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. 50M50G-treated implant developed a fibrous capsule between the coating and the bone when implanted in vivo. The 70M30T coating resulted in good osseointegration, 8 weeks after implantation. The correlation between the in vitro and in vivo results was very poor; there was no explanation for the 50M50G failure in vivo. The protein layer adhered to the surface may have direct implication in implant osseointegration. These proteins may determine in vivo outcome. Therefore, we studied the protein layer adhering to the surface involving direct implications for the tissue response. Proteomic analysis, using mass spectrometry (LC/MS/MS), of serum proteins adsorbed onto the two surfaces was carried out. 171 proteins were identified; 8 of these were enriched on the 70M30T-coated dental implant, and 30 proteins were enriched on the 50M50G implant surface. The latter group included several proteins of the immune complement and blood clotting system. This result suggested that these proteins might trigger the cascade leading to the formation of the fibrous capsule. The implications of these results could open up the possibility to predict the biocompatibility problems in vivo.

Keywords: sol-gel coatings, immune system, C-reactive protein, fibrous capsule, proteomics, osteoimmunology
1. 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 (Navarro et al. 2008).

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 (Juan-Díaz et al. 2016; Martínez-Ibáñez et al. 2016) 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 (Hulsart-Billström et al. 2016).

Given the dense vascularization of organs and tissues, the first fluid to come in contact with an implant is the blood (Drinker et al. 1922), accounting for the formation of the first hydration layer covering the implant surface (Puleo & Nanci 1999). 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 (Horbett 2013). The type and characteristics (hydrophobicity, microtopography, chemical properties) of the constitutive material of the implant (Molino et al. 2012) and the first protein layer on the implant surface will ultimately determine osseointegration,
involving processes like the blood coagulation, inflammation, and humoral immune response (Arvidsson et al. 2007). 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 (Hong et al. 2005). 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 (Romero-Gavilán et al.). These studies open up the exciting possibility of predicting the body reaction after implantation. It is possible that protein deposition studies might provide a major breakthrough in the understanding and prediction of biomaterial behaviour in in vivo environments (Kaneko et al. 2011; Dodo et al. 2013).

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 (Juan-Díaz et al. 2016; Martínez-Ibáñez et al. 2016): 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.
2. Materials and methods

2.1 Preparation of 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.

2.2 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 alkoxy silanes 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 °C and 140 °C, respectively.
2.3 Physicochemical characterisation of 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.

2.4 Cell culture

MC3T3-E1 (mouse-calvaria osteosarcoma cell line) cells were cultured on the 70M30T- and 50M50G-coated Ti discs, at a concentration of 1×10⁴ 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% CO₂, the medium was replaced with an osteogenic medium composed of DMEM with phenol red 1x, 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.
2.5 Cytotoxicity

The biomaterial cytotoxicity was assessed following the ISO 10993-5 norm, using the 96-cell Titer 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.

2.6 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 MgCl₂, pH 10.5) was added to 100 µl of the supernatant obtained from the lysate. After 2 h of incubation in the dark (37 °C, 5% CO₂), 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).

2.7 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 ≤ 0.05 were considered statistically significant.
2.8 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 (Mori et al. 1997). 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 chloride. 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.

2.9 Histological examination and quantification

Four samples for histological examination were processed following the methodology described previously (Martínez-Ibáñez et al. 2016). 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 mm².

2.10 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 °C, 5% CO₂), 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 ddH₂O 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. (Kaneko et al. 2011). 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.

2.11 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 (Anitua et al. 2015). 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 [Glu¹]-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 were considered 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.
3. Results

3.1 Synthesis and physicochemical characterisation

The synthesis conditions described here allowed us to obtain the different coatings. SEM micrographs of 70M30T (Fig. 1a) and 50M50G (Fig. 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 ± 1.82° and 67.59 ± 1.03° for 70M30T and 50M50G coatings, respectively. These data indicate that the 70M30T biomaterial is more hydrophilic than 50M50G.

3.2 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.

3.3 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.

3.4 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 1 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. However, 30 serum proteins adhered differentially to the 50M50G material (Table 2). Proteins related to tissue regeneration and bone metabolism such as plasminogen (Mao et al. 2014; Kanno et al. 2016), proteoglycan 4 (Novince et al. 2012), vitronectin (Salasznyk et al. 2004), Apo E (Newman et al. 2002; Dieckmann et al. 2013), kininogen-1 (Tsuruga et al. 2006; Yamamura et al. 2006) and complement C3 (Maekawa et al. 2014; Matsuoka et al. 2014; Kuo et al. 2015) 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.
4. 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 alkoxy-silane with an epoxy group. In contrast, the TEOS does not possess that group (Schottner 2001). 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 (Hulsart-Billström et al. 2016).

Proteomic analysis using LC/MS-MS identified and quantified the proteins adsorbed onto the two surfaces (Tables 1 and 2). 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 (Varley et al. 1995; Kim et al. 2011). The increased abundance of bone regeneration/repair-related proteins like plasminogen (Mao et al. 2014; Kanno et al. 2016), proteoglycan 4 (Novince et al. 2012), vitronectin (Salasznyk et al. 2004), Apo E (Newman et al. 2002; Dieckmann et al. 2013) and kininogen-1 (Tsuruga et al. 2006; Yamamura et al. 2006) 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 (Dunkelberger & Song 2010). 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 (Zhang et al. 2010; Du Clos 2013). CRP is a well-documented risk factor for cardiac diseases (Zhang et al. 2010). 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 (Siegel et al. 1974). 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 (Engberg et al. 2015). Both C3a and C5b are responsible for the recruitment and activation of the immune cells, such as macrophages, to the activation site (Ricklin et al. 2010). The complement-activated macrophages regulate fibrogenesis by promoting the cytokine activity and cell migration, resulting in the fibroblast proliferation and collagen synthesis (Song et al. 2000). 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 (Anderson et al. 2008).

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 (Nilsson-Ekdahl et al. 1993). Similarly, Engberg et al. have established 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 (Engberg et al. 2015). 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 2 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.

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.

Acknowledgements

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Table 2

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**Table 1.** LC-MS/MS detected proteins differentially predominant in the film adsorbed to the 70M30T 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>
<thead>
<tr>
<th>Description</th>
<th>Ratio 50M50G/70M30T</th>
<th>Ref. bone metabolism or/and immune response</th>
<th>DAVID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropomyosin alpha-3 chain</td>
<td>0.06</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Filagrin-2</td>
<td>0.35</td>
<td>-</td>
<td>5,8</td>
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<tr>
<td>Hornerin</td>
<td>0.40</td>
<td>-</td>
<td>5,8,9</td>
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<tr>
<td>Keratin, type II cytoskeletal 1b</td>
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<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 71</td>
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<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 78</td>
<td>0.60</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Keratin, type I cytoskeletal 10</td>
<td>0.61</td>
<td>-</td>
<td>9</td>
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<tr>
<td>Keratin, type II cytoskeletal 2 epidermal</td>
<td>0.63</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 2. 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).

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Complement C1r subcomponent</td>
<td>1.38</td>
<td>(Conway, 2015)</td>
<td>1,2,5,8</td>
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<tr>
<td>Plasminogen</td>
<td>1.49</td>
<td>(Kanno et al., 2016; Mao et al., 2014)</td>
<td>3,4,5,8</td>
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<td>Ig kappa chain V-II region Cum</td>
<td>1.63</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Apolipoprotein A-IV</td>
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<td>5</td>
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<td>Hemopexin</td>
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<td>Ig lambda-2 chain C regions</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Proteoglycan 4</td>
<td>1.79</td>
<td>(Novince et al., 2012)</td>
<td>7</td>
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<td>Complement factor H</td>
<td>1.82</td>
<td>(Fedarko, 2000)</td>
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<td>Ig kappa chain V-III region VG (Fragment)</td>
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<td>Ig kappa chain V-III region SIE</td>
<td>1.93</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kininogen-1</td>
<td>2.02</td>
<td>(Tsuruga et al., 2006; Yamamura et al., 2006)</td>
<td>1,2,3,4,5,7</td>
</tr>
<tr>
<td>C4b-binding protein alpha chain</td>
<td>2.06</td>
<td>(Conway, 2015)</td>
<td>1</td>
</tr>
<tr>
<td>Complement C1s subcomponent</td>
<td>2.07</td>
<td>(Conway, 2015)</td>
<td>1,2,5,8</td>
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<tr>
<td>Ig kappa chain V-IV region Len</td>
<td>2.08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>2.21</td>
<td>(Salasznyk et al., 2004)</td>
<td>7</td>
</tr>
<tr>
<td>Complement component C7</td>
<td>2.22</td>
<td>(Conway, 2015)</td>
<td>1</td>
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<tr>
<td>Complement C1q subcomponent subunit B</td>
<td>2.24</td>
<td>(Conway, 2015)</td>
<td>1,2</td>
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<tr>
<td>Complement C1q subcomponent subunit C</td>
<td>2.26</td>
<td>(Conway, 2015)</td>
<td>1,2</td>
</tr>
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FIGURE LEGENDS

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

**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 (µg/µl) of cells grown without disc (oblique lines), grown on control Ti discs (horizontal lines), 70M30G (white column) and 50M50G coated Ti discs (black column).

**Figure 3.** In vivo studies. a) Light microscopy images of (EXAKT® cut and Gomori Trichrome stain) in vivo implants 4 and 8 weeks post-implantation of 70M30T and 50M50G sol-gelcoated screw. b) Quantification of the estimated area of fibrous connective tissue for
a n=4. Significant values were considered for a p<0.05 (ANOVA, with Newman-Keuls post-test). Calibration bar 200 µm.