Title

Proteomic analysis of silica hybrid sol-gel coatings: a potential tool for predicting biocompatibility of implants *in vivo*

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Abstract

Biomaterials interact with the host organism, determining the success or failure of an implantation. *In vitro* testing is used to assess the biocompatibility of a material. Unfortunately, *in vitro* and *in vivo* results are not always concordant. New methods for biomaterial characterisation are needed for an effective prediction of *in vivo* outcome. The first layer of proteins conditions the host response. Four distinct hybrid sol-gel biomaterials were tested. No differences *in vitro* were observed, although *in vivo* show distinct material behaviour. To characterise the first layer of proteins adsorbed onto the different surfaces, a mass spectrometry experiment was carried out. Six of the 171 proteins adsorbed onto the surfaces were significantly more abundant on the materials with weak biocompatibility, with known relation to the complement pathway. This could indicate that protein analysis might be a suitable tool for the prediction of the *in vivo* outcome of implantations using new biomaterials.

Keywords: hemocompatibility, osteoimmunology, fibrous capsule, bone regeneration, dental implants, C-reactive protein

Introduction

Newly developed biomaterials used in the field of medical engineering and regenerative medicine require *in vitro* testing to assess their safety and efficacy. One of the major drawbacks of implantations is the induction of foreign body reaction, which might include an acute and chronic inflammation and scar tissue formation (Duffield et al. 2008; Boehler et al. 2011), conditions that cannot be tested *in vitro*. Consequently, the *in vitro* outcome does not predict well the *in vivo* behaviour of a given biomaterial. It is, therefore, necessary to develop new methods for characterisation of biomaterials, with sufficient accuracy to predict the outcome of implantation (Hulsart-Billström et al. 2016).

Biomaterials interact with the host organism on several levels of biological organization, and this interface with the host organism might determine the success or failure of the implant. In particular, the surface properties of the implanted device (*eg*, hydrophilicity, roughness or surface energy) are important for defining a path of tissue remodelling (Molino et al. 2012).

When a biomaterial interacts with the bodily fluids of the host, it triggers a natural immune response to the foreign body. The immune response starts with the activation of the innate immune system, a normal short-term reaction. However, sometimes, the implant might induce chronic inflammation and the formation of a fibrous capsule ((myo)fibroblasts, neutrophils and foreign body giant cells (Damanik et al. 2014)), leading to implant rejection (Boehler et al. 2011).

The understanding of the biological events after the implantation trauma is crucial in the development of new biomaterials to prevent or control blood coagulation, infections, immune response and, ultimately, implant rejection. It has been suggested that the differences between the foreign body reactions induced by different biomaterials are mostly determined by the first layer of serum proteins adsorbed onto the implant surface (Brash & Ten Hove 1984; Anderson et al. 2008). The adsorption mechanisms are not yet completely elucidated (Arvidsson et al. 2007); however, the first protein layer is likely to be responsible for the cell/organism response to the foreign bodies (Engberg et al. 2015).

Proteomics has at its disposal powerful tools to examine the proteins adsorbed on different surfaces (Kaneko et al. 2011). In a previous study, we have reported differences between the compositions of protein layers deposited onto two distinct titanium surfaces (Romero-Gavilán et al. 2017). Some authors have proposed the

C4/C4BP protein ratio as a predictor of biomaterial biocompatibility (Engberg et al. 2015). Other studies indicate that binding of C3 protein onto biomaterials is correlated with their biocompatibility (Nilsson-Ekdahl et al. 1993). It is, therefore, tempting to assume a direct link between the proteins absorbed on the biomaterial surface and the elicited inflammatory response (Chen et al. 2015). However, the mechanisms of the immune response to biomaterials remain largely unknown (Vishwakarma et al. 2016).

The hybrid sol-gel materials synthesised using alkoxysilanes have shown great potential in biomedical applications (P. Chiriac et al. 2011). These biomaterials, during their degradation process, release silicon compounds in the $Si(OH)_4$ form (Juan-Díaz et al. 2016a), imparting the osteoinductive properties (Khan et al. 2014).

Our research group has been working on sol-gel biomaterials applied as coatings on dental implants. A variety of compositions based on mixtures of different alkoxysilanes had been developed and widely studied (Hernández-Escolano et al. 2013; Juan-Díaz et al. 2014; Martínez-Ibáñez et al. 2016; Juan-Díaz et al. 2016b). In our previous studies, a poor correlation between *in vitro* and *in vivo* results has been observed for some of these biomaterials. Whereas only small differences between the two studied surfaces have been found *in vitro*, a completely diverse *in vivo* behaviour has been observed. The objective of the current study was to characterise the first protein layer absorbed on titanium discs coated with four distinct hybrid silica sol-gel formulations. Two of the formulations induced the formation of a fibrous connective tissue surrounding the implant (poor biocompatibility), whereas the other two showed good osteointegration (good biocompatibility). We examined the correlation between the properties of the first adsorbed protein layer with the *in vivo* outcome of implantation for the four tested coatings.

Materials and methods

Titanium discs

Titanium (Ti) discs (12 mm in diameter, 1-mm thick) were made from commercially available, pure, grade-4 Ti bar (Ilerimplant SL, Lleida, Spain). We used sandblasted acid-etched (SAE) Ti discs, abraded with 4-μm aluminium oxide particles and acid-etched by submersion in sulphuric 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 sterilised using UV irradiation.

Sol-gel synthesis and sample preparation

The silica hybrid coatings were obtained using the sol-gel route. The alkoxysilanes precursors used were methyltrimethoxysilane (MTMOS), 3-glycidoxypropyl-trimethoxysilane (GPTMS), tetraethyl orthosilicate (TEOS) and triethoxyvinylsilane (VTES) (Sigma-Aldrich, St. Louis, MO, USA). Four different compositions were synthesised with molar percentages of 70% MTMOS and 30% TEOS (70M30T), 35% MTMOS, 35% GPTMS and 30% TEOS (35M35G30T), 50% MTMOS and 50% GPTMS (50M50G) and 50% VTES and 50% GPTMS (50V50G). 2-Propanol (Sigma-Aldrich, St. Louis, MO, USA) was used as a solvent in the alcohol-siloxane mix (volume ratio 1:1). Hydrolysis of alkoxysilanes was carried out by adding (drop s^{-1}) the corresponding stoichiometric amount of acidified aqueous solution 0.1M HNO_3 (Panreac, Barcelona, Spain). The solution was stirred for 1 h and left to rest for 1 h; the samples were prepared immediately after this stage. SAE titanium discs were used as the substrate. The coating was performed employing a KSV DC dip-coater (Biolin Scientific, Stockholm, Sweden). The discs were introduced into the appropriate sol-gel solution at a speed of 60 cm min^{-1}, for one minute, and removed at a 100 cm min^{-1}. Finally, samples were cured for 2 h (0M30T and 35M35G30T coatings at 80 ºC, and 50M50G and 50V50G, at 140 ºC).

Physicochemical characterisation of coated titanium discs

The contact angle was measured using an automatic contact angle meter OCA 20 (DataPhysics Instruments, Filderstadt, Germany). An aliquot of 10 μL of ultrapure 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

(http://www.dataphysics.de/startseite/produkte/software-module/). Five discs of each material were examined (two drops deposited on each). The surface topography of coated titanium discs was characterised using atomic force microscopy (AFM) (Bruker Multimode 8, MA, USA) under dry conditions. Measurements were carried out at scan size of 60 μm, with a scan rate of 1 Hz. A mechanical profilometer Dektack 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 coatings were studied using scanning electron Leica-Zeiss LEO microscope (SEM; Leica, Wetzlar, Germany) under vacuum. Platinum sputtering was applied to increase conductivity for the SEM.

In vitro assays

Cell culture

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. We used the 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). The cells were incubated for 24 h at 37 ºC in a humidified (95%) atmosphere with 5% CO_2. Then, 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 the cells were incubated again under the same conditions. The culture medium was changed every 48 h. Wells without Ti discs were used as a control of culture conditions.

Cytotoxicity

The cytotoxicity of the biomaterials was assessed following the ISO 10993-5 norm. 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 cells not exposed to the biomaterial extract as a negative control. At the same time, cells incubated with latex (known to be cytotoxic) were used as a positive control; 70% of cell viability was considered the limit below which a biomaterial was considered cytotoxic.

Alkaline phosphatase (ALP) activity

The conversion of p-nitrophenylphosphate (p-NPP) to p-nitrophenol was used to assess the ALP activity. Aliquots of 0.1 mL of cell lysate were used to conduct the assay. Lysate was obtained by adding 100 µL of lysate buffer (0.2% Triton X-100, 10 mM Tris-HCl pH 7.2) to each well. After a period of 7 min of ice, the samples were removed from the wells, transferred to microtubes, and sonicated for 2 min, obtaining the final lysate. Following centrifugation, 100 µL of p-NPP (1mg mL^{-1}) in substrate buffer (50 mM glycine, 1 mM $MgCl_2$, pH 10.5) was added to 100 µL of the supernatant obtained from the lysate. After a 2-h incubation in the dark (37 ºC, 5% CO_2), the absorbance was spectrophotometrically measured using a microplate reader, at a wavelength of 405 nm. ALP activity was read from a standard curve, previously obtained using different solutions of p-nitrophenol and 0.02 mM sodium hydroxide. The results were presented as mmol p-nitrophenol per h (mM PNP h^{-1}). The ALP activity data were normalised to the total protein content (µg $µL^{-1}$) established using Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA), at 7 and 14 days.

In vivo assay

In vivo experimentation

To evaluate the histological response to the selected coatings, dental implants were surgically placed in the tibia of New Zealand rabbits (*Oryctolagus cuniculus*). This implantation model is widely used for examining the osseointegration of dental implants (Mori et al. 1997). All experiments were conducted in accordance with the protocols of Ethical Committee at the University of Murcia (Spain), European guidelines, the legal conditions in R. D. 223/1988 of March 14th and the Order of October 13rd, 1988 of the Spanish Government law on the protection of experimental animals. The rabbits 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*. Dental implants were supplied by Ilerimplant SL (Lleida, Spain). The implants were of internal connection type, made with titanium grade-4 (trademark GMI dental implants, of 3.75-mm diameter and 8-mm length, Frontier model). They had undergone the Advanced Doubled-Grip surface treatment, a combination of white corundum micro-bubble treatment and etching with nitric acid and sulphuric acid solution. We used 40 implants, 20 uncoated (controls) and 5 coated (test samples) with each material. The control samples and test samples were implanted under the same conditions.

We used 20 rabbits (5 for each material) with weights between 2000 and 3000 g. Their age was near the physical closure, which is indicative of an adequate bone volume. The implantation period was 2 weeks. This period was chosen to be able to examine a complete osseointegration process. The implants were inserted in the left and right proximal tibiae, two implants per animal (one control and one test sample). Animals were first sedated (chlorpromazine hydrochloride) and prepared for surgery, and then anesthetized (ketamine chlorhydrate). A coetaneous incision was made in the proximal tibia. The periosteum was removed, and the osteotomy was made using low revolution micromotor and drills of successive diameters of 2, 2.8 and 3.2 mm, with continuous irrigation. Implants were put in by press-fit, and the wound was sutured, washed with saline solution and covered with plastic spray dressing (Nobecutan, Inibsa Laboratories, Barcelona, Spain). At the end of each implantation period, the animals were euthanized by carbon monoxide inhalation to retrieve the screws and to study the surrounding tissues.

Histological quantification

Samples for histological examination were processed following the method described by Peris *et al.* (Peris et al. 1993). Briefly, the samples were embedded in methyl methacrylate, and 25–30 mm thick sections were obtained using EXAKT technique (EXAKT Technologies, Inc., Oklahoma, USA). For optical microscopy examination, all the sections were stained using Gomori Trichrome solution. The region of interest was delimited using the software *ImageJ* (https://imagej.nih.gov/ij/). The area occupied by connective tissue surrounding the implant was measured (mm^2).

Statistical analysis

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

Adsorbed protein layer

Ti discs coated with different sol-gel compositions were incubated in 24-well plates 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 the removal of the serum, 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 a 4% SDS, 100 mM DTT, 0.5 M TEAB solution (Sigma-Aldrich, St. Louis, MO, USA) The experimental method was adopted from a study by Kaneko *et al.* (Kaneko et al. 2011). Four replicates of each biomaterial were used for analysis. 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}.

Proteomic analysis

The eluted protein sample was 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 connected to a SYNAPT G2-Si spectrometer (Waters, Milford, MA, USA). Samples were loaded onto a Symmetry 300 C18 UPLC Trap column of 5 µm, 180 µm × 20 mm (Waters, Milford, MA, USA), connected to a BEH130 C18 column of 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 in a positive ion mode. Data were post-acquisition lock-mass corrected using the double-charged monoisotopic ion of [Glu^1]-fibrinopeptide B. Accurate LC-MS data were collected in HDDA mode, which enhances signal intensities using the ion mobility separation.

Progenesis LC-MS software (Nonlinear Dynamics) was used for differential protein expression analysis. Raw files were imported into the program, 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 peptides detected in all samples was searched against a 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.

Results

Synthesis and physicochemical characterisation

Four different sol-gel compositions were coated onto the surface of SAE titanium discs. The coatings were homogeneous, without cracks, as can be seen on SEM micrographs (Figure 1).The 70M30T biomaterial conserved the initial titanium topography to a greater extent (Figure 1a) than 35M35G30T (b), 50M50G (c) and 50V50G (d). AFM images show that the 70M30T surface is the roughest (Figure 2a). The 35M35G30T (Figure 2b), 50M50G and 50V50G coatings display a smoother topography (Figure 2c and 3d), covering well the initially rough titanium surface. Mechanical profilometer measurements obtained a significantly higher Ra value for 70M30T than for 35M35G30T, 50M50G and 50V50G coatings (Figure 3). This result may be due to the GPTMS in the last three biomaterials, which might affect the coating thickness, allowing an effective masking of the initial rough features. Contact angle measurements showed that the coatings with TEOS (non-organo-modified precursor), 70M30T and 35M35G30T, were the most hydrophilic (Figure 4). The 50M50G and 50V50G coatings had a larger contact angle, probably due to the higher organic matter content.

In vitro assays

None of the biomaterials was cytotoxic (Figure 5a). ALP activity measurements showed no significant differences between the tested materials (Figure 5b). Interestingly, whereas normalised ALP activity of control cells (wells without Ti disc) seemed to decrease with time, the cells grown on Ti and coated Ti discs maintained or even increased this activity.

In vivo assays

Histological analysis showed a good osseointegration pattern (it occurs on direct contact between the new bone and the implant surface) for 70M30T- and 35M35G30T-coated implants (Figure 6b and 6c). However, using 50V50G (Figure 6d) and 50M50G (Figure 6e) coatings resulted in a formation of a distinct area of fibrous connective tissue between the material and the osteoid/newly formed bone. The sizes of these areas were similar for 35M35G30T and 70M30T but were larger in the 50V50G and 50M50G samples (Figure 7). The materials were categorised into two groups according to the *in vivo* results. The first group, comprising 70M30T and 35M35G30T, achieved a good level of osseointegration (high biocompatibility). The coatings in the second group, composed of the 50M50G and 50V50G materials, caused the formation of

fibrous connective tissue surrounding the implant surface, blocking the direct contact between the newly formed bone and the biomaterial (poor biocompatibility).

Proteomic analysis

The eluted proteins were analysed using LC-MS/MS. Progenesis QI software was employed to identify the proteins attached to different materials. The DAVID was used to classify according to their function. We detected and identified 171 proteins for each material. When comparing 70M30T with 35M35G30T (good biocompatibility group), 6 proteins were identified as predominant on the 35M35G30T coating. The largest difference was observed for MYH1 protein (17.19-fold), involved in maintenance of cytoskeletal integrity. The other proteins were associated with the immune system (C1QA and FCN2) and metal-binding (HBA) functions. However, we found 3 proteins that adhered to the 70M30T coating in larger amounts than to 35M35G30T. These were the proteins involved in the protection against the inflammatory disorders (CLUS) (Cunin et al. 2016), coagulation (FAXII) (de Maat & Maas 2016) and lipid transport and carbohydrate binding (APOA5) (Table 1).

It was also carried out a comparative study of proteins adhering to 50V50G and 50M50G surfaces (poor biocompatibility group, Table 2). Two proteins were found adhering in substantially larger amounts to the 50V50G coating, both with metal-binding functions (HORN and DSC1). The enzyme endopeptidase (LCN1) was found predominantly on 50M50G surfaces. The proteomic analysis showed few differences between the coatings in each group. On the two coatings with good biocompatibility, we found 9 differentially predominant proteins and only 3 on the poor biocompatible materials.

To find an explanation for the differences in the *in vivo* outcomes, we compared the proteins differentially attached to each of the materials with good biocompatibility (70M30T and 35M35G30T) with the proteins detected on one of the materials with a negative outcome (poor biocompatibility group), 50M50G. Fifteen proteins were found predominantly adsorbed onto the 50M50G surface in comparison with the other two materials. They included the proteins involved in bone metabolism and regeneration (VTNC (Salasznyk et al. 2004), APOE (Shiraki et al. 1997; Newman et al. 2002; Rodrigues et al. 2012) and KNG1 (Tsuruga et al. 2006; Yamamura et al. 2006)) and proteins related to the immune system and inflammatory response (VTNC , CRP, SAMP, C1QB, C1QC, CO7, C1S and C4BPA). All these proteins seem to favour the complement cascade activation (Ricklin et al. 2010), except for C4BPA and VTNC (which might inhibit this process (Mollnes & Kirschfink 2006)). However, 5 proteins (3

types of keratins, HORN and FILA2) adsorbed more to 70M30T and 35M35G30T than to 50M50G coatings. These proteins play a role in the cytoskeleton integrity and have a peptidase activity (Table 3).

The proteins on the good biocompatibility materials (70M30T and 35M35G30T) were also compared with those found on the 50V50G coating (negative outcome). Table 4 displays the 16 proteins with the highest abundance, among which 15 adhered more to the 50V50G coating. All the proteins characteristically associated with the 50V50G coating are directly involved in the immune response and acute inflammatory response (CRP, CO5, SAMP, C1QB, IC1, CFAH, CO7, C1S, CO3, C1R, VTNC and C1QC). Most of the proteins in this cluster are involved in the complement cascade activation (Ricklin et al. 2010), except for CFAH and VTNC, the important regulators/repressors of the activation of the complement system (Mollnes & Kirschfink 2006; Kishore & Sim 2012). Only one protein, the endopeptidase SPB, was more abundant on both positive-outcome materials than on 50V50G.

To establish which proteins adhere differentially to both 50M50G and 50V50G (poor biocompatibility) in comparison with 35M35G30T and 70M30T (good biocompatibility) sol-gel coatings, the data in Tables 3 and 4 was analysed. The common proteins with increased abundance on the poor biocompatibility materials were looked for (Table 5). Nine such proteins were found, including VTCN, 2 immunoglobulins and 6 proteins related to the acute inflammatory response processes of the immune system (CRP, SAMP, C1QB, C1QC, C1S and CO7).

These results demonstrate that the proteins related to the activation or inhibition of the complement cascade are adhered predominantly to 50M50G and 50V50G coatings. The complement system is a highly complex mechanism with the intricate regulation of inhibition and activation. Table 6 displays the abundance of adsorbed inhibitory proteins (C4BPS, CFAH and VTNC), comparing the materials with good and poor in vivo outcome and their respective protein ratios. The complement-inhibiting proteins C4BPA, CFAH and VTNC and two of the principal activating proteins of the same system, CRP and SAMP, were the main differentially adhering proteins on the poor-outcome materials. Interestingly, the inhibitory proteins were more predominant in the weak biocompatibility material group. Nevertheless, the ratio of complement-system inhibitory proteins to activating proteins was higher for the biomaterials of good biocompatibility (70M30T and 35M35G30T) than for those with the poor biocompatibility (50M50G and 50V50G).

Discussion

Biomaterials and non-biological substances introduced into the human body are exposed to the blood and tissue elements. The first event after their introduction is the deposition of a monolayer of plasma proteins onto the surfaces of foreign materials. This can induce, among other processes, the activation of the complement system and coagulation cascades (Andersson et al. 2005). In extreme cases, it can result in a host reaction to the foreign body, which includes blood-material interactions, provisional matrix formation, acute and/or chronic inflammation, granulation tissue development and the formation of fibrous capsules (Luttikhuizen et al. 2006; Anderson et al. 2008). The physicochemical properties of the different biomaterials used in implants such as their topography, roughness, chemistry and surface energy might affect the types and quantities of adsorbed proteins and even their conformation (Vishwakarma et al. 2016) and, consequently, the body response. This paper focuses on the characterisation of the protein layer adsorbed onto titanium coated with four distinct silica hybrid sol-gel biomaterials (70M30T, 35M35G30T, 50M50G and 50V50G). The coatings had been originally introduced to make titanium implant surface more bioactive and increase its capacity of osteogenic molecule delivery (Martínez-Ibáñez et al. 2016; Juan-Díaz et al. 2016b). The different compositions of the coatings change their physicochemical properties (Romero-Gavilán et al. 2016). Thus, 70M30T formulation resulted in the highest roughness in comparison with the others (all containing GPTMS). 50V50G and 50M50G coatings were more hydrophobic than the other surfaces, probably due to their higher organic compound content. However, compositions containing TEOS (non-organo-modified alkoxysilane) displayed a more hydrophilic behaviour. Apart from dissimilarities in morphology and hydrophilicity, these coatings also differ chemically, depending on the organo-modified alkoxysilanes used in their synthesis (Schottner 2001). All these variations might affect the implant biocompatibility and, therefore, the biological response.

None of the four biomaterials tested *in vitro* was found to be cytotoxic, and the ALP activity assay showed no significant differences. In fact, all the sol-gel compositions showed a good *in vitro* behaviour, even in comparison with the control sandblasted and acid-etched titanium (SAE-Ti), whose good properties are widely known (Wennerberg & Albrektsson 2009; Khorasani et al. 2015).

Although *in vitro* experimentation is largely used to predict the host response to a biomaterial, it cannot accurately predict biocompatibility *in vivo.* This is because, in such experiments, many of the *in vivo* elements are missing (eg, the blood and the immune system). These elements strongly affect the *in vivo* result, especially in the

field of osteoregenerative materials (Anderson 2016; Hulsart-Billström et al. 2016). During the bone regeneration process, the vascularisation, stabilisation, scaffolding, cell signalling and progenitor cells are all required (Giannoudis et al. 2007). Following the implantation, the material is in contact with the blood, which contains the innate immune system cells that might degrade the implanted material or induce the formation of connective tissue surrounding the implant, hampering the osteointegration. The formation of the fibrous capsule surrounding the implant is regarded a natural immune response of the host to a foreign body (Varley et al. 1995).

A fibrous connective tissue was found surrounding 50M50G and 50V50G-coated implants; this did not happen in the cases 70M30T and 35M35G30T coatings even though they showed similar *in vitro* outcomes. Hence, even though *in vitro* tests might offer some guidance, neutral or positive results of such tests do not guarantee similar outcomes *in vivo*.

The implanted biomaterials are exposed to the blood immediately after their insertion. The *in vivo* behaviour of a given biomaterial is difficult to predict; however, it has been proposed that the first layer of adhering serum proteins might be responsible for the different responses of the host organism (Ekdahl et al. 2013).

Employing proteomic analysis using LC/MS-MS and the Progenesis software, the first layer of proteins was studied. Following examination of the proteins predominately adhered to the biomaterials with similar *in vivo* response (Tables 1 and 2), it was acknowledged that the differences within these groups of materials were very limited. However, when the two biomaterials with good biocompatibility were compared to either of the coatings inducing the formation of connective tissue, a significant number of differentially adhering proteins was detected. Interestingly, proteins related to the immune/inflammatory response were found predominantly on the poor biocompatibility surfaces 50M50G (Table 3) and 50V50G (Table 4), when compared simultaneously to 70M30T and 35M35G30T (good biocompatibility).

In parallel, it was also found (Table 5) some proteins differentially adhered to the two good biocompatibility materials (70M30T and 35M35G30T) in comparison with the poor biocompatibility coatings (50M50G and 50V50G). It is compelling that most of these proteins are associated with the immune system, including the pentraxins CRP and SAMP, as well as the complement proteins (C1QB, C1QC, C1S and CO7). The latter belong, according to the DAVID analysis, to a protein cluster related to an acute inflammatory response. Notably, CRP, an activator of the classical pathway (Du Clos 2013) was particularly more abundant on the two materials with poor biocompatibility

than on the 70M30T and 35M35G30T coatings. CRP acts by direct binding of the globular heads of C1q, the first component of the complement system (Ricklin et al. 2010). We found that the amounts of C1q adhering to 50V50G and 50G50G surfaces were larger than on the coatings that achieve a good *in vivo* osseointegration. Upon the activation of C1q, the classical inflammatory pathway follows a series of complement activations, peaking on the generation of C3 and C5 convertases, which results in the production of C3a and C5b fragments. These are chemoattractant proteins with a role in the proliferation of the elements of the innate immunity system, such as macrophages (Ricklin et al. 2010). In turn, the macrophages regulate fibrogenesis by releasing the cytokines and growth factors. Furthermore, the macrophages modulate fibroblast proliferation and collagen synthesis (Song et al. 2000). However, the VTNC, which might participate in the regulation/repression of complement activation (Mollnes & Kirschfink 2006), is vital for interleukin IL-4 adhesion on biomaterial surfaces and induces the switch of the macrophages to their M2 reparative phenotype (Chen et al. 2015).

As this chain of events might lead to the formation of a fibrous capsule (a typical foreign body reaction of the host), it may seem paradoxical that the inhibitors of the complement system such as VTNC are found predominantly adhering onto 50M50G and 50V50G coatings, whose surfaces become surrounded by a layer of connective tissue after implantation. Explanation of this apparently contradictory finding might lie in the ratio of inhibitory to activator proteins on each surface. It is observed that the 70M30T and 35M35G30T biomaterials showed a higher ratio of the complement system inhibitory proteins to activator proteins in comparison with the materials of poor biocompatibility.

Our hypothesis is based on the fact that the equilibrium between the activating and inhibitory proteins regulating the complement system might determine the *in vivo* success of an implantation. Thus, a high ratio of inhibitory to activating proteins should lead to a positive outcome. A low ratio might trigger a disproportionate immune response with consequent excessive and chronic inflammation reaction, culminating in the formation of the fibrous capsule around the implant (Chen et al. 2015). This acute inflammation response might alter the normal balance between the coagulation cascade and the natural anticoagulant pathway, possibly by suppression of the latter (Opal & Esmon 2003). The anticoagulant pathway not only limits the coagulation but also modulates the inflammatory response (eg, by reduction of cytokine expression) (Esmon 2004). The lack of control in both coagulation and inflammatory pathways, a

consequence of the anticoagulant system alteration, might cause a negative *in vivo* response after implantation.

This hypothesis is consistent with other reports, in which the proteins of the complement system have been associated with biocompatibility problems (Mosqueira et al. 2001; Andersson et al. 2005). Engberg *et al*. have even proposed a method for prediction of biocompatibility by evaluating the ratio of C4/C4BP, assumed to reflect the inflammatory response of the host organism to a biomaterial (Engberg et al. 2015). However, in their study, a protein list was preselected; the CRP levels were not analysed in that publication.

The proteomic approach using the LC/MS-MS might have a significant potential for predicting the biocompatibility of biomaterials by analysing the first layer of proteins attached to the tested surfaces.

In summary, we tested four different hybrid sol-gel biomaterials with distinct physico-chemical properties, which might affect the composition of the protein layer adhered to their surfaces. The implants with different coatings had different *in vivo* outcomes. Two of the implantations resulted in the formation of fibrous connective tissue between the implant and new bone (50V50G and 50M50G). The other two biomaterials achieved a successful osseointegration (70M30T and 35M35G30T). The results of our proteomic analysis suggested some reasons for these differences. In this paper is described a cluster of proteins differentially adhering to 50M50G and 50V50G coatings, which might negatively affect the osseointegration. Most of the proteins in this group have been associated with the acute inflammatory response. Therefore, the formation of the fibrous connective structure surrounding the 50M50G and 50V50G materials might be correlated with the adsorption of these proteins. The proteins in this group (CRP, SAMP, C1S, C1QB, C1QC, C7 and VTN) should be useful as biomarkers in the evaluations of material biocompatibility.

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Tables and table captions

Table 1. Proteins differentially predominant in 70M30T vs 35M35G30T sol-gel coating comparatives (Progenesis method). ANOVA (p-value < 0.05). DAVID classification functions were (1) inflammatory/immune response, (2) hydroxylation, (3) blood coagulation, (4) apoptosis regulation, (5) metal binding, (6) phosphorylation, (7) carbohydrate binding, (8) peptidase activity, (9) lipid transport and (10) cytoskeleton integrity.

Description	Accession	Ratio 35M35G30T/70M30T	DAVID
Myosin-1	MYH1_HUMAN	17,19	10
L-lactate dehydrogenase B chain	LDHB_HUMAN	12,18	-
Glutamate dehydrogenase 1, mitochondrial	DHE3_HUMAN	8,65	-
Ficolin-2	FCN2_HUMAN	8,28	1-2-7-5-10
Complement C1q subcomponent subunit A	C1QA_HUMAN	3,06	1-2
Hemoglobin subunit alpha	HBA_HUMAN	1,73	5-10
Clusterin	CLUS_HUMAN	0,61	1-4-9
Coagulation factor XII	FA12_HUMAN	0,58	3-5-8
Apolipoprotein A-V	APOA5_HUMAN	0,53	7-9

Table 2. Proteins differentially predominant in 50M50G vs 50V50G sol-gel coating comparatives (Progenesis method). ANOVA (p-value < 0.05). DAVID classification functions were (1) inflammatory/immune response, (2) hydroxylation, (3) blood coagulation, (4) apoptosis regulation, (5) metal binding, (6) phosphorylation, (7) carbohydrate binding, (8) peptidase activity, (9) lipid transport and (10) cytoskeleton integrity.

Description	Accession	Ratio 50V50G/50M50G	DAVID
Hornerin	HORN_HUMAN	3,41	5-8-10
Desmocollin-1	DSC1_HUMAN	2,14	5
Lipocalin-1	LCN1_HUMAN	0,14	8

Table 3. Proteins differentially predominant in both 70M30T vs 50M50G and 35M35G30T vs 50M50G sol-gel coating comparatives (Progenesis method). ANOVA (p-value < 0.05). DAVID classification functions were (1) inflammatory/immune response, (2) hydroxylation, (3) blood coagulation, (4) apoptosis regulation, (5) metal binding, (6) phosphorylation, (7) carbohydrate binding, (8) peptidase activity, (9) lipid transport and (10) cytoskeleton integrity.

Description	Accession	Ratio 50M50G/ 70M30T	Ratio 50M50G/ 35M35G30T	DAVID
C-reactive protein	CRP_HUMAN	7,83	5,28	1-5-8
Lipocalin-1	LCN1_HUMAN	4,28	5,37	8
Serum amyloid P-component	SAMP_HUMAN	3,48	1,93	1-5-7-8
Apolipoprotein E	APOE_HUMAN	2,40	3,09	3-4-5-6-7-9
Complement C1q subcomponent subunit C	C1QC_HUMAN	2,26	2,12	1-2
Complement C1q subcomponent subunit B	C1QB_HUMAN	2,24	2,05	1-2
Complement component C7	CO7_HUMAN	2,22	1,99	1
Vitronectin	VTNC_HUMAN	2,21	1,77	7
Ig kappa chain V-IV region Len	KV402_HUMAN	2,08	1,93	-
Complement C1s subcomponent	C1S_HUMAN	2,07	2,19	1-2-5-8
C4b-binding protein alpha chain	C4BPA_HUMAN	2,06	2,25	1
Kininogen-1	KNG1_HUMAN	2,02	1,82	1-2-3-4-5-7
Ig lambda-2 chain C regions	LAC2_HUMAN	1,73	1,57	-
Apolipoprotein A-IV	APOA4_HUMAN	1,67	2,23	5
Ig kappa chain V-II region Cum	KV201_HUMAN	1,63	1,79	-
Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	0,63	0,62	10
Keratin, type I cytoskeletal 10	K1C10_HUMAN	0,61	0,55	10
Keratin, type II cytoskeletal 78	K2C78_HUMAN	0,60	0,56	10
Hornerin	HORN_HUMAN	0,40	0,16	5-8-10
Filaggrin-2	FILA2_HUMAN	0,35	0,35	5-8

Table 4. Proteins differentially predominant in both 70M30T vs 50V50G and 35M35G30T vs 50V50G sol-gel coating comparatives (Progenesis method). ANOVA (p-value < 0.05). DAVID classification functions were (1) inflammatory/immune response, (2) hydroxylation, (3) blood coagulation, (4) apoptosis regulation, (5) metal binding, (6) phosphorylation, (7) carbohydrate binding, (8) peptidase activity, (9) lipid transport and (10) cytoskeleton integrity.

Description	Accession	Ratio 50V50G/ 70M30T	Ratio 50V50G/ 35M35G30T	DAVID
C-reactive protein	CRP_HUMAN	15,26	10,29	1-5-8
Complement C5	CO5_HUMAN	10,43	5,78	1-6
Serum amyloid P-component	SAMP_HUMAN	3,33	1,84	1-5-7-8
Complement C1q subcomponent subunit B	C1QB_HUMAN	2,45	2,24	1-2
Ig kappa chain V-IV region Len	KV402_HUMAN	2,45	2,27	-
Plasma protease C1 inhibitor	IC1_HUMAN	2,22	1,66	1-3
Complement factor H	CFAH_HUMAN	2,09	1,73	1
Complement component C7	CO7_HUMAN	2,07	1,86	1
Ig kappa chain V-III region SIE	KV302_HUMAN	2,03	1,83	-
Complement C1s subcomponent	C1S_HUMAN	1,98	2,10	1-2-5-8
Vitronectin	VTNC_HUMAN	1,98	1,58	7
Complement C3	CO3_HUMAN	1,93	1,65	1
Complement C1r subcomponent	C1R_HUMAN	1,92	1,79	1-2-5-8
Ig lambda-2 chain C regions	LAC2_HUMAN	1,72	1,57	-
Complement C1q subcomponent subunit C	C1QC_HUMAN	1,71	1,61	1-2
Serpin B3	SPB3_HUMAN	0,33	0,41	8

Table 5. Proteins differentially predominant at the same time in both 50M50G and 50V50G respect to 35M35G30T and 70M30T sol-gel coatings (Progenesis method). ANOVA (p-value < 0.05).

Description	Accession	Ref. bone metabolism or/and immune response
C-reactive protein	CRP_HUMAN	(Varley et al. 1995; Bottazzi et al. 2016; Ghafouri et al. 2016)
Serum amyloid P-component	SAMP_HUMAN	(Sim et al. 1979; Fujimoto et al. 2015)
Complement C1q subcomponent subunit C	C1QC_HUMAN	(Ricklin et al. 2010)
Complement C1q subcomponent subunit B	C1QB_HUMAN	(Ricklin et al. 2010)
Complement component C7	CO7_HUMAN	(Ricklin et al. 2010)
Vitronectin	VTNC_HUMAN	(Mao et al. 2014)
Ig kappa chain V-IV region Len	KV402_HUMAN	-
Complement C1s subcomponent	C1S_HUMAN	(Ricklin et al. 2010)
Ig lambda-2 chain C regions	LAC2_HUMAN	-

Table 6. Inhibitory/activator protein ratio detected in 70M30T, 35M35G30T, 50M50G and 50V50G biomaterials.

Ratios	70M30T	35M35G30T	50M50G	50V50G
CFAH/CRP	75,19	61,30	17,44	10,32
CFAH/SAMP	2,04	1,37	1,07	1,28
C4BPA/CRP	18,53	11,48	4,88	1,89
C4BPA/SAMP	0,50	0,26	0,30	0,24
VTNC/CRP	76,05	64,09	21,47	9,87
VTNC/SAMP	2,06	1,43	1,31	1,23

Figure captions

Figure 1. SEM micrographs of hybrid sol-gel coatings on SAE titanium discs: 70M30T (a), 35M35G30T (b), 50M50G (c) and 50V50G (d). Calibration bar 10 μm.

Figure 2. AFM images of hybrid sol-gel coatings: 70M30T (a), 35M35G30T (b), 50M50G (c) and 50V50G (d).

Figure 3. Mechanical profilometer measures of Ra. Bars indicate standard deviations.

Figure 4. Contact angle results for sol-gel coatings on titanium discs. Bars indicate standard deviations.

Figure 5. MC3T3-E1 cell viability and mineralization *in vitro*. Percentage of cell survival following the norm ISO 10993-5 (a) ALP activity (mM PNP h^{-1}) normalized to the amount of total protein (μg μL^{-1}) levels of the MC3T3-E1 cells cultivated on titanium discs treated with 70M30T (dotted column), 35M35G30T (checkered column), 50M50G (diagonal striped column) and 50V50G (horizontal striped column) 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. There were no statistically significant differences between the different formulations at the times measured.

Figure 6. Light microscopy representative images (EXAKT® cut and Gomori Trichrome stain) *in vivo* implants 2 weeks post-implantation of: a) representative photo of the area chosen to analyse the osseointegration state b) 70M30T, c) 35M35G30T, d) 50M50G and e) 50V50G sol-gel coated screws. The white arrows point to the area where the fibrous connective tissue was being formed.

Figure 7. Area in mm^2 occupied by the fibrous connective tissue ocuppied by the four tested coatings. Significantly differences between 70M30T/35M35G30T and 50M50G/50V50G were found (ANOVA $p \leq 0.05$ with a Kruskal-Wallis post-test).