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Cell response to collagen-calcium phosphate cement scaffolds investigated for nonviral gene delivery

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Abstract Collagen-hydroxyapatite (HA) scaffolds for the non-viral delivery of a plasmid encoding the osteoinductive protein bone morphogenetic protein (BMP)-7 were developed. The collagen-HA was obtained by the combination of calcium phosphate cement in a collagen template. The effect on cell behavior of increasing amounts of HA in the scaffolds was evaluated. Collagen-HA scaffolds containing 13, 23 or 83 wt% HA were prepared. Cell proliferation was reduced in the 83% HA scaffold after 1 day compared to 13 and 23% HA, but by 14 days the number of cells in 83% HA considerably increased. Alkaline phosphatase (ALP) activity was 8 times higher for the 83% HA scaffolds. BMP-7 plasmid was incorporated into the 83% HA scaffold. The transfection was low, although significant levels of BMP7 were expressed, associated with an increase in cell proliferation.

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1 Introduction

Synthetic bone grafting materials are an issue of current research because of the problems of availability and the donor site morbidity when using autografts, allografts and xenografts. The key elements present in the natural bone grafts, which are responsible for their good performance in bone regeneration applications, are: a 3D network that provides support volume and attachment sites to the cells, namely the extracellular matrix, growth factors, that may induce osteogenesis and vascularization, and the presence of cells with osteogenic potential that will be delivered in vivo [1]. The design of synthetic bone grafts should aim at the development of constructs able to develop the same functions, and therefore should cover not only the intrinsic properties of the material itself, but also include specific biological functionalities directed to its osteogenic potential.

The formation of three dimensional scaffolds made of collagen has been widely used for the regeneration of several tissues [2–5]. The size and directionality of the pores can be controlled depending on the freezing rates and temperatures [6, 7]. Collagen scaffolds have proven to have good biological properties for tissue engineering, but presenting low stiffness. The incorporation of calcium phosphates into the scaffold can be used as a strategy not only to increase their stiffness, but also their osteoconductive potential [8, 9].

Furthermore, the combination of three dimensional scaffolds with growth factors has proven to be a system for the regeneration of bone, being able to make the scaffolds

osteoinductive [10]. One of the main challenges is to be able to deliver the growth factor (such as bone morphogenetic proteins, BMPs) in vivo at a therapeutic level due to the short lives of the molecules and its high cost [11]. Gene therapy has appeared as an alternative, in which the cells are able to produce a specific protein when a gene is properly transferred into the cells [12].

In the gene therapy approach, two main mechanisms by which the cells are transfected can be found: viral and non-viral vehicles. Both systems have been employed to incorporate high doses of proteins for tissue engineering applications. Viral vectors are known to have higher transfection efficiency, although they present high immunogenicity, making non-viral transfection the most appropriate gene delivery vehicle for tissue engineering applications [13].

Calcium phosphate (CaP) based approaches are an attractive option for non-viral plasmid DNA transfection into cultured cells [14]. The purpose of this work was to develop a collagen-hydroxyapatite (HA) scaffold for the non-viral delivery of the plasmid encoding the osteoinductive protein BMP-7. The intention was to provide a local and prolonged release of this growth factor by the cells seeded or migrating within the scaffold. As an innovative route, the collagen-HA scaffold was obtained by the combination of a calcium phosphate cement (CPC) in a collagen template. The CPC was composed of α-tricalcium phosphate (α-TCP), which upon hydrolysis, transforms into hydroxyapatite (HA), therefore leading to the precipitation of HA nanometric crystals within the collagen matrix.

The specific aim of this study was to evaluate the effect on cell behavior of increasing amounts of CPC in the scaffolds: 13, 23 or 83 wt% HA. The delivery of the BMP-7 plasmid incorporated in the scaffolds into mesenchymal stem cells (MSCs) in vitro and the expression of the protein was also assessed when varying the amount of plasmid incorporated.

2 Materials and methods

2.1 Collagen preparation

A mixture of porcine collagen type I and type III, named as collagen type I/III (obtained from Geistlich Biomaterials, Wolhousen, Switzerland), was used. In order to solubilize the collagen, the collagen powder was introduced in a 10 mM HCl solution at a 1% wt concentration. The solution was then blended at 14.000 rpm in order to solubilize the collagen and maintained at 4°C through a refrigerating system. The collagen was blended for 6 h for the complete dissolution of the powder.

2.2 Calcium phosphate cement powder

The CPC consisted of α-TCP, obtained by sintering at 1400°C and subsequent quench of the appropriate mixture of calcium hydrogen phosphate ($CaHPO_4$, Sigma-Aldrich C7263) and calcium carbonate ($CaCO_3$, Sigma-Aldrich C4830). α-TCP was milled in a planetary mill (Pulverisette 6, Fritsch GmbB), and a 2 wt% of precipitated HA crystallites (Merck ref.1.02143) was added as seeds for the precipitation reaction. The α-TCP had a median particle size of 5.2 μm as calculated with laser diffraction, and a specific surface area of 1.228 m^2/g measured by nitrogen adsorption with the BET method.

2.3 Preparation of scaffolds

The α-TCP powder was dispersed in 1 ml of distilled water. The α-TCP suspension was then incorporated in the dissolved collagen. The collagen and the α-TCP were mixed in the overhead blender at 6,000 rpm for 1 h at 4°C. The following samples were prepared: 13, 23 and 83 wt% HA (g HA/g composite); and the collagen without TCP (0% HA). After the fabrication process, the samples were immediately placed in the freeze-dryer (Virtis Advantage) and freeze dried for 20 h at −40°C and 200 mTorr. Once the freeze-drying process was done, the samples were crosslinked with a dehydrothermal method at 105°C and 30 mm Hg with vacuum for 24 h. This treatment leads to the formation of covalent linkages among the polypeptide chains of the collagen fibers by slowly removing the water, resulting in an increase in the strength of the collagen [15, 16]. The samples were then cut with a punch to obtain 8 mm diameter and 2 mm thick samples. The scaffolds were then hydrated by immersion in graded alcohols: 100, 96, 90, 70% and water. Further crosslinking was performed with 14 mM 1-ethyl-3-(3-dimethylaminpropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co, St Louis, MO, USA) applied for 30 min at room temperature. The excess of crosslinking agent was washed out by rinsing in PBS.

The scaffolds were subsequently immersed in water for 5 days for the complete reaction of the α-TCP into HA according to the reaction:

$$3Ca_3(PO_4)_{2(s)} + H_2O_{(l)} \rightarrow Ca_9(HPO_4)(PO_4)_5(OH)_{(s)}$$

2.3.1 Scaffold characterization

The different composite scaffolds were analyzed by scanning electron microscopy (SEM; FEI/Philips XL30 FEG ESEM) in order to observe the differences in the morphology of the samples. The specimens were coated with gold-platinum in order to increase the conductivity of the samples.

In order to identify the mineral phase formed in the scaffold, the samples were digested in 83 μg/ml proteinase K solution dissolved in Tris–HCl overnight at 37°C in order to degrade the collagen matrix. The crystals were then crushed in order to obtain a powder and were analyzed by X-ray diffraction (XRD, Philips MRD) to confirm the presence of HA. Ni-filtered Cu K_α radiation was used. The step-scanning was performed with an integration time of 50 s at intervals of 0.017° (2θ). Indexing of the peaks was carried out by means of Joint Committee on Powder Diffraction Standards (JCPDS) cards 29-359 for α-TCP and 9-432 for HA.

2.4 Plasmid preparation

Multiplication of the pcBMP7 plasmid (Fig. 1) (obtained from Stryker Biotech, Hopkinton, MA) was performed by heat-shock transformation into *E. coli* DH5α competent cells grown overnight in LB medium containing ampicillin. It was then purified using QIAfilter plasmid Mega kit (Qiagen; Valencia, CA). The absorption ratio at 260 and 280 nm was used to determine the plasmid concentration and purity. The plasmid integrity was verified with polyacrylamide gel electrophoresis. The size of the pcBMP7 was 6.7 kb.

2.5 MSC culture

Mesenchymal stem cells isolated from goat marrow were used. Cells were thawed and expanded using low glucose DMEM supplemented with 1% Pen/Strep and 10% FBS (non-osteogenic medium) as the medium. When confluence was achieved, cells were trypsinized and counted with trypan blue. Cells were sub cultured in a tissue culture flask or seeded onto the scaffolds.

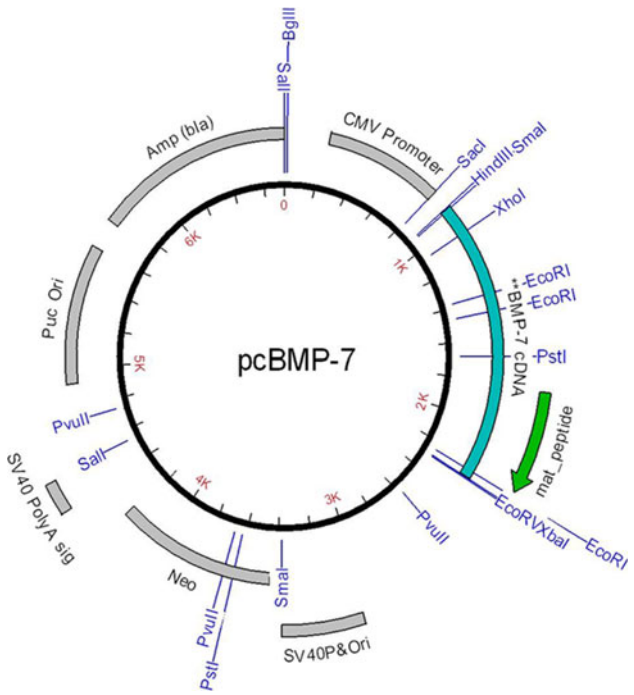


Fig. 1 Map of the pcBMP7 plasmid incorporated in the scaffolds

Twenty-four well plates were coated with 1% agarose solution in order to prevent cell attachment to the bottom of the dishes. Scaffolds were placed on top of the agarose coating. Cells were seeded on top of the scaffolds at a concentration of 10^6 cells per scaffold. Cells were left for a period of time to attach to the scaffolds, which were maintained wet, after which, 1 ml of expansion medium was added. After 24 h, the expansion medium was replaced with osteogenic medium consisting of α-MEM supplemented with 0.173 μM L-ascorbic acid, 10 mM β glycerophosphate and 100 nM Dexamethasone, as well as 1% penicillin/streptomycin (pen/strep) solution and 10% fetal bovine serum (FBS). The medium was changed every 2 days.

2.6 In vitro behavior of scaffolds containing different HA content

The effect of having different amounts of HA incorporated in the collagen scaffold was verified. The four types of scaffolds prepared were: Collagen scaffold, Collagen-13% HA scaffold, Collagen-23% HA scaffold and collagen-83% HA scaffold. Cell proliferation in the 4 groups of scaffolds was determined by measuring the DNA content ($n = 6$), and the differentiation by means of alkaline phosphatase (ALP) activity ($n = 6$), after 1, 7 and 14 days. Cell morphology was assessed by SEM and histology at 7 and 14 days.

2.6.1 Proliferation

Cell proliferation was measured by means of the DNA picogreen assay at 1, 7 and 14 days. For that purpose, scaffolds were washed in PBS at each time and frozen without liquid. In order to measure the DNA, samples were freeze-dried overnight and digested afterwards in a 83 μg/ml proteinase K solution dissolved in Tris–HCl. The assay was done using the Quant-iT PicoGreen dsDNA assay kit (Molecular Probes P7589). The method consisted in a fluorimetric assay measured in a 96-well plate (Black Isoplate, Wallac #1450-571) in a plate reader (Wallac) recording the excitation/emission at 485/535 nm.

2.6.2 Differentiation

The alkaline phosphatase activity was measured at 1, 7 and 14 days. The samples were washed twice in phosphate buffered saline (PBS), freezing the samples afterwards with PBS and a drop of Tween 20. The samples were then

freeze-thawed three times in order to enhance cell lysis. Afterwards, samples were analyzed using the QuantiChrom Alkaline phosphatase assay kit (BioAssay Systems, DALP-250). The reading was then performed at 405 nm.

2.6.3 Cell morphology

Cell-seeded scaffolds were washed twice in PBS, and then immersed in a 2.5% glutaraldehyde (Sigma-Aldrich G400-4) solution in PBS. The samples were then treated in a 1% solution of osmium tetraoxide (Sigma-Aldrich 201013) in order to increase the electron-contrast of the samples. Graded ethanol solutions were used to dehydrate the samples (50, 70, 90, 96 and 100% ethanol).

Samples for histology were immediately fixed at the determined time points in 4% paraformaldehyde solution and embedded in paraffin. Consecutive sections (6 μm thick) were cut and stained with Masson's trichrome [17] in order to visualize cell morphology as well as the collagen scaffold.

2.7 Plasmid incorporation

In the second part of the study, the effect of incorporating the plasmid in the scaffold was determined for the collagen-83% HA construct. The amount of plasmid incorporated varied from 0 to 50 μg. 20 μl of the solution containing the appropriate amount of plasmid were pipetted into the surface of the scaffold. The low amounts of plasmid incorporated were for safety reasons.

The amount of BMP-7 contained within the scaffolds after 1, 4 and 7 days ($n = 3$) was determined by freeze-thawing them three times and physically breaking them down in order to release the BMP-7 produced. The amount of BMP-7 was measured by a sandwich ELISA kit for BMP-7 protein (DuoSet ELISA Development System human BMP-7 DY354). In order to also observe the effect of the plasmid on cell proliferation, a DNA assay was performed as previously described. The values for BMP-7 expression were represented as normalized values with respect to the amount of DNA obtained for the proliferation assay.

In order to make sure that in the case of the quantification of DNA, what was being read was because of the cells, an internal control was done. The previously described procedure was applied in a series of scaffolds without incorporating the cells in order to see if the DNA quantification technique would also quantify the DNA from the plasmid.

2.8 Statistical analysis

The cell experiments were performed using six replicates for each scaffold composition. Statistical analysis was carried out with significance of 5% or less. One-factor analysis of variance (ANOVA) with Fisher's post-hoc test was conducted. The data are expressed as mean value ± standard error.

3 Results

3.1 Microstructure of the scaffolds

Homogeneous slurries were obtained with the collagen type I/III and α-TCP. It was important to stir the system for time enough so that the α-TCP and the collagen would intimately mix, avoiding problems of heterogeneity. As shown in Fig. 2, the XRD patterns revealed that the transformation of the initial α-TCP into HA was complete after 5 days immersion in water, so that HA was the only mineral phase present in the scaffolds.

The microstructures of the collagen-13% and collagen-23% scaffolds were very similar (Fig. 3a and b), demonstrating collagen fibers with some crystals incorporated into the matrix. The morphology of the scaffolds containing 83% HA was completely different (Fig. 3c). In this case, the collagen fibers were completely covered by the HA crystals. The matrix of the collagen (Fig. 3d) was seen as completely smooth with no particles dispersed.

3.2 Cell response to collagen scaffolds with different HA contents

The 1-day DNA content, reflecting the initial attachment of the mesenchymal stem cells to the scaffolds, was substantially lower for the collagen-83% HA scaffold compared to the 13% HA and 23% HA constructs, which were comparable (Fig. 4a). The collagen scaffolds which did not contain HA, contained about 40% fewer cells than the 13 and 23% HA samples (Fig. 4a).

After 7 days, the amount of DNA in the collagen and 13 and 23% HA scaffolds increased dramatically compared to the 1-day values, indicating considerable cell proliferation (Fig. 4a). While the number of cells in the 83% HA constructs increased more than 2-fold compared to the 1-day measurements, the cell content was less than 13% of that in the other scaffold groups (Fig. 4a). There were no significant differences in the DNA contents among the 0, 13 and 23% HA groups after 7 days.

No increase in cell proliferation was observed at 14 days for the scaffolds containing 0, 13 and 23% HA, compared to the 7-day results (Fig. 4a). There was, however, almost a 5-fold increase in the number of cells in the collagen-83% HA scaffolds from 7 to 14 days (Fig. 4a). There was no significant difference in cell number between the 13 and 23% HA scaffolds at 14 days, but the 13 and 23% HA

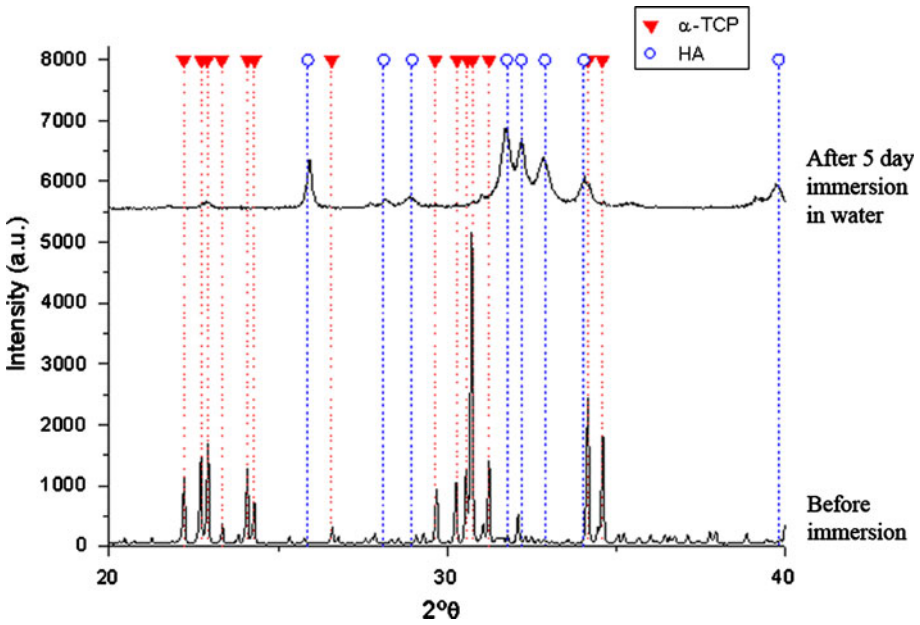


Fig. 2 X-ray diffraction patterns of 83% HA scaffold before (*bottom*) and after (*top*) 5 days immersion in water. Initially the scaffold contained only α-TCP as a mineral phase, which completely hydrolysed to HA after 5 days

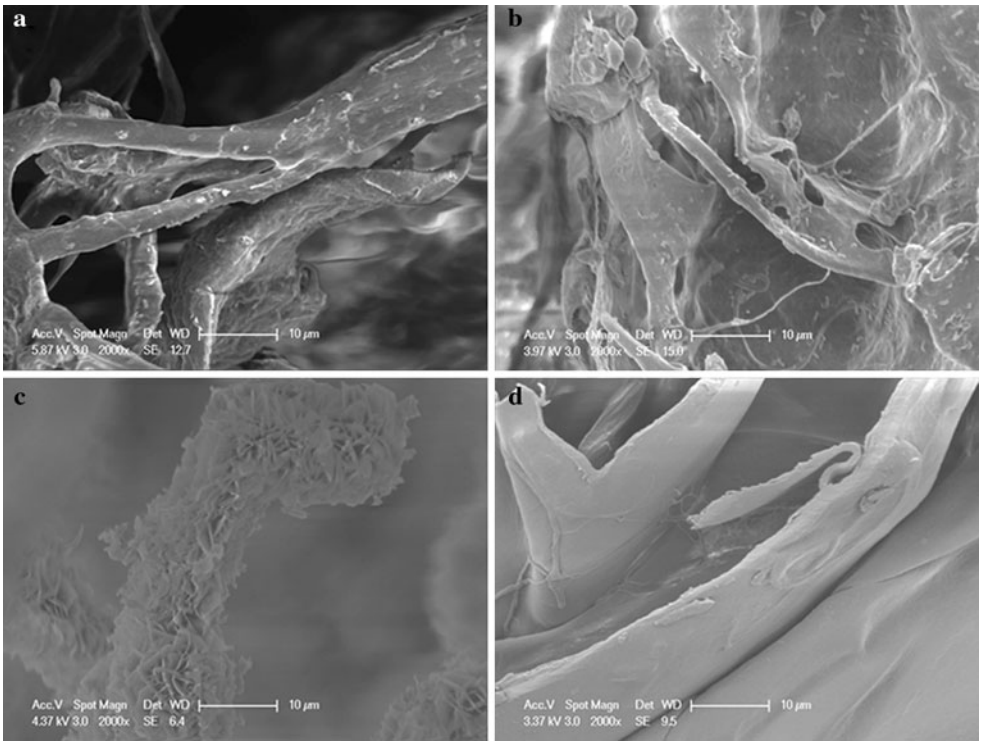


Fig. 3 SEM images of collagens prepared with different % HA in wt **a** 13%, **b** 23%, **c** 83% and **d** 0%

scaffolds had significantly higher DNA contents than the 0% and 83% HA scaffolds.

After 7 days, the ALP activity, normalized to the number of cells in the scaffolds, was 7-fold higher for the 83% HA scaffolds compared to the constructs in the 3 other groups (Fig. 4b). The ALP values for the 13 and 23% HA groups were comparable, and the collagen scaffold group displayed about 60% of their value (Fig. 4b). Between the 7- and 14-day periods, the ALP content of the collagen scaffolds increased about 2-fold, while the values for the HA containing scaffolds all decreased. After 14 days the ALP/cell was higher for the collagen scaffolds compared to the 13 and 23% HA groups, but the highest ALP content was still found for the collagen-83% HA scaffolds.

Monolayers of cells were found on the walls of the 13 and 23% HA scaffolds after 7 and 14 days (Fig. 5a and b).

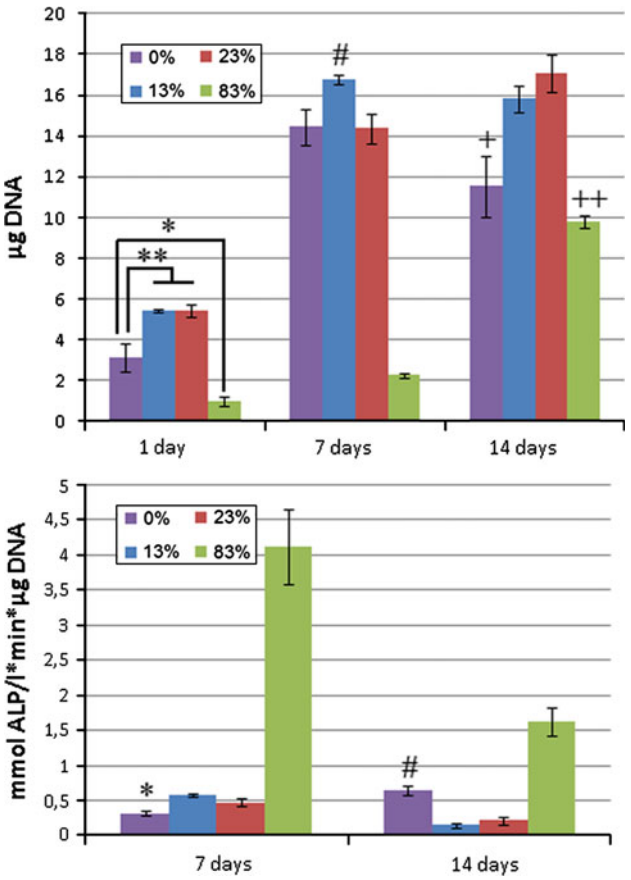


Fig. 4 **a** Cell proliferation measured by means of DNA quantification. * indicates significant differences between 0% HA scaffolds and 83% scaffolds at 1 day. ** indicates significant differences between 0% HA and 13 and 23% HA scaffolds at 1 day. # indicates significant difference between 13% HA and the other scaffolds at 7 days. + indicates significant differences between 0% HA and 13 and 23% HA at 14 days and ++ indicates significant differences between the 13 and 23% HA scaffolds and the 83% HA scaffold ($P < 0.05$). **b** Alkaline phosphatase activity for the scaffolds with different amounts of HA incorporated.* denotes significant differences between 0% HA and the other compositions at 7 days. # denotes significant differences between 0% HA and the other compositions

The cells were well spread, presenting filipodia extending throughout the scaffold. In the case of the 83% HA scaffolds, there were differences when comparing 7 and 14 days. After 7 days, there were few cells on the scaffold, with few contact points with the material, presenting an elongated shape (Fig. 5c). After 14 days, a higher number of cells were observed on the scaffold, with a more spread morphology, presenting higher amounts of expanding filipodia. One of the main differences with the 13 and 23% HA scaffolds, was the presence of deposits on top of the cells, probably corresponding to extracellular matrix (Fig. 5d).

Similar trends were observed through the Masson's trichrome staining of the histological sections (Fig. 6). Qualitatively, higher numbers of cells were observed in the 13 and 23% HA scaffolds (Fig. 6a and b), having in general an elongated morphology and creating an entangled cell network. In the case of the 83% HA, the number of cells was lower with fewer protrusions and filipodia (Fig. 6c). In the case of the collagen scaffold alone (0% HA; Fig. 6d), the cells tended to agglomerate in certain parts of the scaffold, and their morphology was similar to that found in the 13 and 23% HA scaffolds. Interestingly, according to the histological and SEM studies, the cells were able to penetrate throughout the whole thickness of the scaffold, in all the series studied. Of note was that the scaffold itself stained red, instead of blue, with the Masson trichrome stain, related to the state of strain in the collagen at the time of fixation. When collagen is in a relaxed state at the time of paraformaldehyde fixation, Masson trichrome stains the collagen blue; collagen that is under tension at the time of fixation is stained red [18].

3.3 BMP-7 expression

In this part of the study, the plasmid encoding BMP-7 was incorporated in the 83% HA scaffolds, using different plasmid loadings. First, it was assessed that the plasmid did not interfere with the DNA technique used to quantify cell proliferation. This was done by incorporating the plasmid in the scaffolds without seeding cells and afterwards applying the procedure of DNA quantification, and no signal was obtained.

As shown in Fig. 7a, cell proliferation was higher in the cases in which the plasmid was present. In all cases the trend shown was that the higher doses of plasmid increased the amount of cells. The increase in proliferation was dose dependent, except in the case of the 20 and 50 µg dose in which the proliferation was the same.

Regarding the expression of the BMP-7 protein, the highest levels of protein were found in the scaffolds after 1 day and, interestingly, the highest amount was found for 20 µg of incorporated plasmid (Fig. 7b). At 4 days, the highest BMP-7 content was obtained for the 5 µg dosed scaffolds. But after 14 days, the 20 µg dosed scaffolds yielded again the highest BMP-7 levels in the matrix (Fig. 7b). The BMP-7 accumulated in the scaffolds over the 7-day period was higher for the plasmid groups, compared to the non-transfected controls (0.264 ng of BMP-7 for the 20 µg group, compared to 0.139 ng of BMP-7 for the 0 µg group).

The values of the BMP-7 measured in the scaffolds were normalized by the DNA content of the specimens (Fig. 7c). After 1 and 4 days, the control scaffolds which were not supplemented with plasmid contained the highest amounts of BMP-7 normalized to DNA. By 7 days, however, the collagen-83% HA scaffold dosed with 5 µg of plasmid had the highest amount of BMP-7 (Fig. 7c).

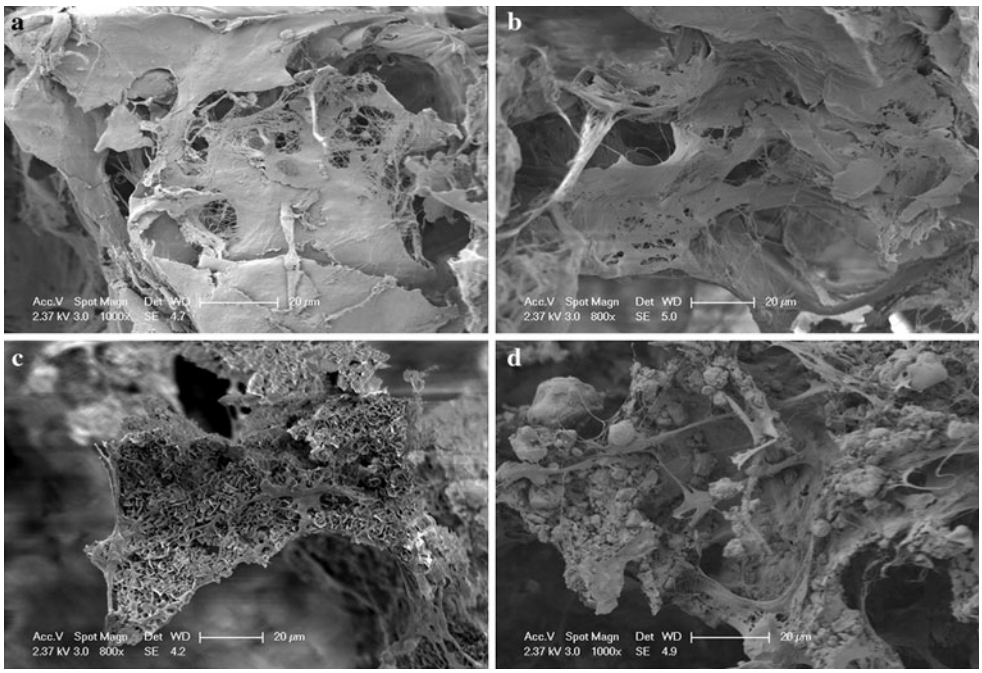


Fig. 5 SEM images of the cell morphology on the different scaffolds for **a** 13% HA (14 days), **b** 23% HA (14 days), **c** 83% HA (7 days) and **d** 83% HA (14 days)

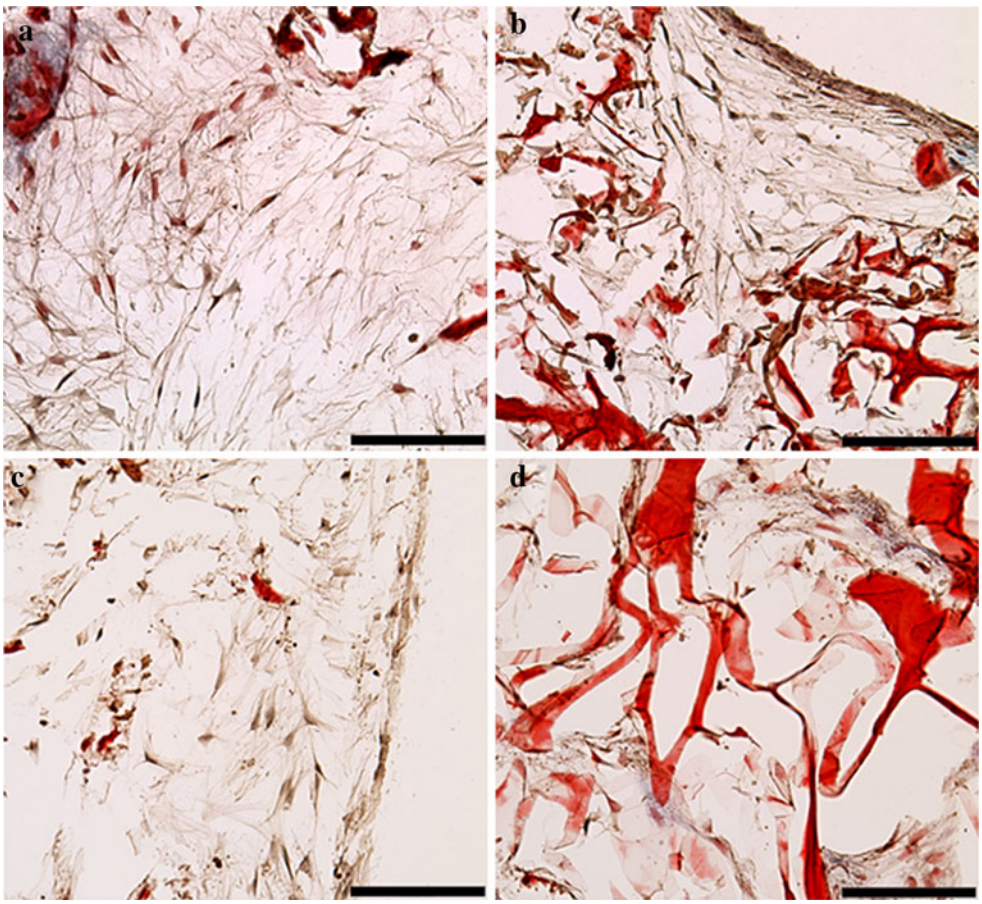
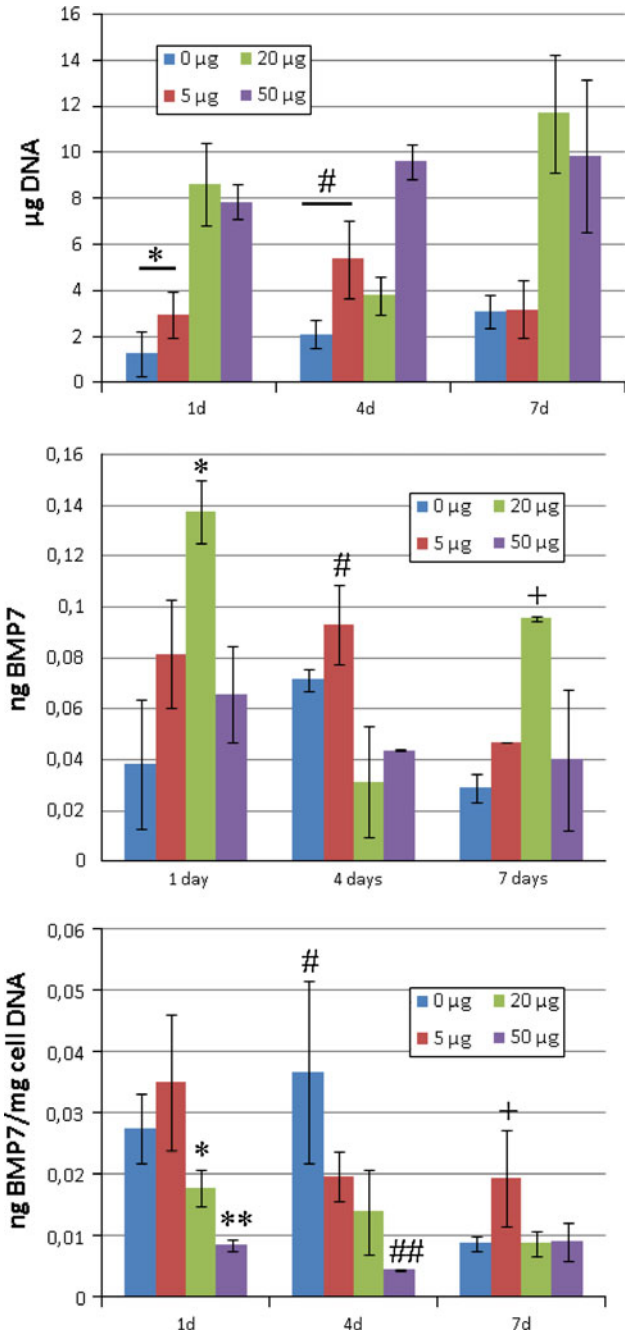


Fig. 6 Histogical sections of the different scaffolds stained with Masson's trichrome, being **a** 13% HA, **b** 23% HA, **c** 83% HA and **d** 0% HA. Scale bar = 100 µm



◀ **Fig. 7** **a** Proliferation measured by means of DNA quantification for scaffolds composed of 83% HA containing different amounts of plasmid: 0, 5, 20 and 50 µg. * indicates significant differences between 0 and 5 µg of plasmid incorporated at 1 day. # indicates significant differences between 0 and 5 µg of plasmid incorporated at 4 days ($P < 0.05$). **b** BMP7 expression for the 83% HA scaffolds with different amounts of plasmid incorporated during 7 days. * denotes significant differences between the 20 µg plasmid loaded scaffolds and the other plasmid loadings at 1 day. # denotes significant differences between the 5 µg plasmid loaded scaffolds and the other plasmid loadings at 4 days. + denotes significant differences between the 20 µg plasmid loaded scaffolds and the other plasmid loadings at 7 days. **c** BMP7 expression normalized by the DNA content from cell proliferation for the 83% HA scaffolds with different amounts of plasmid incorporated during 7 days. * denotes significant differences between the 20 µg plasmid loaded scaffolds and the other plasmid loadings at 1 day. ** denotes significant differences between the 50 µg plasmid loaded scaffolds and the other plasmid loadings at 1 day. # denotes significant differences between the 0 µg plasmid loaded scaffolds and the other plasmid loadings at 4 days. ## denotes significant differences between the 50 µg plasmid loaded scaffolds and the other plasmid loadings at 4 days. + denotes significant differences between the 5 µg plasmid loaded scaffolds and the other plasmid loadings at 4 days

4 Discussion

The present work allowed the development of macroporous collagen-HA scaffolds with different HA contents. Initially, the collagen/α-TCP slurry was blended at 4°C, avoiding the formation of bundles due to collagen precipitation. The hydrolysis of α-TCP has an increase of the pH associated which, near neutral pH, may induce the collagen precipitation. This would lead to a heterogeneous slurry, and therefore to heterogeneous scaffolds. By decreasing the temperature, the hydrolysis rate was reduced, which combined with the blending process, reduced the risk of bundle formation.

A recent study of collagen-glycosaminoglycan scaffolds [19] has shown that at the temperature used for the dehydrothermal treatment of the freeze-dried collagen/α-TCP (105°C), about 25% of collagen is denatured. It will be interesting in future work to evaluate the degree of collagen denaturation in the collagen/α-TCP, to determine if the calcium phosphate may play a protective role in reducing the collagen denaturation. One would not expect a reaction between the collagen and the calcium phosphate during the dehydrothermal treatment at 105°C. The calcium phosphate is not altered in any way at this temperature, being completely stable. It would also be of interest in future studies to investigate how the EDAC-crosslinked material is degraded in vivo, with respect to the ultimate clinical applications.

The characterization of the scaffolds by XRD showed the transformation of the mineral phase into HA. SEM observations revealed that at low amounts of incorporated HA, the HA precipitates were found in the fibers of the collagen and not in the voids. At higher amounts of incorporated HA, the structure revealed a similar trend, observing HA completely covering the collagen fibers. Once again, the HA was precipitating on top of these collagen fibers and the crystalline structure of the mineral phase corresponded to that previously seen in previous work in which the α-TCP had been hydrolyzed [20]. An interesting fact is that the porous structure of the collagen network was maintained similar in all cases, being the main difference, the mineralization of the collagen fibers. Further physico-chemical characterization will be assessed for the different scaffolds (e.g., mechanical properties, FTIR, pore size distribution).

The proliferation of the mesenchymal stem cells and their differentiation to osteogenic cells were comparable for the collagen scaffold and the scaffolds containing 13 and 23% HA. The main difference was observed for the higher content (83%) of HA. In this case, the proliferation was significantly lower compared to the other types of scaffolds, owing in part to the fewer cells initially adhered to the scaffold. The main benefit of the higher amounts of HA, was the significant increase in ALP activity, suggesting the high efficiency of the HA to induce the differentiation of the MSC into osteoblasts. This observation was not seen in similar types of scaffolds, in which it was shown that the incorporation of different ratios of HA on the collagen scaffolds did not change the ALP activity [21].

The lower proliferation of the 83% HA initially can be attributed to the fact that, when cells are seeded, collagen plays an important role in initial adhesion. In this case, the cells lack this benefit since they only enter in contact with the HA and not with the underlying collagen. Moreover, it has been previously reported that for a set CPC analogous to the one used in this work, the microstructure of the HA had a direct effect on cell proliferation and differentiation [22]. It was seen that, depending on the microstructure cell proliferation could be delayed. Although there were lower rates of proliferation in the scaffolds with the highest HA content, cell differentiation was increased.

The SEM observation of the cell morphologies revealed the trend previously mentioned for proliferation and differentiation. Cell morphology was clearly different for the 13 and 23% HA scaffolds and the 83% HA scaffolds (Figs. 5 and 6). In the first two cases, the cells presented a spread morphology creating a cell layer on the scaffold which was probably near confluence, as shown by the proliferation assay. In the case of the 83% HA, cells are only able to attach to the HA, and this adhesion is slower than in the case of collagen due to the absence of the cell adhesion motifs sequences present in collagen. This is revealed by the cell morphology observed for the 83% HA, where the cells presented elongated morphology with few contact points.

It is also worth highlighting that after 14 days, cells were able to proliferate, as was seen by the proliferation assay, and were shown to cover most of the scaffold surface, presenting expanded cells on the 83% HA scaffolds. The main difference was the presence of a higher extracellular matrix formation in the case of the 83% HA compared to the other two cases, which would be in agreement with the ALP results obtained. Future studies will address later time points in order to assess the biological behavior of the scaffolds at longer culture times.

In order to increase the osteoinduction ability of the scaffolds, BMP is widely used in the field of bone regeneration [10, 23, 24]. In the present work, the approach is different, since the BMP-7 was not incorporated into the scaffold, being expressed by the cells after transfection of a specific gene encoding the BMP-7 protein. The plasmid used in the present work has already been previously used in vivo, presenting promising results [25]. The BMP-7 has a higher affinity for HA, that is why the BMP-7 levels were measured directly on the scaffolds by breaking them [26, 27]. Actually, the amount of BMP-7 was also measured in preliminary studies in the medium, but the values obtained were even lower and therefore, the results were analyzed in the scaffold. The amount of BMP-7 produced was higher in the 20 μg plasmid loading after 1 and 7 days and it was also found that the accumulated values in this case was 0.264 ng. Nevertheless, a surprising fact was that when the values of BMP7 were normalized by the cell content, it was seen that the amount of BMP7 per cell content was higher in the cases in which no plasmid was present. This also means that the combination of the scaffolds with the MSCs in the osteogenic media is expressing the BMP-7 protein. Therefore, the main effect of the plasmid was the increase on cell proliferation, not directly on protein expression. As mentioned in the results, it was discarded that the higher proliferation rates measured with the DNA kit could correspond to the signal coming from the gene DNA, since the incorporation of the gene without cells in the scaffold and the subsequent DNA reading gave no value.

The intention of the work was to incorporate a CaP in the collagen structure since CaP have been widely used for more than 30 years as gene delivery vehicles, having low efficiencies, but showing few problems of cytotoxicity. In these cases, the CaP have been used as nanoparticles [28–31]. These nanoparticles are usually in the range of 300 nm and the mechanism by which the cells are transfected is by endocytosis of the nanoparticles [31]. There are a large number of factors that affect the transfection efficiency, such as, time of contact between the material with the plasmid and the cells, the cell type used, the Ca/P ratio, as well as the morphology of the precipitate among others [28, 32, 33].

The use of CaP in the form of nanoparticles is challenging for use as tissue engineering scaffolds [28–30]. These nanoparticles have been combined with alginate hydrogels as an implantable device, but not combined with cells [34]. That is why the use of tissue engineering constructs incorporating HA was thought as a promising system to deliver the plasmid and to be able to combine them with cells. Unfortunately, the amount of protein was low and in some cases was comparable to the values in the absence of plasmid. This means that the plasmid might not have been incorporated inside the cells, although the possible mechanism by which the plasmid could have been incorporated was discussed.

In the case of having the HA in the collagen matrix, a continuous surface of HA crystals is created. Therefore, it

was hypothesized that the plasmid could have been incorporated as naked DNA or as part of the crystals. Because HA may present electrostatic charge, the gene is able to physically attach to the surface of the crystal. These crystals may be detached from the structure by manipulation (e.g. media changing) or degradation of the scaffold [35] or a CaP may be precipitated on the surface of the existing HA due to the supersaturation of calcium and phosphates in the medium. The size of these HA crystals is in the range of several hundreds of nanometers to several microns. That is why some HA may break and detach from the structure, being in the range of sizes which the cells are able to endocytosise.

It seems that even if the plasmid was able to enter the cells, the transfection was still low probably due to the aggressive environment found inside the cell cytoplasm [36, 37]. The more protected the gene is, the higher the transfection efficiency will be, although it has been shown that the efficiencies are less than 2% even when the gene is protected with a CaP [28].

5 Conclusions

Collagen-CPC scaffolds may serve as macroporous scaffolds for MSC proliferation and differentiation. The scaffolds produced were shown to homogenously distribute the HA on the collagen fibers. The different HA contents affected cell proliferation and differentiation. The use of the scaffolds as gene delivery vehicles for the production of BMP7 must be improved although significant levels of BMP7 were expressed, associated with an increase in cell proliferation.

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