Title

The effect of strontium incorporation into sol-gel biomaterials on their protein adsorption and cell interactions

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Abstract

It is known strontium can both inhibit the osteoclast formation and stimulate the osteoblast maturation, so biomaterials containing this element can favour bone structure stabilisation. The addition of Sr to biomaterials could affect their interactions with proteins and cells. Here, a silica-hybrid sol-gel network doped with different amounts of $SrCl_2$ and applied as coatings on titanium discs was examined. *In vitro* analysis was performed to determine the potential effect of Sr in the coatings, showing enhanced gene expression of osteogenic markers (alkaline phosphatase and transforming growth factor-β) in MC3T3-E1 incubated with Sr-doped biomaterials. The examination of inflammatory markers (tumour necrosis factor-α and interleukin 10) in RAW 264.7 macrophages revealed an anti-inflammatory potential of these materials. Proteins adsorbed onto the coatings incubated with human serum (3 h at 37 ºC) were also analysed; mass spectrometry was used to characterise the proteins adhering to materials with different Sr content. Adding Sr to the coatings increased their affinity to APOE and VTNC proteins (associated with anti-inflammatory and osteogenic functions). Moreover, the proteins involved in coagulation processes, such as prothrombin, were more abundant on the coatings containing Sr than on the base sol-gel surfaces. Correlations between gene expression and proteomic results were also examined.

Keywords:

Dental implants, apolipoprotein E, vitronectin, osteogenesis, bone regeneration, proteomics

1. Introduction

Strontium is a bone-seeking trace metal, found in the bone in relatively small amounts (only 3.5% of the Ca content) [1]. It stimulates bone growth and reduces its resorption, and has a positive effect on osteogenesis. Even minor quantities of this element can help in the stabilisation of bone structure as it inhibits the osteoclast activity and stimulates osteoblast maturation [2]. Sr was pointed out on its potential use for osteoporosis treatment [3]. In particular, strontium ranelate and strontium chloride have been used in osteoporosis to decrease the risk of bone fractures [4].

The increasing interest in the effect of Sr on bone tissue regeneration has been reflected in the design and studies of new Sr-releasing biomaterials [5]. The silica sol-gel hybrid networks have been showing consistently encouraging results in biomedical applications [6]. These materials are bioactive, biocompatible and biodegradable. The sol-gel technique makes it easy to produce coated materials, allowing surface functionalisation of metal implants such as dental prostheses [7]. Moreover, the versatility of the sol-gel method permits the control of the degradation kinetics, making the materials of this family perfect candidates for controlled release vehicles [8]. The organic-inorganic sol-gel networks have been already used to develop new biomaterials that can release Sr, displaying a promising osteogenic behaviour [9–11].

Immediately after its implantation, a biomaterial begins to interact with surrounding tissues and fluids. A complex sequence of events is initiated in a cascade, oriented primarily towards the tissue repair and integration of the foreign body into the organism or its rejection [12]. One of the first steps in this process is the interaction between the biomaterial and bodily fluids, such as the blood. As a result, various proteins adhere to the surface of the implant through a competitive displacement mechanism (Vroman effect) [13]. The physico-chemical properties of biomaterials, such as surface energy, topography, the degree of hydrophilicity and chemical functionalisation, may control the surface-protein interactions, depending on the protein affinity. Thus, these biomaterial

properties will determine the composition, concentration and conformation of the protein layer adsorbed to it [14]. The activation and progression of biological processes after the implantation is largely dependent on this first layer of proteins formed after initial contact with the blood [15].

Inflammation processes triggered by the complement system pathway activation and the immune cell recruitment become a part of the microenvironment formed after the surgical procedure [12]. The specific proteins adhering onto the biomaterial might mark the initiation and intensity of this reaction [16]; the composition of the protein layer adhering to the biomaterial might be related to its biocompatibility [17].

Additionally, the earliest events in tissue repair can be associated with thrombogenesis. The thrombus has to grow as an organised structure to seal the wound effectively. It also protects the underlying tissue from oxidation, provides a reservoir of mitogens and tissue repair mediators and a provisional scaffold for the tissue repair [18].

Thus, to ensure the success of implantation, the coagulation and immune and inflammatory responses should result in an appropriate response to the foreign body implantation, favouring the best possible tissue regeneration and good osseointegration [19].

The protein layer adsorbed on implant surfaces clearly plays an important role in the bone healing process, and the Sr-containing biomaterials are increasingly frequently used in various medical applications. Therefore, the effect of strontium incorporation on the patterns of the protein binding to such surfaces is of considerable interest and, potentially, of significant practical value. The hybrid sol-gel compounds doped with various amounts of Sr can be easily used for coating the dental implants, supplying an ideal system for such analysis. Here, it was assessed the effect of increasing content of Sr on the osteogenic potential of these biomaterials and their interactions with immune cells (macrophages). Furthermore, mass spectrometry (LC-MS/MS) analysis was

conducted to identify the protein patterns affected by Sr addition and relative quantification approximation. The interactions between the biomaterials, proteins and cells and the correlations between gene expression and proteomic results are discussed.

2. Materials and methods

2.1. Substrate

Ti discs (12 mm in diameter, 1-mm thick) were made from a bar of commercially available, pure, grade-4 Ti (Ilerimplant-GMI S.L., Lleida, Spain) and then subjected to sandblasting and acid-etching (SAE). The discs were abraded with 4-μm aluminium oxide particles and acid-etched by submersion in sulfuric acid for 1 h. Then, they were washed with acetone, ethanol and 18.2-Ω purified water (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 coating preparation

The Sr-doped silica-hybrid materials were obtained using the sol-gel route. The precursors were the alkoxysilanes methyltrimethoxysilane (M), 3-(glycidoxypropyl)-trimethoxysilane (G) and tetraethyl orthosilicate (T) (Sigma-Aldrich, St. Louis, MO, USA). The molar percentages of these precursors in the material selected as a Sr-release vehicle were 35 % M, 35 % G and 30 % T, respectively. The material, 35M35G30T, was chosen on the basis of previous studies [8].

Table 1. Chemical compositions of Sr-doped coatings expressed as $SrCl_2$ weight percentage.

Reference	Sol-gel network	$SrCl_2$ (%)
0Sr	35M35G30T	0
0.25Sr	35M35G30T	0.25
0.5Sr	35M35G30T	0.5
1Sr	35M35G30T	1
1.5Sr	35M35G30T	1.5

Table 1 shows the different coating compositions. 2-Propanol (Sigma-Aldrich) was used as a solvent in the synthesis, at a volume ratio (alcohol:siloxane) of 1:1. The hydrolysis of alkoxysilanes was carried out by adding (at a rate of 1 drop s^{-1}) the corresponding stoichiometric amount of aqueous solution of 0.1M HNO_3 (Panreac, Barcelona, Spain). The solution also contained the required quantities of strontium chloride (Sigma-Aldrich). The preparations were kept for 1 h under stirring and then 1 h at rest. The samples were prepared immediately afterwards. SAE-titanium was used as the substrate for the sol-gel coatings. The coating was performed employing a dip-coater (KSV DC; KSV NIMA, Espoo, Finland). The discs were immersed in the sol-gel solutions 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.

2.3. *Physico-chemical characterisation*

The surface topography of the samples was examined using 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 for the SEM observations. An optical profilometer (interferometric and confocal) PLm2300 (Sensofar, Barcelona, Spain) was used to determine the roughness. Three discs of each type 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 was deposited on the disc surfaces at a dosing rate of 27.5 µL s^{-1} at room temperature. Contact angles were determined using SCA 20 software (DataPhysics Instruments). Six discs of each material were studied, after depositing two drops on each disc.

2.4. In vitro Assays

2.4.1. Cell culture

MC3T3-E1 (mouse calvaria osteosarcoma cell line) and RAW 264.7 (mouse murine macrophage cell line) were cultured on the Sr-doped discs at a concentration of 1×10^4 cells/well, in 24-well culture NUNC plates (Thermo Fisher Scientific, Waltham, MA, USA). The medium contained 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). After incubation for 24 hours at 37 ºC in a humidified (95%) atmosphere of 5% CO_2, the MC3T3-E1 cell line 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. In parallel, cells were allowed to differentiate for 7, 14 and 21 days before being harvested for RNA isolation.

2.4.2. *RNA isolation and cDNA synthesis*

Total RNA was prepared from both cell lines grown on the sol-gel coated titanium discs, using Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany), after digestion with DNase I (Qiagen), according to the manufacturer's instructions. The quantity, integrity and quality of the resulting RNA were measured using NanoVue® Plus Spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). For each sample, about 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.

2.4.3. Quantitative Real-time PCR

Before the RT-qPCR reaction, the primers for assessing the expression levels of the osteogenic markers ALP and TGF-β and inflammatory markers TNF-α and IL-10 were designed. To do so, specific DNA sequences for these genes available from NCBI (https://www.ncbi.nlm.nih.gov/nuccore) were used, employing the PRIMER3plus software tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The expression levels were measured using primers purchased from Life Technologies S.A. (Gaithersburg, MD). These were: GADPH sense, TGCCCCCATGTTTGTGATG; GADPH anti-sense, TGGTGGTGCAGGATGCATT; ALP sense, CCAGCAGGTTTCTCTCTTGG; ALP anti-sense, CTGGGAGTCTCATCCTGAGC; TGFβ sense, TTGCTTCAGCTCCACAGAGAS; TGFβ anti-sense, TGGTTGTAGAGGGCAAGGACD; TNF-α sense, AGCCCCCAGTCTGTATCCTT; TNF-α anti-sense, CTCCCTTTGCAGAACTCAGG; IL-10 sense, CCAAGCCTTATCGGAAATGA and IL-10 anti-sense, TTTTCACAGGGGAGAAATCG. The cDNAs from MC3T3-E1 cultures with different materials were used for RT-qPCR with the ALP and TGFβ primers. The TNF-α and IL-10 primers were used to assess the expression levels in the RAW 264.7 cell culture. All primers are listed from 5′ to 3′. GADPH was used as a housekeeping gene to normalise the data obtained from the RT-qPCR and calculate the relative fold-change between the conditions. qPCR reactions were carried out using SYBR PREMIX Ex Taq (Tli RNase H Plus) (TAKARA Bio Inc.), in an Applied Biosystems StepOne Plus™ Real-Time PCR System (Foster City, CA, USA). The cycling parameters were as follows: an initial denaturation step at 95 ºC for 30 s, followed by 95 ºC for 5 s and 60 ºC for 34 s for 40 cycles. The final melting curve stage comprised a cycle at 95 ºC for 15 s and at 60 ºC, for 60 s.

2.5. Adsorbed protein layer

Sr-doped and non-doped sol-gel biomaterials were incubated for 180 min with 1 mL of human blood serum from male AB plasma (Sigma-Aldrich) in a 24-well plate in a

humidified atmosphere (37 ºC, 5% CO_2). 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 of dithiothreitol (DTT). Four independent experiments were carried out for each type of surface; in each experiment, four discs of each material were processed. The protein content was quantified before the experiment (Pierce BCA assay kit; Thermo Fisher Scientific), obtaining a value of 49 mg mL^{-1}.

2.6. Proteomic analysis

Proteomic analyses were performed in cuadruplicate as described by Romero-Gavilán *et al.* [14], with minor variations. Briefly, the eluted proteins were in-solution digested, following the FASP protocol established by Wisnewski *et al.* [20], and loaded onto a nanoACQUITY UPLC system connected online to an 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 [14], and the functional annotation of the proteins was performed using DAVID Go annotation programme (https://david.ncifcrf.gov/).

2.7. Statistical analysis

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

3. Results

3.1. Synthesis and physico-chemical characterisation

The sol-gel synthesis was carried out successfully, and suitable coatings were obtained. These adhered well to the substrates, without forming $SrCl_2$ precipitates, as can be observed in the SEM micrographs (Figure 1). However, the incorporation of more than 1% of $SrCl_2$ in the sol-gel network caused some defects. Figure 1e and 1f show small craters in the 1Sr coating and small pores in the 1.5Sr coating.

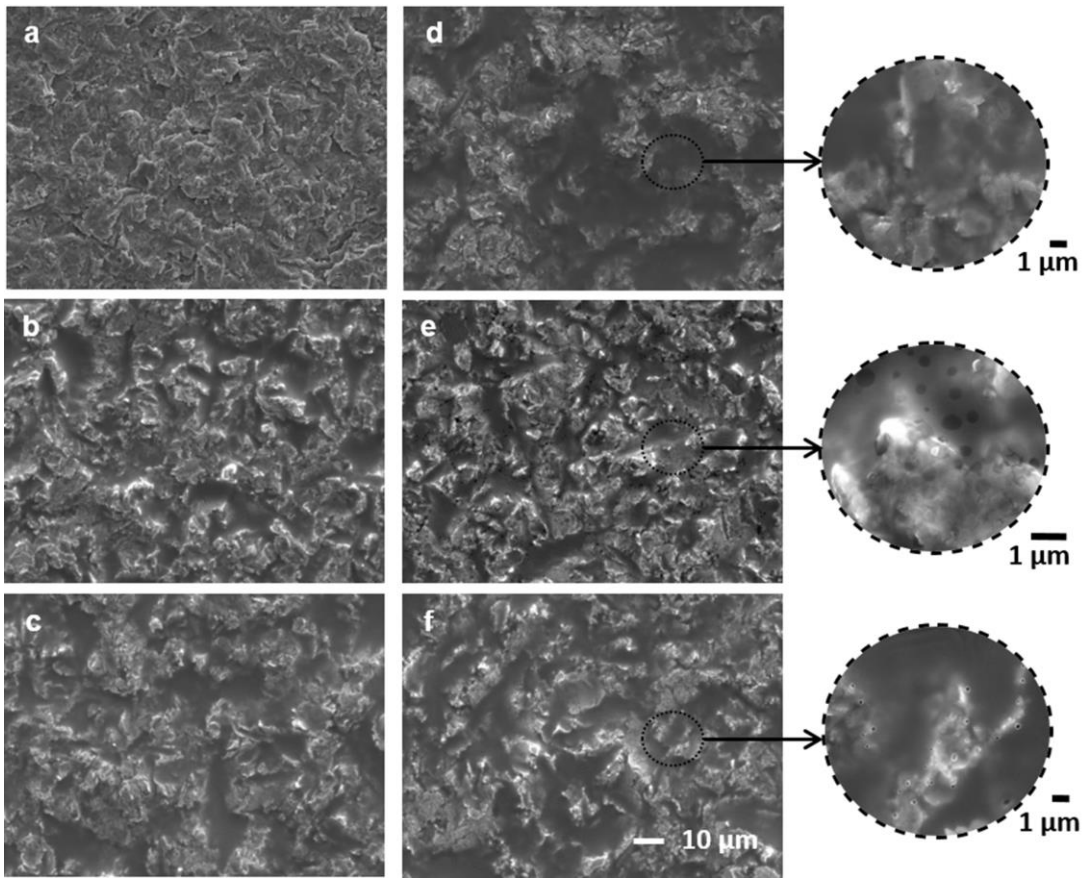

Figure 1. SEM micrograph of SAE-Ti (a), 0Sr (b), 0.25Sr (c), 0.5Sr (d), 1Sr (e) and 1.5Sr (f). Scale bar: a-f: 10 µm.

The sol-gel materials covered the initial SAE roughness on the Ti discs. This caused a decrease in Ra parameter obtained using the optical profilometer (Figure 2a). However, the Ra value increased significantly when Sr was incorporated (in comparison with 35M35G30T formulation).

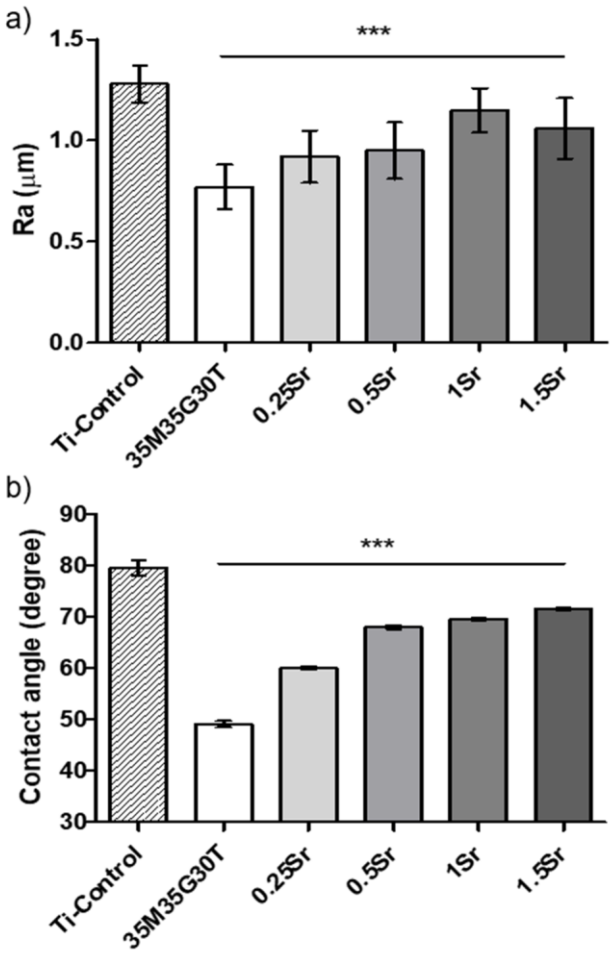

Figure 2. Roughness (a) and contact angle (b) results. Bars indicate standard deviations. Statistical analysis was performed using one-way ANOVA with a Kruskal-Wallis post-hoc test (***, $p < 0.001$), showing significant differences in comparison with the 35M35G30T base material.

1Sr and 1.5Sr coatings had the highest Ra values, most likely associated with the observed defects (craters and pores). The hydrophilicity of these Sr-doped coatings was determined using the contact angle measurements (Figure 2b). The 35M35G30T base material displayed a hydrophilic behaviour with a contact angle of approximately 50º. The addition of $SrCl_2$ to this material caused a significant increase in the contact angle (reaching the values between 60º and 70º as more Sr was added), resulting in a moderately hydrophilic behaviour.

3.2. In vitro assay

3.2.1. mRNA expression levels

In the MC3T3-E1 cells cultured with the Sr-containing hybrid sol-gel materials, the gene expression of osteogenic markers ALP and TGF-β tend to increase for some concentrations in comparison with cells grown on the 35M35G30T base material. This effect was clearly significant for the 0.25Sr (TGF- β) and 0.5Sr (ALP) samples after 14 days of culture (Figure 3).

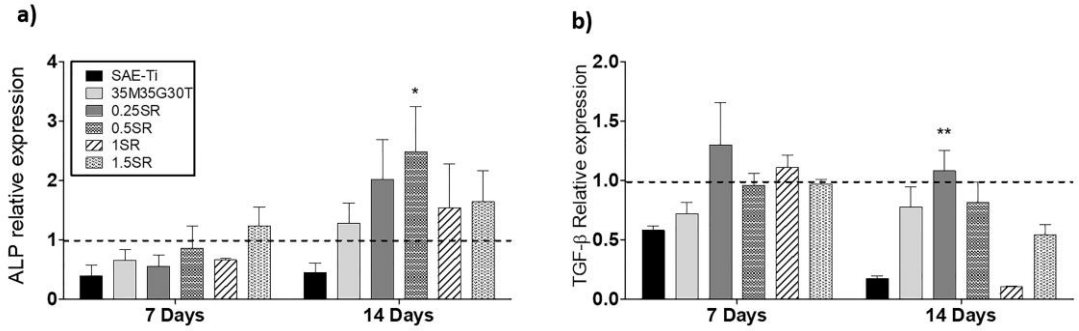

Figure 3. Relative gene expression levels of the osteogenic markers ALP, a), and TGF-β, b), in the MC3T3-E1 cells cultured on the different Sr-containing coatings. The dotted black line represents the control (the well containing cells only, no disc), assigned the value of 1 as a reference. The relative mRNA expression was determined by RT-PCR after 7 and 14 days of culture. Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis post-hoc test (*, $p < 0.05$; **, $p < 0.01$). Significant differences in expression were found between the cultures grown on Sr-discs and the base material (35M35G30T).

The expression levels of TNF-α, a standard marker of early stages of inflammation, decreased significantly ($p < 0.001$) after 7 days of culture with most of the Sr-supplemented materials (except for the 1Sr formulation) compared to reference sample, indicating that the Sr might decrease the pro-inflammatory effect of the 35M35G30T base material (Figure 4a). Otherwise, the expression levels of the IL-10, one of the main anti-inflammatory markers, increased in the cells grown on all the Sr-supplemented coatings

(Figure 4b). This was observed for both experimental periods and was particularly clear after 14 days of culture.

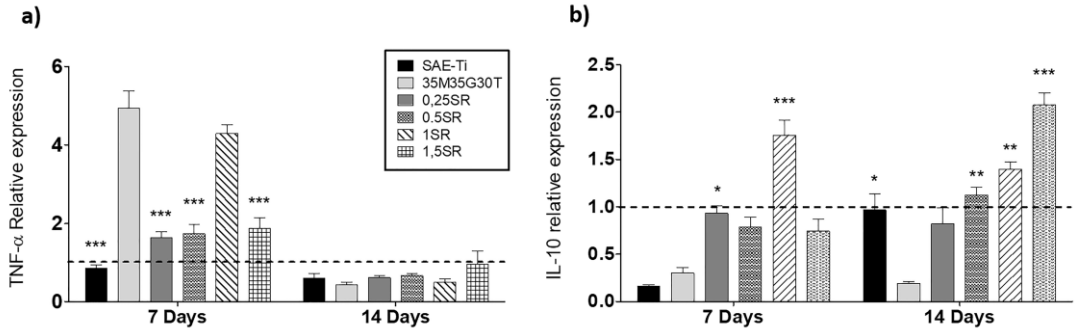

Figure 4. Relative gene expression levels of the inflammatory markers TNF-α, a), and IL-10, b), in the RAW 264.7 cells cultured on the different tested formulations. The dotted black line represents the control (cells only, no disc), assigned the value of 1 as a reference. The relative mRNA expression was determined by RT-PCR after 7 and 14 days of cell culture. Statistical analysis was performed using one-way ANOVA with a Kruskal-Wallis post-hoc test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Significant differences were found for the comparisons with the non-supplemented base material (35M35G30T).

3.3. Proteomic analysis

The LC-MS/MS analysis identified 136 distinct proteins attached to the studied surfaces. Relative protein quantification among the Sr-doped coatings and the base sol-gel material was carried out using the Progenesis QI software. The DAVID bioinformatics resource and database was employed to classify proteins according to their functions.

Table 2 shows the proteins listed as a result of the Progenesis comparative analysis. Two main affinity trends were observed in the patterns of adsorbed proteins depending on progressively increasing amounts of $SrCl_2$ incorporated in the material. A group of 14 proteins (Table 2) was more abundant on Sr-doped coatings than on the control coating (0Sr). However, there was also a group of 9 proteins whose surface-binding affinity diminished as a result of adding the $SrCl_2$ to the sol-gel network. Among the proteins

with preferentially increased affinity to Sr-surfaces, the apolipoproteins APOA, APOA5 and APOE were associated with lipoprotein metabolic processes by DAVID analysis. The CLUS, SAMP, C1QC and C1S were classified as the innate immune response proteins. The proteins THRB and FA5 were related to blood coagulation. VTNC, DSC1 and PF4V were classified as glycoproteins, and H4 and HBB, as binding proteins.

The proteins CO6 and IGLL5 (also associated with the innate immune response by DAVID) were found in smaller amounts on the Sr-coatings than on the non-supplemented surfaces. KNG1 and FIBA, involved in blood coagulation, and the glycoproteins ITIH4 and FHR2 also showed preferential binding to the base-material surfaces. Proteins RET4, ALBU and AACT displayed similar behaviour.

4. Discussion

Strontium has an important effect on bone metabolism, increasing and maintaining bone formation by boosting the osteoblast differentiation and maturation [21] while inhibiting bone resorption by osteoclasts [22]. In dental applications, in particular, strontium is considered a factor potentially enhancing and shortening the recovery after implantation. It is now increasingly used in clinical bone healing treatments, and in bone diseases such as osteoporosis [23]. It has also been shown that the first layer of proteins adsorbed onto the surfaces after the implantation has a key role in the bone healing homeostasis and in the development of inflammatory processes [17].

The work presented here examined the effects of incorporating a strontium salt, at increasing concentrations, in a specific biomaterial (silica-hybrid sol-gel 35M35G30T) on protein adsorption and gene expression in cell cultures. Two cell lines involved in specific stages of bone healing were used: a macrophage line associated with the initial inflammation after the implantation and an osteoblast line involved in the osteogenesis.

The addition of Sr to a sol-gel network altered the physico-chemical characteristics of the base-material surface. It caused an increase in both the wettability and roughness of

the surface (Figure 2). Such changes in the physical and chemical properties might alter the affinity between the tested materials and the serum proteins. This would affect the protein deposition, and define and condition the initial behaviour of the cells involved in the bone healing processes [14].

Rechendorf *et al.* have found that an increase in surface roughness can be associated with a rise in total protein adsorption [24]. Nevertheless, the effect of changes in roughness on the adsorption of specific proteins is not linear, and the affinities of various proteins can be affected differently [25]. Changes in wettability can also affect protein adsorption [26]. However, it is difficult to correlate the exact effects of changes in these physico-chemical properties on the adsorption of specific proteins when complex multi-protein systems, such as human serum, are studied. This is particularly true for the studies in which several properties are modified at the same time.

The proteomic analysis (LC-MS/MS) identified some changes in the patterns of proteins attached to the sol-gel, caused by increasing amounts of $SrCl_2$ incorporated into the base network material (Table 2). The DAVID bioinformatic tool was used to relate these differentially attached proteins to their functions. A cluster of proteins involved in the innate immune response was identified; complement proteins C1S and C1QC and a pentraxin protein SAMP were more abundant on the materials with more than 1% of $SrCl_2$. These proteins have a direct role in the activation of the complement cascade pathway [27]. However, the complement protein CO6 and the immunoglobulin IGLL5 showed a reduced affinity to the biomaterial surfaces containing Sr compounds. The adhesion of this type of proteins to the surface of a biomaterial could initiate an immune/inflammatory reaction, which could affect the development of the bone healing process [28]. CLUS adsorbed onto Sr-doped coatings in larger amounts than to the Sr-free materials from 0.5Sr. This protein is related to the regulation of inflammation and immunity, such as inhibition of complement cascade [29].

The adsorption of some proteins with a role in blood coagulation could also be affected by the $SrCl_2$ incorporation into the sol-gel; FA5 and THBR were more abundant on Sr-containing formulations. Both of these proteins play a role in the coagulation pathway. THRB, also known as coagulation factor II, can trigger blood clotting through its conversion to the active protease thrombin, activating platelet formation [30]. FA5 has a regulatory function in both pro- and anti-coagulant pathways [31]. The KNG1 and ALBU proteins tended to be adsorbed less onto the coatings with Sr than onto the base sol-gel material. The high molecular weight KNG1 belongs to the kallikrein-kinin system, a surface-activating coagulation system [32]. FIBA is associated with different biological functions, such as blood clotting and fibrinolysis [33]. In contrast, H4, which preferentially adhered to the 1.5Sr material, might be associated with the promotion of platelet aggregation and thrombosis. However, ALBU has been described as an inhibitor of this particular H4 function [34]. It is difficult to predict the final effects of the implant surfaces adsorbing various proteins, which might play opposing roles in a living organism. The real magnitude of each effect cannot be easily evaluated, and there may be some synergistic relationships coming into play.

Apolipoproteins APOA, APOA5 and APOE showed stronger affinity to Sr-materials and differentially adsorbed onto Sr-modified surfaces. This family of proteins have functions in lipid metabolism, but they also can prevent the initiation of innate immunity processes [35]. The APOE is known for its potent anti-inflammatory effects, resulting from its ability to promote macrophage conversion from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype [36]. Moreover, this protein could play a role in bone metabolism through its effect on the vitamin K uptake into osteoblasts [37] and its induction upon mineralization [38]. APOE gene deficiency was also related to the bone formation reduction due to the stimulation of p53-mediated osteoblast apoptosis in a high-fat diet condition [39]. Additionally, Kim et al. found APOE could play an inhibitory role in osteoclast differentiation through the suppression of RANK-dependent activation

of nuclear factor κB and induction of c-Fos and nuclear factor of activated T cell c1 [40], which might prevent the overstimulation of osteoclastogenesis and its consequent excessive bone resorption, involved in bone diseases such as osteoporosis. Interestingly, Calciolari et al. studied the proteins and signalling pathways differently regulated following a treatment with guide bone regeneration in the calvaria of healthy and osteoporotic rats, founding an APOE overexpression in osteoporotic conditions [41]. This relationship between APOE with osteoporosis and its greater affinity to Sr-biomaterials should be further investigated to elucidate the potential of this protein in bone regeneration around implants.

Similarly, the incorporation of $SrCl_2$ increased the affinity of VTNC to the coatings. This protein is a known inhibitor of the complement cascade activation (as is CLUS) and therefore could regulate the inflammation and immune response [42]. VTNC also favours the polarisation to the M2 macrophage phenotype [43]. This is a protein involved in the bone metabolism as it can promote the osteogenic differentiation of mesenchymal stem cells [44]. Moreover, it is also associated with the coagulation system, contributing to thrombus formation, and participates in the vascular homoeostasis and tissue regeneration [18].

Many of the deposited proteins might play an important role in the bone healing processes, and the differences in the physico-chemical properties of the various formulations affect their abundance and distribution. It seems reasonable to assume that the cell behaviour will also be affected by such changes. Analysis of relative gene expression was conducted (using qRT-PCR) for all the tested formulations to examine the effect of $SrCl_2$ incorporation on the behaviour of macrophage and osteoblast cells.

Interestingly, the gene expression of osteogenic markers ALP and TGF-β showed a tendency to increase in cells incubated with coatings 0.5Sr and 0.25Sr, respectively, in comparison with the 35M35G30T formulation (Figure 3). The effect of Sr on cell differentiation, in particular on the synthesis of osteoblastic mRNAs, is well documented.

This is especially clear in the case of the ALP, where Sr increases the expression of its gene in a dose-dependent manner [23]. Sr also enhances the TGF-β expression in chondrocytes and osteoblasts, suggesting its active role in bone-cell metabolism and activity [45]. It is tempting to assume a correlation between the increased affinity of osteogenic reference proteins (such as APOE and VTNC) to Sr-containing coatings and the enhanced expression of these markers. The osteogenic potential of Sr-enriched materials has also been suggested by *in vivo* studies. Neves *et al.* have reported the effectiveness of Sr-doped biomaterials in stimulation of bone regeneration in animal models [5]. Similarly, Shy *et al.* have concluded that Sr-modified implant surfaces have a significantly positive effect on osseointegration *in vivo* [46].

The macrophage mRNA synthesis seems to be affected by the incorporation of $SrCl_2$ into the base materials. There was a dose-dependent effect, similar to that for the osteogenic genes. In this case, a significant decrease was observed in the expression of the pro-inflammatory gene TNF-α after 7-day culture with Sr-containing sol-gel material (at all concentrations, except for 1Sr; Figure 4a). The gene for the anti-inflammatory protein IL-10 displays a clear dose-dependent response to the Sr-supplemented formulations, in particular after 14 days culture; the IL10 expression increases with increasing concentration of added $SrCl_2$ (Figure 4b). Interestingly, these results could be correlated with the changes in the proteomic patterns observed using LC-MS/MS analysis. Ali K *et al.* have found an increase in plasma levels of pro-inflammatory cytokines, such as TNF-α, in APOE-knockout mice [47]. This is consistent with the reduction in the TNF-α expression observed on the material with a stronger affinity to APOE. The enhanced expression of IL10, a cytokine generated by M2 macrophage switch, concurs with an increased affinity to proteins like APOE and VTNC, favouring the macrophage differentiation from pro-inflammatory M1 to anti-inflammatory M2 phenotype [36,43].

5. Conclusion

In summary, incorporation of $SrCl_2$ into a hybrid sol-gel biomaterial significantly changes its physico-chemical properties (hydrophilicity and roughness) and affects protein affinity profiles and cell responses. The LC-MS/MS analysis revealed that the addition of Sr modified significantly the attachment profiles of proteins involved in the inflammation and coagulation processes. Remarkably, it increased the adsorption of APOE and VTNC, proteins related to osteogenic and anti-inflammatory processes, onto the biomaterial surface. Both the osteoblast and macrophage cells showed $SrCl_2$ dose-dependent behaviour. An increase in the gene expression of osteogenic markers ALP and TGF-β was observed in the MC3T3-E1 cells. While, a decrease in the expression of inflammation marker TNF-α and an increase in anti-inflammatory IL-10 expression were seen in the macrophage cell line. The proteomic and gene expression analyses showed that the introduction of Sr into the biomaterials might improve their osteogenic and anti-inflammatory potential.

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