities to predict the biocompatibility problems *in vivo*.

Keywords: sol-gel coatings, immune system, C-reactive protein, fibrous capsule, proteomics, osseoimmunology

a. INTRODUCTION

In the design of implantable devices, the foreign body reaction, stress shielding, biocompatibility and (recently introduced) bioactivity and osteoinduction are the required features of the selected biomaterials and surface treatments [1].

Titanium (Ti) and its alloys are commonly used in dental implants with very good results due to their biocompatibility and biochemical properties. However, an increasing number of new biomaterials are being developed and applied to Ti surfaces as coatings [2,3] to improve their existing properties or add new useful features (e.g., osteoinduction).

New approaches must be assessed using reliable and comparable methods (*in vitro* and *in vivo* testing) to be rapidly translated into the clinical practice. Thus, there is an urgent need for proven *in vitro* assays to reduce the burden of animal testing. Unfortunately, the correlation between the *in vitro* and *in vivo* assessments of biomaterials is surprisingly poor, reinforcing the need for further development of relevant *in vitro* assays [4].

Given the dense vascularization of organs and tissues, the first fluid to come in contact with an implant is the blood [5], accounting for the formation of the first hydration layer covering the implant surface [6]. Examination of the constitution of the adsorbed protein layer and its effect on the bone-tissue-implant microenvironment might be crucial in the assessment of the success of an implant [7]. The type and characteristics (hydrophobicity, microtopography, chemical properties) of the constitutive material of the implant [8] and the first protein layer on the implant surface will ultimately determine osseointegration, involving processes like the blood coagulation, inflammation, and humoral immune response [9]. Hong *et al.* have studied the Ti properties in terms of its thrombogenic potential; it is one of the metals with high biocompatibility even though it lacks the bioactive properties [10]. On the other hand, in previous studies of our group using mass spectrometry (LC/MS/MS), we have shown that two different Ti surface treatments, with slightly different *in vivo* behaviour, display variations in the adsorbed first protein layer [11]. These studies open up the exciting possibility of predicting the body reaction after implantation. It is possible that protein deposition studies might provide in the future a major breakthrough in the understanding and prediction of biomaterial behaviour in *in vivo* environments [12,13].

Our present study focuses on the characterisation of the protein layer adsorbed onto Ti discs (blasted and acid-etched) coated with two distinct sol-gel hybrid coating formulations [2,3]; 50% methyltrimethoxysilane : 50% 3-glycidoxypropyl-trimethoxysilane (50M50G) and 70% methyltrimethoxysilane : 30 % tetraethyl orthosilicate (70M30T) and the correlation between their *in vitro* and *in vivo* behaviour. Our results show that the biomaterial that induces scar tissue in *in vivo* implants is associated with a distinct map of adsorbed proteins. Most of these proteins are related to the immune response, suggesting that this protein layer might be responsible for the formation of the fibrous capsules.

b. MATERIALS AND METHODS

i. Preparation of the coated titanium discs

Ti discs (12 mm in diameter, 1-mm thick) were made from a bar of commercially available, pure, grade-4 Ti (Ilerimplant SL, Lleida, Spain). Sandblasted acid-etched (SAE) Ti discs were abraded with 4 µm aluminium oxide particles and acid-etched by submersion in sulfuric acid for 1 h, to simulate a moderately rough implant surface. The discs were then washed with acetone, ethanol and 18.2 Ω purified water (for 20 min in each liquid) in an ultrasonic bath and dried under vacuum. Finally, all Ti discs were sterilised using UV radiation.

ii. Sol-gel synthesis and sample preparation

The silica hybrid coatings were obtained using the sol-gel route. The synthesised sol-gel compounds were 70% MTMOS: 30% TEOS (70M30T) and 50% MTMOS: 50% GPTMS (50M50G) (Sigma-Aldrich, St. Louis, MO, USA) (molar percentages). 2-propanol (Sigma-Aldrich, St. Louis, MO,

USA) was used as a solvent at a volume ratio of alcohol to siloxane of 1:1. Hydrolysis of alkoxysilanes was carried out by adding (at a rate of a drop per second) the corresponding stoichiometric amount of acidified aqueous solution 0.1M HNO3 (Panreac, Barcelona, Spain). The solution was stirred for 1 h and then left to rest for 1 h. The samples were prepared immediately afterwards. SAE Ti discs were used as a substrate. The coating was performed employing a dip coater (KSV instrument-KSV DC). Discs were immersed in a sol-gel solution at a speed of 60 cm min^{-1}, left for one minute, and removed at a 100 cm min^{-1}. Finally, 70M30T- and 50M50G-coated samples were cured for 2 h at 80 ºC and 140 ºC, respectively.

iii. Preparation of the coated titanium discs

The contact angle was measured using an automatic contact angle meter (DataPhysics, OCA 20). An aliquot of 10 µl of ultra-pure water W04 was deposited on the sol-gel coated surface at a dosing rate of 27.5 µl s^{-1} at room temperature. Contact angles were determined using SCA 20 software. Five discs of each material were studied after depositing two drops on each. The surface topography of the coatings was characterised by scanning electron microscopy (SEM) using a Leica-Zeiss LEO equipment under vacuum. Platinum sputtering was applied to make the samples more conductive for the SEM observations.

iv. Cell culture

MC3T3-E1 (mouse-calvaria osteosarcoma cell line) cells were cultured on the 70M30T- and 50M50G-coated Ti discs, at a concentration of 1×10^4 cells/well. The culture took place in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red (Thermo Fisher Scientific, Waltham, MA, USA), 1% 100× penicillin/streptomycin (Biowest Inc., Riverside, KS, USA) and 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). After an incubation for 24 h at 37 ºC in a humidified (95%) atmosphere of 5% CO_2, the medium was replaced with an osteogenic medium composed of DMEM with phenol red 1×, 1% penicillin/streptomycin, 10% FBS, 1% ascorbic acid (5 mg mL^{-1}) and 0.21% β-glycerol phosphate. The cells were incubated again under the same conditions. The culture medium was changed every 48 hours. Cells at the same concentration were used as a control of culture conditions on each plate.

v. Cytotoxicity

The biomaterial cytotoxicity was assessed following the ISO 10993-5 norm, using the 96-cell Titter Proliferation Assay (Promega®, Madison, WI, USA) according to manufacturer's guidelines. Cells only (blank well) is considered maximal viability. Cells incubated in latex were the control for highly toxic compound. Cell viability of 70% was considered the limit.

vi. Alkaline phosphatase (ALP) activity

The protocol of conversion of p-nitrophenylphosphate (p-NPP) to p-nitrophenol was used to assess the ALP activity at the indicated times. Aliquots of 0.1 mL were used to measure the ALP activity. One hundred µl of p-NPP (1 mg mL^{-1}) in the substrate buffer (50 mM glycine, 1mM $MgCl_2$, pH 10.5) was added to 100 µl of the supernatant obtained from the lysate. After 2 h of incubation in the dark (37 ºC, 5% CO_2), the absorbance was measured in a microplate reader at a wavelength of 405 nm. ALP activity was read from a standard curve obtained using different solutions of p-nitrophenol and sodium hydroxide (0.02 mM). The results were presented as millimoles of p-nitrophenol/h (mmol PNP h^{-1}), and the data were expressed as ALP activity normalised to the total protein content (µg μL^{-1}). Protein concentration was quantified using Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

vii. Statistical analysis

The data were submitted to one-way analysis of variance (ANOVA) and to a Newman-Keuls multiple comparison post-test, when appropriate. Differences with $p \leq 0.05$ were considered statistically significant.

viii. *In vivo* experiments

To evaluate the histological response to the biomaterials described, the implants were surgically placed in the tibia of New Zealand rabbits (*Oryctolagus cuniculus*). This implantation has been widely used in the studies of osseointegration of dental implants [14]. All our experiments were performed in accordance with the protocols of Ethical Committee at the University of Murcia (Spain), European guidelines and the legal conditions formulated in R. D. 223/1988 of March 14th and the Order of October 13rd, 1988, of the Spanish Government law on the protection of animals used for experimentation and other scientific purposes. Briefly, 20 rabbits (2-3Kg) were kept under 12-h span darkness-light cyclic conditions; room temperature was set at 20.5 ± 0.5 °C and the relative humidity ranged between 45 and 65%. The animals were individually caged and fed a standard diet and filtered water *ad libitum*. The dental implants were supplied by Ilerimplant SL

(Lleida, Spain). The implants were internal-connection made with Ti grade IV, trademark GMI dental implants, 3.75 mm in diameter, 8-mm long, Frontier model with ADS (Advanced Doubled-Grip Surface) treatment, a combination of white corundum micro-bubble treatment and acid etching with nitric acid and sulfuric acid solution. 40 implants were used, 20 uncoated as controls, and 5 coated as test samples for each material and each time. 5 rabbits were used for each material and time. The implantation periods of the experimental model were 4 and 8 weeks. Implants were inserted in the left and right proximal tibiae (one control and one test sample). Animals were anesthetized with chlorpromazine hydrochloride and ketamine chlorhydrate. The periosteum was removed, and the osteotomy was performed using a low-revolution micromotor and drills of successive diameters of 2, 2.8 and 3.2 mm, with continuous irrigation. Implants were inserted and press-fit and the surgical wound was sutured, washed with saline solution and covered with plastic spray dressing (Nobecutan, Inibsa Laboratories, Barcelona, Spain). After each examined implantation period, the animal was euthanized by carbon monoxide inhalation, to retrieve the screws and study the surrounding tissues.

ix. Histological examination and quantification

Four samples for histological examination were processed following the methodology described previously [2]. Briefly, the samples were embedded in methyl methacrylate, and 25–30 µm sections were obtained using EXAKT technique (EXAKT Technologies, Inc., Oklahoma, USA). For optical microscopy examination, all the sections were stained using Gomori Trichrome solution.
Fibrous connective tissue was quantified using ImageJ software (https://imagej.nih.gov/ij/). The results are expressed as the estimate area occupied by fibrous connective tissue per area in mm^2.

x. Adsorbed protein layer

Ti discs coated with 70M30T (n = 4) and 50M50G (n = 4) were incubated in a 24-well plate for 180 min in a humidified atmosphere (37 ºC, 5% CO_2), after the addition of 2 mL of human blood serum from male AB plasma (Sigma-Aldrich, St. Louis, MO, USA). After removing the serum, to remove the remaining non-adhered proteins, the discs were rinsed five times with ddH_2O and once with 100 mM NaCl, 50 mM Tris-HCl, pH 7.0. The adsorbed protein layer was collected by washing the

discs in the solution of 4% SDS, 100 mM DTT and 0.5M TEAB. The experimental method was adapted from a previous study by Kaneko *et al.* [12]. Four replicates of each biomaterial were obtained. Total protein content was quantified before the experiment (Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA)), obtaining the value of 51 mg mL^{-1}.

xi. Proteomic analysis

The eluted protein samples were resolved on 10% polyacrylamide gels, using a Mini-Protean II electrophoresis cell (Bio-Rad®, Hercules, CA, USA). A constant voltage of 150 V was applied for 45 min. The gel was then stained using SYPRO Ruby stain (Bio-Rad®, Hercules, CA, USA) following the manufacturer's instructions. The gel was washed, and each lane was cut into 4 slices. Each of these slices was digested with trypsin following a standard protocol [15]. The resulting peptides were resuspended in 0.1% formic acid, separated using online NanoLC and analysed using electrospray tandem mass spectrometry. Peptide separation was performed on a nanoACQUITY UPLC system (Waters, Milford, MA, USA) connected to a SYNAPT G2-Si spectrometer (Waters, Milford, MA, USA) Samples were loaded onto a Symmetry 300 C18 UPLC Trap column with 5 µm, 180 µm × 20 mm connected to a BEH130 C18 column with 1.7 µm, 75 µm × 200 mm (Waters, Milford, MA, USA). The column was equilibrated in 3% acetonitrile and 0.1% FA. Peptides were eluted at 300 nl min^{-1} using a 60-min linear gradient of 3−50% acetonitrile.

A SYNAPT G2-Si ESI Q-Mobility-TOF spectrometer (Waters, Milford, MA, USA) equipped with an ion mobility chamber (T-Wave-IMS) for high definition data acquisition analyses was used for the analysis of the peptides. All analyses were performed using electrospray ionization (ESI) in a positive ion mode. Data were post-acquisition lock-mass corrected using the double charged monoisotopic ion of [Glu^1]-fibrinopeptide B. The accurate LC-MS data were collected in HDDA mode, which enhances the signal intensities using the ion mobility separation.

Progenesis LC-MS software (Nonlinear Dynamics, Newcastle, UK) was used for differential protein expression analysis. Raw files were imported into the programme, and one of the samples was selected for a reference run to which the precursor masses in all the other samples were aligned. Abundance ratio between the run to be aligned and the reference run were calculated for all features at given retention times. These values were then logarithmised and the programme,

based on the analysis of the distribution of all ratios, automatically calculated a global scaling factor. Once normalised, the samples were grouped into the appropriate experimental categories and compared. A peak list containing the detected peptides in all samples was searched against the Swiss-Prot database using the Mascot Search engine (www.matrixscience.com). Peptide mass tolerance of 10 ppm and 0.2-Da fragment mass tolerance were used for the searches. Carbamidomethylation of cysteines was selected as the fixed modification and oxidation of methionine as a variable modification for tryptic peptides. Proteins identified with at least two peptides with an FDR < 1% were kept for further examination. Proteins were quantified based on the intensity of their 3 most abundant peptides, when available. Proteins with ANOVA $p < 0.05$ and a ratio higher than 1.3 in either direction was considered as significantly different.

Finally, the data were entered in the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources to classify the Progenesis differential protein list into functionally related clusters.

c. RESULTS

i. Synthesis and physicochemical characterisation

The synthesis conditions described here allowed us to obtain the different coatings. SEM micrographs of 70M30T (**Figure 1a**) and 50M50G (**Figure 1b**) coatings show distinct topographies. 70M30T surface conserves the SAE-Ti roughness properties and is rougher than the 50M50G surface. In the latter, the initial SAE-Ti irregularities are covered, possibly due to an increased thickness. The contact angle measurements gave values of 50.78 ± 1.82º and 67.59 ± 1.03º for 70M30T and 50M50G coatings, respectively. These data indicate that the 70M30T biomaterial is more hydrophilic than 50M50G.

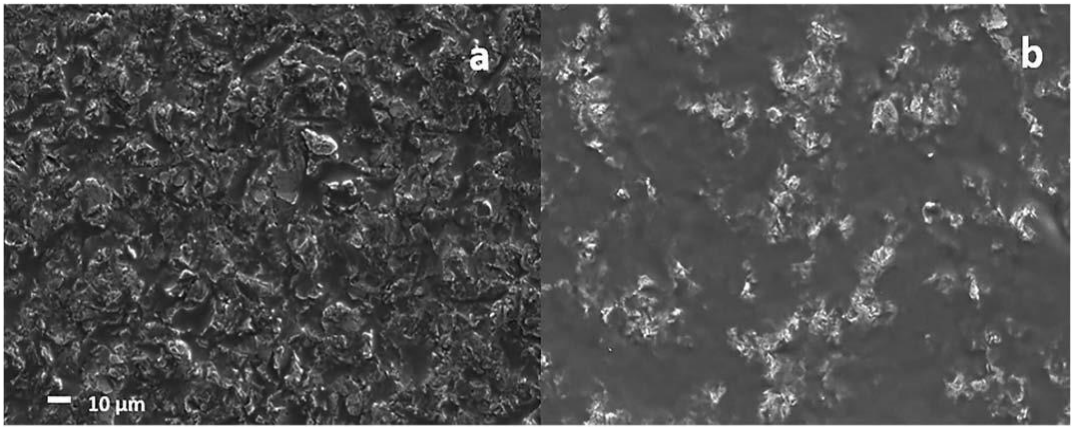

Figure 1. SEM images of sol–gel coated disc surface. 70M30T (a) and 50M50G (b). Calibration bar 10 mm.

ii. *In vitro* culture

Neither of the materials was cytotoxic (**Figure 2a**). The mineralisation analysis, performed by measuring ALP activity, showed that *in vitro*, 70M30T and 50M50G did not affect the osteoblast cell differentiation significantly, at 7 and 14 days (**Figure 2b**). The two biomaterials behaved similarly in terms of the metabolic and differentiation processes of MC3T3-E1 cells *in vitro.*

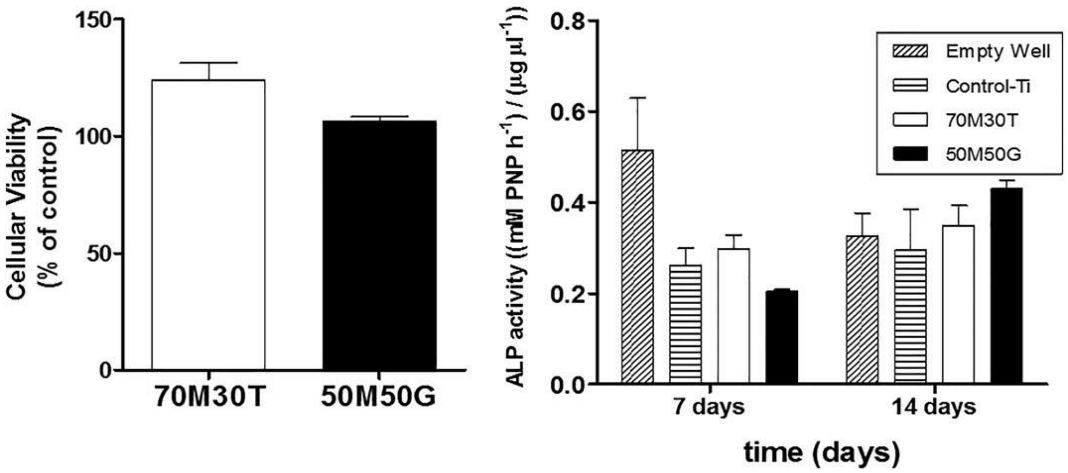

Figure 2. MC3T3-E1 cell viability and mineralization *in vitro*. Percentage of cell survival following the norm ISO 10993–5 (a). ALP activity (mM PNP/h) normalized to the protein concentration (mg/mL) of cells grown without disc (oblicue lines), grown on control Ti discs (horizontal lines), 70M30G (white column) and 50M50G coated Ti discs (black column).

iii. *In vivo* assay

Four and eight weeks after the implantation, some differences between the materials were observed (**Figure 3a**). Whereas 70M30T-coated screws displayed good osseointegration on the implant-cortical bone interface, the 50M50G-coated implants were surrounded by a thick fibrous capsule. This result is support by the graph on **Figure 3b** displaying that the area occupied by fibrous connective tissue is approximately 4-fold higher on the 50M50G material, compared to the 70M30T, for both times. In light of these results, we can conclude that 50M50G-coated implants provoked an immune/inflammatory response, which might prevent implant integration, bone formation and ultimately cause the implant rejection.

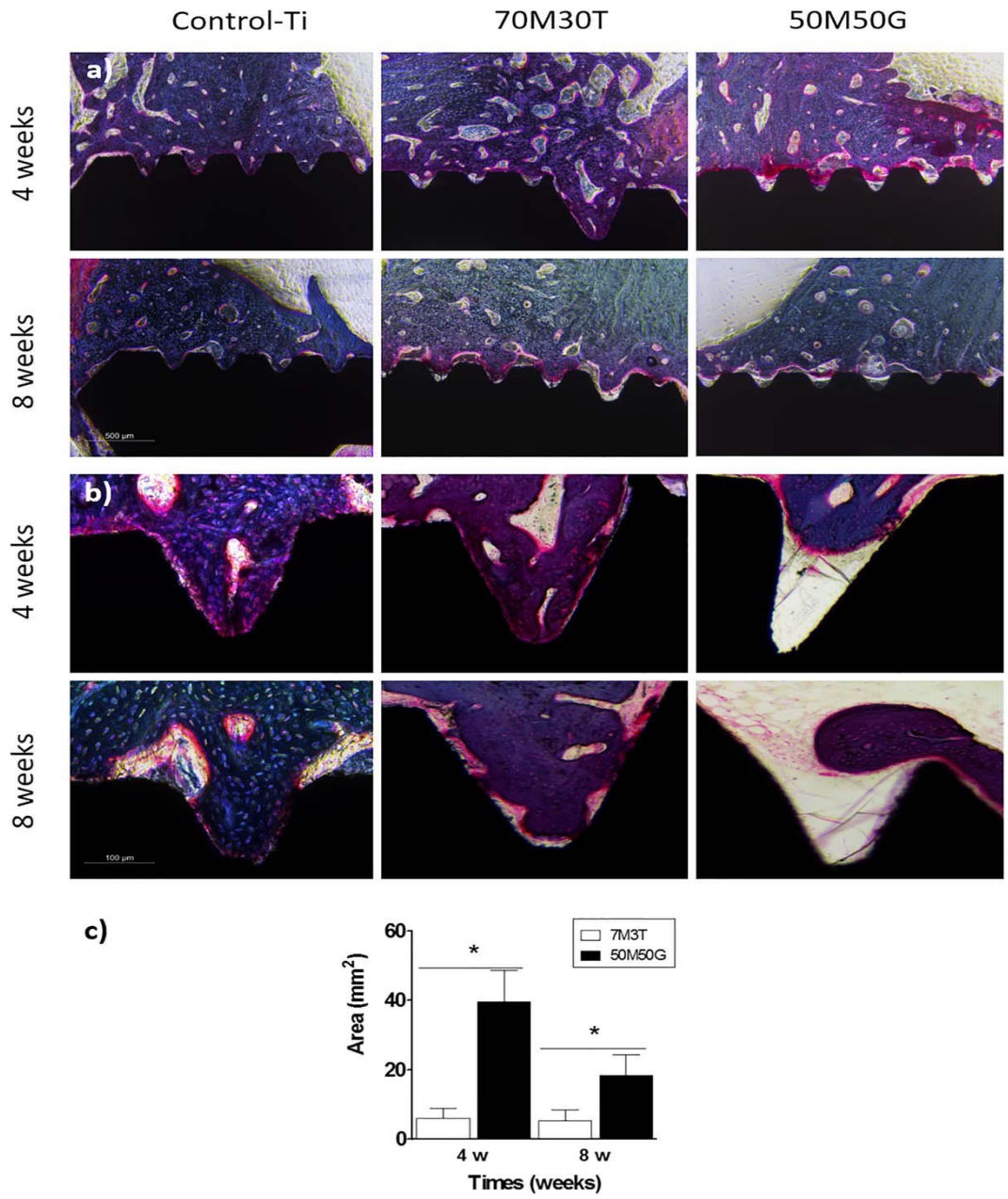

Figure 3. *In vivo* studies. Light microscopy images (EXAKT® cut and Gomori Trichrome stain) from *in vivo* implants 4 and 8 weeks postimplantation of Control-Ti, 70M30T and 50M50G sol–gel coated screw; (a) Calibration bar 500 mm; (b) Calibration bar 100 mm. (c) Quantification fibrous of the connective tissue area (n=4). Significant values were considered for $p<0.05$ as analysed by ANOVA followed by Newman-Keuls post-test.

iv. Proteomic analysis

The protein layers absorbed onto 70M30T and 50M50G coatings were analysed by LC-MS/MS, which identified 171 different proteins. The data were also analysed (n = 4) using the Progenesis QI software, to find out which proteins were differentially predominant on the two materials. Moreover, the DAVID was used to obtain a functional classification of the proteins. **Table I** shows eight enriched proteins identified in the 70M30T film. Keratins, hornerin, filaggrin-2 and tropomyosin alpha-3 chain were substantially more abundant on this material. All these proteins are related to peptidase activity and/or the integrity of the cytoskeleton.

Table I. LC-MS/MS Detected Proteins Differentially Predominant in the Film Adsorbed to the 70M30T Sol–Gel Biomaterial (Progenesis Method)

Description	Ratio 50M50G/70M30T	Ref. bone metabolism or/and immune response	DAVID
Tropomyosin alpha-3 chain	0,06	-	9
Filaggrin-2	0,35	-	5,8
Hornerin	0,40	-	5,8,9
Keratin, type II cytoskeletal 1b	0,49	-	9
Keratin, type II cytoskeletal 71	0,51	-	9
Keratin, type II cytoskeletal 78	0,60	-	9
Keratin, type I cytoskeletal 10	0,61	-	9
Keratin, type II cytoskeletal 2 epidermal	0,63	-	9

The averages are the result of 4 independent replicates. Differences were considered significant with an ANOVA p values<0.05. DAVID classification functions were inflammatory/immune response (1), hydroxylation (2), blood coagulation (3), apoptosis regulation (4), metal binding (5), phosphorylation (6), carbohydrate binding (7), peptidase activity (8), and cytoskeleton integrity (9).

However, 30 serum proteins adhered differentially to the 50M50G material (**Table II**). Proteins related to tissue regeneration and bone metabolism such as plasminogen [16,17], proteoglycan 4 [18], vitronectin [19], Apo E [20,21], kininogen-1 [22] and complement C3 [24–26] were more abundant on that coating. Increased amounts of many proteins related to the immune system and inflammatory response were also found on this material. Complement C1r subcomponent, complement factor H, C4b-binding protein alpha chain, C-reactive protein, complement C3, complement C5, complement component C7, serum amyloid P-component, complement C1q subcomponent subunits A, B and C, complement C1s subcomponent and plasma protease C1 inhibitor proteins were found and classified by DAVID database analysis belonging to a protein cluster related to an acute inflammatory response.

Table II. LC-MS/MS detected proteins differentially predominant in the film adsorbed to the 50M50G sol-gel biomaterial (Progenesis method). The averages are the result of 4 independent replicates. Differences were considered significant with an ANOVA p-value < 0.05. DAVID classification functions were inflammatory/immune response (1), hydroxylation (2), blood coagulation (3), apoptosis regulation (4), metal binding (5), phosphorylation (6), carbohydrate binding (7), peptidase activity (8) and cytoskeleton integrity (9). (Table continues next page)

Description	Ratio 50M50G/ 70M30T	Ref. bone metabolism or/and immune response	DAVID
Complement C1r subcomponent	1,38	[41]	1,2,5,8
Plasminogen	1,49	[16,17]	3,4,5,8
Ig kappa chain V-II region Cum	1,63	-	-
Apolipoprotein A-IV	1,67	-	5
Hemopexin	1,70	[45]	5,6
Ig lambda-2 chain C regions	1,73	-	-
Proteoglycan 4	1,79	[18]	7
Complement factor H	1,82	[41]	1
Ig kappa chain V-III region VG (Fragment)	1,90	-	-
Ig kappa chain V-III region SIE	1,93	-	-
Kininogen-1	2,02	[28]	1,2,3,4,5, 7
C4b-binding protein alpha chain	2,06	[41]	1
Complement C1s subcomponent	2,07	[41]	1,2,5,8
Ig kappa chain V-IV region Len	2,08	-	-
Vitronectin	2,21	[19]	7
Complement component C7	2,22	[41]	1
Complement C1q subcomponent subunit B	2,24	[41]	1,2
Complement C1q subcomponent subunit C	2,26	[41]	1,2
Complement C3	2,27	[23;24]	1
Plasma protease C1 inhibitor	2,34	[44]	1,3
Apolipoprotein E	2,40	[20;21]	3,4,5,6,7
Leucine-rich alpha-2-glycoprotein	2,54	[42]	5
Complement C5	2,55	[41]	1,6
Serum amyloid P-component	3,48	[43]	1,5,7,8
Complement C1q subcomponent subunit A	3,60	[41]	1,2

Description	Ratio 50M50G/ 70M30T	Ref. bone metabolism or/and immune response	DAVID
Myosin-1	4,22	-	9
Lipocalin-1	4,28	-	8
C-reactive protein	7,83	[31]	1,5,8
Glutamate dehydrogenase 1, mitochondrial	13,47	-	-
L-lactate dehydrogenase B chain	14,30	-	-

d. DISCUSSION

This study focused on the characterisation of the protein layer adsorbed onto the Ti discs (blasted and acid-etched) coated with two distinct biomaterials, 70MTMOS:30TEOS (70M30T) and 50MTMOS:50GPTMS (50M50G). Moreover, it was analysed the correlation between their *in vitro* and *in vivo* behaviour. The application of these biomaterials onto the disc surfaces changed the biological and physicochemical properties of Ti.

Distinct precursors were used to synthesise the two coatings. Both materials are composed by MTMOS, being the main chemical difference between them the presence of TEOS and GPTMS. GPTMS is an organo-modified alkoxysilane with an epoxy group. In contrast, the TEOS does not possess that group [25]. The more pronounced organic features of 50M50G sol-gel matrix increase the hydrophobic properties of this material, reflected in the contact angle results. These differences in the chemical, hydrophilic and morphologic characteristics might affect the response and behaviour of the material in a biological context. However, *in vitro* experiments show no significant differences between the two types of coated discs. Both materials were found to be non-cytotoxic or/and even did influence nor positively nor negatively the ALP activity compared to non-coated SLA titanium. Nonetheless, it was observed drastic differences between the *in vivo* behaviour of these coatings. Our results showed the formation a layer of fibrous connective tissue surrounding the 50M50G, between the bone and the implant, which was not found on the 70M30T surface.

Although there is a considerable need for proven *in vitro* assays to reduce the burden of animal testing, a recent multicentre review has shown no significant overall correlation between the *in vitro* and *in vivo* effects of biomaterials used for bone regeneration. The inadequacies of the current *in vitro* assessments highlight the urgent need for novel approaches to the *in vitro* biomaterial testing and the lack of validated pre-clinical studies [4].

Proteomic analysis using LC/MS-MS identified and quantified the proteins adsorbed onto the two surfaces (**Tables I** and **II**). The results displayed a distinct cluster of proteins, closely related to the immune and/or inflammatory response, predominant on the 50M50G biomaterial (in comparison with 70M30T). This observation might explain the *in vivo* outcome. The formation of a fibrous connective tissue in *in vivo* experiments has been reported and attributed to the natural immune and inflammatory response to a foreign body [26,27] The increased abundance of bone regeneration/repair-related proteins like plasminogen [16,17], proteoglycan 4 [18], vitronectin [19], Apo E [20,21] and kininogen-1 [28,29] observed on the 50M50G-coated implants might be required for osseointegration. Interestingly, it was also found increased levels of proteins of the classical complement system on this material in comparison with the 70M30T coating. The complement system plays a crucial role in an immediate immune response to the pathogens [30]. We are prone to speculate that the first layer of the proteins adsorbed onto the surface induces a fast-immune response. This response might be induced by the increased levels (7-fold) of CRP (C-reactive protein), a protein with an important role in the immune response pathways [31,32]. CRP is a well-documented risk factor for cardiac diseases [32]. It belongs to a family of serum proteins with a pentameric structure, pentraxins, which can recognize antigens, activate the immune system (*e.g.* immunoglobulins), and interact with the complement system. In fact, one of the first reported CRP functions is its ability to trigger the whole classical complement system [33]. It acts by direct binding of the C1q, the first component of this system; C1q levels were also augmented on the 50M50G coating (2-fold). The binding of C1q to CRP activates a cascade of complements. C1r and C1s are activated, activating C4 and C2 in turn, followed by the generation of C3 convertases. The C3 convertases cleave C3 into C3a and C3b. C3a has both pro- and anti-inflammatory effects. C3b functions as an opsonin and activates the cleavage of C5 into anaphylatoxins C5a and C5b, ultimately forming the C5b-9 complex [34]. Both C3a and C5b are responsible for the recruitment and activation of the immune cells, such as macrophages, to the

activation site [35]. The complement-activated macrophages regulate fibrogenesis by promoting the cytokine activity and cell migration, resulting in the fibroblast proliferation and collagen synthesis[36]. Specifically, they act by secreting pro-fibrogenic factors, increasing fibrogenesis by fibroblasts and inducing the formation and development of the fibrous capsule around the implanted material. The thickness of the formed fibrous capsule can interfere with the function of the biomaterial, depending on the intensity of the immune/inflammatory response [37].

Thus, it is tempting to correlate the presence of these proteins with the *in vivo* response observed in this experimental work. Other authors have discussed the possibility of this binomial behaviour. Ekdahl *et al*. have reported that the binding of C3 protein on the surface of biomaterials might be negatively correlated with their biocompatibility [38]. Similarly, Engberg *et al.* have stablished a correlation between the absorption of proteins such as C3, C4, C5, C1q, factor H or C4BP and the inflammatory response induced by biomaterials. They have proposed the high C4/C4BP protein ratio as a predictor of low biocompatibility [39]. These studies have been carried out by pre-selecting the proteins to be detected. However, we believe that the proteomics methodology used in our study may improve our understanding of the role of proteins in the osseointegration processes. The results shown in **Table II** link the formation of the fibrous capsule with the cluster of proteins related to an acute inflammatory response. The presence of CRP within this cluster might be important in the activation of the immune reaction. Thus, within the limitations of this study, CRP might be proposed as a marker of poor biocompatibility, if it is found on the biomaterial surfaces at substantially increased levels.

However, we found intracellular proteins adhered to the biomaterial surface. This finding may be a consequence of the plasma isolation process, where cellular breakdown might occur. The presence of such proteins may or may not influence the biomaterial *in vivo* outcome, since these proteins should be mostly expressed intracellularly. It is generally accepted that intra-cellular and membrane proteins or fragments are commonly found serum and plasma [40]. We cannot exclude a possible cellular breakdown *in vivo*, even as a consequence of the surgery itself.

To summarise, we found that surfaces with distinct physico-chemical properties, such as 70M30T and 50M50G sol-gel coatings, could produce different *in vivo* responses. These responses might depend on the bodily fluids (serum/blood) in contact with the implant surface. We showed that

the majority of the specific serum proteins adhering to the 50M50G biomaterial belong to a cluster of proteins related to the immune/inflammatory response. Thus, it is plausible that the fibrous connective tissue surrounding the 50M50G material might be the consequence of the adsorption of various complement system proteins. The increased abundance of CRP, one of these proteins, might significantly affect the success of osseointegration.

e. ACNOWLEDGEMENTS

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5. Chapter 2

CHAPTER 2

Osseointegration mechanisms: a proteomic approach

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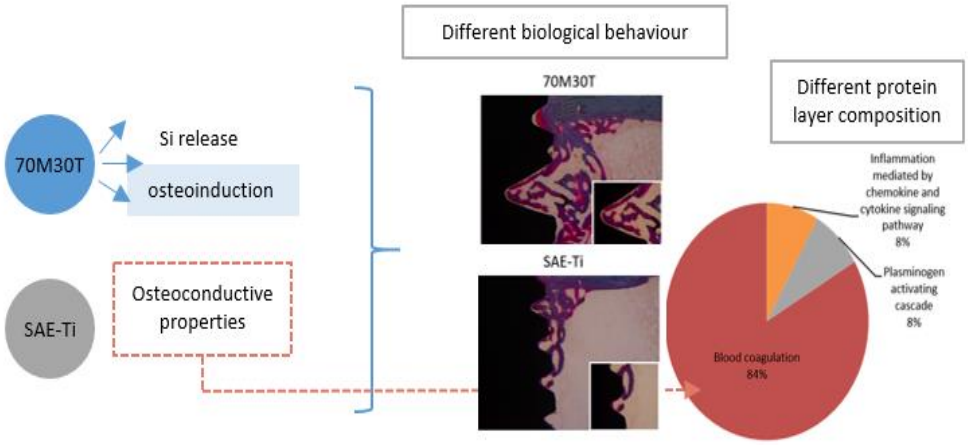
GRAPHICAL ABSTRACT



ABSTRACT

The prime objective in the development of biomaterials for dental applications are to improve the quality of osseointegration and to short the time needed to achieve it. Design of implants nowadays involve changes in the surface characteristics to obtain a good cellular response. Incorporating osteoinductive elements is one way to achieve the best regeneration possible post-implantation. This study examined the osteointegrative potential of two distinct biomaterials: sandblasted acid-etched titanium and a silica sol-gel hybrid coating, 70% MTMOS-30% TEOS. *In vitro*, *in vivo* and proteomic characterisation of the two materials were conducted. Enhanced expression levels of ALP and IL-6 in the MC3T3-E1 cells cultured with coated discs, suggest that growing cells on such surfaces may increase mineralisation levels. 70M30T-coated implants, showed improved bone growth *in vivo* compared to uncoated titanium. Complete osseointegration was achieved on both. However, coated implants displayed osteoinductive properties, while uncoated implants demonstrated osteoconductive characteristics. Coagulation-related proteins attached predominantly to SAE-Ti surface. Surface properties of the material might drive the regenerative process of the affected tissue. Analysis of the proteins on the coated dental implant showed that few proteins specifically attached to its surface, possibly indicating that its osteoinductive properties depend on the silicon delivery from the implant.

Keywords:

Dental implants, osteogenesis, bone regeneration, coagulation, osseointegration, osteoinduction, biointerfaces, protein deposition

a. INTRODUCTION

Osseous tissue undergoes continuous remodelling, which depends on the balance between the activities of highly specialised cells, the osteoblasts and osteoclasts. This balance is adaptive; the system responds to mechanical stresses and is affected by the processes involved in the maintenance of bone health and bone regeneration [1].

The terms osteoinduction and osteoconduction are often used in the field of orthopaedics. They are commonly discussed in dental implantology practice, where various implants have been employed with long-term success rates of around 95% [2]. As the demand for this type of treatment is growing and the number of such surgeries is increasing, even these high rates seem to be insufficient. New surface types specifically designed for dental implants could improve the success ratios. They should also achieve better and faster osseointegration than the traditional materials [3]. This is especially important in cases with compromised bone regeneration capability (*e.g.* smokers, osteoporotic and diabetic patients, etc.).

Osteoinduction (the process by which osteogenesis is induced) involves stimulation of undifferentiated cells, resulting in the development of bone-forming cell lineage [4]. Osteoconduction is the property of a material serving as a scaffold for the growth of bone tissue. The osteoconductive potential of a surface is affected by its roughness, microtopography, nanotopography and porosity [5]. These two processes (osteoinduction and osteoconduction) are important for osseointegration, i.e. a direct structural and functional connection between the newly formed bone and the biomaterial [6].

The clinical success of a dental implant strongly depends on a short-term osseointegration. Good osseointegration rate of the titanium dental implants is necessary for a successful early clinical outcome [7]. Such implants, apart from supporting the correct healthy bone integration, should promote the activation of osteoblasts in the impaired tissue, stimulating the osteogenesis. This should facilitate surgical implantations in patients with regenerative limitations.

Thus, the design of new dental implants should consider both chemical and physical surface modifications. Such modifications affect surface implant topography, hydrophobicity and the

chemical properties of the implant material (and especially, of its surface). The purpose is to enhance the biological interaction of the living tissue with the material [8].

The sol-gel hybrid materials synthesised using alkoxysilanes are being increasingly used as coatings for biomedical applications. They are being developed as coatings for titanium dental implants [9,10]. These biomaterials release silicic acid compounds ($Si(OH)_4$), which impart osteoinductive properties to the implant [11,12].

The application of these coatings onto an implant surface affects its physical and chemical attributes, and, consequently, alters the conformation, type and quantity of proteins adsorbed immediately after implantation [13]. These are the proteins that might determine the initiation and intensity of the immune and inflammatory response and coagulation [14] and activate processes triggering osteogenesis, leading to effective osseointegration. Thus, the studies of the adsorbed proteins are of primary importance for the orthopaedics and other associated medical fields. Such studies should contribute to new insights into the mechanisms governing the microenvironment of the protein–biomaterial surface interactions.

This article presents *in vitro*, *in vivo* and proteomic characaterisation of two different surfaces type (SAE-Ti, and sol-gel coating). The bone regeneration mechanism and potential of the two surfaces are studied and compared.

b. MATERIALS AND METHODS

i. Preparation of the titanium discs

Ti discs (12 mm in diameter, 1-mm thick) were made from a bar of commercially available, pure, grade-4 Ti (Ilerimplant S.L., Lleida, Spain). To obtain the sandblasted, acid-etched (SAE) Ti, the discs were abraded with 4-µm aluminium oxide particles and acid-etched by submersion in sulfuric acid for 1 h, to simulate a moderately rough implant surface. The discs were then washed with acetone, ethanol and 18.2-Ω purified water (for 20 min in each liquid) in an ultrasonic bath and dried under vacuum. Finally, all Ti discs were sterilised using UV radiation.

ii. Sol-gel synthesis and sample preparation

The silica hybrid sol-gel material was synthesised from the alkoxysilane precursors: methyltrimethoxysilane (MTMOS) and tetraethyl orthosilicate (TEOS) (Sigma-Aldrich, St. Louis, MO, USA) in molar percentages of 70% and 30%, respectively. This composition was adopted on the basis of the previous results [9].

2-Propanol (Sigma-Aldrich, St. Louis, MO, USA) was used as a solvent in the process at a volume ratio (alcohol:siloxane) of 1:1. Hydrolysis of alkoxysilanes was carried out by adding (at a rate of 1 drop. s^{-1}) the corresponding stoichiometric amount of 0.1 M aqueous solution of HNO_3 (Panreac, Barcelona, Spain). The mixture was kept for 1 h under stirring followed by 1 h at rest. Coated samples were prepared immediately afterwards with SAE-Ti as a substrate. The samples were coated employing a KSV DC dip coater (Biolin Scientific, Stockholm, Sweden). Discs and implants were immersed in the sol-gel solution at a speed of 60 $cm.min^{-1}$, left immersed for one minute, and removed at a 100 $cm.min^{-1}$. Finally, the samples were cured for 2 h at 80 ºC.

iii. Physico-chemical characterisation of the coated titanium discs

The surface topography of samples was examined by scanning electron microscopy (SEM), employing the Leica-Zeiss LEO equipment under vacuum (Leica, Wetzlar, Germany). Platinum sputtering was applied to make the materials more conductive. A mechanical profilometer Dektack 6M (Veeco Instruments, Plainview, NY, USA) was used to assess the material roughness. Two coated discs of each composition were tested. Three measurements were performed for each disc to obtain the average values of the Ra parameter. The contact angle was measured using an automatic contact angle meter OCA 20 (DataPhysics Instruments, Filderstadt, Germany). Aliquots of 10 µL of ultrapure water W04 were deposited on the disc surfaces at a dosing rate of 27.5 µL. s^{-1} at room temperature. Contact angles were determined using the SCA 20 software. Six discs of each material were studied, after depositing two drops on each disc.

iv. *In vitro* assays

1.1. Cell culture

Mouse calvaria osteosarcoma cells (MC3T3-E1) were cultured on the sol-gel coated titanium discs at a concentration of 1×10^4 cells/well. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with phenol red (Gibco-Life Technologies, Grand Island, NY, USA), 1% penicillin/streptomycin solution 100× (Biowest Inc., Riverside, KS, USA) and 10% fetal bovine serum (FBS) (Gibco-Life Technologies). After incubation for 24 hours at 37 ºC in a humidified (95%) atmosphere with 5% CO_2, the medium was replaced with an osteogenic medium composed of DMEM with phenol red 1×, 1% penicillin/streptomycin, 10% FBS, 1% ascorbic acid (5 mg mL^{-1}) (Sigma-Aldrich) and 0.21% β-glycerol phosphate (Sigma-Aldrich), and incubated again under the same conditions. The culture medium was changed every 48 hours. In each plate, an empty well with cells at the same concentration (1×10^4 cells), was used as a control of culture conditions. For RNA isolation, the cells were allowed to differentiate for 7 and 14 before being harvested.

1.2. Cytotoxicity

The biomaterial cytotoxicity was evaluated following the ISO 10993-5 norm; it was assessed using spectrophotometry, after incubation of the cells with the material extract, obtained after following the norm. The *CellTiter* 96 Proliferation Assay (Promega®, Madison, WI, USA) was employed to measure cell viability after 24 h incubation. We used a negative control (the empty cell well) and a positive control with latex, known to be toxic to the cells. Seventy-percent cell viability was the limit below which a biomaterial was considered cytotoxic.

1.3. Cell proliferation

For measuring cell proliferation, the commercial cell-viability assay alamarBlue® (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) was used. The kit measures the cell viability on the basis of a redox reaction with resazurin. The cells were cultured in wells with the discs (3 replicates per treatment) and examined following the manufacturer's protocol after 1, 3, 5 and 7 days of culture. The results (percentage of reduced resazurin) were used to evaluate cell proliferation.

1.4. Alcaline Phosphatase (ALP) activity

The conversion of p-nitrophenylphosphate (p-NPP) to p-nitrophenol was used to assess the activity. The culture medium was removed from the wells, which were then washed 3 times with 1 × DPBS (Dulbeccos' phosphate-buffered saline- Thermofisher Scientific), and 100 µL of lysis buffer (0. 2% Triton X-100, 10 mM Tris-HCl, pH 7.2) (Sigma-Aldrich) was added to each well. Sample aliquots of 0.1 mL were used to conduct the assay. One hundred µL of p-NPP (1 mg mL-1) in substrate buffer (50 mM glycine, 1mM MgCl2, pH 10.5) was added to 100 µL of the supernatant obtained from the lysate. After two hours of incubation in the dark (37 ºC, 5% CO_2), the absorbance was measured, using a microplate reader, at a wavelength of 405 nm. ALP activity was read from a standard curve obtained using different solutions of p-nitrophenol and 0.02 mM sodium hydroxide (Sigma-Aldrich). The results were presented as mmol of p-nitrophenol/hour (mmol PNP h^{-1}). The data were

expressed as ALP activity normalised to the total protein content ($\mu g.\mu L^{-1}$) obtained using Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) after 7 and 14 days of culture.

1.5. RNA isolation and cDNA synthesis

Total RNA was prepared from the cell lysates grown on the sol-gel coated titanium discs, using Qiagen RNeasy Mini kit (Hilden, Germany), following digestion with DNase I (Qiagen), according to the manufacturer's instructions. The quantity, integrity and quality of the resulting RNA were assessed using NanoVue® Plus Spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). For each sample, approximately 1 µg of total RNA was converted to cDNA using PrimeScript RT Reagent Kit (Perfect Real Time) (TAKARA Bio Inc., Shiga, Japan). The resulting cDNA was diluted in DNAse-free water to a concentration suitable for reliable RT-PCR analysis.

1.6. Quantitative real-time PCR

Before the qRT-PCR reaction, primers for ALP, IL6, Col I and OCN genes were designed from specific DNA sequences available from NCBI (https://www.ncbi.nlm.nih.gov/nuccore), using PRIMER3plus software tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Expression levels were measured using primers purchased from Life Technologies S.A. (Gaithersburg, MD); GADPH sense, TGCCCCCATGTTTGTGATG; GADPH antisense, TGGTGGTGCAGGATGCATT; alkaline phosphatase sense, CCAGCAGGTTTCTCTCTTGG; alkaline phosphatase antisense, CTGGGAGTCTCATCCTGAGC; IL6 sense, AGTTGCCTTCTTGGGACTGA; IL6 antisense, TCCACGATTTCCCAGAGAAC; COLIAI sense, CCTGGTAAAGATGGTGCC; COLI antisense, CACCAGGTTCACCTTTCGCACC; OCN sense, GAACAGACTCCGGCGCTA and OCN antisense, AGGGAGGATCAAGTCCCG. All primers are listed from 5´ to 3´. GADPH was used as a housekeeping gene to normalise the data obtained from the qRT-PCR and calculate the relative fold change between the conditions. qPCR reactions were carried out using SYBR Premix Ex Taq (Tli RNase H Plus) (TAKARA), in a StepOne Plus™ Real-Time PCR System (Applied Biosystems, Foster City, California, USA). The cycling parameters were as follows: an initial denaturation step at 95 ºC for

30 s; followed by 95ºC for 5s and 60 ºC for 34 s for a total of 40 cycles. The final melt curve stage comprised a cycle of 95 ºC for 15 s and 60 ºC for 60 s.

1.7. Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) and to a Newman-Keuls multiple comparison post-test, when appropriate. Differences with $p \leq 0.05$ were considered statistically significant.

1.8. *In vivo* experimentation

The *in vivo* procedures and histological evaluation of the two tested materials, SAE-Ti and 70M30T, were carried out using the previously described methods [9], with the tibia of New Zealand rabbits (*Oryctolagus cuniculus*) as the experimental model. All the experiments were conducted in accordance with the protocols of Ethical Committee of the Valencia Polytechnic University (Spain), the European guidelines and legal conditions in R. D. 223/1988 of March 14th, and the Order of October 13th, 1988 of the Spanish Government on the protection of animals used for experimentation and other scientific purposes. Briefly, the dental implants, supplied by Ilerimplant S.L. (Lleida, Spain), were the Frontier model (3.75-mm diameter and 8-mm length) with SAE surface treatment. Twenty implants were used, 10 uncoated (SAE-Ti) and 10, coated (70M30T). The implantation periods of the experimental model were 2 and 4 weeks. Five rabbits were used for each material; the implants were inserted into the tibiae of the animals. The samples for histological examination were embedded in methyl methacrylate using EXAKT technique (EXAKT Technologies, Inc., Oklahoma, USA). For optical microscopy examination, all the sections were stained using Gomori Trichrome solution.

1.9. Adsorbed protein layer

Both 70M30T-coated and uncoated SAE titanium discs were incubated in a 24-well plate for 180 min in a humidified atmosphere (37 ºC, 5% CO_2), after the addition of 1 mL of human blood serum from male AB plasma (Sigma-Aldrich, St. Louis, MO, USA).

The serum was removed, and, to eliminate the non-adsorbed proteins, the discs were rinsed five times with ddH_2O and once with 100 mM NaCl, 50 mM Tris-HCl, pH 7.0. The adsorbed protein layer was collected by washing the discs in 0.5 M triethylammonium bicarbonate buffer (TEAB) with 4% of sodium dodecyl sulphate and 100 mM dithiothreitol (Sigma-Aldrich). Four independent experiments were carried out for each coating (n = 4); in each experiment, each elution was obtained from the incubation of serum of four discs, for each formulation. The protein content was quantified (Pierce BCA assay kit; Thermo Fisher Scientific), obtaining a value of 51 mg mL^{-1}.

1.10. Proteomic analysis

Proteomic analysis was performed as described by Romero-Gavilán *et al.* [15], with minor variations. Briefly, the eluted protein was resolved in polyacrylamide gels; then, the bands were cut out. Each of the slices was digested with trypsin and loaded onto a nanoACQUITY UPLC system connected online to a SYNAPT G2-Si MS System (Waters, Milford, MA, USA). Each material was analysed in quadruplicate. Differential protein analysis was carried out using Progenesis software (Nonlinear Dynamics, Newcastle, UK) as described before [15]. The functional annotation of the proteins was performed using PANTHER (http://www.pantherdb.org) and DAVID Go annotation programmes (https://david.ncifcrf.gov/).

c. RESULTS

i. Synthesis and physicochemical characterisation

SEM micrographs (**Figure 1**) demonstrated that the sol-gel preparation was carried out correctly and a homogenous coating was obtained. Some morphological differences between the SAE-Ti surfaces and 70M30T coatings were observed; the initial SAE-Ti roughness was diminished after coating. The Ra, measured using a mechanical profilometer, was lower for the coated samples. The Ra for SAE-Ti was 0.98 ± 0.09 µm and for 70M30T sol-gel coating, 0.87 ± 0.13 µm. The sol-gel treatment also caused a decrease in the contact angle. The angle was 79.55 ± 7.51º for SAE-Ti and 50.78 ± 1.82º for 70M30T, showing a significant increase in hydrophilicity after coating.

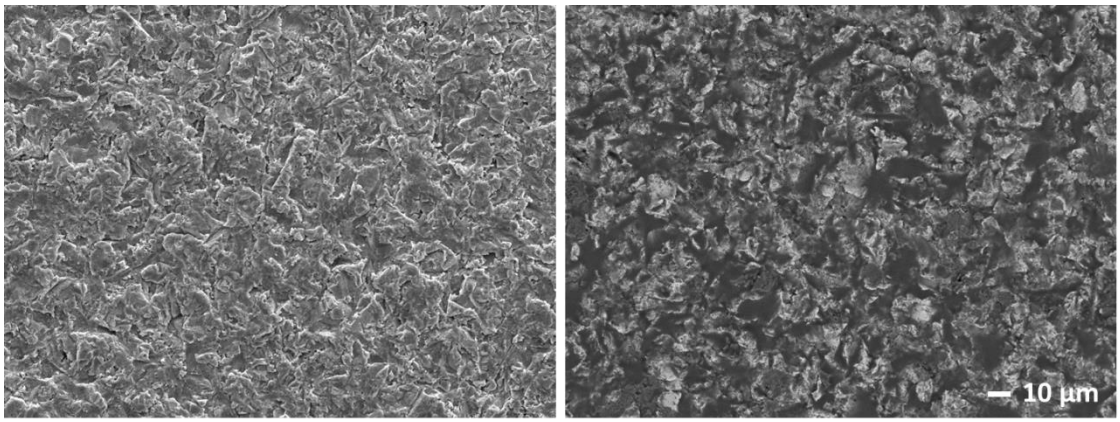


Figure 1. SEM micrographs of SAE-Ti surfaces and 70M30T sol-gel coating. Calibration bar, 10 µm.

ii. *In vitro* assays

1.1. Cytotoxicity, proliferation and ALP activity

Neither of the tested materials was cytotoxic (data not shown). Cell proliferation results did not show significant differences between the tested materials (**Figure 2A**). There were no differences between ALP activities for the two materials after 7 and 14 days of incubation (**Figure 2B**).

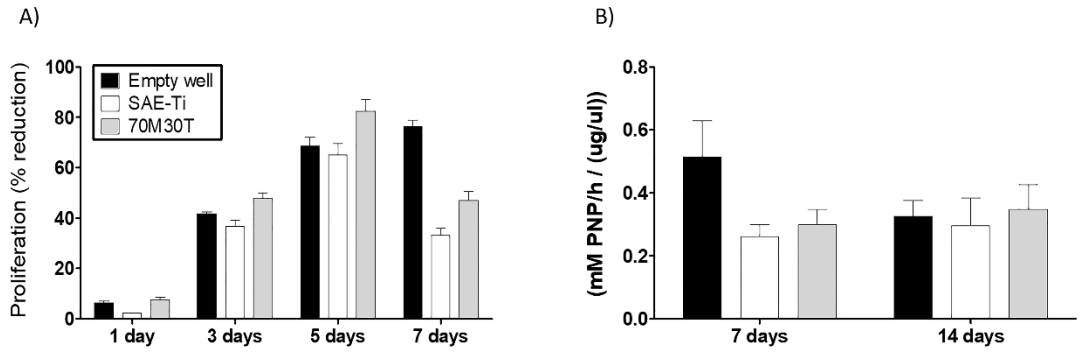


Figure 2. MC3T3-E1 *in vitro* assays: A) MC3T3-E1 cell proliferation after 1, 3, 5 and 7 days of incubation with SAE-Ti (white bar) and 70M30T (grey bar) materials. B) ALP activity (mM PNP h^{-1}) normalised to the amount of total protein (µg μL^{-1}) levels in the MC3T3-E1 cells cultivated on SAE-Ti (white bar) and 70M30T formulation (grey bar). Cells incubated without discs were used as a positive control (black bar).

1.2. mRNA expression levels

The mRNA expression levels for ALP and IL-6 genes show a distinctive and significant response of the osteoblasts to the coating. After 14 days of culture, the expression of these genes was substantially higher for the cells grown on the coated surfaces than on the non-coated titanium (**Figure 3 a** and **b**). These results suggest an enhanced cell mineralisation when cultured on the coated implants [16,17].

COL I expression was similar for the two materials throughout the experiment. After 7 days of culture, the OCN expression levels were significantly higher for the non-coated titanium than for the coated surfaces (**Figure 3 c** and **d**). OCN is a pre-osteoblastic marker; its diminished expression levels on the 70M30T surfaces supports the hypothesis that the sol-gel material accelerates the osteogenesis processes [18].

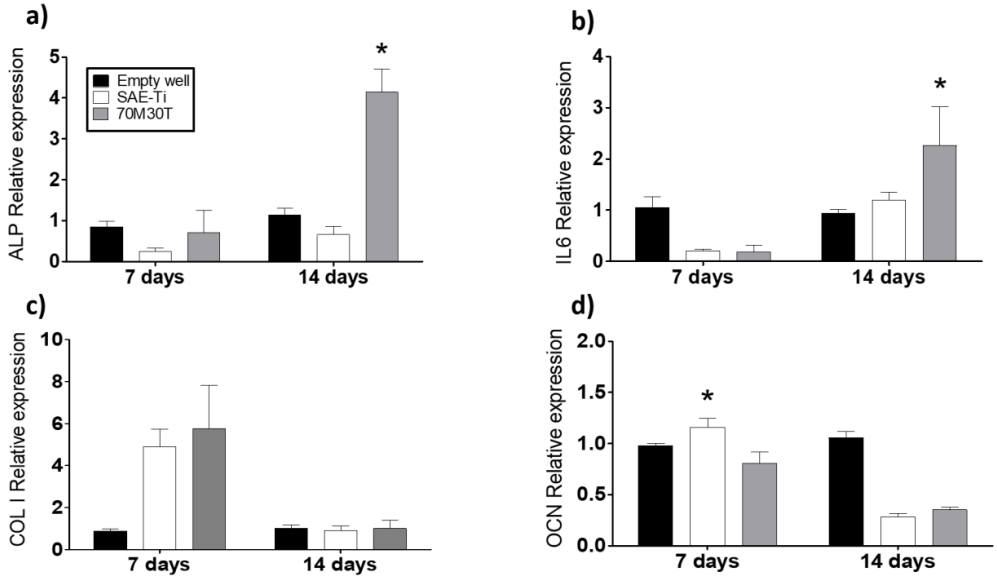


Figure 3. Gene expression of osteogenic markers (a) ALP, (b) IL6, (c) COL I and (d) OCN in MC3T3-E1 osteoblastic cells cultured on SAE-Ti (white bar) and 70M30T (grey bar). The relative mRNA expression was determined by RT-PCR after 7 and 14 days of culture. Statistical analysis was performed using one-way ANOVA with a Kruskal-Wallis post-test (* $p \leq 0.05$).

iii. *In vivo* assays

The results of *in vivo* experiments showed similar regeneration behaviours for the two implants tested. SAE-Ti implants displayed, as expected, good osteointegration after 2 and 4 weeks (**Figure 4**). In some of the roots of the threads of the 70M30T-coated implants, an unstained material corresponding to the remaining sol-gel coating was observed. The 70M30T implants also showed good osteointegrative properties, and qualitatively, the osteogenic activity seemed higher than on the SAE-Ti surfaces. As shown in **Figure 5**, 70M30T-coated surface induced the growth of new bone tissue spicules from the cortical region into the medullary cavity.

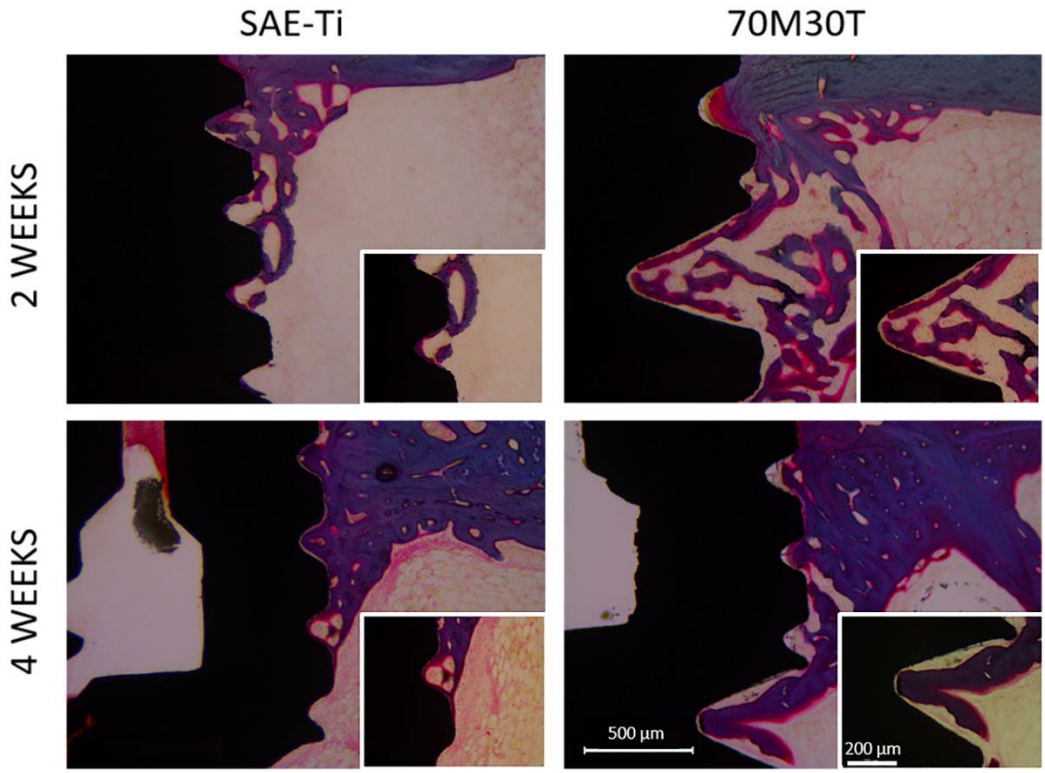


Figure 4. Microphotographs of samples of SAE-Ti and 70M30T implants. The main panels show 4× magnification images of regions close to the cortical bone (up) and the bone marrow cavity (down). In the inserts (lower-right corners), 10× images of the same regions are shown.

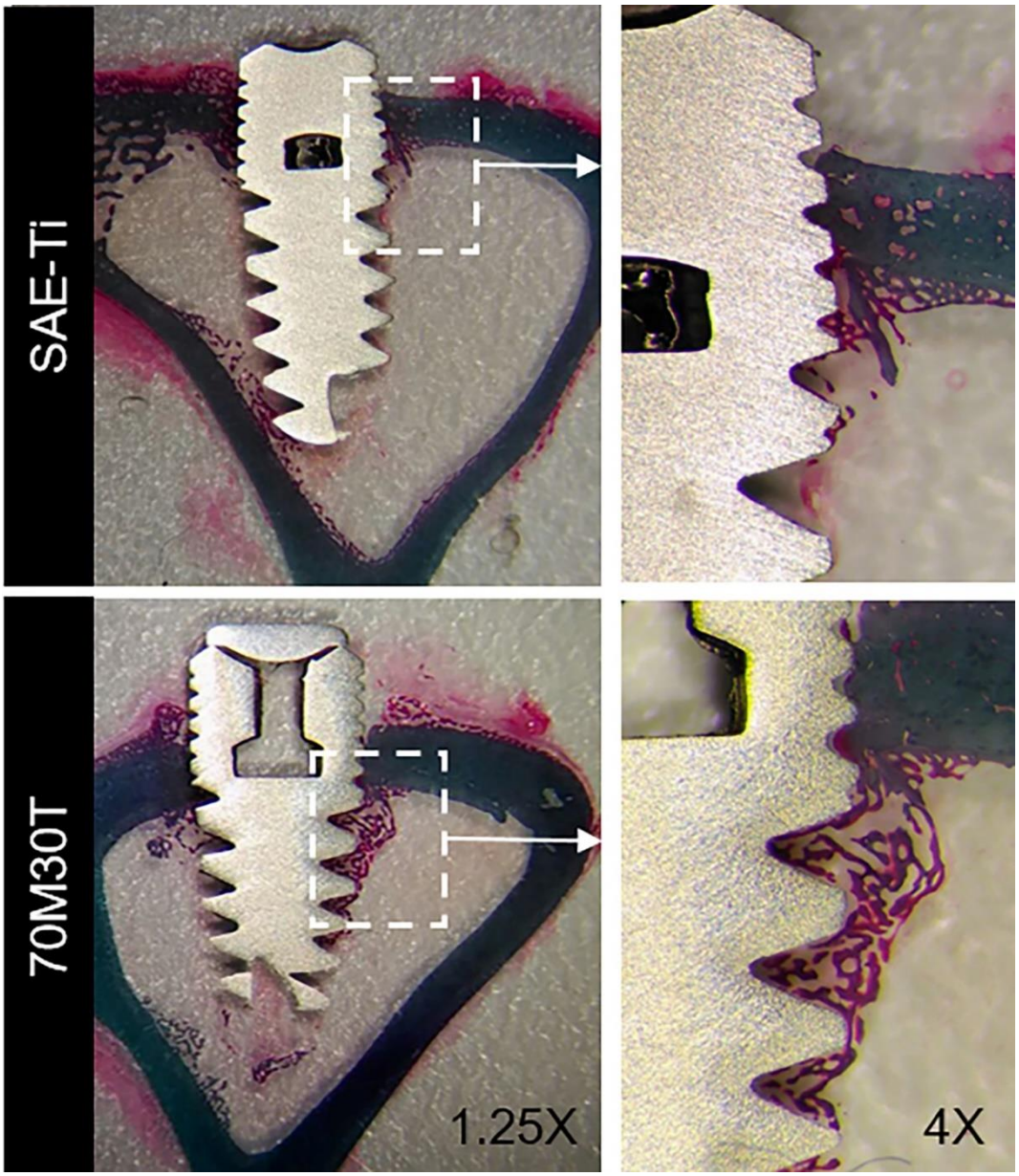


Figure 5. Bone tissue growth 4 weeks after implantation. Panoramic (left) and detailed (right) microphotographs of SAE-Ti and 70M30T implants show the bone tissue generated around the implant surface.

iv. Proteomic analysis

The LC-MS/MS analysis identified 113 proteins. The statistical comparison of the results obtained for SAE-Ti and 70M30T materials was carried out using the Progenesis QI software. The DAVID and PANTHER programmes were employed to classify the detected proteins according to their function.

The comparison between the proteins identified on the two tested materials revealed that only 1 protein preferentially adsorbed onto the 70M30T surface (CLUS, classified as a glycoprotein by DAVID). However, 31 proteins favoured the SAE-Ti (**Table I**). Within this group, a large number of lipoproteins were found, such as apolipoproteins APOA2, APOA5, APOC1, APOC3, APOC4, APOE, APOL1 and the SAA4, a high-density lipoprotein particle. Several proteins associated with blood coagulation functions were also identified. The coagulation factors FA5, FA10 and FA11, THRB, ANT3, PLMN, PROC and PROS belong to this set of proteins. Moreover, DAVID listed HRG, PLMN, PROS, THRB and KLKB1 as proteins involved in fibrinolysis process. TETN, SEPP1, PF4V and VTNC were classified as glycoproteins. Intracellular proteins such as keratins K2C1B and K2C4 were also found.

Table I. Proteins differentially attached to 70M30T and Ti (Progenesis analysis). ANOVA (p-value < 0.05) (Table continues next page)

			Normalized abundance			
Description	**Accession**	**Confidence score**	**Anova (p)**	**Average 70M30T**	**Average Ti**	**Ratio Ti/70M30T**
Clusterin	CLUS_HUMAN	596,70	3,95E-03	7,50E+05	4,89E+05	0,65
Keratin, type II cytoskeletal 1b	K2C1B_HUMAN	292,46	7,61E-03	4,81E+03	6,41E+03	1,33
Apolipoprotein C-III	APOC3_HUMAN	212,82	4,42E-02	4,51E+05	7,69E+05	1,71
Apolipoprotein L1	APOL1_HUMAN	235,45	8,28E-04	9,45E+04	1,64E+05	1,73
Plasminogen	PLMN_HUMAN	781,12	1,62E-02	2,36E+05	4,32E+05	1,83
Ig lambda chain V-III region SH	LV301_HUMAN	120,13	1,25E-02	3,67E+04	7,51E+04	2,05
Coagulation factor V	FA5_HUMAN	192,62	2,04E-05	9,83E+03	2,04E+04	2,08
Apolipoprotein A-V	APOA5_HUMAN	194,49	1,14E-03	8,91E+03	2,41E+04	2,70
Vitamin K-dependent protein S	PROS_HUMAN	78,23	9,83E-03	4,21E+03	1,20E+04	2,85
Ig kappa chain V-III region SIE	KV302_HUMAN	153,44	5,26E-03	8,45E+04	2,56E+05	3,03
Plasma kallikrein	KLKB1_HUMAN	86,19	1,60E-04	2,91E+03	9,45E+03	3,24
Tetranectin	TETN_HUMAN	206,13	3,01E-03	8,11E+03	2,72E+04	3,35
Selenoprotein P	SEPP1_HUMAN	208,07	2,92E-05	1,94E+04	6,99E+04	3,60
Apolipoprotein A-II	APOA2_HUMAN	144,49	1,00E-03	6,31E+04	2,77E+05	4,39
Antithrombin-III	ANT3_HUMAN	488,69	3,49E-04	6,55E+04	3,14E+05	4,80
Apolipoprotein E	APOE_HUMAN	1831,27	5,56E-07	1,82E+06	9,32E+06	5,11
Prothrombin	THRB_HUMAN	369,68	9,15E-04	4,62E+04	2,77E+05	5,99
Keratin, type II cytoskeletal 4	K2C4_HUMAN	155,48	1,60E-02	8,74E+02	5,76E+03	6,59
Serum amyloid A-4 protein	SAA4_HUMAN	135,14	7,04E-04	4,94E+04	3,45E+05	6,98
Kininogen-1	KNG1_HUMAN	350,85	6,48E-06	5,49E+04	4,16E+05	7,59
Coagulation factor XI	FA11_HUMAN	296,94	1,56E-05	1,78E+04	1,96E+05	11,00
Platelet basic protein	CXCL7_HUMAN	82,00	1,28E-06	2,48E+03	2,90E+04	11,70
Apolipoprotein C-I	APOC1_HUMAN	185,56	9,22E-08	2,51E+05	2,97E+06	11,81
Apolipoprotein C-IV	APOC4_HUMAN	96,41	3,11E-06	6,24E+03	7,86E+04	12,60
Creatine kinase M-type	KCRM_HUMAN	146,67	2,33E-05	9,78E+02	2,26E+04	23,08
Histidine-rich glycoprotein	HRG_HUMAN	527,94	4,86E-07	3,90E+04	9,09E+05	23,33
Coagulation factor X	FA10_HUMAN	81,54	5,51E-06	8,72E+02	2,31E+04	26,53

Description	Accession	Confidence score	Anova (p)	Average 70M30T	Average Ti	Ratio Ti/70M30T
Vitamin K-dependent protein C	PROC_HUMAN	70,62	1,74E-03	1,90E+02	5,96E+03	31,28
Vitronectin	VTNC_HUMAN	307,15	3,10E-03	7,52E+04	2,99E+06	39,76
Platelet factor 4 variant	PF4V_HUMAN	117,94	2,64E-05	3,19E+02	2,68E+05	840,27

The PANTHER pie chart in **Figure 6** shows the functional classification of the proteins adhering more to the SAE-Ti than to 70M30T surface. The most common biological functions were related to cellular processes (19%), biological regulation (14%) and response to stimulus (14%). Notably, a proportion of associated functions were represented by the immune system processes (3%). Among the proteins linked to various pathway processes, 84% were associated with blood coagulation. Two small groups of proteins (8%) were linked to the plasminogen activating cascade and inflammation.

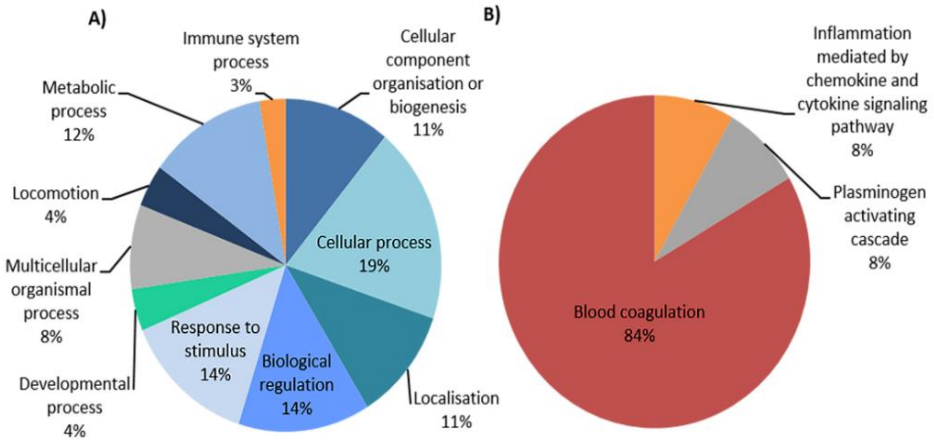


Figure 6. PANTHER pie charts with the biological process (A) and pathway (B) functions for the proteins adhering predominantly to SAE-Ti in comparison with 70M30T surfaces.

d. DISCUSSION

In the recent years, improving the bioactivity of materials has become an utmost standard field of study for biomaterial studies, in particular in the field of dental science. The successful incorporation of an implant into a living organism involves a series of unknown biological mechanisms, including processes like coagulation and immune response, leading to a desired correct bone regeneration [19]. These processes are triggered by the first layer of proteins adsorbed on the biomaterial surfaces, conditioning and determining cell behaviour during the post-implantation recovery [14]. After a surgical procedure, there are some immediate interactions between those proteins and the biomaterial. The extent and type of these interactions largely depend, apart from the biochemistry of the organism, on the physical and chemical surface characteristics of the implant. These characteristics often determine a specific type, quantity and conformation of the proteins attaching to the implant surface via competitive displacement, known as the Vroman effect [20]. Hence, this experimental work focused on the interactions between the implant surface and serum proteins.

The intrinsic physico-chemical characteristics of the tested surfaces can condition the cellular behaviour and, consequently, modulate the adaptation to the implanted foreign body. The analysis of the materials examined here showed clear differences between their chemical composition and physico-chemical properties (hydrophilicity, topography and contact angle).

These differences were not unexpected given the characteristic, distinct cell behaviour in response to each surface type. The 70M30T coating triggered stronger cell responses, particularly noticeable on the mRNA expression levels. The expression of ALP and IL-6, the major biomarkers of osteogenic differentiation [16, 17], was enhanced in the cells exposed to the coated surface in comparison with the uncoated SAE-Ti surfaces. The difference was sufficiently significant to infer that this coating affected the cell behaviour.

However, in the *in vivo* experiments, these differences were not so clear-cut (**Figure 4**). These results suggest that the processes underlying the osseointegration for these two surface types are distinct or regulated by different mechanisms (**Figure 5**).

In the case of SAE-Ti, the regeneration process might be based on osteoconduction, while the bone repair achieved using the 70M30T implants might be primarily based on osteoinduction. The differences between the compositions of the respective protein layers suggest a correlation between the attached proteins and the particular mechanism of bone regeneration. The examination of the proteins adsorbed onto different surfaces might help to find this correlation.

Only one protein, CLUS, was adsorbed preferentially to the 70M30T-coated surface. This protein has been associated with several functions. Among those, there are some processes with a role in inflammation and immunity, such as the regulatory activity of complements [21]. In contrast, 31 proteins were significantly and predominantly attached to the SAE-Ti surface. A substantial number of apolipoproteins were part of this group. Apolipoproteins, besides their function in lipid metabolism, might prevent the initiation of innate immunity [22]. Immunoglobulins LV301 and KV302 were two of the characteristic proteins attached to the titanium surface; this could be related to the immune system process functions (3%) shown in **Figure 6**. Although the proteomic study was carried out using human serum, some intracellular proteins such as keratins were obtained during the elution. The presence of these molecules could be an artefact of the industrial process used to purify the serum. According to DAVID classification, some of the proteins with more affinity to SAE-Ti than to 70M30T (HRG, PLMN, PROS, THRB and KLKB1) are involved in the fibrinolysis process. These proteins, as well as FA5, FA10, FA11, PROC, ANT3 and KNG1, are also related to the coagulation system. The PANTHER classification (pathway functions) indicates that 84% of the proteins preferentially adhering to SAE-Ti are associated with the blood coagulation (**Figure 6**). Both fibrinolysis and coagulation are the processes necessary to achieve a correct bone tissue repair [23]. Among the SAE-Ti-associated proteins, FA5, FA10, FA11 and THBR might play a role in blood coagulation pathway, promoting blood clotting. ANT III, PROC and PROS are involved in the regulation of this pathway [24]. PLMN has an important role in the plasminogen system activation, the key step in the fibrinolysis process. After trauma, the coagulation system is one of the main initiators of the development of blood clots. Following this event, the plasminogen system acts during the extracellular matrix degradation and the consequent tissue remodelling and angiogenesis, leading to correct tissue healing [25]. The HRG protein is associated with blood coagulation, fibrinolysis and innate immune systems. It could function as both anticoagulant and antifibrinolytic modulator and might regulate platelet function *in vivo* [26]. KLKB1 is involved in the

regulation of multiple proteolytic cascades, such as the intrinsic pathway of coagulation, as well as the fibrinolytic system and the complement pathways [27]. In addition, KLKB1, in association with FA12 and high-molecular-weight KNG1, forms the kinin–kallikrein surface-activated coagulation system [28].

Some of the SAE-Ti-attached proteins, such as VTNC, TETN and APOE, might have osteogenic activity. APOE has a role in the vitamin K uptake into osteoblasts [29]. VTNC could promote the human osteoblast attachment and proliferation on the Ti implants, accelerating the osseointegration process [30]. The TETN protein has been linked to correct bone tissue development; TETN-knock-out mice have kyphosis and show the symptoms of osteoporosis [31].

On the basis of the available data and the *in vitro* and *in vivo* results obtained here, it is tempting to suggest a relationship between the type and function of these proteins and different mechanisms of osseointegration *in vivo*. The 70M30T material, releasing Si compounds into the implant surroundings might stimulate the undifferentiated osteoblasts, leading to the formation of bone tissue [32, 33]. This would represent a case of osteoinduction in the tissue surrounding the implant; such assumption is supported by the overexpression of osteogenesis-related genes, ALP and IL-6, demonstrated here. This mechanism could not be proven by the proteomic analysis of the eluate from the implant surface.

However, the predominance of coagulation- and fibrinolysis-related proteins adsorbed onto the SAE-Ti surface could indicate an ongoing osteoconduction process with the participation of key proteins such as PLMN and VTNC. This result illustrates the validity of the proteomic analysis, reflecting the *in vivo* outcome. Moreover, the coagulation might be the result of the kallikrein-kinin system activation. This is consistent with the hypothesis that the coagulation and, consequently, the regeneration, spreading from the titanium surface to the medullar area of the implanted bone, are based on an osteoconductive process.

e. CONCLUSIONS

Two different dental implants, sandblasted acid-etched titanium (SAE-Ti) and a silica sol-gel hybrid coating (70M30T), were characterised. The results suggest two different mechanisms of bone regeneration. The SAE-Ti surfaces display osteoconductive properties. However, the *in vivo* results for silica sol-gel implants suggest osteoinductive behaviour. These results were confirmed by *in vitro* testing. The 70M30T coating displayed strong cell activation properties. The mRNA expression levels for ALP and IL-6, important biomarkers of osteogenic differentiation, were higher for 70M30T than for SAE-Ti surfaces. The results of proteomic analysis could explain some differences observed in bone healing. In particular, the effect of surface properties on cell behaviour could shed some light on the osteoconduction phenomenon. It is tempting to infer that certain proteins related to coagulation processes take part in the initial regenerative events on the biomaterial surface.

f. ACKNOWLEDGEMENTS

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6. Chapter 3

CHAPTER 3

Silica-gelatin hybrid sol-gel coatings: A proteomic study with biocompatibility implications.

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ABSTRACT

Osseointegration, including the foreign body reaction to biomaterials, is an immune-modulated, multifactorial, and complex healing process in which various cells and mediators are involved. The buildup of the osseointegration process is immunological and inflammation-driven, often triggered by the adsorption of proteins on the surfaces of the biomaterials and complement activation.

New strategies for improving osseointegration use coatings as vehicles for osteogenic biomolecules delivery from implants. Natural polymers, such as gelatin, can mimic collagen I and enhance the biocompatibility of a material. In this experimental study, two different base sol-gel formulations and their combination with gelatin, were applied as coatings on sandblasted, acid-etched titanium (SAE-Ti) substrates and their biological potential as osteogenic biomaterials was tested. We examined the proteins adsorbed onto each surface and their *in vitro* and *in vivo* effects. *In vitro* results showed an improvement in cell proliferation and mineralization in gelatin-containing samples. *In vivo* testing showed the presence of a looser connective tissue layer in those coatings with substantially more complement activation proteins adsorbed, especially those containing gelatin. Vitronectin and FETUA, proteins associated with mineralization process, were significantly more adsorbed in gelatin coatings.

Keywords:

Dental implants; biocompatibility; biomaterial, immunology; complement pathway; bone regenerati

a. INTRODUCTION

The regenerative processes in the bone entail responses to continuous biological challenges. The sequence of bone induction and conduction events, involving various types of cells and signaling pathways in a determined order, is necessary to achieve an ideal regeneration [1]. The implants used in the field of bone regeneration have been continuously studied and optimized since the last century. The results of implantation depend largely on the deposition of signaling proteins onto the surface of a biomaterial and will define the magnitude and type of the reaction (specially inflammatory, immune, and coagulation) of the host to the foreign body implantation [2].

The complement cascade is involved in a variety of physiological and pathophysiological processes, apart from its role as an immune effector. This cascade also regulates the cellular turnover, healing, proliferation, and regeneration [3]. The disproportionate long-term effects are generally interpreted as implant rejection events. These responses involve mostly uncontrolled blood coagulation processes, the development of infection, and the formation of immune structures (*e.g.* fibrous capsule) surrounding the foreign body and infected or damaged tissue.

Biomaterials are manufactured and tested to improve the life quality of the patient by minimizing the impact of the implanted foreign body and achieving the recovery in the shortest time possible [4,5]. Assessing the viability of biomaterials involves a battery of extensive tests before the final product can be released to the market; these tests normally entail both *in vitro* and *in vivo* procedures. *In vivo* testing is the ideal standard in new biomaterial trials as it examines their effects on a living organism. However, *in vitro* testing is the first and necessary step to exclude the immediate damage to the organism; it can also help to avoid the ethical problems and minimize the costs. However, the *in vivo* results often do not reflect the *in vitro* outcomes. This problem is invariably emphasized by the experts in the field of regenerative bone engineering. Thus, new approaches and tools are needed to avoid detrimental side effect and predict the efficacy of biomaterials [6].

Silica sol-gel hybrid materials are often used in biomedical applications due to the relative ease of controlling their degradation kinetics and the network pore size. These materials degrade by releasing silicon compounds in the $Si(OH)_4$ form to the surrounding microenvironment, providing

a good osteogenic setting for the new bone tissue formation [7–9]. Moreover, the process itself results in a good grade of purity at low temperatures. Gelatin is occasionally embedded in the surface of the biomaterial to favour biocompatibility, cell adhesion, proliferation and differentiation [10,11] because it can mimic the chemical and biological functions of collagen I in a living organism [12].

In the silicon networks, gelatin can be effectively crosslinked with an inorganic sol-gel alkoxysilane matrix without losing its osteogenic properties [13]. This is useful for controlling the degradation rate, and gelatin can be used as a therapeutic agent in the matrix under mild conditions [14,15]. Different gelatin-silica composites have been developed and studied, and good biocompatibility of these systems has been demonstrated [16–19]. Lei et al. have set silica-gelatin hybrid implants using 3-glycidoxypropyl-trimethoxysilane (GPTMS) and tetraethoxysilane (TEOS) alkoxysilanes as precursors. Biological assays have shown that these materials have good biocompatibility and they enhance cell proliferation [16,17].

In a previous work sol-gel compositions with gelatine physically or chemically entrapped were sinthetized, achieving different physico-chemical properties [20]. The protein adsorption onto these gelatin-silica networks was studied with quartz crystal microbalance using monoprotein solutions and distinct affinities were detected when the gelatin was present [21].

In this experimental study, two sol-gel coating bases and their silicon-gelatin correspondents were applied as coatings on sandblasted, acid-etched titanium disc substrates/implants (SAE-Ti), and their biological potential as biomaterials was tested. The compositions with gelatin and their respective base materials were incubated with human serum, simulating a more real setup. Their effects on the adsorbed protein layer using mass spectrometry (LC-MS/MS) were examined, and the *in vitro* and *in vivo* outcomes were studied.

b. MATERIALS AND METHODS

i. Titanium discs

Ti discs (12 mm in diameter, 1-mm thick) were made from a bar of commercially available, pure, grade-4 Ti (Ilerimplant S.L., Lleida, Spain). To obtain the sandblasted, acid-etched (SAE) Ti, the discs were abraded with 4-µm aluminum oxide particles and acid-etched by submersion in sulfuric acid for 1 h to simulate a moderately rough implant surface. Discs were then washed in acetone, ethanol, and 18.2-Ω purified water (for 20 min in each liquid) in an ultrasonic bath and dried under vacuum. Finally, all Ti discs were sterilized using UV radiation.

ii. Sol-gel synthesis and sample preparation

The silica-gelatin hybrid coatings were obtained through the sol-gel route. The precursor alkoxysilanes used were methyltrimethoxysilane (MTMOS), 3-(glycidoxypropyl)-trimethoxysilane (GPTMS) and tetraethyl orthosilicate (TEOS) (Sigma-Aldrich, St. Louis, MO, USA). Four different compositions were synthesized. We obtained two silica materials with molar percentages of 70% MTMOS and 30% TEOS (70M30T) and 35% MTMOS, 35% GPTMS, and 30% TEOS (35M35G30T). Their respective composites (70M30T-GEL and 35M35G30T-GEL) were made with 0.9% (weight relative to the amount of alkoxysilane) of gelatin from porcine skin (Sigma-Aldrich, St. Louis, MO, USA). The two gelatin-free compositions were synthesized using 2-Propanol (Sigma-Aldrich, St. Louis, MO, USA) as solvent at a volume ratio (alcohol: siloxane) of 1:1. Hydrolysis of alkoxysilanes was carried out by adding (at a rate of 1 drop s^{-1}) the corresponding stoichiometric amount of 0.1M HNO_3 acid aqueous solution (Panreac, Barcelona, Spain). The solution was kept for 1 h under stirring and then 1 h at rest. The materials with gelatin were prepared using a mixture of 50% 2-Propanol and 50% distilled water as a solvent at a volume ratio (solvent: siloxane) of 1:1. After adding the alkoxysilane precursors, the hydrolysis was carried out by adding (at 1 drop s^{-1}) the stoichiometric amount of 0.1M HCl acidified aqueous solution (Panreac, Barcelona, Spain) with the dissolved gelatin. The solution was kept 1 h under stirring and then 1 h at rest, at 37 ºC. The samples were prepared immediately afterward. SAE-titanium was used as a substrate. The coating was

performed employing a dip coater (KSV instrument-KSV DC). Discs and implants were immersed in the corresponding sol-gel solution at a speed of 60 cm min^{-1}, left immersed for one minute, and removed at a 100 cm min^{-1}. Finally, the samples were cured for 2 h at 80 ºC.

iii. Physico-chemical characterisation of the coated titanium discs

A mechanical profilometer Dektak 6M (Veeco, NY, USA) was used to determine the roughness. Two coated discs of each composition were tested. Three measurements were performed for each disc to obtain the average values of the Ra parameter. The contact angle was measured using an automatic contact angle meter OCA 20 (Dataphysics Instruments, Filderstadt, Germany). Ten µL of ultrapure water W04 were deposited on the sol-gel coated surface at a dosing rate of 27.5 µL s^{-1} at room temperature. Contact angles were determined using SCA 20 software. Five discs of each material were studied, after depositing two drops on each disc. The surface topography of the coatings was characterized using scanning electron microscopy (SEM) employing the Leica-Zeiss LEO equipment under vacuum (Leica, Wetzlar, Germany). Platinum sputtering was applied to make the samples more conductive for the SEM observations.

iv. *In vitro* assays

MC3T3-E1 (mouse calvaria osteosarcoma cell line) cells were cultured on the sol-gel coated titanium discs at a concentration of 1×10^4 cells/well, in Dulbecco's modified Eagle's medium (DMEM) with phenol red (Gibco-Life Technologies, Grand Island, NY, USA), 1 % 100× penicillin/streptomycin (Biowest Inc., Riverside, KS, USA), and 10 % fetal bovine serum (FBS) (Gibco-Life Technologies, Grand Island, NY, USA). After incubation for 24 hours at 37 ºC in a humidified (95 %) atmosphere of 5 % CO_2, the medium was replaced with an osteogenic medium composed of DMEM with phenol red 1×, 1 % penicillin/streptomycin, 10 % FBS, 1 % ascorbic acid (5 mg mL^{-1}), and 0.21 % β-glycerol phosphate, and incubated again under the same conditions. The

culture medium was changed every 48 hours. In each plate, a well with cells at the same concentration (1×10^4 cells) was used as a control of culture conditions.

The biomaterial cytotoxicity was assessed following the ISO 10993-5 norm, measured by spectrophotometry, by contact of the material extract with the cell line. The 96-Cell Titter Proliferation Assay (Promega®, Madison, WI, USA) was employed to measure the cell viability after 24-h incubation of the cells with the extract. We used one negative control (empty cell well) and a positive control with latex, known to be toxic to the cells. Seventy-percent cell viability was the limit below which a biomaterial was considered cytotoxic.

For measuring cell proliferation, the commercial cell viability assay alamar Blue® (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) was used. This kit measures the cell viability on the basis of a redox reaction with resazurin. The cells were cultured in wells with the discs (3 replicates per treatment) and examined following the manufacturer's protocol after 4 days, 8 days, and 14 days. The percentage of reduced resazurin was used to evaluate cell proliferation.

To obtain the samples for total protein measurement (BCA) and ALP activity, the culture medium was removed from the wells, the wells were washed three times with 1 × DPBS, and 100 µL of lysis buffer (0. 2 % Triton X-100, 10 mM Tris-HCl, pH 7.2) were added to each well, obtaining the cell lysate. After being kept on ice for 10 min, the lysate was sonicated and centrifuged for 7 min at 13,300 rpm and the supernatant was used to measure the total protein content and the ALP activity. Each sample was pipetted in triplicate (5 µL per well).

The total protein content was calculated from a standard curve for bovine albumin and expressed as $\mu g\ \mu L^{-1}$, following the manufacturer's instructions, using the colorimetric measurement of BCA at 570 nm on a microplate reader Multiskan FC® (Thermo Scientific®).

The conversion of p-nitrophenylphosphate (p-NPP) to p-nitrophenol was used to assess the alkaline phosphatase (ALP) activity. Sample Aliquots of 0.1 mL were used to carry out the assay. One hundred µL of p-NPP (1 mg mL^{-1}) in substrate buffer (50 mM glycine, 1mM $MgCl_2$, pH 10.5) was added to the 100 µL of the supernatant obtained from the lysate. After two hours of incubation in the dark (37 ºC, 5 % CO_2), absorbance was measured using a microplate reader at a wavelength of 405 nm. ALP activity was obtained from a standard curve obtained using different solutions of p-nitrophenol and 0.02 mM sodium hydroxide. Results were presented as mmol of p-

nitrophenol/hour (mmol PNP h^{-1}), and data were expressed as ALP activity normalized by the total protein content (μg μL^{-1}) obtained using Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) after 7 and 14 days.

ii. Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) and to a Newman-Keuls multiple comparison post-test, when appropriate. Differences with $p \leq 0.05$ were considered statistically significant.

iii. *In vivo* experimentation

To assess the *in vivo* behaviour to the selected coatings, coated dental implants were surgically placed in the tibia of New Zealand rabbits (*Oryctolagus cunicu*lus). This implantation model is widely used to study the osseointegration of dental implants [22]. All the experiments were conducted in accordance with the protocols of Ethical Committee of the Valencia Polytechnique University (Spain), the European guidelines and legal conditions laid in R. D. 223/1988 of March 14th, and the Order of October 13rd, 1988 of the Spanish Government on the protection of animals used for experimentation and other scientific purposes. The rabbits were kept under 12-h span darkness-light cycle; room temperature was set at 20.5 ± 0.5 °C, and the relative humidity ranged between 45 and 65 %. The animals were individually caged and fed a standard diet and filtered water *ad libitum*. Dental implants were supplied by Ilerimplant S.L. (Lleida, Spain). They were the internal-connection dental implants, made with titanium grade 4, (trademark GMI), of 3.75-mm diameter and 8-mm length. We used the Frontier model, with SAE surface treatment. Overall, 40 implants were used, 20 uncoated (control) and 5 coated (test samples) with each material. The control and test samples were implanted under the same conditions.

We used 20 rabbits, 5 for each material, with weights between 2000 and 3000 g, of the age near the physical closure (indicative of an adequate bone volume). The implantation period for the experimental model was 2 weeks. Implants were inserted in both left and right proximal tibiae, each animal receiving two implants (one control sample and one test sample). Animals were sedated (chlorpromazine hydrochloride) and prepared for surgery, and then anesthetized (ketamine hydrochloride). A coetaneous incision was made in the implantation site in the proximal tibia. The periosteum was removed, and the osteotomy was performed using a low revolution micromotor and drills of successive diameters of 2, 2.8, and 3.2 mm, with continuous irrigation. Implants were placed by press-fit, and surgical wound was sutured by tissue planes, washed with saline and covered with plastic spray dressing (Nobecutan, Inibsa Laboratories, Barcelona, Spain). After each implantation period, the animal was euthanized by carbon monoxide inhalation, and the implant screws were retrieved to study the surrounding tissues.

Samples for histological examination were processed following the method described by Peris *et al.* [23]. Briefly, the samples were embedded in methyl methacrylate, and 25–30 μm thick sections were obtained using EXAKTtechnique (EXAKT Technologies, Inc., Oklahoma, USA). For optical microscopy examination, all the sections were stained using Gomori Trichrome solution.

iv. Adsorbed protein layer

Sol-gel coated titanium discs were incubated in a 24-well plate for 180 min in a humidified atmosphere (37 ºC, 5 % CO_2), after the addition of 2 mL of human blood serum from male AB plasma (Sigma-Aldrich, St. Louis, MO, USA).

The serum was removed, and, to eliminate the non-adsorbed proteins, the discs were rinsed five times with ddH_2O and once with 100 mM NaCl, 50 mM Tris-HCl, pH 7.0. The adsorbed protein layer was collected by washing the discs in 0.5 M Triethylammonium bicarbonate buffer (TEAB) with 4 % of sodium dodecyl sulfate (SDS) and 100 mM of Dithiothreitol (DTT). The experimental method was adopted from a study by Kaneko et al. [24]. Four replicates for each biomaterial were obtained. The total protein content of the serum employed to this study was quantified before the experiment (Pierce BCA assay kit; Thermo Fisher Scientific, Waltham, MA, USA), obtaining a value of 51 mg mL^{-1}.

v. Protein analysis

Proteomic analysis was performed as described by Romero-Gavilán et al. [25], with minor variations. Briefly, the same amount of sample (2/10 of the eluted material) was loaded in each lane for the same experimental conditions. The eluted protein was resolved in polyacrylamide gels; the gels were cut into slices. Each of these slices was digested with trypsin and loaded onto a nanoACQUITY UPLC system connected online to a SYNAPT G2-Si MS System (Waters, Milford, MA, USA). Differential protein analysis was carried out using Progenesis software (Nonlinear Dynamics, Newcastle, UK) as described before [25], and the functional annotation of the proteins was performed using PANTHER (www.pantherdb.org/) and DAVID Go annotation programs (https://david.ncifcrf.gov/). Uniprot (http://www.uniprot.org/) nomenclature was adopted to name the proteins without the ending "_HUMAN".

c. RESULTS

i. Synthesis and physicochemical characterisation

Our chosen synthesis parameters allowed us to obtain different materials, all with homogenous surfaces, as can be seen in the SEM micrographs (**Figure 1**). The 70M30T coating had different morphology in comparison with 35M35G30T. However, no differences in morphology were detected when gelatin was incorporated in the networks. This agrees with the data obtained using the mechanical profilometer. 70M30T and 70M30T-GEL materials had an average surface roughness (Ra) of 0.77 ± 0.13 and 0.79 ± 0.07 µm, respectively. The compositions 35M35G30T and 35M35G30T-GEL exhibited lower roughness (Ra of 0.51 ± 0.14 µm and 0.58 ± 0.15 µm, respectively).

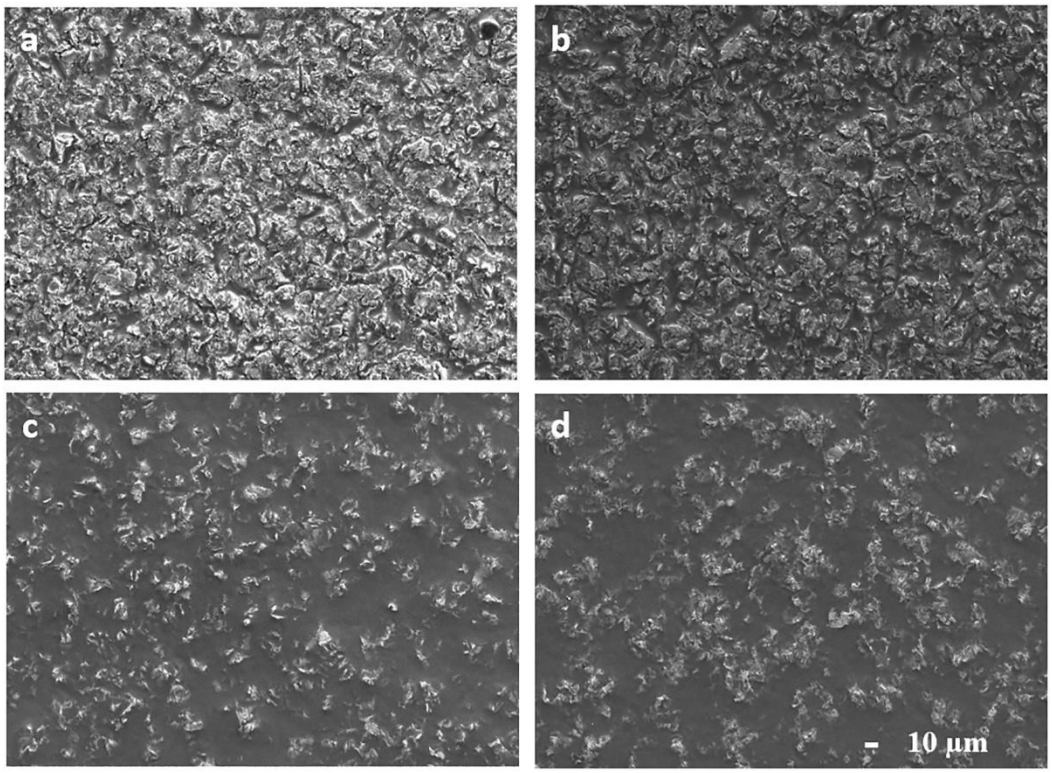

Figure 1. Scanning electron microscopy images of hybrid sol–gel coatings onto titanium discs: 70M30T (a), 70M30T-GEL (b), 35M35G30T (c), and 35M35G30T-GEL (d). Calibration bar 10 µm.

The contact angle measurements (**Figure 2**) gave similar values for 70M30T and 35M35G30T coatings. However, the addition of gelatin caused a decrease in wettability on both materials.

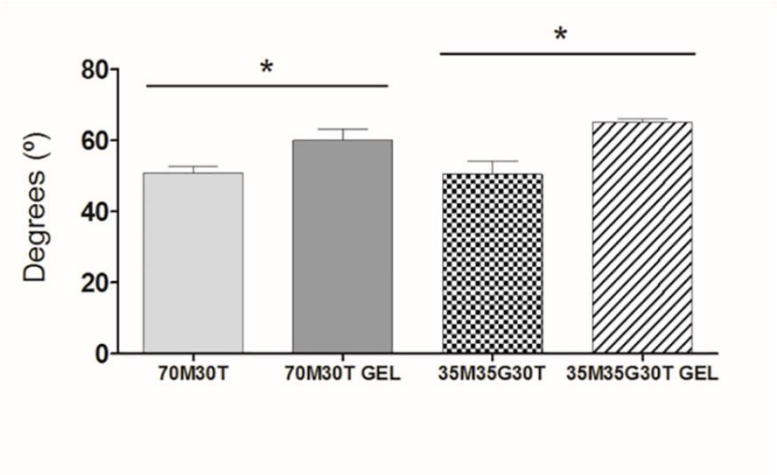

Figure 2. Contact angle measurements of 70M30T, 70M30T-GEL, 35M35G30T, and 35M35G30T-GEL sol–gel coatings. ANOVA (p-value < 0.05).

ii. *In vitro* assays

None of the biomaterials tested was cytotoxic. After 7 days of incubation, we found no differences between the ALP activities for the examined materials or even between these materials and SAE-Ti. Interestingly, after 14 days, we observed a significant increase in the ALP activity on the material 35M35G30T-GEL in comparison with the other formulations even though this material had the lowest activity after 7 days (**Figure 3**). After 14 days, cell proliferation increased slightly on the formulations with gelatin in comparison with their base materials; the cultures grown on the formulation 70M30T-GEL showed higher levels of proliferation than the control cells. We noted that the proliferative potential of 70M30T base material was better than the proliferative properties of 35M35G30T.

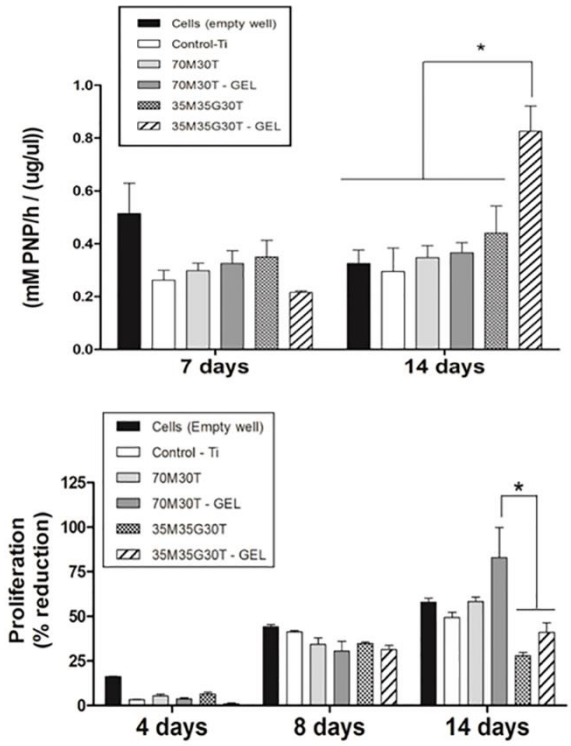

Figure 3. MC3T3-E1 *in vitro* results. (a) Alkaline phosphatase activity (mM p-nitrophenol [PNP]/hr) normalized to the amount of total protein (μg/μl) levels and (b) proliferation results of the cells cultivated on titanium discs treated with 70M30T, 70M30T-GEL, 35M35G30T, 35M35G30T-GEL formulations. Cells on an empty well without disc were used as a positive control (black column), whereas uncoated titanium discs (white column) were used as a negative control. ANOVA (p-value < 0.05).

iii. *In vivo* assays

Titanium implant coatings generated a distinctive tissue response at the experimental time tested. In the screw grooves corresponding to the cortical region no new bone tissue was observed in 70M30T and 35M35G30T coated implants. When implants were coated with a mixture of gelatin combined with either of the two sol-gel solutions new bone tissue growing was observed filling the grooves. From the cortical bone new bone trabeculae grew towards the implant surface region located in the medullary cavity. The relative length of the trabeculae as well as their density was slightly higher for the 70M30T implants when compared to the other 3 experimental groups (**Figure 4**). Furthermore, in the medullary cavity a fibrous connective tissue was developed also around the

implant surface of the 70M30T samples, containing arterial vessels. The connective tissue was looser and the arterial vessels density lower around 35M35G30T coated implants and those implants coated with a formulation containing gelatin. The inflammatory response was lower for the 70M30T coating considering the relative density and size of giant multinucleated cells laying the implant coating.

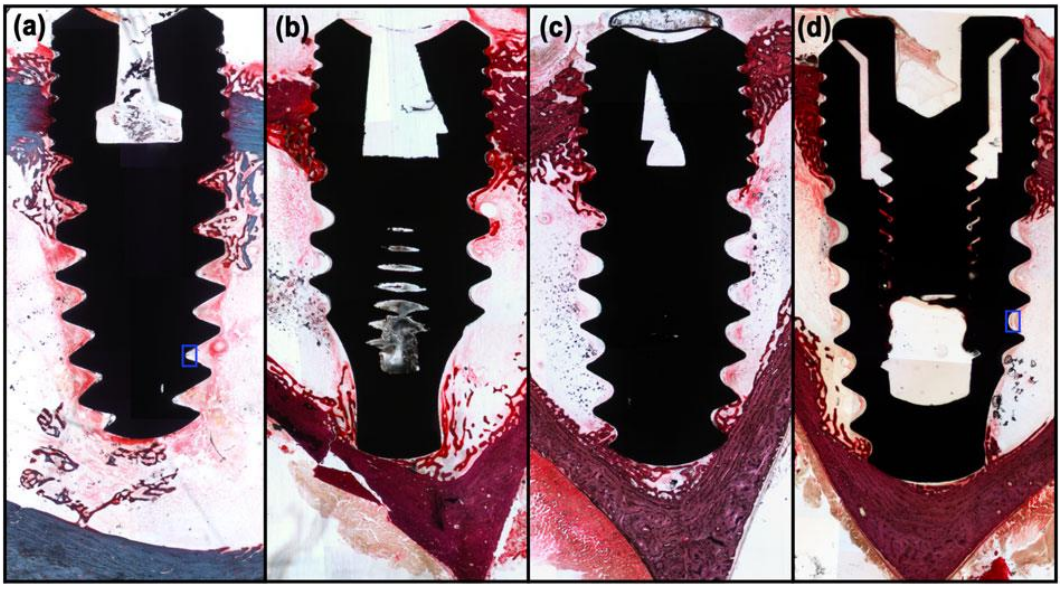

Figure 4. Microphotographs of titanium implants. Panoramic images of (a) 70M30T, (b) 70M30T-GEL, (c) 35M35G30T, and (d) 35M35G30TGEL implants. The delineated regions (blue rectangles) in the medullary cavity of (a) and (d) images are shown magnified in **Figure 5.**

Thus, few osteoclast-like and multinucleated giant cells, most of them of a small size were observed in the grooves of the implant (**Figure 5a**). The relative density of giant cells was slightly higher for the 35M35G30T-coated implants. The addition of gelatin to the sol-gel coating was related to an evident increase of the giant cells size and cell density, that was about 3- and 4-times higher for the 70M30T- and 35M35G30T-GEL (**Figure 5b**) coatings, respectively.

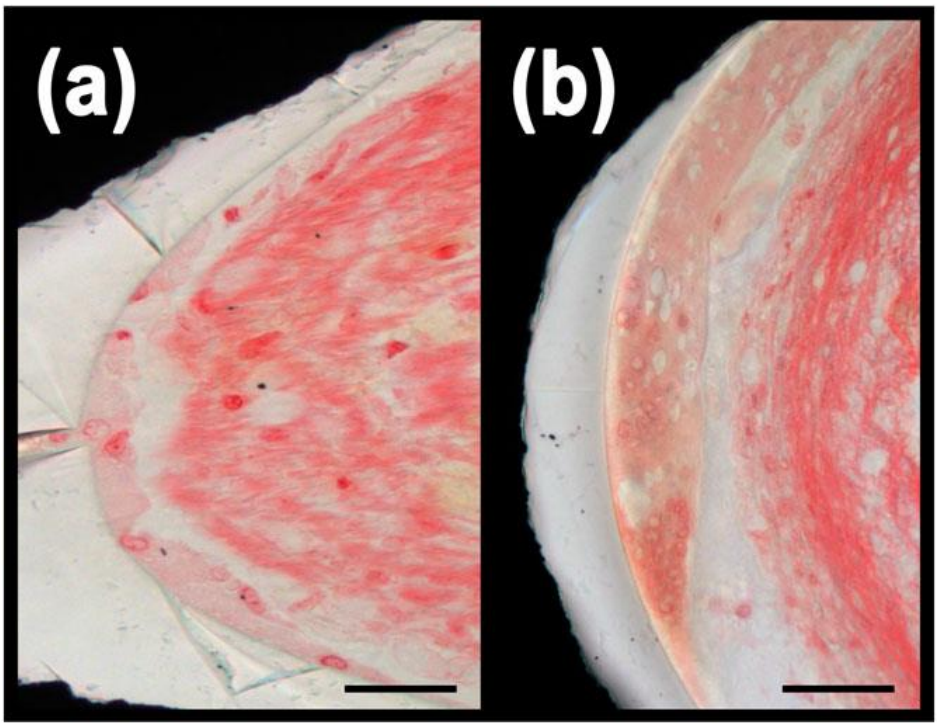

Figure 5. Microscopic detail of areas corresponding to the medullary cavity. Multinucleated cells layering the groove surface of (a) 70M30T and (b) 35M35G30T-GEL implants. The areas shown correspond to those delineated in **Figure 4**. Scale bar, 0.05 mm.

iv. Proteomic analysis

The proteins eluted from each biomaterial were studied using LC-MS/MS. The Progenesis QI software was employed to compare the characteristic proteins adhering to the different surfaces. One hundred seventy-one proteins were detected and quantified for each surface coating.

The comparison of identified proteins on the 70M30T and 35M35G30T materials displays 6 proteins more absorbed onto the 35M35G30T coating (**Table I**), while CLUS, FA12 and APOA5 proteins are more abundant on the 70M30T coating.

Table I. The comparison of proteins differentially adhered to 70M30T and 35M35G30T sol-gel coatings (Progenesis analysis). ANOVA (p-value < 0.05).

Description	Accession	70M30T	35M35G30T	35M35G30T/ 70M30T
Myosin-1	MYH1_HUMAN	5,71E+02	9,81E+03	17,19
L-lactate dehydrogenase B chain	LDHB_HUMAN	1,00E+04	1,22E+05	12,18
Glutamate dehydrogenase 1, mitochondrial	DHE3_HUMAN	8,24E+02	7,13E+03	8,65
Ficolin-2	FCN2_HUMAN	7,05E+03	5,84E+04	8,28
Complement C1q subcomponent subunit A	C1QA_HUMAN	3,17E+04	9,68E+04	3,06
Hemoglobin subunit alpha	HBA_HUMAN	3,75E+04	6,50E+04	1,73
Clusterin	CLUS_HUMAN	6,81E+05	4,15E+05	0,61
Coagulation factor XII	FA12_HUMAN	1,34E+05	7,80E+04	0,58
Apolipoprotein A-V	APOA5_HUMAN	5,41E+03	2,85E+03	0,53

The comparison of the data obtained for the 70M30T and 70M30T-GEL materials reveals 5 proteins with increased adsorption to the composition with gelatin (C1QA, FINC, FETUA, LDHB, and CO8B), while only one (K2C71) is more abundant on the 70M30T coating (**Table II**).

Table II. The comparison of proteins differentially adhered to 70M30T and 70M30T-GEL hybrid coatings (Progenesis analysis). ANOVA (p-value < 0.05).

Description	Accession	70M30T	70M30T-GEL	70M30T-GEL/70M30T
Complement C1q subcomponent subunit A	C1QA_HUMAN	3.17E+04	6.71E+04	2.12
Fibronectin	FINC_HUMAN	8.26E+03	1.54E+04	1.86
Alpha-2-HS-glycoprotein	FETUA_HUMAN	2.25E+05	3.91E+05	1.74
L-lactate dehydrogenase B chain	LDHB_HUMAN	1.00E+04	1.74E+04	1.73
Complement component C8 beta chain	CO8B_HUMAN	1.04E+04	1.50E+04	1.45
Keratin, type II cytoskeletal 71	K2C71_HUMAN	8.38E+03	5.90E+03	0.70

The PANTHER diagram showing classification by function is displayed in **Figure 6**. Although the 70M30T material yielded only one differentially adhering protein (keratin), adding gelatin to the matrix induced the adhesion of proteins related with the immune system (14%) and the biological adhesion (14%).

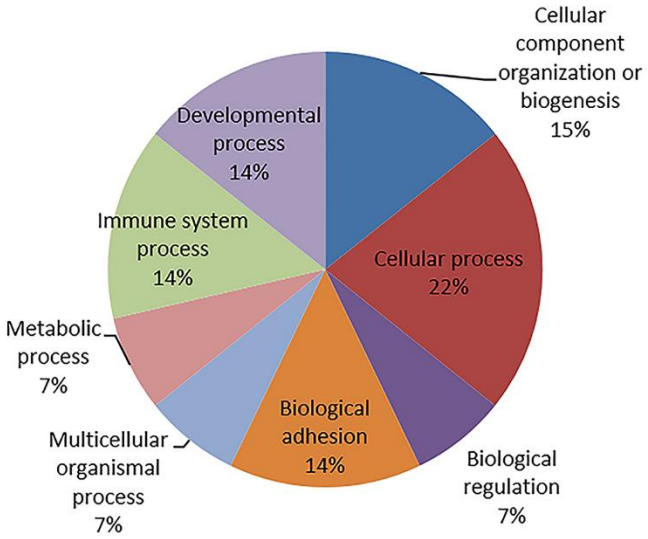

Figure 6. PANTHER diagram with the biological process of the proteins differentially adhered to 70M30T-GEL, respect 70M30T.

Similarly, **Table III** shows the comparison between the compositions 35M35G30T and 35M35G30T-GEL. In this case, 9 proteins were identified as more abundant on the composition with gelatin; CFAD, CO6, CRP, CO8B, and APOM were among them. However, the levels of adhering IGJ, CATD, HORN, and FCN2 were significantly higher for the 35M35G30T biomaterial. The GO functional classification of the proteins was performed using PANTHER system.

Table III. The comparison of proteins adhered to 35M35G30T and 35M35G30T-GEL hybrid coatings (Progenesis analysis). ANOVA (p-value < 0.05).

Description	Accession	35M35G30T	35M35G30T-GEL	35M35G30T-GEL /35M35G30T
Ig kappa chain V-I region Roy	KV116_HUMAN	2.73E+04	7.34E+04	2.69
Complement factor D	CFAD_HUMAN	2.14E+04	4.98E+04	2.33
Complement component C6	CO6_HUMAN	2.15E+04	4.91E+04	2.28
C-reactive protein	CRP_HUMAN	6.62E+03	1.35E+04	2.04
Complement component C8 beta chain	CO8B_HUMAN	1.05E+04	2.13E+04	2.02
Ig gamma-3 chain C region	IGHG3_HUMAN	8.18E+04	1.47E+05	1.80
Ig kappa chain V-II region Cum	KV201_HUMAN	5.71E+05	9.69E+05	1.70
Apolipoprotein M	APOM_HUMAN	2.84E+04	4.74E+04	1.67
Ig kappa chain V-IV region Len	KV402_HUMAN	3.46E+05	5.28E+05	1.53
Immunoglobulin J chain	IGJ_HUMAN	9.79E+04	6.99E+04	0.71
Cathepsin D	CATD_HUMAN	3.65E+04	1.66E+04	0.46
Hornerin	HORN_HUMAN	8.79E+03	2.25E+03	0.26
Ficolin-2	FCN2_HUMAN	5.84E+04	8.79E+03	0.15

Figure 7a and **7b** show the biological functions of the proteins differentially adsorbed onto 35M35G30T and 35M35G30T-GEL, respectively. It is noteworthy that while the 35M35G30T proteins were only involved in 4 functions, after adding gelatin, we found differentially adhering proteins participating in 9 biological processes. Moreover, for the formulations with gelatin, the proportion of functions associated with immune system processes increased from 14 % to 20 %.

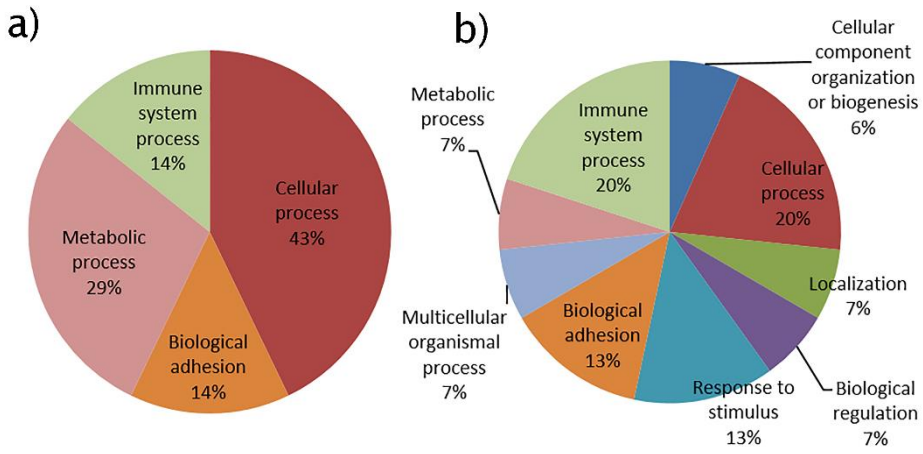

Figure 7. PANTHER diagram with the biological processes of the proteins differentially adhered to 35M35G30T (a) and 35M35G30T-GEL (b).

The comparison between **Table 2** and **3**, give us the unique common differentially adhering protein common to the two materials with gelatin, a complement protein C08B. The comparison between the gelatin compositions is presented in **Table IV**. Sixteen proteins were preferentially adsorbed onto the 35M35G30T-GEL coating (e.g. S10A9, CFAD, CRP, SAMP, C1QC, and VTNC), while only 4 were more abundant on the 70M30T-GEL (APOA, CLUS, APOA5, and IGJ).

Table IV. The comparison of proteins differentially adhered to 35M35G30T-GEL and 70M30T-GEL (Progenesis analysis). ANOVA (p-value < 0.05).

Description	Accession	70M30T-GEL	35M35G30T-GEL	35M35G30T-GEL/70M30T-GEL
Glutamate dehydrogenase 1, mitochondrial	DHE3_HUMAN	1.35E+03	1.67E+04	12.39
L-lactate dehydrogenase B chain	LDHB_HUMAN	1.74E+04	1.57E+05	9.01
Myosin-1	MYH1_HUMAN	2.18E+03	6.79E+03	3.11
Protein S100-A9	S10A9_HUMAN	3.74E+04	9.33E+04	2.49
Complement factor D	CFAD_HUMAN	2.13E+04	4.98E+04	2.34
C-reactive protein	CRP_HUMAN	6.08E+03	1.35E+04	2.22
Serum amyloid P-component	SAMP_HUMAN	2.54E+05	5.29E+05	2.09
Ig kappa chain V-I region Roy	KV116_HUMAN	3.79E+04	7.34E+04	1.93
Ig kappa chain V-III region SIE	KV302_HUMAN	2.44E+06	4.71E+06	1.93
Complement C1q subcomponent subunit C	C1QC_HUMAN	1.09E+06	1.90E+06	1.75
Ig kappa chain V-II region Cum	KV201_HUMAN	5.93E+05	9.69E+05	1.63
Vitronectin	VTNC_HUMAN	3.45E+05	5.50E+05	1.60
Gelsolin	GELS_HUMAN	1.58E+06	2.39E+06	1.51
Complement C1s subcomponent	C1S_HUMAN	2.43E+05	3.51E+05	1.45
Ig gamma-3 chain C region	IGHG3_HUMAN	1.03E+05	1.47E+05	1.43
Actin, cytoplasmic 1	ACTB_HUMAN	4.88E+04	6.78E+04	1.39
Immunoglobulin J chain	IGJ_HUMAN	9.78E+04	6.99E+04	0.71
Apolipoprotein(a)	APOA_HUMAN	3.07E+04	1.78E+04	0.58
Clusterin	CLUS_HUMAN	9.81E+05	5.11E+05	0.52
Apolipoprotein A-V	APOA5_HUMAN	7.21E+03	3.71E+03	0.51

d. DISCUSSION

The implantation of bone biomaterials triggers an immediate host response, provided by the immune system. Multi-directional pathways and mechanisms are activated, ultimately determining the integration or rejection of the biomaterial. These responses involve interactions between three types of components: the host immune cells, the host bone cells, and the materials themselves [26]. The initial layer of proteins adsorbed onto the biomaterial surface will ultimately define its biocompatibility, triggering, among other processes, coagulation, immune and angiogenesis signaling cascades. Hence, each biomaterial, depending on its chemical and physical composition, conformation, and intrinsic characteristics, can adsorb distinct sets and quantities of proteins to its surface. Titanium has been widely used as the base material for implants because of its bioinertia and osteoconductive characteristics [27]. Nowadays, various coatings are deposited onto this material to confer bioactive properties that enhance and accelerate the osseointegration in a living organism [28]. Our experimental work focused on the characterisation of the protein layer adsorbed onto four distinct biomaterials coated on the titanium discs (*in vitro* assays) or implants (*in vivo* experiments): 70M30T, 70M30T-GEL, 35M35G30T, and 35M35G30T-GEL, and their correlation with *in vitro* and *in vivo* experimentation results. These silica sol-gel hybrid materials were selected because they confer bioactive properties to the titanium surface [8,29].

Gelatin-containing formulations were used to examine potential improvements in the biocompatibility as it might enhance the adhesion of the cells by mimicking the behaviour of collagen I. Some studies have combined gelatin with other materials with positive bone regeneration properties, such as calcium phosphates or silicon [16–18,30] improving the *in vitro* results [31].

Gelatin was incorporated in both sol-gel base compositions (70M30T and 35M35G30T). In 70M30T, the gelatin is kept in the silica network due to hydrogen bonds between amino and carboxyl groups from gelatin and silanol groups [32]. However, in 35M35G30T, it is anchored to the structure through covalent bonds formed by the reaction with the epoxy ring of the GPTMS precursor [18].

The main chemical difference between the tested base materials (70M30T and 35M35G30T) is the presence of the GPTMS organic groups in the 35M35G30T. In general, physico-chemical results

display a decrease on roughness when GPTMS is added, whereas the incorporation of gelatin in both base materials shows an increase on hydrophobicity, possibly due to the special distribution of hydrophobic groups at the surface. Regardless of these differences, the *in vitro* results show non-significant or non-existent divergences between both base materials. The only exceptions were the increased cell proliferation on the 70M30T-GEL samples (result that it is described in the bibliography [16,17], and the significant increase in the ALP activity on 35M35G30T-GEL (**Figure 3**). However, it is worth mentioning the *in vitro* strategy adopted (using a single immortalized cell line), is not the ideal to simulate the whole *in vivo* setup of an implantation procedure, in which various biological systems and cues are involved, but it is the generally accepted standard for testing biomaterials nowadays. This *in vitro* setup gives the experimentation some clues about the material influence directly on the osteoblastic cell behaviour but does not consider parameters like the immune response and coagulative systems, which is something to take into account as a future perspective [33].

Regarding proteomic analysis, it is interesting to observe the correlation between the base material 35M35G30T and 70M30T (**Table I**), *in vivo* outcomes and the adsorbed layer of proteins formed onto each surface. In particular, 35M35G30T-coating shows the formation of a thin fibrous connective tissue surrounding the material. In the mentioned comparative **Table I**, it is clear the greater adsorption of mainly two proteins directly related with the complement pathway: involved in the classical pathway (C1QA) and the lectin pathway (FCN-2), respectively [34].

Interestingly, between 70M30T and 70M30T-GEL (**Table II**), is notable a slightly greater adsorption of C1QA when the base material is supplemented with gelatin, which can explain the existence of a very thin sheet of fibrous connective tissue (**Figure 4c**). At the same time, it is clear the greater adsorption of FINC on the material with gelatin, which is a protein widely described to be involved on cellular adhesion and proliferative processes [35,36]. Notably, the proteins FETUA, CO8B, and C1QC were more abundant on the 70M30T-GEL than on its base material. C1QC and CO8B are pro-inflammatory proteins. However, it is interesting to note that FETUA has been described as a modulator of macrophage opsonization, displaying anti-inflammatory activity, among its other functions. Moreover, this protein has a role in the fibril mineralization and may promote bone tissue formation [37].

The comparison and characterisation of the layers of proteins formed on 35M35G30T and 35M35G30T-GEL coated surfaces (**Table III**) showed more proteins related to the immune system adsorbed onto the 35M35G30T-GEL material (**Figure 7**), in particular, the proteins CO6, CFAD, CO8B, and CRP, a complement system activator [38]. Hence, in this aspect and at this point it is important to confirm the correlation between the greater adsorption of complement proteins and what is observed in regard to the increased presence of multinucleated giant cells around the gelatin-doped materials (**Figure 5**), that means that the incorporation of gelatin molecule supposes an increase in the immune response associated to the biomaterial.

It was also observed the increased levels of adsorption (approximately 2-fold) of pro-inflammatory proteins onto the 35M35G30T-GEL in comparison with 70M30T-GEL, namely S10A9 [39], CFAD [40], CRP, SAMP, C1S, and C1QC [41]. Worthy of note is the enhanced adsorption of VTNC (1.60-fold increase) on the 35M35G30T-GEL. This protein induces the osteogenesis by promoting the osteoblast differentiation, in the same way as collagen I [42]. This different protein adsorption could be related to both the differences in base material characteristics and the distinct gelatin linking strategies, which could condition the gelatin conformational organization in the network and then the exposure of its functional groups to the serum proteins.

In the *in vivo* experiments connective tissue developed and remained around regions of the implant surfaces not situated in the proximity of bone tissue. Thus, around the medullary cavity portion of 70M30T-coated implants a more fibrous layer was observed. All materials with differentially adsorbed proteins related to the complement system or with complement system activator proteins (35M35G30T and gelatin formulations) developed a looser connective tissue around the implant. Despite of the connective tissue formation, the histology of these materials showed proper bone tissue developing and direct bone-implant contact in some areas. The incorporation of gelatin indeed had some effect on the induction of a better implant integration, as new bone tissue was observed filling the screw grooves on the cortical zone, which is concordant with the hypothesis established above, as far as it enhances the osteoblast proliferation and differentiation. The increased abundance of complement proteins on some of the materials might be sufficiently high to promote the formation of a loose connective tissue layer (e.g. in comparison with more fibrous capsules) but not too high to prevent partially good osseointegration. A non-chronic immune response is not always undesirable as it favours the tissue growth and

regeneration around the implant [3]. In fact, the role of cytokines is not uniquely limited to the inflammatory response, as they are described to play a role on osteoblastic activation and/or on osteoclast inhibition, thus enhancing bone formation processes [43].

One way of measuring the grade of the immune reaction to these materials, might be the establishment of an inhibitory/activator ratio of the identified anti-inflammatory proteins, as VTNC, in comparison with the pro-inflammatory protein CRP. Applying these criteria, the data obtained from the proteomic analysis shows a decrease on these ratios, in particular on the materials incorporating gelatin. For example, the ratio VTNC/CRP on the 70M30T material reaches a value of 76.05, whilst the same material supplemented with gelatin as a value of 56.71. The same finding is observed with the 35M35G30T material, although not having such high values.

This might be related with the differences between the base materials. Having into account these ratios, an appropriate equilibrium between anti- and pro-inflammatory proteins can be desirable to avoid a chronic inflammatory response and fibrotic tissue formation. The results obtained from the analysis of this ratio are consistent with the *in vivo* results, in the sense that is visible an increase on the inflammatory reaction with a greater presence of multinucleated giant cells around the gelatin-supplemented coating on both base materials. This might be explained due to the smaller ratio due to the greater adsorption of CRP on them. This fact comes to reinforce the potential of proteomic analysis when addressing material biocompatibility, as documented in a previous study [44].

e. CONCLUSIONS

In summary, it has been shown that the base material 35M35G30T may induce overall a higher immune response than the other base material 70M30T *in vivo*. Although *in vitro* results are not concordant with this behaviour, proteomic analysis show effectively more adsorption of proteins related to the immune/inflammatory response on the base material 35M35G30T. The *in vivo* behaviour displays that 70M30T base material produces a lower immune response at the period tested that increases when adding gelatine while the 35M35G30T formulation induces a higher response that also increases when gelatin is added. Overall, the addition of gelatin on each material's matrix, provide an even greater immune response, supported by the fact of the adsorption of having more pro-inflammatory proteins adsorbed on the gelatin-silica hybrid sol-gel formulations, in particular the CRP, a great activator of the complement cascade. On the equilibrium between pro and anti-inflammatory adsorbed proteins may reside the key for a prediction of *in vivo* outcome.

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7. Chapter 4

8. General Discussion

8. GENERAL DISCUSSION

This doctoral thesis has been focused on the possible development and assessment of new methodologies *in vitro* to be applied to biomaterials development, specifically in the dental implantology field. These methodologies can represent a significant breakthrough at the moment of testing a determined biomaterial for dental application, as they can be potential tools to shirk considerable economical and ethical costs related to experimentation.

Pointedly, the need for an improvement on the standard *in vitro* experimentation has come due to the lack of a well-documented *in vitro-in vivo* correlation. The *in vitro* experimentation is critical to enable the selection of the materials to be tested *in vivo*. Moreover, it is a required step to reduce the animal experimentation burden.

The standard *in vitro* assays to test a new biomaterial for dental application do not ponder the whole biological complex processes involved on bone healing post-implantation, as generally only osteoblastic cell lines are used, thus focusing more on the osteogenic potential of the material.

The immune response to a material can be even more defining of implant successfulness on the biological context.

This outcome might be dependent of the type, amount and conformation of the proteins attached to the implant on the following moments (days, weeks, months…) after the surgery. Moreover, these proteins might condition the osteogenic cellular response to the implant through intricate pathways and interplay amongst the proteomic content of the organism adsorbed onto the surface of the device. This proteomic content and attachment can condition the immune cell behaviour, impairing the healing of the implanted tissue.

Hence, this thesis was focused on the study of the proteins absorbed in the biomaterials surface during first moments post-implantation and its correlation with *in vitro* and *in vivo* experimentation.

To this effect, the three first chapters of this thesis have the focal point on finding some patterns regarding finding protein correlations with the *in vivo* response between materials with a known outcome, while the fourth and last chapter was dedicated to the assessment of macrophage

phenotypic changes and how to combine this information with proteomic analysis to a better *in vivo* behaviour prediction.

a) Proteomic analysis as a biocompatibility methodology

Chapter 1 was focused on characterisation of proteins adhered to two biomaterials with distinct outcome: one boosting good biocompatibility and the other with a bad *in vivo* outcome. At first glance, both materials displayed to be non-cytotoxic and presenting good osteoblastic properties, making them good candidates to be tested on a living organism. However, the result of assaying those materials *in vivo* did not correlate with the *in vitro* data. The proteomic assays using blood serum deposition onto both materials and consequent LC-MS/MS analysis had sufficient sensitivity to show a significant number of proteins more attached to the bad biocompatibility surface, most of those directly related to the immune response processes. It was observed a predominance of complement-related proteins, namely the increased abundance of CRP (7-fold), a pentraxin described to be one of the major activators of the complement system by its binding to C1q.

This chapter was the first step to validate the possibility of adopting this method as a biocompatibility prediction method, as the results were satisfactory.

b) Validation of the methodology

Hence, the second study, **Chapter 2,** was done with the intent of checking if there were any differences in regard to the layer of proteins formed onto the surface when comparing two biocompatible materials, non-coated bioinert Titanium (SAE-Ti), and a sol-gel hybrid biocompatible material 70M30T, the same as used on the first chapter, having in account the immune response proteins. However, as expected, considering the outcome on the biological organism context, in regard to the cluster of immune response proteins described on the point **a)**, no differences were found and osseointegration was achieved on both, which validates the results from the first study. Moreover, interestingly, the sol-gel hybrid coating employed showed that the bone healing process had some osteoinductive properties, which was corroborated by the gene expression analysis. This fact was explainable by the intrinsic $Si(OH)_4$ ion release onto the implant surroundings, enhancing the osteoblastic activity, at the same time it explains the lack of differential proteins adsorbed onto

the coating. On the other hand, the proteomic analysis showed the greater attachment of proteins related to the coagulation and fibrinolysis system on the SAE-Ti, namely PMLN and VTNC, that might validate once more the well-documented osteoconductive properties of the material. This study not only gave validity to the methodology in the biocompatibility sense, but also made clear that this method is sensible enough to make some broad correlations with bone healing processes like coagulation and fibrinolysis.

c) Equilibrium immune response/osteogenesis

On **Chapter 3** our group tested and compared through proteomic characterisation the biocompatible material used on the previous chapters (70M30T), against another base material developed by our group (35M35G30T), distinguishable by having a GPTMS percentage incorporated onto its network. GPTMS has the characteristic of having an epoxy ring on its composition, enabling the coating to have bioactive compounds incorporated on its composition. Moreover, the main goal of this chapter was to check whether if the incorporation of a molecule like gelatin, capable of mimicking collagen I properties, had some effect on protein adsorption, *in vitro* and *in vivo* experimentation outcome, following the same protocol established for the first two chapters. The results displayed an improvement on *in vitro* osteoblastic cell behaviour (enhanced proliferation and mineralization) when gelatin was incorporated. The proteomic analysis displayed a greater attachment of proteins related to mineralization on those materials, namely Vitronectin (VTNC) and Alpha-2-HS-glycoprotein (FETUA), supporting the osteoblastic assays. At the same time, there was increased adsorption of complement-related proteins, among those the CRP, which underlies too that gelatin provokes an increased immune response. All of these results were validated through the *in vivo* experimentation, in which is observable a greater presence of multinucleated giant cells all around the materials with gelatin, in particular when incorporated on the 35M35G30T matrix. However, and although the existence of an increased immune reaction, all of the materials reached osseointegration, which could underlie a potential equilibrium on the adsorption between anti- and pro-inflammatory to occur the rejection of a biomaterial on a biological context. That hypothesis was formulated on this study by establishing the ratio between VTNC and CRP.

Altogether, the results of this chapter show that the implant outcome could be dependent on an established balance between anti- and pro- inflammatory proteins. Moreover, that an increased inflammatory response could be favourable to the bone formation processes.

d) Binomial protein adsorption-macrophage polarization

Finally, the study of **Chapter 4** was made with the intent to: I) prove that the epoxy ring belonging to the GPTMS group was the causing agent for the increased inflammation and II) checking if the relationship between adsorbed type of inflammation-related proteins and predominance of pro- or anti- inflammatory macrophage phenotype. To achieve that, we have tested three materials with distinct concentrations of GPTMS, regarding proteomic analysis, *in vitro* and *in vivo* behaviour concerning inflammatory response, in particular focusing on the macrophage phenotype shift when exposed to those materials. Interestingly, our hypothesis was once again validated, showing that the materials with GPTMS on its composition have greater affinity, on a dose-dependent manner (GPTMS concentration), to immune response proteins. Ficolin-2, CRP, and other complement proteins were found significantly more attached on the material with the greatest concentration of GPTMS. Moreover, immunocytochemistry and ELISAs assays showed greater predominance of M1 macrophages and increased liberation of TNF-α, apart from the slow and worst osseointegration around the coating as the amount of GPTMS increases.

All things considered, once again the proteomic analysis was proven as a potential useful tool to predict the inflammatory properties of a determined material. Furthermore, its clear-cut correlation with the differentiative and functional macrophage polarization imposes the possibility of changes on *in vitro* testing on a near future.

9. Conclusions

9. Conclusions / Final remarks

From the analysis of the research contained in this doctoral thesis it was possible to partially reply to the questions formulated on the objectives:

- Explicitly, we were able to establish the close relationship between proteins adsorbed initially on the biomaterial surface and biological response, not only comprising the osteogenesis processes, but also the whole set of processes belonging to bone healing post-implantation.

- Inflammation-related proteins, such as CRP and the whole set of complement proteins, belonging to the distinct pathways, seem to be intrinsically associated with the emergence of biocompatibility problems.

- The macrophage polarization shifts were proven to have some association with the type of proteins adsorbed onto a determined biomaterial, with increased inflammatory potential onto materials having greater presence of complement proteins on it.

- It might be possible to predict the *in vivo* outcome of a material regarding inflammatory response through assessment of macrophage polarization *in vitro*.

9. Conclusiones

A partir del análisis de la investigación contenida en esta tesis doctoral, se pudo responder parcialmente a las preguntas formuladas en el apartado de objetivos:

• Explícitamente, pudimos establecer la estrecha relación entre las proteínas adsorbidas inicialmente en la superficie del biomaterial y la respuesta biológica, que no solo comprenden los procesos de osteogénesis, sino también todo el conjunto de procesos que pertenecen a la curación ósea después de la implantación.

• Proteínas relacionadas con la inflamación, como la CRP y todo el conjunto de proteínas del complemento, pertenecientes a distintas vías, parecen estar intrínsecamente asociadas con la aparición de problemas de biocompatibilidad.

• Se demostró que los cambios en la polarización de los macrófagos tienen cierta relación con el tipo de proteínas adsorbidas en un determinado biomaterial, con un mayor potencial inflamatorio en los materiales que tienen una mayor presencia de proteínas del complemento.

• Podría ser posible predecir el resultado *in vivo* de un material en relación a su respuesta inflamatoria a través de la caracterización de la polarización de macrófagos *in vitro*.

9. Conclusions

A partir de l'anàlisi de la investigació continguda en aquesta tesi doctoral, es va poder respondre parcialment a les preguntes formulades en l'apartat d'objectius:

• Explícitament, vam poder establir l'estreta relació entre les proteïnes adsorbides inicialment en la superfície del biomaterial i la resposta biològica, que no només comprenen els processos d'osteogènesi, sinó també tot el conjunt de processos que pertanyen a la curació òssia després de la implantació.

• Proteïnes relacionades amb la inflamació, com la CRP i tot el conjunt de proteïnes del complement, pertanyents a diferents vies, semblen estar intrínsecament associades amb l'aparició de problemes de biocompatibilitat.

• Es va demostrar que els canvis en la polarització dels macròfags tenen certa relació amb el tipus de proteïnes adsorbides en un determinat biomaterial, amb un major potencial inflamatori en els materials que tenen una major presència de proteïnes del complement.

• Podria ser possible predir el resultat *in vivo* d'un material en relació a la seva resposta inflamatòria a través de la caracterització de la polarització de macròfags *in vitro*.

10. Future perspectives

10. Future perspectives

The experimental work developed on this thesis has allowed to remark and underline the importance of the type of proteins adhered to a biomaterial surface on post-implantation bone tissue healing scenario, especially on the immune response context. The characterisation of the proteins adsorbed onto a biomaterial has established a close relationship between them and macrophage polarization shift patterns, which are proven to be accountable for an implantation successful outcome.

In that sense, and following the strategy adopted thesis future works to assess macrophage polarization onto biomaterials are planned to be carried out, not only focusing on the use of a single macrophage behaviour, but also the interaction with, for example, osteoblastic cell lines, on a co-culture context. Moreover, our research group has the intention to adopt the use of 3D-cultures, as they represent a hallmark on this subject, simulating more accurately cell demeanor and assess the macrophage plasticity. Moreover, making use and optimizing distinct techniques such as flow cytometry analysis, may serve useful to appraise this phenom, as it identifies precisely the macrophage phenotype by the surface markers present on each.

The use of biocompatible sol-gel materials supplemented with bioactive compounds and respective proteomic analysis, which our group has been carried out for the last months, is undergoing and will undergo in the near future, aiming to understand the biomaterial-protein-cell-tissue microenvironment interactions, and how it may affect the bone regenerative processes post-implantation.

On the other hand, studying the layer of proteins adsorbed onto biomaterials regarding other processes involved in bone healing, like protein clusters specifically involved on coagulation and fibrinolysis may allow us to have a whole perspective to predict whether if a material is more or less successful on a biological context. In that sense, right of this moment it is being planned by our group an *in vivo* experimentation to be carried out in osteoporotic models, with the final goal of correlating them with proteomic analysis and establishing protein patterns influencing coagulation pathways.

11. Annex

a. Publication listing

Below is a list of the papers published in international journals indexed as a result of the experimental work exposed in this work, as well as other related lines of research developed during this period:

- **N. Araújo-Gomes**, F. Romero-Gavilán, A.M. Sánchez-Pérez, M. Gurruchaga, M. Azkargorta, F. Elortza, M. Martinez-Ibañez, I. Iloro, J. Suay, I. Goñi, Characterization of serum proteins attached to distinct sol–gel hybrid surfaces, J. Biomed. Mater. Res. - Part B Appl. Biomater. 106 (2018) 1477–1485. doi:10.1002/jbm.b.33954

- **N. Araújo-Gomes**, F. Romero-Gavilán, I. García-Arnáez, C. Martínez-Ramos, A.M. Sánchez-Pérez, M. Azkargorta, F. Elortza, J.J.M. de Llano, M. Gurruchaga, I. Goñi, J. Suay, Osseointegration mechanisms: a proteomic approach, J. Biol. Inorg. Chem. 23 (2018) 459– 470. doi:10.1007/s00775-018-1553-9

- **N. Araújo-Gomes**, F. Romero-Gavilán, I. Lara-Sáez, F. Elortza, M. Azkargorta, I. Iloro, M. Martínez-Ibañez, J.J. Martín de Llano, M. Gurruchaga, I. Goñi, J. Suay, A.M. Sánchez-Pérez, Silica-gelatin hybrid sol-gel coatings: a proteomic study with biocompatibility implications, J. Tissue Eng. Regen. Med. (2018) 1–11. doi:10.1002/term.2708

- F. Romero-Gavilán, **N.C. Gomes**, J. Ródenas, A. Sánchez, M. Azkargorta, I. Iloro, F. Elortza, I. García Arnáez, M. Gurruchaga, I. Goñi, J. Suay, Proteome analysis of human serum proteins adsorbed onto different titanium surfaces used in dental implants, Biofouling. 33 (2017) 98–111. doi:10.1080/08927014.2016.1259414

- F. Romero-Gavilán, A.M. Sanchez-Pérez, **N. Araújo-Gomes**, M. Azkargorta, I. Iloro, F. Elortza, M. Gurruchaga, I. Goñi, J. Suay, Proteomic analysis of silica hybrid sol-gel coatings: a potential tool for predicting the biocompatibility of implants in vivo, Biofouling. 33 (2017) 676–689. doi:10.1080/08927014.2017.1356289

- F. Romero-Gavilan, **N. Araújo-Gomes**, A.M. Sánchez-Pérez, I. García-Arnáez, F. Elortza, M. Azkargorta, J.J.M. de Llano, C. Carda, M. Gurruchaga, J. Suay, I. Goñi, Bioactive potential of silica coatings and its effect on the adhesion of proteins to titanium implants, Colloids Surfaces B Biointerfaces. 162 (2017) 316–325. doi: 10.1016/j.colsurfb.2017.11.072

- F. Romero-Gavilán, **N. Araújo-Gomes**, I. García-Arnáez, C. Martínez-Ramos, F. Elortza, M. Azkargorta, I. Iloro, M. Gurruchaga, J. Suay, I. Goñi, The effect of strontium incorporation into sol-gel biomaterials on their protein adsorption and cell interactions, Colloids Surfaces B Biointerfaces. Under review, May 2018

- B. Palla-Rubio, **N. Araújo-Gomes**, M. Fernández-Gutiérrez, L. Rojo, J. Suay, M. Gurruchaga, I. Goñi, Synthesis and characterization of silica-chitosan hybrid materials as antibacterial coatings for titanium implants, Carbohydrate Polymers. Under Review, pending major revisions, June 2018

- F. Romero-Gavilán, **N. Araújo-Gomes**, I. García-Arnáez, C. Martínez-Ramos, F. Elortza, M. Azkargorta, I. Iloro, M. Gurruchaga, J. Suay, I. Goñi, Proteomic analysis of calcium enriched sol-gel biomaterials. In process, 2018

- **N. Araújo-Gomes**, F. Romero-Gavilán, Y. Zhang, C. Martinez-Ramos, A.M. Sánchez-Pérez, F. Elortza, M. Azkargorta, J.J. Martín de Llano, M. Gurruchaga, I. Goñi, J.J.J.P. van den Beucken, J. Suay, Complement pathways and macrophage polarization regulation: proteomic interfaces. In process, 2018.

b. Image of the first page of the published articles exposed in the results and discussion section

CHAPTER 1

Society For Biomaterials

Characterization of serum proteins attached to distinct sol–gel hybrid surfaces

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Abstract: The success of a dental implant depends on its osseointegration, an important feature of the implant biocompatibility. In this study, two distinct sol–gel hybrid coating formulations [50% methyltrimethoxysilane: 50% 3-glycidoxypropyl-trimethoxysilane (50M50G) and 70% methyltrimethoxysilane with 30% tetraethyl orthosilicate (70M30T)] were applied onto titanium implants. To evaluate their osseointegration, *in vitro* and *in vivo* assays were performed. Cell proliferation and differentiation *in vitro* did not show any differences between the coatings. However, four and eight weeks after *in vivo* implantation, the fibrous capsule area surrounding 50M50G-implant was 10 and 4 times, respectively, bigger than the area of connective tissue surrounding the 70M30T treated implant. Thus, the *in vitro* results gave no prediction or explanation for the 50M50G-implant failure *in vivo*. We hypothesized that the first protein layer adhered to the surface may have direct implication in implant osseointegration, and perhaps correlate with the *in vivo* outcome. Human serum was used for adsorption analysis on the biomaterials, the first layer of serum proteins adhered to the implant surface was analyzed by proteomic analysis, using mass spectrometry (LC-MS/MS). From the 171 proteins identified; 30 proteins were significantly enriched on the 50M50G implant surface. This group comprised numerous proteins of the immune complement system, including several subcomponents of the C1 complement, complement factor H, C4b-binding protein alpha chain, complement C5 and C-reactive protein. This result suggests that these proteins enriched in 50M50G surface might trigger the cascade leading to the formation of the fibrous capsule observed. The implications of these results could open up future possibilities to predict the biocompatibility problems *in vivo*. J Biomed Mater Res Part B: Appl Biomater, 106B: 1477–1485, 2018.

Key Words: sol–gel coatings, immune system, C-reactive protein, fibrous capsule, proteomics, osseoimmunology

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JBIC Journal of Biological Inorganic Chemistry (2018) 23:459–470
https://doi.org/10.1007/s00775-018-1553-9

ORIGINAL PAPER

Osseointegration mechanisms: a proteomic approach

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Abstract
The prime objectives in the development of biomaterials for dental applications are to improve the quality of osseointegration and to short the time needed to achieve it. Design of implants nowadays involves changes in the surface characteristics to obtain a good cellular response. Incorporating osteoinductive elements is one way to achieve the best regeneration possible post-implantation. This study examined the osteointegrative potential of two distinct biomaterials: sandblasted acid-etched titanium and a silica sol–gel hybrid coating, 70% MTMOS-30% TEOS. In vitro, in vivo, and proteomic characterisations of the two materials were conducted. Enhanced expression levels of ALP and IL-6 in the MC3T3-E1 cells cultured with coated discs, suggest that growing cells on such surfaces may increase mineralisation levels. 70M30T-coated implants showed improved bone growth in vivo compared to uncoated titanium. Complete osseointegration was achieved on both. However, coated implants displayed osteoinductive properties, while uncoated implants demonstrated osteoconductive characteristics. Coagulation-related proteins attached predominantly to SAE-Ti surface. Surface properties of the material might drive the regenerative process of the affected tissue. Analysis of the proteins on the coated dental implant showed that few proteins specifically attached to its surface, possibly indicating that its osteoinductive properties depend on the silicon delivery from the implant.

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RESEARCH ARTICLE

WILEY

Silica–gelatin hybrid sol–gel coatings: A proteomic study with biocompatibility implications

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Abstract

Osseointegration, including the foreign body reaction to biomaterials, is an immune-modulated, multifactorial, and complex healing process in which various cells and mediators are involved. The buildup of the osseointegration process is immunological and inflammation-driven, often triggered by the adsorption of proteins on the surfaces of the biomaterials and complement activation. New strategies for improving osseointegration use coatings as vehicles for osteogenic biomolecules delivery from implants. Natural polymers, such as gelatin, can mimic Collagen I and enhance the biocompatibility of a material. In this experimental study, two different base sol–gel formulations and their combination with gelatin were applied as coatings on sandblasted, acid-etched titanium substrates, and their biological potential as osteogenic biomaterials was tested. We examined the proteins adsorbed onto each surface and their in vitro and in vivo effects. In vitro results showed an improvement in cell proliferation and mineralization in gelatin-containing samples. In vivo testing showed the presence of a looser connective tissue layer in those coatings with substantially more complement activation proteins adsorbed, especially those containing gelatin. Vitronectin and FETUA, proteins associated with mineralization process, were significantly more adsorbed in gelatin coatings.

KEYWORDS

biocompatibility, biomaterial, bone regeneration, complement pathway, dental implants, immunology

c. Presentations at national and international conferences

Below is a list of the communications published in national and international conferences during the period of development of the doctoral thesis:

2016

- J. Suay; F. Romero-Gavilán; **N. Araújo-Gomes**; J. Rodenas; I. García; M. Gurruchaga; I. Goñi; A. Sánchez; Proteome analysis of human serum proteins adsorbed onto different titanium surfaces used in dental implants. 9th meeting of Scandinavian Society for Biomaterials (ScSB), 1-3 June 2016, Reykjavik, Iceland.

2017

- **N. Araújo-Gomes**, F. Romero-Gavilán, A.M. Sánchéz-Pérez, F. Elortza, M. Gurruchaga, I. Goñi, J. Suay; Biocompatibility problems: an alternative method to approach in vitro testing? 10th annual meeting of the Scandinavian Society for Biomaterials (ScSB), 15-17 March 2017, Hafjell, Norway (Oral presentation)

- F. Romero-Gavilán, **N. Araújo-Gomes**, A.M. Sánchéz-Perez, F. Elortza, M. Gurruchaga, I. Goñi, J. Suay; Proteomic identification of predictive markers related with biocompatibility problems; 10th annual meeting of the Scandinavian Society for Biomaterials (ScSB),), 15-17 March 2017, Hafjell, Norway (Poster)

- F. Romero-Gavilán, **N. Araújo-Gomes**, A.M. Sánchéz-Perez, F. Elortza, M. Gurruchaga, I. Goñi, J. Suay; Proteomic analysis as a biocompatibility predictor on hybrid sol-gel biomaterials. 5th International Conference on Multifunctional, Hybrid and Nanomaterials, 6-10 March 2017, Lisbon, Portugal (Poster)

- **N. Araújo-Gomes**, F. Romero-Gavilán, A.M. Sánchez-Pérez, F. Elortza, M. Gurruchaga, I. Goñi, J. Suay; Estudio de la biocompatibilidad de materiales mediante marcadores de inflamación. XL Congreso Sociedad Ibérica de Biomecánica y Biomateriales, 10-11 November 2017, Barcelona, Spain (Oral communication)

- F. Romero-Gavilán, **N. Araújo-Gomes**, A.M. Sánchez-Pérez, F. Elortza, M. Gurruchaga, I. Goñi, J. Suay; Identificación de marcadores relacionados con problemas de biocompatibilidad mediante proteómica. XL Congreso Sociedad Ibérica de Biomecánica y Biomateriales, 10-11 November 2017, Barcelona, Spain (Oral communication)

- J.J. Martín de Llano, **N. Araújo-Gomes**, F. Romero-Gavilán, A.M. Sánchez-Pérez, M. Azkargorta, F. Elortza, M. Gurruchaga, I. Goñi, R. Salvador-Clavell, L. Millián, J. Suay, C. Cardá; TIssue response to titanium implants with a hybrid silica sol-gel in a rabbit tibia model. Congreso Iberoamericano de Histología 2017 - Sociedad Española de Histologia y Inginíeria Tisular (SEHIT), 5-8 September 2017, Santiago Compostela, España (Poster)

- **N. Araújo-Gomes**, F. Romero-Gavilán, A.M. Sánchez-Pérez, F. Elortza, I. Goñi, M. Gurruchaga, J. Suay; The Role of Proteomics in Prediction of Biomaterials Biocompatibility. 28th Anual Conference of the European Society for Biomaterials, 4-8 September 2017, Athens, Greece (Oral communication)

- J. Suay; F. Romero-Gavilán; **N. Araújo-Gomes**; A.M. Sánchez-Pérez; F. Elortza; I. Goñi; M. Gurruchaga; J. Franco; Predictive markers associated with dental implants biocompatibility using proteomic analysis. 26 th Annual Scientific Meeting of the European Association for Osseointegration (EAO2017), 5-7 October 2017, Madrid, Spain. In: Clin Oral Impl Res. 2017, 28 (14) pp. 67. ISSN 0905-7161. doi:10.1111/clr.66_13042.

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d. Abbreviation listing

- ALP Alkaline Phosphatase
- ANOVA Analysis of variance
- BCA Bicinchoninic acid
- CLR Calcitonin-like receptor
- COL I Collagen I
- CR Complement receptors
- CRP C-reactive protein
- DAVID Database for Annotation, Visualization and Integrated Discovery´
- DMEM Dulbecco's Modified Eagle's Medium
- DTT Dithiothreitol
- ECM Extracellular Matrix
- ESI Electrospray ionization
- FBR Foreign Body Reaction
- FBS Fetal Bovine Serum
- FGF Fibroblast Growth Factor
- GPTMS 3-glycidoxypropyl-trimethoxysilane
- HA Hydroxyapatite
- IGF-1 Insulin growth factor 1
- Ig Immunoglobulins
- IL Interleukins
- LC-MS/MS Liquid Chromatography- Mass Spectromety and tandem mass spectrometry
- MAC Membrane attack complex

- MAPK Mitogen-activated kinase pathway
- MASP Mannan-binding lectin serine protease
- MBL Mannan-binding lectin
- MMP Matrix metalloproteases
- MSC Mesenquimal stem cells
- MTMOS Methyltrimethoxysilane
- OCN Osteocalcin
- PANTHER Protein ANalysis THrough Evolutionary Relationships
- PDGF Platelet derived growth factor
- p-NPP p-nitrophenylphosphate
- PRR Pattern recognition receptor
- SAE Sandblasted acid-etched
- SDS Sodium dodecyl sulfate
- SEM Scanning electron microscope
- TEAB Triethylammonium bicarbonate buffer
- TEOS Tetraethyl-orthosilicate
- TF Tissue factors
- TGF-β Transforming growth factor β
- Th T helper cells
- Ti Titanium
- TLR Toll-like receptor
- TNF-α Tumor necrosis factor alfa
- tPA Tissue plasminogen activator

- uPA Urokinase plasminogen activator
- VEGF Vascular Endothelial Growth Factor
- β-TCP β-Tricalcium phosphate

e. Figure listing

Introduction

Chapter 1

Figure 1. SEM images of sol–gel coated disc surface. 70M30T (a) and 50M50G (b). Calibration bar 10 mm.

Figure 2. MC3T3-E1 cell viability and mineralization *in vitro*. Percentage of cell survival following the norm ISO 10993–5 (a). ALP activity (mM PNP/h) normalized to the protein concentration (mg/mL) of cells grown without disc (oblicue lines), grown on control Ti discs (horizontal lines), 70M30G (white column) and 50M50G coated Ti discs (black column).

Figure 3. *In vivo* studies. Light microscopy images (EXAKT® cut and Gomori Trichrome stain) from *in vivo* implants 4 and 8 weeks postimplantation of Control-Ti, 70M30T and 50M50G sol–gel coated screw; (a) Calibration bar 500 mm; (b) Calibration bar 100 mm. (c) Quantification fibrous of the connective tissue area (n=4). Significant values were considered for $p<0.05$ as analysed by ANOVA followed by Newman-Keuls post-test.

Chapter 2

Figure 1. SEM micrographs of SAE-Ti surfaces and 70M30T sol-gel coating. Calibration bar, 10 µm.

Figure 2. MC3T3-E1 *in vitro* assays: A) MC3T3-E1 cell proliferation after 1, 3, 5 and 7 days of incubation with SAE-Ti (white bar) and 70M30T (grey bar) materials. B) ALP activity (mM PNP h^{-1}) normalised to the amount of total protein (µg μL^{-1}) levels in the MC3T3-E1 cells cultivated on SAE-Ti (white bar) and 70M30T formulation (grey bar). Cells incubated without discs were used as a positive control (black bar).

Figure 3. Gene expression of osteogenic markers (a) ALP, (b) IL6, (c) COL I and (d) OCN in MC3T3-E1 osteoblastic cells cultured on SAE-Ti (white bar) and 70M30T (grey bar). The relative mRNA expression was determined by RT-PCR after 7 and 14 days of culture. Statistical analysis was performed using one-way ANOVA with a Kruskal-Wallis post-test (* $p \leq 0.05$).

Figure 4. Microphotographs of samples of SAE-Ti and 70M30T implants. The main panels show 4× magnification images of regions close to the cortical bone (up) and the bone marrow cavity (down). In the inserts (lower-right corners), 10× images of the same regions are shown.

Figure 5. Bone tissue growth 4 weeks after implantation. Panoramic (left) and detailed (right) microphotographs of SAE-Ti and 70M30T implants show the bone tissue generated around the implant surface.

Chapter 3

Figure 1. Scanning electron microscopy images of hybrid sol–gel coatings onto titanium discs: 70M30T (a), 70M30T-GEL (b), 35M35G30T (c), and 35M35G30T-GEL (d). Calibration bar 10 µm.

Figure 2. Contact angle measurements of 70M30T, 70M30T-GEL, 35M35G30T, and 35M35G30T-GEL sol–gel coatings. ANOVA (p-value < 0.05).

Figure 3. MC3T3-E1 *in vitro* results. (a) Alkaline phosphatase activity (mM p-nitrophenol [PNP]/hr) normalized to the amount of total protein (µg/µl) levels and (b) proliferation results of the cells cultivated on titanium discs treated with 70M30T, 70M30T-GEL, 35M35G30T, 35M35G30T-GEL formulations. Cells on an empty well without disc were used as a positive control (black column), whereas uncoated titanium discs (white column) were used as a negative control ANOVA (p-value < 0.05).

Figure 4. Microphotographs of titanium implants. Panoramic images of (a) 70M30T, (b) 70M30T-GEL, (c) 35M35G30T, and (d) 35M35G30TGEL implants. The delineated regions (blue rectangles) in the medullary cavity of (a) and (d) images are shown magnified in **Figure 5.**

Figure 5. Microscopic detail of areas corresponding to the medullary cavity. Multinucleated cells layering the groove surface of (a) 70M30T and (b) 35M35G30T-GEL implants. The areas shown correspond to those delineated in Figure 4. Scale bar, 0.05 mm.

Figure 6. PANTHER diagram with the biological process of the proteins differentially adhered to 70M30T-GEL, respect 70M30T.

Figure 7. PANTHER diagram with the biological processes of the proteins differentially adhered to 35M35G30T (a) and 35M35G30T-GEL (b).

Chapter 4

Figure 1. SEM micrographs of 70M30T (**a**), 35M35G30T (**b**) and 100G (**c**) sol-gel coated surfaces. Calibration bar 10 µm. (**d**) Ra values of each formulation (6 measurements). Statistical analysis was performed by one-way ANOVA with Newman-Keuls pos-test. *P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001.

Figure 2. MC3T3-E1 *in vitro* assays: **a)** MC3T3-E1 cell survival assay following the norm ISO 10993-5 standard. Cells in a well without disc were used as a positive control, corresponding to 100% of cell viability. **b)** ALP activity (mM PNP h^{-1}) normalised to the total protein levels (µg $µl^{-1}$) in the MC3T3-E1 cells cultivated on titanium discs with the different formulations tested. There were no statistically significant differences between the different formulations.

Figure 3. Gene expression of osteogenic markers **a**) IL6, **b**) TGF-β and **c**) COL I in MC3T3-E1 osteoblastic cells cultured onto the different formulations. Relative mRNA expression was determined by RT-PCR after 7 and 14 days of culture. Statistical analysis was performed by one-way ANOVA with Newman-Keuls pos-test. *P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001.

Figure 4. Cytokine expression of IL1-β (**a**), TGF-β (**b**), TNF-α (**c**) and IL10 (**d**) by RAW 264.7 macrophages at 24h and 72h timepoints. Statistical analysis was performed by one-way ANOVA with Newman-Keuls pos-test. *P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001.

Figure 5. Immunostaining of different types of macrophages cultured onto the 70M30T, 35M35G30T and 100G sol-gel hybrid formulations after 72h. IL7-R (**a'-c'**) was used as a pro-inflammatory M1 marker, while CD206 (**a''-c''**) was used an anti-inflammatory M2 marker.

Figure 6. Microphotographs of titanium implants. Panoramic images of (a) 70M30T, (b) 35M35G30T, and (c) 100G implants showing the cortical bone region and the medullary cavity. Yellow dashed line in (b) delimits the metal layer of the implant that detached when processing the sample.

f. Tables listing

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Table IV. The comparison of proteins differentially adhered to 35M35G30T-GEL and 70M30T-GEL (Progenesis analysis). ANOVA (p-value < 0.05).

Chapter 4

Table I. Progenesis analysis of complement-related proteins attached to 70M30T, 35M35G30T and 100G. Grey background was applied to significant values (ANOVA p<0.05).