



Title: New Scaffolds Encapsulating TGF- β 3/BMP-7 Combinations

Driving Strong Chondrogenic Differentiation

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Funding:



Highlights:

- The sustained delivery of BMP-7 enhances the chondrogenic activity of TGF- β 3.
- BMP-7 and TGF- β 3 can be entrapped in polymers matrices as PEG-heparin complexes.
- Scaffolds delivering TGF- β 3 and BMP-7 drive the chondrogenesis of cell progenitors.

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**New Scaffolds Encapsulating TGF- β 3/BMP-7 Combinations Driving Strong
Chondrogenic Differentiation**

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Abstract

The regeneration of articular cartilage remains an unresolved question despite the current access to a variety of tissue scaffolds activated with growth factors relevant to this application. Further advances might result from combining more than one of these factors; here, we propose a scaffold composition optimized for the dual delivery of BMP-7 and TGF- β 3, two proteins with described chondrogenic activity. First, we tested in a mesenchymal stem cell micromass culture with TGF- β 3 whether the exposure to microspheres loaded with BMP-7 would improve cartilage formation. Histology and qRT-PCR data confirmed that the sustained release of BMP-7 cooperates with TGF- β 3 towards the generation of chondrogenic differentiation. Then, we optimized a scaffold prototype for tissue culture and dual encapsulation of BMP-7 and TGF- β 3. The scaffolds were prepared from poly(lactic-co-glycolic acid), and BMP-7/TGF- β 3 were loaded as nanocomplexes with heparin and Tetronic 1107. The scaffolds showed the sustained release of both proteins over four weeks, with minimal burst effect. We finally cultured human mesenchymal stem cells on these scaffolds, in the absence of exogenous chondrogenic factor supplementation. The cells cultured on the scaffolds loaded with BMP-7 and TGF- β 3 showed clear signs of cartilage formation macroscopically and histologically. RT-PCR studies confirmed a clear upregulation of cartilage markers SOX9 and Aggrecan. In summary, scaffolds encapsulating BMP-7 and TGF- β 3 can efficiently deliver a cooperative growth factor combination that drives efficient cartilage formation in human mesenchymal stem cell cultures. These results open attractive perspectives towards *in vivo* translation of this technology in cartilage regeneration experiments.

1. Introduction

Scaffolds for tissue engineering integrating growth factors (GFs) are expected to provide important medical benefits for tissue regeneration; this has been confirmed by pioneering technologies such as Infuse[®] (Medtronic), a type 1 collagen sponge that contains rhBMP-2 and is intended for bone regeneration. Despite some medical limitations [1], Infuse[®] results in excellent clinical benefits for on-label use. However, there is a consensus that for the regeneration of some complex tissues, the delivery of multiple GFs will be necessary. This is because the tissue development pathways that we aim to recapitulate usually integrate more than one cellular signal. For instance, Mooney et al. [2] designed poly(lactic-co-glycolic acid) (PLGA) scaffolds for the dual delivery of Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) as a proangiogenic device. They showed that the dual delivery of GFs produces larger and more mature vessels than those produced by single GFs. In 2004, the same research group used alginate hydrogels for the dual delivery of Bone Morphogenetic Protein-2 (BMP-2) and Transforming Growth Factor- β 3 (TGF- β 3) to differentiate rat bone marrow stromal cells into bone cells. Again, the combination of the two GFs in the same delivery system led to effective bone formation compared to the delivery of the individual factors on their own [3].

Articular cartilage regeneration is an unresolved problem for tissue engineering where several researcher groups have recently presented scaffolds for the co-delivery of GFs in an attempt to improve regenerative outcomes. For instance, the group of Mikos tried to improve cartilage repair using TGF- β 1 and Insulin-like Growth Factor-1 (IGF-1) loaded into a hydrogel. While histological scoring showed minimal differences between the co-delivery and the single delivery, the dual delivery group had a higher proportion of subchondral bone repair, greater bone growth at the defect margins, and lower bone

specific surface [4,5]. Du et al. developed a dual-release hydrogel system of basic Fibroblast Growth Factor (bFGF) and TGF- β to enhance human mesenchymal stem cell (hMSC) differentiation [6]. Recently, Han et al. developed a conically graded chitosan-gelatin hydrogel/PLGA scaffold for the dual-delivery of TGF- β 1 and BMP-2, which showed preliminary indications of chondrogenic differentiation [7]. Globally, it is clear that the success of a dual-delivery strategy rests on the rational selection of the combination of growth factors, and the use of a scaffold capable of providing suitable release kinetics for the therapeutic action of these proteins.

TGF- β isoforms have been particularly studied for their ability to produce chondrogenic differentiation of hMSCs in serum-free media and high cellular concentrations; in fact, the TGF- β 3 supplemented medium is used in current standard protocols for this purpose [8–10]. However, devices integrating only TGF- β 3 delivery are suboptimal for articular cartilage regeneration [11], which has sparked the interest of delivering a second GF for improved outcomes [12,13].

Factors of the family of bone morphogenetic proteins (BMPs) are known to act as inducers of chondrogenic differentiation [14,15]; BMP-7 is considered one of the most important anabolic factors of chondrocytes [16,17]. Shen et al. found that BMP-2 is able to cooperate with TGF- β 3 for improved cartilage formation [13]. However, whether this finding would translate from BMP-2 to BMP-7 was unclear, since the former is a member of the Dpp subclass of BMPs, and the latter of the 60A subclass; this ultimately results in both molecules having preferential affinity for different receptors [18]. The same research group later showed that the combined application of BMP-7 and TGF- β 3 substantially improves the chondrogenic and osteogenic *in vitro* differentiation of hMSCs compared to BMP-7 alone [19]. Unfortunately, this study did not clarify whether this combination improved cartilage formation compared to TGF- β 3 alone, the

gold standard GF for chondrogenic differentiation. Besides, these researchers did not study any sustained release formulation, necessary to maintain precise levels of the GFs for potential *in vivo* use. The importance of controlled release is highlighted by our knowledge to the *in vivo* response to different concentrations of TGF- β 3. A prolonged exposure to high doses of this GF produces undesirable effects like fibrosis, hypertrophic scars [20] and osteophyte formation [21].

We recently studied the development of biodegradable microspheres where BMP-7 is encapsulated through a so-called nanocomplex encapsulation method. In this procedure, the protein, which has to present a heparin-binding domain, is complexed with a heparin-type molecule, and later with a low-charge cationic derivative of polyethylene glycol. The integration of the proteins in these structures improve protein loading in the final polymeric system [22]. These complexes are freeze-dried and can be resuspended directly in an organic polymer solution used for casting the final vehicle (i.e. microspheres, nanoparticles, scaffolds, etc.). Microspheres prepared by this method showed very high BMP-7 encapsulation efficiency (>85%), better than that achieved with the standard double-emulsion method, and sustained release of the protein for 2–3 months in bioactive form [23]. We recently implanted these microspheres *in vivo*, and confirmed the activation of the BMP-7 signaling route (p-Smad 1/5/8) at the region of implantation for over two months [24]. The same method can be adapted to a variety of biodegradable polymers, but biodegradable polyesters in general, and PLGA in particular, are of special interest due to their history of medical use, versatility to form different structures and widely studied physicochemical properties [25,26]. Since both TGF- β 3 and BMP-7 are heparin-binding proteins, we proposed that this method could be suitable for the dual delivery of these GFs in cartilage regeneration devices.

This study aimed at three objectives: (i) to check whether there is an improvement in combining the sustained release of BMP-7 over TGF- β 3 supplementation in cartilage formation studies; (ii) to study the possibility of applying the nanocomplex encapsulation method for the preparation of scaffolds optimized for tissue culture and for the delivery of BMP-7/TGF- β 3 combinations; and (iii) to evaluate whether the dual delivery of BMP-7 and TGF- β 3 from the scaffolds could by itself drive chondrogenic cell differentiation and cartilage formation.

2. Materials and Methods

2.1 Materials

Soy lecithin, heparin sodium porcine mucosa, ethylenediamine tetrakis(propoxyethyl-*block*-ethoxylate) tetrol (Tetronic 1107[®], T1107), polyoxyethylene (20) sorbitan monolaureate (Tween[®] 20), cottonseed oil, polycaprolactone (PCL, Mn 70000 – 90000 Da), poly(methyl vinyl ether-*alt*-maleic) anhydride (PVM/MA, Mn 80000 Da), methoxypolyethylene glycol amine 5000 (PEGAm), dextran sulfate sodium salt (Mw > 500000) were all purchased from Sigma-Aldrich (Missouri, USA). Poly(lactic-co-glycolic acid) (PLGA) was obtained from Boehringer-Ingelheim (Petersburg, USA). Recombinant proteins BMP-7 and TGF- β 3 were bought from Peprotech (USA); their respective ELISA kits were bought from Abnova (Taiwan) and Usen Life Science Inc. (China). The remaining reagents and solvents used were of analytical grade or better. In all the experiments, ultrapure water was employed (Milli-Q, Millipore).

2.2 Microsphere preparation and characterisation

BMP-7 microspheres were prepared by the nanocomplex encapsulation method (**Figure S1**), a procedure optimized for the encapsulation of heparin-binding proteins, and previously reported by us [23,24]. Briefly, 2 μ g of BMP-7 (theoretical loading of 0.01% w/w) and the same amount of heparin were dissolved in 300 μ L of ultrapure water and incubated for 30 min at room temperature. Then, 2.5 mg of Tetronic 1107 (T1107) was added, and the components were allowed to interact for another 30 min to ensure the formation of the BMP-7/heparin/T1107 nanocomplexes. The resulting system was frozen at -20 °C and freeze-dried using the following program: primary drying at -35 °C

for 24 h, secondary drying at 0 °C for 24 h, and 14 h at room temperature (freeze-drier Genesis 25 ES, VirTis Model-Wizard 2.0, SP Industries, USA).

The solid cake was resuspended in a 20 mg PLGA solution in 400 µL of acetonitrile (final ratio of PLGA:T1107 8:1 w/w). After that, the organic phase was added dropwise to 4 mL of 0.5% (w/v) soybean lecithin in cottonseed oil. Next, the suspension was sonicated for 20 s using a Branson 250 sonicator (40W, Danbury, CT, USA) and stirred at 500 rpm for 30 min in a fume hood. After this time, 2 mL of petroleum ether was added to harden the immature microspheres, and the mixture was stirred for 10 more minutes. Finally, the microspheres were collected using a nitrocellulose membrane (25 mm, 0.22 µm). The microspheres retained in the filter were washed with abundant petroleum ether, freeze-dried and stored under a vacuum in a desiccator until use.

2.3 Polymeric film preparation

The films were fabricated by a solvent casting-evaporation method, where the proteins were added to the polymer organic solution in the form of a nanocomplex with heparin and a PEG cationic derivative. Briefly, 233 µL of ultrapure water, 40 µL of a BMP-7 aqueous solution (0.1 mg/mL) and 2 µL of a 2 mg/mL polysulfated polymer aqueous solution (for a 1:1 w/w protein:polymer ratio) were added to a low binding plastic microtube. After incubation for 30 minutes with mild shaking at room temperature, the samples were incubated with 25 µL of a PEG cationic derivate solution at 50 mg/mL, and incubated again for 30 min. The complexes resulting from these incubation steps were freeze-dried and stored in a desiccator at room temperature until use. The polysulfated polymers tested in this study were heparin and dextran sulfate; the studied PEG cationic derivatives were T1107 and PEGAm.

Films were prepared for screening multiple conditions in parallel by a high-throughput approach. During film casting, each freeze-dried sample (i.e. four possible combinations of polysulfated polymer and cationic PEG derivative) was redissolved in 400 μL of acetonitrile (solution A). In parallel, a 100 mg/ml solution of each biodegradable polymer (PCL, PLGA and PVM/MA) was prepared in an organic solvent (acetonitrile for PLGA and PVM/MA and dichloromethane for PCL, solution B). Then, 100 μL of solution B was placed in the wells of a polypropylene 96-well plate. Immediately, 10 μL of the corresponding solution A was added to each well while shaking horizontally. The 96-well plate setup was optimised to test all the possible combinations of the three biodegradable polymers (PCL, PLGA, PVM/MA), two polysulfated polymers (heparin, dextran sulfate) and two cationic PEG derivatives, i.e. 12 different conditions, $n=3$ (**Figure S2**). The multiwell plate was kept at room temperature for 5 h, and in a desiccator under a vacuum for another 19 h for complete solvent removal. For all the samples, the final amount of biodegradable polymer was 10 mg/well and the loading of BMP-7 0.1 μg /well.

2.4 Scaffold preparation and characterisation

The scaffolds were prepared through a solvent casting/salt leaching procedure [27], adapted for loading the proteins through a nanocomplex encapsulation method similar to those described before. First, 20 μg of BMP-7, 600 ng of TGF- β 3 and 20.6 μg of heparin were dissolved in 300 μL of ultrapure water (growth factors: heparin ratio 1:1 w/w). This solution was incubated for 30 minutes at room temperature. After this incubation, 10 mg of T1107 was added and allowed to interact with the remainder of the components for another 30 min at room temperature. The resulting samples were frozen at $-20\text{ }^{\circ}\text{C}$ and freeze-dried (same program as described above). The freeze-dried cakes

were dissolved in 400 μL of acetone containing 40 mg or 80 mg of PLGA (for 10 or 20% w/v scaffolds, respectively). This solution was mixed with 480 mg of sieved NaCl (180–250 μm particle diameter) and transferred to four cylindrical molds (9 mm diameter, 1–1.5 mm height). The molds were left in the fume hood for 4 hours until the complete evaporation of the solvent. After this time, the scaffolds were taken from the molds and the imperfections in their surface removed with a scalpel (**Figure S3**). To eliminate the porogen, the scaffolds were immersed in distilled water for one week, renewing the water every 24 hours. Scaffolds with a theoretical loading of 4 μg of BMP-7 and 120 ng of TGF- β 3 per unit were obtained with this method. The morphology of the scaffolds was characterized through scanning electron microscopy (EVO LS 15 microscope).

2.5 *In vitro* release of BMP-7 from the polymeric films

For release studies, 200 μL of PBS BSA 1% was added to each well of the multiwell plate where the films had been prepared. The plate was sealed with a plastic cover and kept at 37 °C under mild horizontal shaking. At established time points, the release medium was collected and replaced with fresh medium. The BMP-7 concentration in the release media was quantified using ELISA following the kit manufacturer's instructions (Abnova).

2.6 BMP-7 and TGF- β 3 encapsulation efficiency

The scaffolds were prepared as described in section 2.4 with a theoretical composition of 4 μg BMP-7 and 120 ng of TGF- β 3. The content of growth factors was analyzed at time 0 (just after the 1 week washing step in water) and after 30 days of release in PBS (section 2.7). With that purpose, scaffolds were dissolved in 150 μL of DMSO. For a

complete degradation of the PLGA structure, the scaffold was kept under horizontal shaking for 15 min. Then, 400 μ L of SDS 0.5 % in water were added and the samples were shaken for 30 more minutes. Finally, BMP-7 and TGF- β 3 were quantified in the degraded samples using their corresponding ELISA kits. Encapsulation efficiency (EE%) was calculated by the following equation:

2.7 *In vitro* release of BMP-7 and TGF- β 3 from scaffolds

The scaffolds were placed in a glass tube containing 1.5 mL of PBS, 1% BSA, 2% penicillin/streptomycin and 0.2% fungizone. The tubes were covered with a lid and kept at 37 °C with mild shaking. Every day for 30 days, the release medium (1.5 mL) was removed and frozen for future analysis. After the removal, the same volume of fresh medium was added to the scaffolds. At the end of the 30 days, samples for every three days were pooled and the BMP-7 and TGF- β 3 content was quantified using their corresponding ELISA kit, following the manufacturer's instructions.

2.8 Cell culture

Human Mesenchymal Stem Cells (hMSCs) obtained from adipose tissue were a kind gift from Advanced in Vitro Cell Technologies SL, Spain. On arrival, hMSCs were thawed, and plated in a 10 cm dish in Mesencult Media supplemented with penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. The cells' identity and differentiation capability were tested before use.

2.9 hMSCs characterisation

HMSCs characterization was carried out by immunophenotypic analysis of surface proteins by flow cytometry and by their differentiation capacity to adipose, bone and cartilage tissue.

For immunophenotypic analyses, 2×10^5 hMSCs (passage 4) were harvested and resuspended in PBS. HMSC were incubated for 15 minutes with fluorescein isothiocyanate (FITC)-conjugated anti-CD90, FITC-conjugated anti-CD44, FITC-conjugated anti-CD45, FITC-conjugated anti-CD34 and phycoerythrin (PE)-conjugated anti-CD105 (all from Becton Dickinson Biosciences, San Jose, CA, USA). Sample acquisition was performed in a FACScan™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). The CellQuest software program (Becton Dickinson Biosciences) was used for the acquisition.

For the adipogenic differentiation assay, 80% confluent hMSCs, seeded previously in a 100-plate (BD Falcon), were subsequently incubated in adipogenic medium (DMEM High glucose, 10 μ g/ml Insulin, 100 μ M Indomethacin, 500 μ M Isobutyl-1-methylxanthine; all from Sigma). The medium was changed twice a week for 21 days. Then, the cells were washed and fixed, and adipogenesis was measured by the accumulation of neutral lipids in fat vacuoles, stained with Nile Red solution (Sigma) and quantified by flow cytometry (Becton Dickinson Biosciences).

For osteogenic differentiation, 2×10^5 hMSCs were pelleted in a 2 ml screw cap microtube (BD Falcon) and incubated in specific osteogenic medium: DMEM High Glucose, 100 μ g/ml Ascorbic acid 2-phosphate, 20 nM dexamethasone, 20 mM Glycerophosphate (all from Sigma) and 2 μ g/ml BMP-2 (Peprotech, Inc., UK). The medium was replaced every 3–4 days. After 21 days, the pellets were washed, fixed and embedded in paraffin. Bone formation was analyzed by histology with von Kossa staining.

For chondrogenic differentiation, 2×10^5 hMSCs were pelleted in a 2 ml screw cap microtube (BD Falcon) and incubated in specific chondrogenic medium: DMEM-high glucose, 40 $\mu\text{g/ml}$ proline (Sigma), 1% ITS solution (BD Biosciences), 5.35 $\mu\text{g/ml}$ linoleic acid, 100 nM dexamethasone (Sigma), 1.25 mg/ml BSA (Sigma), and 10 ng/ml of recombinant human TGF- β 3 (Peprotech Inc., UK). The medium was replaced every 3–4 days. After 21 days, the pellets were washed, fixed and embedded in paraffin. Cartilage formation was analyzed by histology with Safranin-O staining.

2.10 Chondrogenic differentiation on BMP-7 loaded microsphere pellets

Chondrogenic differentiation of hMSCs was induced in a 3D culture based on the copelletisation of the cells with microspheres, either blank (control) or loaded with BMP-7. Specifically, 200,000 hMSCs were suspended together with 20 mg of microspheres (0.01% loading of BMP-7) in 1 mL of chondrogenic differentiation medium (described in Section 2.9). This suspension was precipitated by centrifugation (350·g) in the form of a pellet, and cultured for three weeks at 37 °C in a humidified atmosphere containing 5% CO₂ and with the same differentiation medium. The chondrogenic medium was replaced twice a week.

2.11 Chondrogenic differentiation on BMP-7/TGF- β 3 loaded scaffolds

One week before cell seeding, the scaffolds were hydrated with sterile PBS with antibiotics (penicillin and streptomycin) and exposed to UV for 12 h to sterilize them. The sterilized scaffolds were pinned to non-treated cell culture plates and 10^6 hMSCs were seeded on each scaffold. The cells were cultured for three weeks in DMEM high glucose, supplemented with 1% ITS solution (BD Biosciences), 5.35 $\mu\text{g/ml}$ linoleic acid, 40 $\mu\text{g/ml}$ proline (Sigma), 100 nM dexamethasone (Sigma), 1.25 mg/ml BSA

(Sigma), penicillin and streptomycin (Gibco) and fungizone (Gibco). The culture medium was changed twice a week, and the incubator was set as 37°C in a humidified atmosphere containing 5% CO₂. Two experimental groups were compared: hMSC on blank scaffolds (control) and hMSC on BMP-7/TGF-β₃ loaded scaffolds. Cartilage formation was analyzed by histology (Safranin-O staining) and qRT-PCR for chondrogenic markers.

2.12 Histology

Samples from the chondrogenesis experiments with microspheres and scaffolds (Sections 2.10 and 2.11) were collected after three weeks of culture. The pellets were included in a drop of HistoGel (ThermoScientific) and, once solidified, rinsed in PBS and embedded in paraffin. The scaffolds were directly embedded in paraffin. Sections of 5 μm thickness were cut and mounted on glass slides. Before staining, these sections were dewaxed in xylene and hydrated with graded ethanol.

Both the pellets and scaffolds were stained with Safranin-O (Sigma) for cartilage tissue observation. Following deparaffinisation, the samples were stained with haematoxylin for five minutes. Then, the samples were rinsed with acid alcohol and stained with 0.02% Fast Green solution for three minutes. The samples were rinsed with 1% acetic acid for 30 s, stained with 0.1% Safranin-O for 10 minutes, rinsed again with distilled water, and finally mounted after dehydration. The mounted slides were analyzed using an optical microscope (Olympus BX43F).

2.13 Reverse transcription and real-time PCR analysis

Total RNA was isolated from the chondrogenesis experiments (Sections 2.10 and 2.11) after three weeks of culture using a NucleoSpin[®] RNA kit (Macherey-Nagel) following

the manufacturer's protocol. Total RNA (500 ng) was then converted to cDNA with MML-V retrotranscriptase (Invitrogen). All real-time quantitative polymerase chain reactions (PCR) were performed on a Taqman RealPlex System (Eppendorf) in 20 μ l reaction volume containing 1x Universal Master Mix (Applied Biosystems), 1x Taqman Assay, cDNA and RNase free water. The expression of the following genes was examined: Sox9 and Aggrecan. Actin B and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes. The primer sequences for all genes are listed in the Supplementary Material (Table S1). The PCR reaction was performed for 1 min at 95 °C, followed by 40 amplification cycles (15 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C). Each PCR was processed in triplicate. Relative transcript levels were calculated using the 2-delta delta ct (cycle-threshold) method.

2.14 Statistical analysis

Unless otherwise indicated, the experiments performed here were repeated at least three times. The experimental data are presented as mean \pm standard deviation. A Mann-Whitney Test was used to determine the statistical significance. The differences were considered significant for * $p < 0.05$, and very significant for ** $p < 0.01$. All the statistical analyses were carried out with GraphPad Prism Version 5.0 software.

3. Results and Discussion

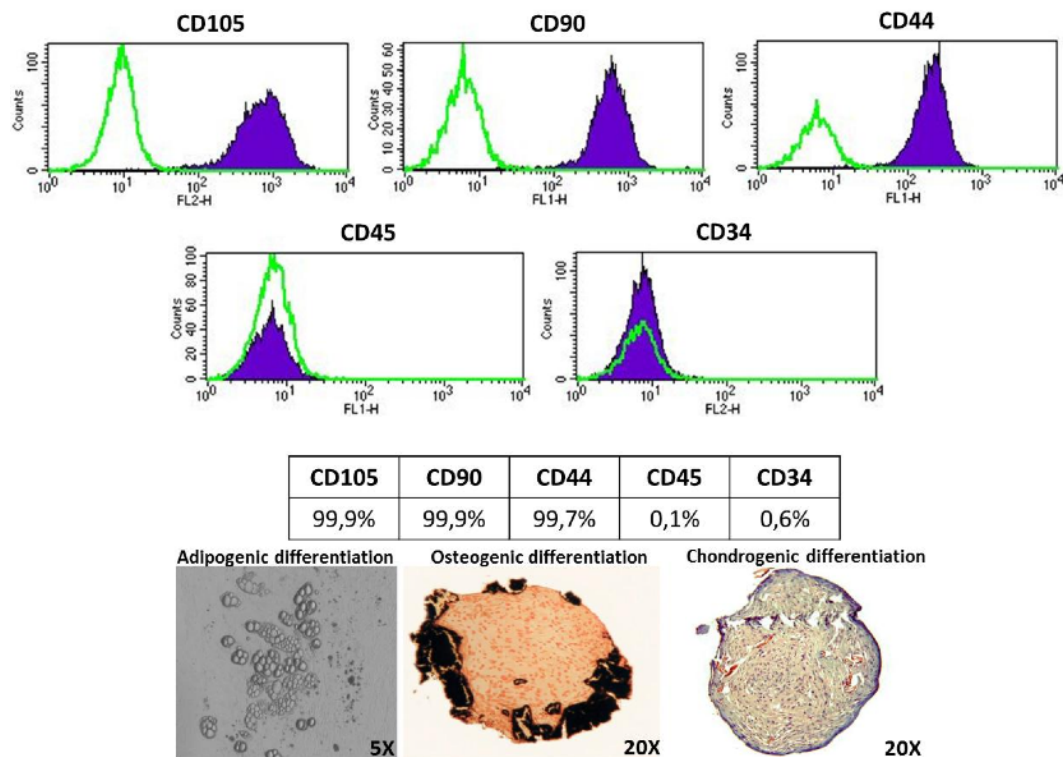


Figure 1: (A) Characterization of hMSCs by flow cytometry (filled purple plot) and a negative control (empty green plot). The hMSC population presented markers CD105, CD90 and CD44, and the absence of CD45 and CD34. The table insert indicates the percentage of positive cells for each marker. (B) Confirmation of functional differentiation of hMSC in vitro. Histological sections after: adipogenic (left), osteogenic (middle), and chondrogenic differentiation of hMSCs (right).

The integration of growth factors (GFs) in controlled release systems is a useful and much-explored method to improve their efficacy *in vivo* [28–32]. However, it is assumed that inducing the repair of complex tissues, such as cartilage, might only be possible through the orchestrated co-delivery of two or more GFs. Here, we aimed at designing a platform for the co-delivery of bone morphogenetic protein 7 (BMP-7) and transforming growth factor β 3 (TGF- β 3), since both proteins are known to be

chondrogenic on their own and in combination [19]. However, there is no data in the literature indicating whether this combination provides any improvement over the use of TGF- β 3 alone, the standard protocol of hMSC differentiation to cartilage.

To perform the cartilage differentiation experiments, we used adipose tissue derived human mesenchymal stem cells (hMSCs). Before starting, the hMSC population was fully characterized to confirm the characteristics that are required for lineage identification. The cell population was positive for CD105, CD90 and CD44, associated with this mesenchymal population, and negative for haematopoietic markers CD45 and CD34. The capacity of hMSCs to undergo differentiation to bone, fat and cartilage cells was also confirmed (**Figure 1**).

3.1 Effect of BMP-7 release on TGF- β 3 induced chondrogenesis

We have previously described microspheres capable of providing over two months of sustained protein release for BMP-7 [23,24]; here, we took advantage of this technology and studied the cartilage formation from hMSCs after co-pelletisation of the cells with microspheres, in the presence of a classical cartilage formation medium that contains TGF- β 3. The tissue formed in pellets with BMP-7 loaded microspheres was compared to that of pellets with cells cultured with blank microspheres (control).

Macroscopic observation of the pellets after 21 days of culture gave an initial indication of the beneficial effect of BMP-7. Pellets formed with BMP-7 loaded microspheres showed more dense tissue, a viscous appearance and darker red color than those with the blank microspheres (**Figures 2A, 2B**). In both cases, it was possible to observe the presence of microspheres still remaining in the pellets. This was expected since complete microsphere hydrolysis lasts more than one month, according to previous studies [24]. Histological images showed tissue deposition in both cases, but only the

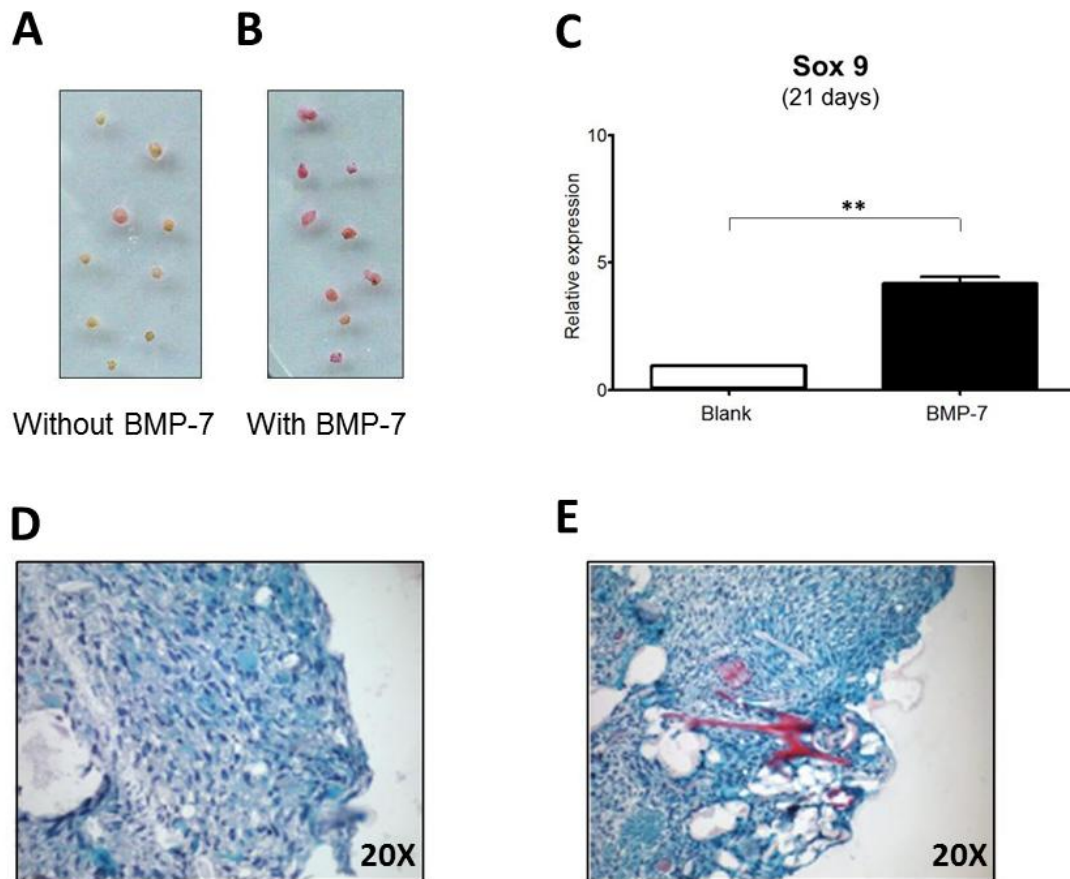


Figure 2: Representative photograph of the macroscopic appearance of hMSCs pellets cultured with microparticles without BMP-7 (A) and with BMP-7 (B) after 21 days in culture. (C) Relative expression of SOX9 in pellets cultured on BMP-7 microspheres relative to pellets cultured on blank microspheres. Data represents mean \pm SD (n=6); **p<0.01 (Mann Whitney test). Histology sections (Safranin-O staining) of pellets cultured on blank microspheres (D), and pellets cultured on BMP-7 microspheres (E).

pellets cultured with BMP-7 loaded microspheres showed tissue regions with intense red staining (**Figure 2E**), characteristic of highly charged proteoglycans. These regions were absent in the control pellets (**Figure 2D**). The chondrogenic marker expression was quantified by qRT-PCR; we observed that pellets with BMP-7 loaded microspheres had significantly higher SOX9 expression than the control pellets (i.e. a five-fold increase, p<0.01, **Figure 2C**). We could not detect Aggrecan mRNA in any of the

samples, a negative result probably due to the limited amount of sample obtained from the low cell mass used.

Based on the evidence gathered, we concluded that sustained levels of BMP-7 cooperate with TGF- β 3 to induce chondrogenic differentiation in adipose derived MSCs. This finding is in agreement with the results of Estes et al., who demonstrated that adipose-derived stem cells differentiate to chondrocytes when treated with a mixture of TGF- β 3 and BMP-6 [15]. These authors also suggested that the efficiency on the differentiation to chondrocytes might be highly dependent on the specific cell source, being adipose-derived stem cells especially appropriate for this GF combination. Our results also support previous observations from Shen et al., who showed that BMP-7 cooperates with TGF- β 3 for cartilage formation [19]. However, no data was previously available comparing this GF combination with the gold standard treatment, TGF- β 3 alone. Due to the chondrogenic activity observed for the BMP-7/TGF- β 3 combination over TGF- β 3, we established a rationale for the co-delivery of these GFs from cartilage regeneration implants. We hypothesized that the microspheres would not be the optimal presentation for this tissue-engineering device; consequently, we decided to adapt our delivery technology to a polymeric scaffold format.

3.2 BMP-7 release kinetics from different polymer compositions

To formulate a scaffold suitable for the co-delivery of the GFs, we decided to apply the nanocomplex encapsulation method used in the microspheres [23,24] to other polymeric device forms and compositions. The method is highly flexible and can be applied to a variety of materials. Therefore, before generating a final scaffold prototype, we decided to explore the effect of a range of compositions in the release properties.

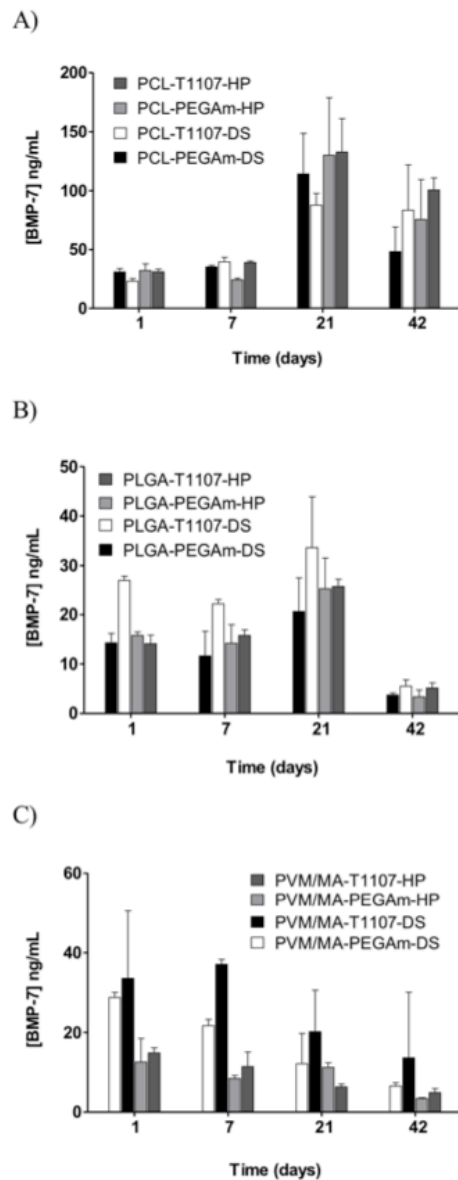


Figure 3: BMP-7 released from films generated from different polymer combinations. In vitro release was performed in PBS, at 37°C in sealed multiwell plates with horizontal shaking. The films were prepared from three different biodegradable polymers: (A) PCL, (B) PLGA and (C) PVM/MA. In each film preparation one polysulfated polymer was included, heparin (HP) or dextran sulfate (DS), and one PEG cationic derivative, Tetronic 1107 (T1107) or methoxypolyethyleneglycol amine (PEGAm). Results represent mean \pm SD, n=3.

Compositions made by the nanocomplex encapsulation method can change: (1) the polysulfated polymer used to complex the protein; (2) the cationic PEG derivative used to coat the protein:polysulfate complex; and (3) the biodegradable polymer used for encapsulation of these complexes. We decided to check how all these variables influence the release kinetics, but assuming a number of simplifications to improve experimental execution. First, we would test the release from thin films; this was done to generate a more homogeneous morphology among all samples. Even more importantly, we adapted this morphology to a high-throughput method that we intended to use. With this experimental setup, we were able to test 12 possible material combinations for protein release in a streamlined experiment (**Figure S2**).

A second simplification was that we only encapsulated BMP-7. From our previous experience, it is not possible to extrapolate release kinetics totally between different GFs. However, due to the similar physicochemical properties of both proteins (BMP-7, MW 28.8 kDa, calculated pI 6.04; TGF- β 3 MW 25 kDa, calculated pI 6.11), we expected that the general tendencies observed in BMP-7 would replicate in TGF- β 3. Finally, to maintain sampling to a reasonable number, we reduced the release data time points to just four (24 hours and 1, 3 and 6 weeks).

Release data were obtained for the 12 different films (**Figure 3**). Fragmentation of the dataset indicated some minor significant contributions of the polysulfated polymer and the cationic PEG derivative, under very defined conditions; however, it is probable that such contributions are only random noise, a byproduct of the large number of measurements. The multi-variant analysis performed in the complete dataset indicated that the factors “polysulfated polymer” and “cationic PEG derivative” did not significantly contribute to BMP-7 release kinetics. This observation suggests that dextran sulfate can play a similar role in the binding of heparin-binding proteins as

heparin itself. Similar conclusions have been reported in the literature for this and other heparin-mimics [33,34]. The lack of differences between T1107 and PEGAm is more surprising, but we have to consider that, in general, both molecules are expected to bear one cationic charge under physiological conditions, both are hydrophilic, and both are characterized by long PEG chains.

As expected, the biodegradable polymer was an important factor determining the release from the films. BMP-7 release from PVM/MA films showed comparatively high burst and a minimum release over time (**Figure 3C**), while the films themselves showed macroscopic signs of failure. This result is consistent with previous data showing the fast hydrolysis of this polymer [35,36]. Due to this release and degradation properties, these films were not finally considered for scaffold design. The profile of PCL and PLGA was similar, but the latter showed in general the lowest burst release. PLGA release data also seemed to be more constant (**Figure 3B**), while the release from PCL films clearly peaked at 21 days (**Figure 3A**). The reduced burst effect is a typical feature of our nanocomplex encapsulation method, and has been described before in microsphere formulations [23]. When analyzing the release from the films made with the slow degrading polyesters, it was interesting to note that the polymer with the theoretical slower degradation (PCL) seemed to be the fastest to release the protein. This tendency suggests that, under the current conditions, there might be other factors affecting protein release. For instance, the lower glass transition temperature of PCL could result in facilitated mobility for the nanocomplexes in this polymeric matrix.

For the purpose of an *in vitro* cartilage formation experiment such as that described in Section 3.4, PCL release could have been more promising; however, considering a possible translation to *in vivo* experiments, we decided to keep the PLGA/T1107/heparin composition that showed sustained release and a minimum burst. Globally, this

high-throughput experiment allowed us to define the relevant composition parameters affecting BMP-7 release, and to define the overall tendencies between several types of compositions.

3.3 Scaffolds for the sustained release of BMP-7 and TGF- β 3

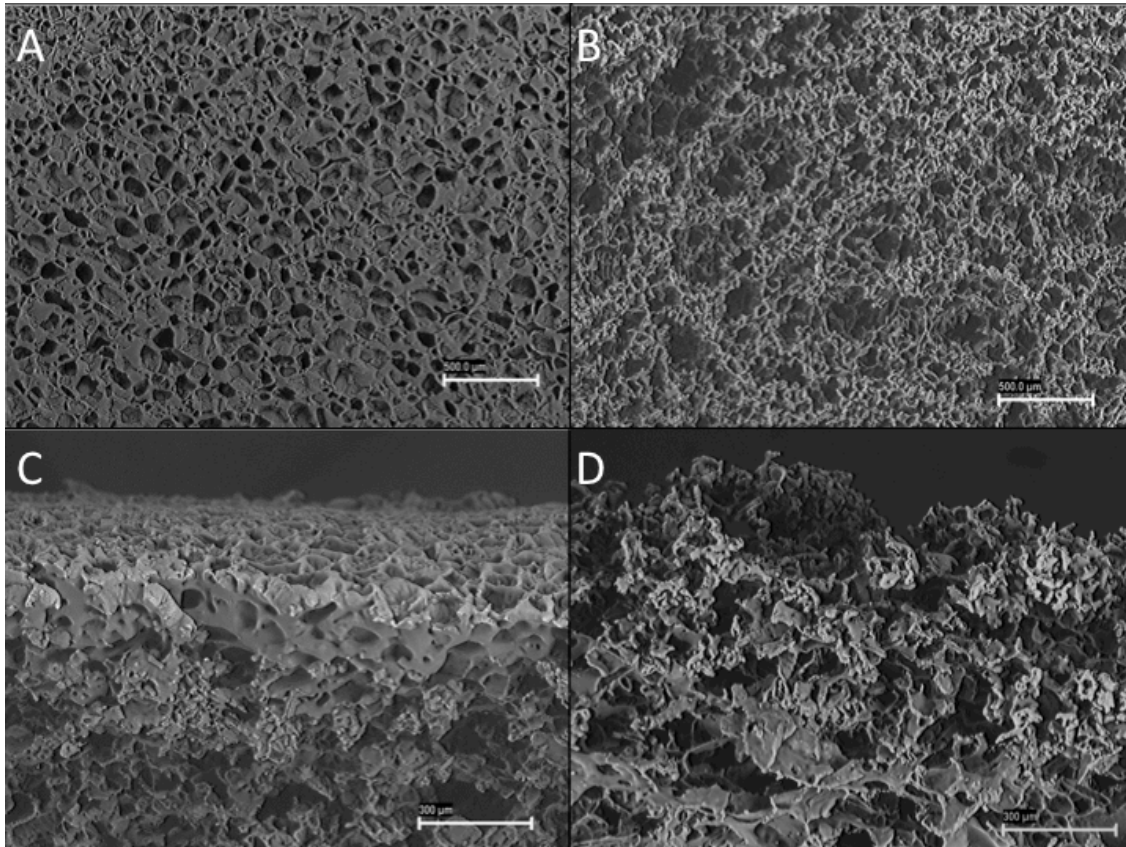


Figure 4: SEM images of surface (A and B) and vertical sections (C and D) of the scaffolds (PLGA, Tetronic 1107, heparin preparation). Scaffolds were prepared from 20% (w/v) solutions (A and C) or from 10% (w/v) solutions of PLGA. The images show a more porous structure in the scaffolds prepared from 10% (w/v) polymer solutions.

In a further step, we aimed to apply the selected composition for the development of scaffolds suitable for tissue culture in 3D and the co-delivery of BMP-7 and TGF- β 3. To this end, we adapted the nanocomplex encapsulation method to introduce the GFs in a

classical salt-leaching scaffolding technique [27]. Two percentages of PLGA (10 and 20% w/v) in the polymer casting solution were tested to optimize the scaffold morphology. The freeze-dried nanocomplex cake was easily resuspended in the polymer casting solutions, and the scaffolds were prepared in cylindrical molds. In this study, scaffolds with 9 mm diameter and 1–1.5 mm thickness were prepared. As expected, the scaffolds had a regular shape and a sponge-type porous complexion (**Figure S3**).

SEM images of the top view and inner sections of the scaffolds confirmed this porous structure (**Figure 4**). In all cases, there were macropores in the structure of 100–200 μm size, suitable for cell colonization and tissue formation. Together with these pores, the structure also showed other smaller pores. In both scaffold types, pore interconnectivity was clear. However, the porosity and the interconnectivity of the scaffold prepared with 10% w/v PLGA was higher than that prepared with 20% w/v polymer. A higher porosity could be beneficial for cell growth, but the scaffolds with 10% polymer were too fragile for continuous manipulation. Based on this consideration, we decided to test the 20% prototype in subsequent studies.

In a next step, the encapsulation efficiency of both GFs in the scaffold was determined after destruction of the PLGA structure with DMSO for direct quantification by ELISA. The study indicated that $79 \pm 7\%$ of TGF- β 3 and $50 \pm 13\%$ of BMP-7 were encapsulated. Because this method is based on direct GF determination requiring a previous extraction step, we believe that these figures are probably underestimates of the real GF payloads. However, some GFs might have been lost during scaffold preparation, for example due to premature release in the porogen elimination step.

Protein release kinetics from polymeric scaffolds are highly dependent on scaffold composition and structure. For example, IGF-1 and TGF- β 1 loaded into scaffolds made of oligo(poly(ethylene glycol fumarate)) present a parallel release profile, with a burst

within the first 3–4 days and a prolonged release for up to 28 days [4]. Similar behavior is shown by BMP-7 and TGF- β 3 loaded into chitosan nanoparticles embedded into alginate hydrogels. In this case, the burst release is not as pronounced, and 15–30% of the factors are released within the first three weeks [37]. Loo et al. achieved different release profiles for bFGF and BMP-2 loaded into oppositely charged gelatin nanospheres that form a gel *in situ* [38]. The maximum release of bFGF was achieved at approximately two weeks (around 20%); meanwhile, BMP-2 was continuously released and reached less than 20% of the payload at four weeks. Finally, Han et al. determined the release of TGF- β 1 and BMP-2 from scaffolds containing PLGA and a chitosan/gelatin hydrogel. In this case, the profile of the two factors differs during the six weeks of the experiment, the release of BMP-2 being slower at the beginning, and increasing after the second week [7].

Our *in vitro* release studies confirmed that BMP-7 and TGF- β 3 could be co-delivered from the scaffolds (**Figure 5**), and that the release kinetics observed showed several beneficial characteristics. First, the release of both GFs lacked any burst effect. In fact, we observed an almost two-week release lag-time for TGF- β 3. Secondly, if we neglect the lag time of TGF- β 3, both GFs are released with near zero-order kinetics, which is expected to be ideal for most tissue engineering applications. The linearity of BMP-7 release is particularly outstanding and could be related to the intricate morphology of the scaffold and the particular composition used in the nanocomplex encapsulation method, which has already resulted in sustained release kinetics of this GF from polymeric microspheres [23]. Finally, our scaffolds were able to provide at least 30 days of sustained release for both GFs. Because ELISA was used to quantify the GFs, we can confirm that the proteins are, at least released, in an antigenically active conformation.

At the end of the release experiments, the tested scaffolds were destroyed with DMSO for protein extraction and the remaining GFs quantified by ELISA. The remaining TGF- β 3 was 28.2% of the theoretical payload, while the remaining BMP-7 was 11.1%. These results imply a release of 51.9% of TGF- β 3 and 39.14% of BMP-7 during the 30 days of the experiment. Within some deviation, this is in accordance with the release data presented previously.

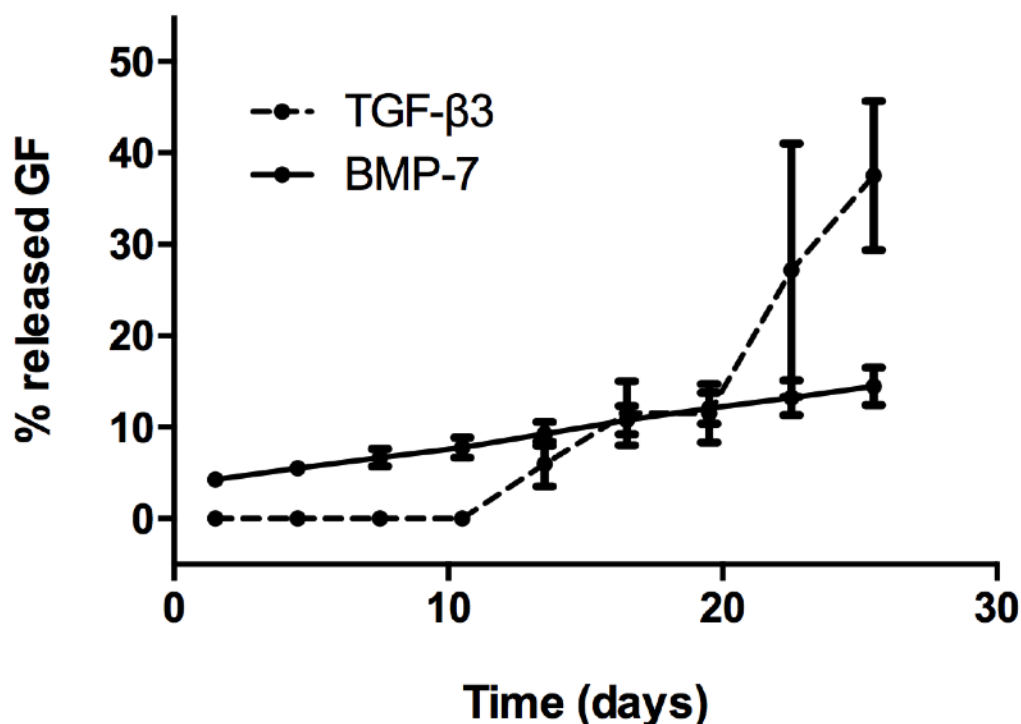


Figure 5: Release profile of BMP-7 and TGF-3 loaded as complexes with heparin and T1107 in PLGA scaffolds. In vitro release experiments were performed in PBS 1% BSA, 2% penicillin/streptomycin and 0.2% fungizone, at 37°C with horizontal shaking at 300 rpm. The experiment confirmed the sustained release of both growth factors (GFs). Release is represented as % of the theoretical payload, and data represent mean \pm SD, n=3.

A pending question is whether the GFs are released in a free form or as complexes with heparin (and T1107). If this would be the case, as suggested by the work of d'Angelo et al., it could result in enhanced stability of the released GFs complexes [39], and in another added advantage for this formulation. However, this remains a hypothesis requiring further verification.

3.4 Chondrogenic differentiation in scaffolds delivering TGF- β 3 and BMP-7

Considering the morphological and pharmaceutical properties of the scaffolds, we aimed to test whether the GFs delivered by the scaffolds would suffice to direct hMSCs towards the formation of cartilage. Therefore, hMSCs were cultured for 21 days on scaffolds, either blank or loaded with BMP-7/TGF- β 3, and in the absence of any external GF supplementation in the culture medium. Tissue deposition in the constructs over the 21 days in culture was evident, even at the macroscopic level (**Figure 6C**). Histological analysis of the tissues provided the first indications of efficacy for the BMP-7/TGF- β 3 constructs. Where the controls did not show red-stained proteoglycan rich areas (**Figure 6D1**), those were evident on the GF-loaded scaffolds (**Figure 6D2**). Analysis of the gene expression provided further evidence of the efficacy of the GF-loaded scaffolds. After 21 days of culture, the GF-loaded scaffolds showed a nine-fold upregulation of the chondrogenic master transcription factor SOX9 ($p < 0.05$, **Figure 6A**) and a 10-fold upregulation of the cartilage-specific proteoglycan Aggrecan ($p < 0.01$, **Figure 6B**). Upregulation of cartilage markers continued even after 28 days in culture (**Figure S4**). Therefore, we confirmed the delivery of biologically active GFs from our scaffolds, and that the released GFs are sufficient by themselves to drive hMSC cultures towards the formation of cartilage-type tissue.

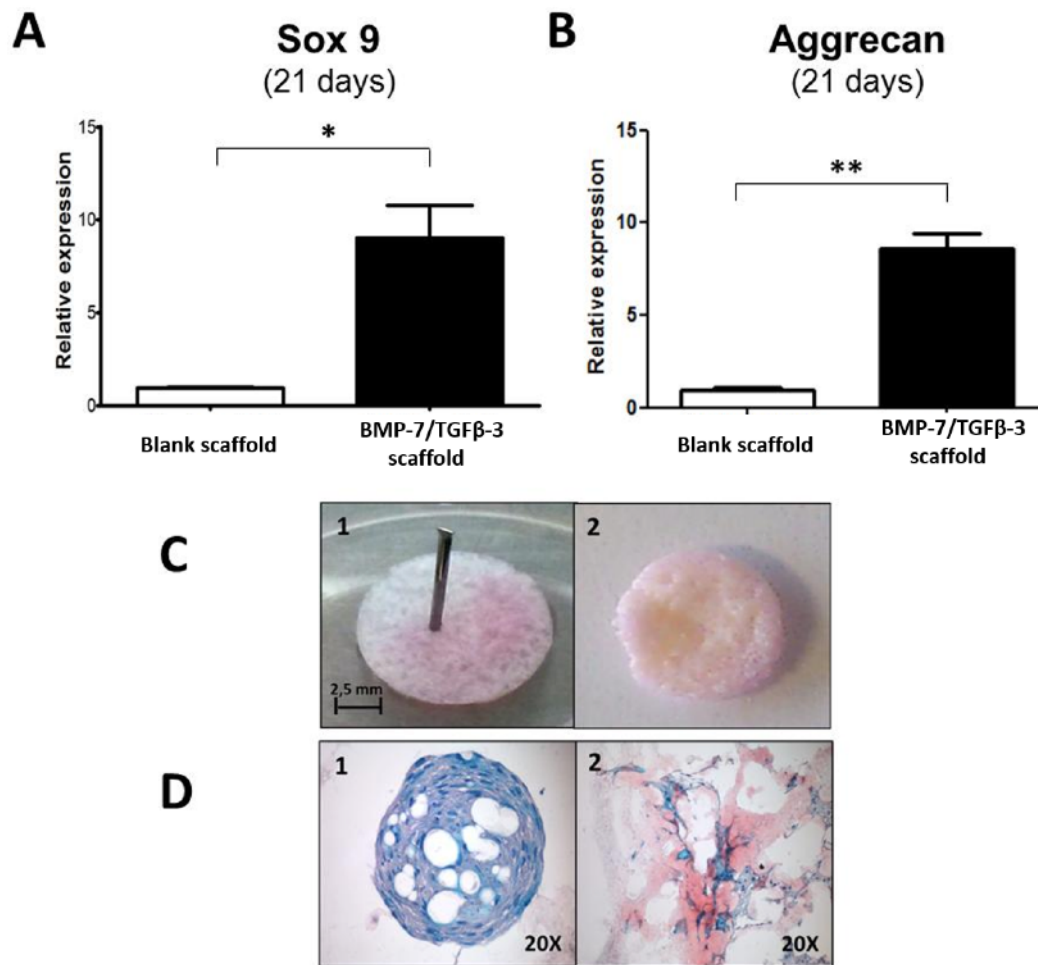


Figure 6: Relative expression of Sox9 (A) and Aggrecan (B) in hMSCs cultured on scaffolds loaded with growth factors (BMP-7/TGF-β3) compared to cells cultured on scaffolds without growth factors (control). Cells were cultured for 3 weeks for differentiation and gene expression was analyzed by qRT-PCR. Data represents mean ± SD, n=3; *p <0.05, **p < 0.01 (Mann Whitney test). Photographs showing the macroscopic appearance of scaffolds loaded with BMP-7/TGF-β3 at the beginning of the experiment (C1) and after 21 days in culture (C2). Histology images (Safranin-O staining) from representative sections of a control construct (D1) and a growth factor-loaded construct (D2) after 21 days in culture. Highly charged glycosaminoglycan areas typical of cartilage are stained in red.

While several works have shown that the combination of 3D cultures with controlled delivery of GFs is a potential strategy to induce cartilage regeneration [40], this study is one of the first testing cartilage formation driven only by the two GFs released from the scaffold. We believe that the sustained delivery of the GFs from the scaffold could result in a transport advantage over media supplementation already under *in vitro* culture conditions: once the extracellular matrix starts to form, it could prevent the transport of the GFs to the cells in the inner regions of the scaffold. In contrast, GFs are uniformly distributed in the scaffold, thus signaling that later stages should be more homogeneous. These might be partially responsible for the effective response observed in our *in vitro* tests; such beneficial effects should translate to the *in vivo* situation.

Overall, our results support the strategy of activating scaffolds for the co-delivery of rationally selected GFs, as observed elsewhere [4,5,7,41]. We have also made a strong case for combining TGF- β 3 with BMP-7 in a suitable delivery system for cartilage repair.

4. Conclusions

We have shown that controlled supplementation of BMP-7 can improve the chondrogenic effect of TGF- β 3, and that scaffolds loaded with this combination of growth factors can induce cartilage formation in hMSC cultures, even in the absence of other external differentiation factors. Scaffolds loaded with TGF- β 3/BMP-7 by the nanocomplex encapsulation method have a suitable morphology and beneficial controlled release properties for cartilage regeneration applications.

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Author Disclosure Statement

No competing financial interests exist.

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Supporting Information

Table S1: Reference of the Taqman Assay sequences used for qRT-PCR (ThermoFisher Scientific).

<u>Taqman Assay</u>	Reference
Sox9	Hs01001343_g1
<u>Aggrecan</u>	Hs00153936_m1
<u>Actin B</u>	Hs99999903_m1
GAPDH	Hs99999908_m1

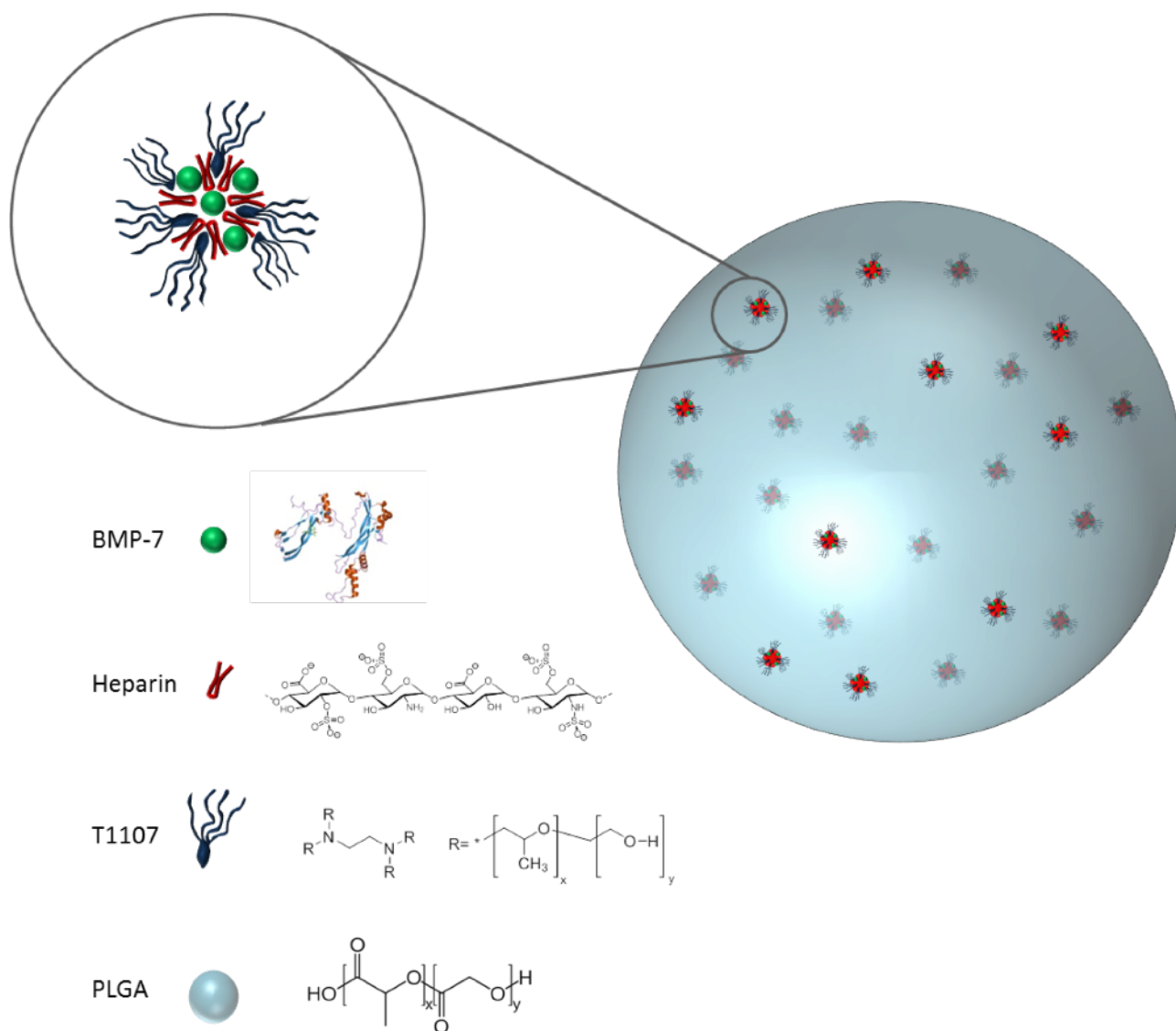
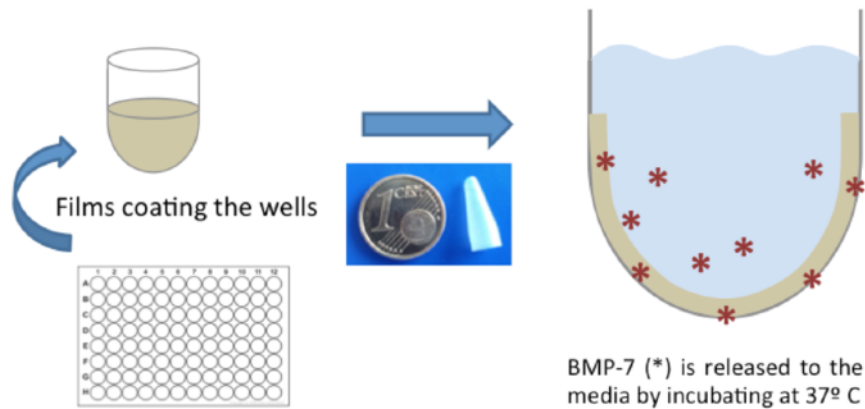


Figure S1: The nanocomplex encapsulation technique. In this stabilization and encapsulation strategy, the growth factor, here BMP-7, is complexed sequentially with heparin and Tetronic 1107. The resulting nanocomplexes can be freeze-dried and loaded in PLGA microparticles.

A



B

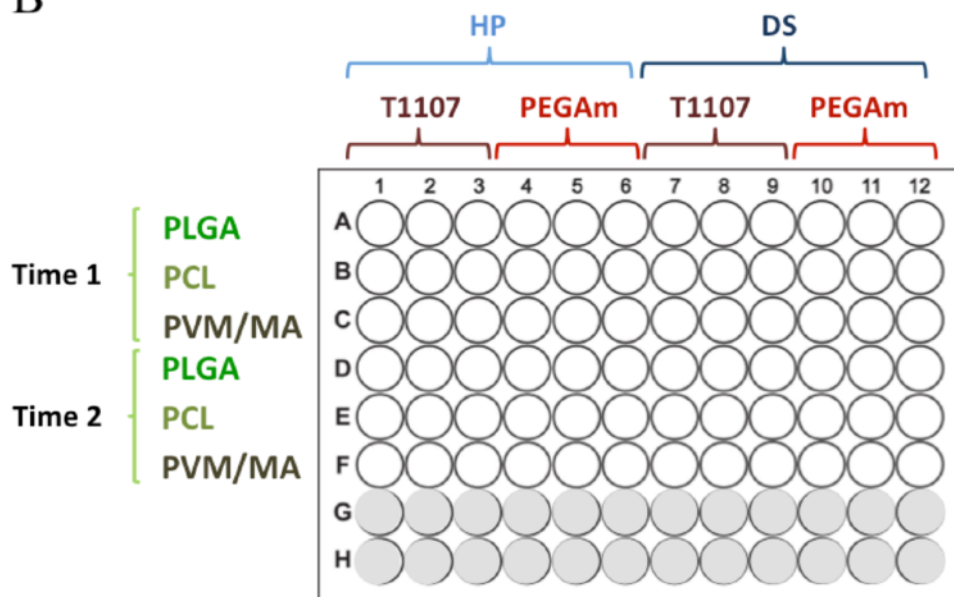


Figure S2: (A) The experimental strategy for high throughput screening of different BMP-7 controlled release compositions. Films based on different material combinations are prepared on a 96-well plate. Once dried, the films on the plate are used to perform an in vitro release test. (B) Experimental setup: films were prepared using different combinations of (i) biodegradable polymers, (ii) polysulfated polymers, and (iii) cationic PEG derivatives. The biodegradable polymers tested were polycaprolactone (PCL), poly(lactic-co-

glycolic acid) (PLGA) and poly(methyl vinyl ether-alt-maleic) anhydride (PVM/MA). The polysulfated polymers tested were dextran sulfate (DS) and heparin (HP). The PEG cationic derivatives tested were methoxypolyethylene glycol amine 5000 (PEGAm) and ethylenediamine tetrakis(propoxylate-block-ethoxylate) (T1107). By combining these variables up to 12 different conditions could be tested in triplicate and for two release timepoints per plate. Two rows were left on the plate for calibration curve and reference wells.

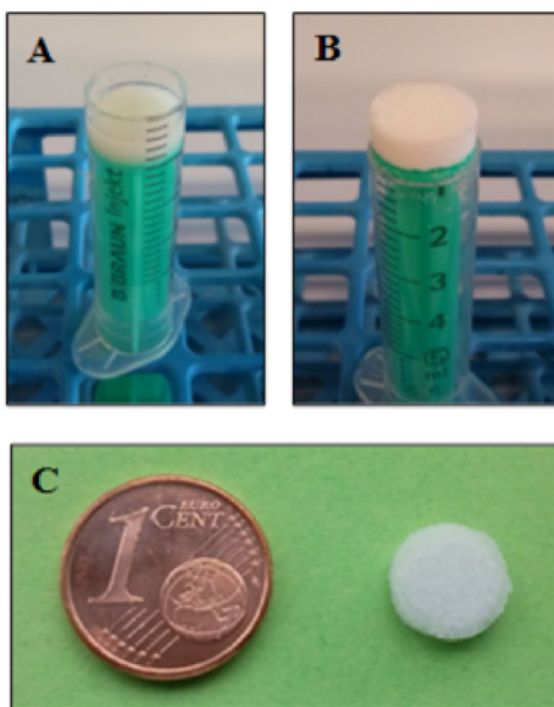


Figure S3: Preparation of scaffolds by the solvent casting/salt leaching method. The mixtures of the polymers dissolved in an organic solvent are added to a known amount of salt, homogenized and put into a mold (A). The elimination of the solvent by evaporation at room temperature leads to a polymeric matrix filling the salt mask (B). After that, the porogen mask is removed by immersing the scaffold into ultrapure water. Finally, the scaffolds are dried at room temperature. The prepared scaffolds were regular and homogenous, with 9 mm diameter (C).

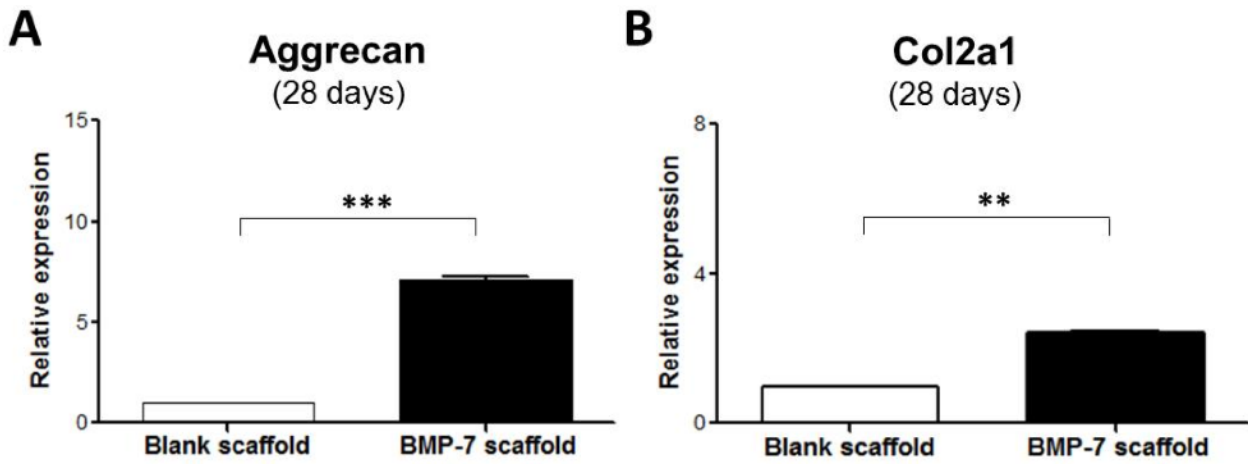


Figure S4: Relative expression of Aggrecan (A) and Col2a1 (B) genes in hMSC cultured on scaffolds loaded with growth factors (BMP-7/TGF- β 3 scaffold) compared to those grown on blank scaffolds (control) over 4 weeks of culture. Gene expression was determined by RT-PCR.