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Covalent Binding of Bone Morphogenetic Protein-2 and Transforming Growth Factor-β3 to 3D Plotted Scaffolds for Osteochondral Tissue Regeneration

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Engineering the osteochondral tissue presents some challenges mainly relying in its function of transition from the subchondral bone to articular cartilage and the gradual variation in several biological, mechanical, and structural features. A possible solution for osteochondral regeneration might be the design and fabrication of scaffolds presenting a gradient able to mimic this transition. Covalent binding of biological factors proved to enhance cell adhesion and differentiation in two-dimensional culture substrates. Here, we used polymer brushes as selective linkers of bone morphogenetic protein-2 (BMP-2) and transforming growth factor-β3 (TGF-β3) on the surface of 3D scaffolds fabricated via additive manufacturing (AM) and subsequent controlled radical polymerization. These growth factors (GFs) are known to stimulate the differentiation of human mesenchymal stromal cells (hMSCs) toward the osteogenic and chondrogenic lineages, respectively. BMP-2 and TGF-β3 were covalently bound both homogeneously within a poly(ethylene glycol) (PEG)-based brush-functionalized scaffolds, and following a gradient composition by varying their concentration along the axial section of the 3D constructs. Following an approach previously developed by our group and proved to be successful to generate fibronectin gradients, opposite brush-supported gradients of BMP-2 and TGF-β3 were finally generated and subsequently tested to differentiate cells in a gradient fashion. The brush-supported GFs significantly influenced hMSCs osteochondral differentiation when the scaffolds were homogenously modified, yet no effect was observed in the gradient scaffolds. Therefore, this technique seems promising to maintain the biological activity of growth factors covalently linked to 3D scaffolds, but needs to be further optimized in case biological gradients are desired.

1. Introduction

In the last decades regenerative medicine relied on the use of 3D structures, namely scaffolds, to support cell adhesion and tissue growth. Several studies provided a number of techniques to generate scaffolds, such as gas foaming,[1] salt leaching,[2] freeze drying,[3] or solvent casting.[4] All these techniques present well known limitations, such as the lack of pore interconnectivity, the impossibility to fine tune the pore size and the geometry of the constructs. In alternative to these well-established fabrications, additive manufacturing (AM) allows the generation of scaffolds with a fully interconnected pore size, a desired structural design, and enables fine tuning of scaffold properties during production.[5,6] Whereas it is possible to functionalize scaffolds with biological factors to improve cell attachment and differentiation, AM scaffolds offer the appealing advantage to create gradients thanks to the capillary forces originated in the pore network.[7] In our previous study, we exploited this advantage to let the scaffold's pore network be filled by a fibronectin solution. Poly(ε-caprolactone) (PCL) scaffolds were modified by surface-initiated atom transfer radical polymerization (SI-ATRP) of poly(oligo (ethylene glycol)

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methacrylate) (POEGMA).[7] The POEGMA nanobrushes covered the whole scaffold allowing further functionalization with fibronectin. When the scaffolds were placed on a protein solution reservoir, an axial gradient was generated via diffusion due to capillary forces.[7] Growth factors such as bone morphogenetic protein (BMP) and transforming growth factor β (TGFβ) are known to trigger the differentiation of human mesenchymal stromal cells (hMSCs) toward the osteogenic or chondrogenic lineage.[8–11] A number of studies applied scaffolds in combination with growth factors (GFs)[12–16] to improve hMSCs differentiation. These techniques are highly expensive, due to the great dosage of GFs used,[17] which can also lead to adverse effects.[18–19] On the other hand, linking GFs to the surface[20–22] ensures a continuous mono-dose treatment, which may also continuously trigger the underlying signaling pathways, instead of continued infusion of GFs during the culture time.

GFs in combination with hMSCs were applied in the attempt to regenerate the osteochondral tissue. The osteochondral interface can be seen as a gradient tissue allowing the transition from the mineralized bone to the soft and highly hydrated cartilage.[23] Within the osteochondral tissue, gradual variations of growth factors, cell number, collagen orientation, and mineralization are present.[24] Due to this complex variation in structure, mechanical properties, and biological components, the regeneration of this interface remains an open challenge in the field of tissue regeneration. The degeneration of osteochondral tissue progresses with age. Therefore, the need of scaffolds and techniques to support an effective regeneration will increase in the following years with the increase of life expectancy. In order to mimic the progressive variation of chemical characteristics of the osteochondral extra-cellular matrix (ECM), we propose here a scaffold fabrication based on AM of PCL, to create fully interconnected 3D supports, later on modified with POEGMA brushes applying SI-ATRP from initiator functions on the scaffold surface. The functionalizable POEGMA brushes on the scaffolds were subsequently functionalized with BMP-2 and TGF-β3. The constant presence of BMP-2 or TGF-β3 should determine a continuous stimulus for the hMSCs to differentiate, as we hypothesized that the underlying biological signaling pathways would be continuously activated. We also linked the BMP-2 and TGF-β3 in a double gradient fashion in order to stimulate the differentiation of hMSCs within the construct toward an osteochondral like tissue interface in vitro. The GF homogenously linked to the surface of the 3D scaffolds outperformed the simple addition of their soluble form to the cell culture media, determining the overexpression of osteogenic and chondrogenic markers in the shorter time span of 10 days, when normally the expression takes between 3 and 4 weeks with the soluble factors.

2. Experimental Section

2.1. Scaffold Preparation

Scaffolds made of poly(ε-caprolactone) (PCL) were produced by additive manufacturing (Bioscaffolder, SysENG, Germany). The polymer was loaded in a stainless steel syringe and processed at 100 °C. The molten polymer was extruded through a cartridge unit by applying a nitrogen flow (5 bar) and an extrusion screw rotating at 200 rpm. During plotting, fiber spacing, needle diameter, layer thickness, and translation speed of the syringe were maintained constant at 650, 200, 150, and 180 mm min^{-1}, respectively. Squared-shaped pores (0–90° fiber architecture) were fabricated as a model scaffold as a proof of concept that covalently binding of growth factors may be effective in the differentiation of hMSCs. The scaffolds were plotted in blocks of 20×20 mm and 4 mm in height. The tested samples were punched out from the blocks with cylindrical shape, having a size of 4×4 mm. Punching the scaffolds out from a bigger square shaped block was done in order to: a) simulate the actual punching of the surgeon when the damaged cartilage is removed; b) allow us to limit the scaffold-to-scaffold variation; and c) have less time consumption compared to the fabrication of custom shaped scaffolds, at the same time allowing the polymer to reside for less time in its molten state in the syringe, thus being less prone to thermal degradation during fabrication.

2.2. Atom Transfer Radical Polymerization of OEGMA

3D scaffolds were activated as explained elsewhere.[7] Purified OEGMA monomer (5 g, 9.5 mmol) and 2,2′bipyridine (81.7 mg, 0.52 mmol) were mixed with water (5 mL) and methanol (1,26 mL). The solution was flushed with argon for 30 min. CuCl (18.75 mg, 0.19 mmol) and CuBr 2 (2 mg, 0.009 mmol) were added into another reaction flask and purged with argon as well. Monomer, ligand, and catalyst were then pooled and stirred for another 30 min to enable the formation of the organometallic complex. This solution was then moved into flasks containing PCL-Br substrates. The flasks were sealed with rubber septa and kept at room temperature under nitrogen. Following 10 min of reaction time, the substrates were rescued from the polymerization solution, thoroughly rinsed with water to wash away any unreacted compound, and finally dried under a nitrogen stream. Brush thickness was measured by ellipsometry, as previously described.[7]

2.3. Functionalization of PCL-POEGMA Scaffolds

POEGMA brushes on PCL scaffolds were activated by placing them in a solution based on dry DMSO, 200 mM of N,N′-disuccinimidyl carbonate (DSC, Sigma–Aldrich, 98%) and 4-dimethylaminopyridine (DMAP, Sigma–Aldrich, ≥99%). Afterwards, the samples were incubated in a protein solution containing either 2.5 μg mL^{-1} BMP2 or TGF-β3 (R&D Systems, Abingdon, United Kingdom). Scaffolds homogeneously covered by proteins were manufactured by placing a scaffold in a 1 mL Eppendorf vial filled with 100 μL of either one of the protein solutions. The axial protein gradients along the z axis of the scaffolds were produced by placing the DSC-activated PCL-POEGMA scaffolds on top of a micro porous paper sheet, which was previously imbibed with 10 μL of either one of the protein solutions. A weight of 7.5 g was used to ensure the contact between the reservoir and the scaffolds during the gradient formation. Following 60 min of reaction time, the scaffolds were extensively washed with milli-Q water, and finally blow-dried with a nitrogen stream. For the double axial gradients, first a single BMP-2 gradient was produced according to the procedure

Table 1. Table showing the genes analyzed for osteogenic and chondrogenic differentiation, and the forward and reverse primers used for the PCR.

Gene	Forward primer	Reverse primer
β2 microglobulin	ACAAAGTCACATGGTTCACA	GACTTGTCTTTCAGCAAGGA
Aggrecan	AGGCAGCGTGATCCTTACC	GGCCTCTCCAGTCTCATTCTC
Sox9	TGGGCAAGCTCTGGAGACTTC	ATCCGGGTGGTCCTTCTTGTG
Collagen IIα	CGTCCAGATGACCTTCCTACG	TGAGCAGGGCCTTCTTGAG
Runt-related transcription factor 2	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
Alkaline phosphatase	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
Bone sialoprotein	CCCCACCTTTTGGGAAAACCA	TCCCCGTTCTCACTTTCATAGAT
Osteocalcin	TGAGAGCCCTCACACTCCTC	CGCCTGGGTCTCTTCACTAC/

previously mentioned. Subsequently, the scaffold was inverted and placed on top of another micro porous paper sheet previously soaked with a TGF-β3 solution.

2.4. ELISA Assay

The amount of BMP-2 and TGF-β3 attached on the scaffolds was quantified via BMP-2 and TGF-β3 ELISA kits (Sigma–Aldrich, Zwijndrecht, the Netherlands). Briefly, the supernatant of the functionalization solution was retrieved after the functionalization process. The remaining BMP-2 and TGF-β3 in solution was quantified following the supplier protocol. The measured amount was subtracted from the original concentration.

2.5. Cell Expansion and Seeding on Scaffolds

hMSCs (male, age 22) were retrieved from the Institute of Regenerative Medicine (Temple, Texas), after ethical approval from the local and national authorities and donor written consent. The procedure is briefly described in our previous study.[6] Cells were seeded on the scaffolds following the procedure previously published elsewhere.[25] All the scaffolds in this study were cultured for 10 days in basic medium, consisting of minimal essential medium (α-MEM, Life Technologies, Bleiswijk, the Netherlands) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U mL^{-1} penicillin (Life Technologies), 10 μg mL^{-1} streptomycin (Life Technologies), 2 mM L-glutamin

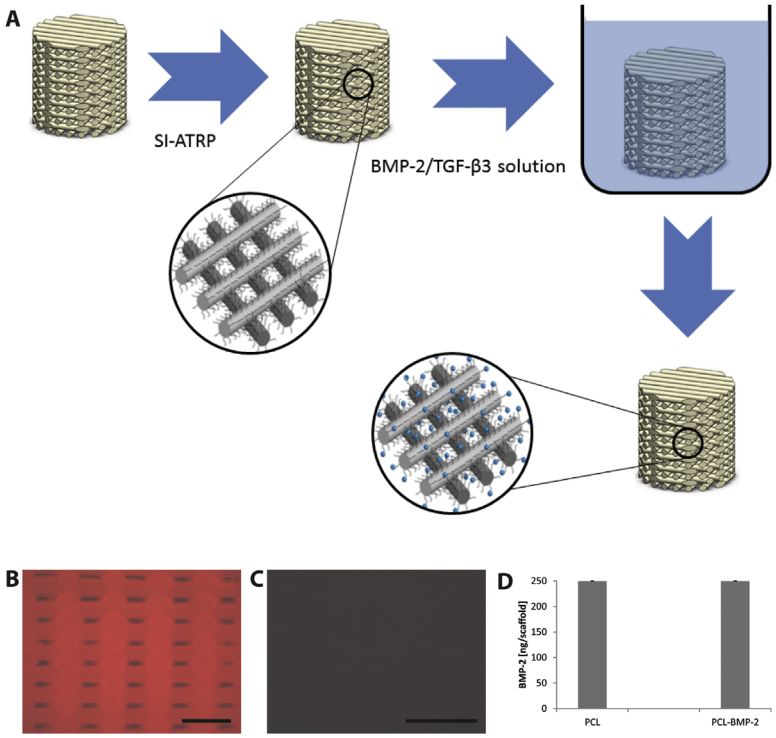

Figure 1. Homogeneous covalent binding of GFs on 3D scaffolds. Cartoon summarizing the functionalization process (A), fluorescent micrographs of BMP-2 fully functionalized (B), and bare (B) PCL scaffolds, and plot showing the BMP2 amount unspecifically adsorbed on PCL scaffolds and linked to the PCL-POEGMA brushes (PCL-BMP-2) before the washing steps after the functionalization (D). Scale bar 650 μm (B) and 1 mm (C).

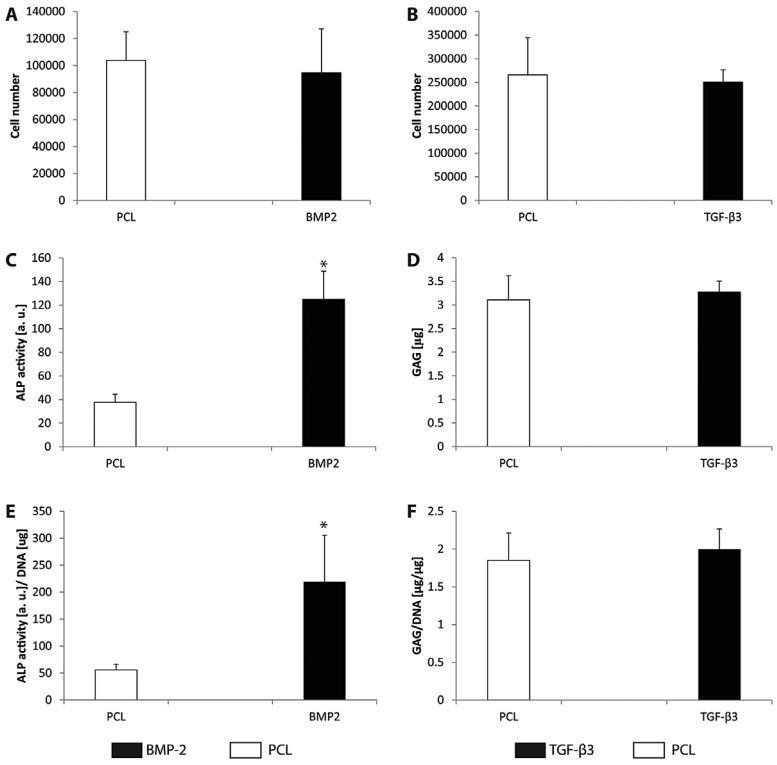

Figure 2. Biological characterization of hMSCs differentiation on homogenenously functionalized 3D scaffolds. Graphs displaying the cell number on PCL scaffolds and scaffolds functionalized with BMP2 and TGF-β3 (A and B). ALP activity and ALP activity normalized by DNA of BMP-2 functionalized scaffolds with respect to PCL scaffolds. (C and E) Glycosaminoglycans (GAGs) amount and GAG normalized by DNA of TGF-β3 functionalized scaffolds with respect to PCL scaffolds (D and F). *Statistical significance $p < 0.05$.

(Life Technologies), and 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma–Aldrich). Scaffolds cultured with the growth factors in soluble form were cultured in basic medium supplemented with 10 ng mL^{-1} BMP-2 or TGF-β3 (R&D System).

2.5.1. DNA Analysis

The cell number per scaffold was calculated using a Cyquant DNA assay kit (Life Technologies) following the protocol delivered by the supplier. Four samples per condition were tested.

2.5.2. ALP Activity and GAG Amount Quantification

To evaluate hMSCs differentiation toward the osteogenic lineage, alkaline phosphatase (ALP) content was detected using a CDP star kit (Roche, Woerden, the Netherlands). From the supernatant of the DNA assay, the ALP assay was performed following the supplier protocol. To evaluate the differentiation toward the chondrogenic lineage, the glycosaminoglycan (GAG) amount was quantified using 1,9-Dimethyl Methylene Blue (DMMB) assay from the DNA assay lysates, as previously published.[6] Four scaffolds per condition were tested.

2.6. Gene Expression Analysis

For gene expression analysis, a procedure previously published was followed.[25] Ct values were normalized by the β2 microglobulin (B2M) reference gene and ΔCt ((average of Ct control)-Ct value). Results were represented as fold induction in mRNA expression normalized to the gene expression of the PCL control scaffolds cultured in basic medium. Four scaffolds per condition were tested (**Table 1**).

2.7. Fluorescent Staining

After functionalization, the PCL scaffolds were cut in halves and incubated with PBS-BSA for 30 min. The same procedure was performed on non-functionalized PCL scaffolds. A goat polyclonal antibody against human BMP-2 was resuspended according to the supplier in a PBS–BSA solution up to a concentration of 200 μg mL^{-1}. The scaffolds were covered with a PBS-BSA solution containing the antibody with a dilution of 1:200 and incubated 1 h at room temperature. Scaffolds were incubated for another hour at room temperature in a PBS-BSA solution containing a polyclonal rabbit anti-goat coupled with the fluorophore Alexa 568, at a dilution of 1:1000. POEGMA-functionalized scaffolds and PCL scaffolds were incubated in a solution containing the growth factor. One scaffold per condition was stained and analyzed.

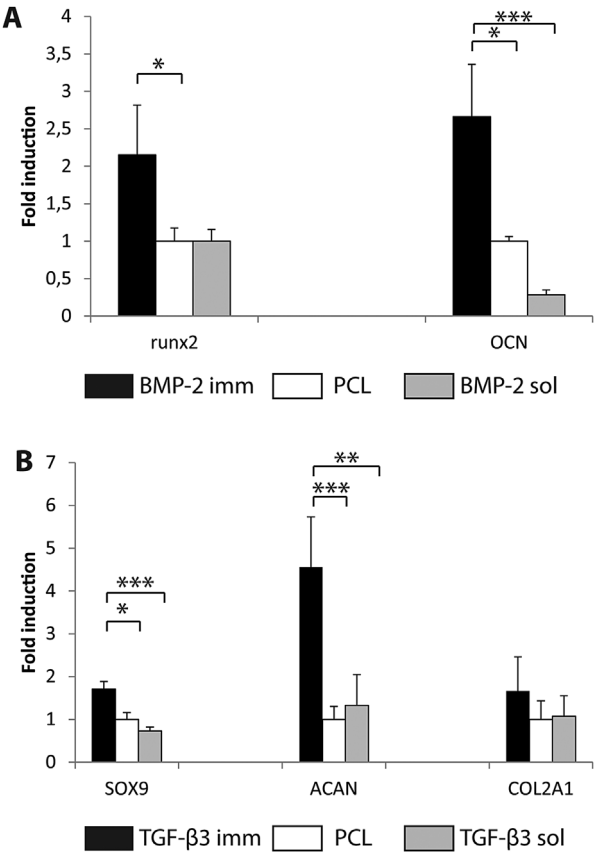

Figure 3. Gene analysis of hMSCs differentiation on homogenenously functionalized 3D scaffolds. Plots showing the fold induction of osteogenic (runx2, OCN) and chondrogenic (sox9, ACAN, coll2a1) genes in hMSCs cultured on scaffolds functionalized with BMP-2 and TGF-β3 with respect to hMSCs cultured on PCL scaffolds and PCL scaffolds with the cell culture media supplemented with the growth factor (A and B). *Statistical significance $p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

2.8. Statistical Analysis

All the quantitative data are expressed as mean ± standard deviation. Statistics were accomplished using IBM SPSS Statistics 20. A two-way ANOVA with Tukey as post hoc test were used. A statistical significance level of $p < 0.05$ was used to discriminate differences between experimental groups, indicated with (*).

3. Results and Discussion

3.1. Homogeneous Scaffold Functionalization

Figure 1 depicts the general strategy of the proposed fabrication. The scaffolds were plotted using an extrusion-based additive manufacturing system, following a computer aided design/computer aided manufacturing (CAD/CAM) program. After plotting, PCL scaffolds were fully functionalized with POEGMA chains.[7] Scaffolds were modified by SI-ATRP of OEGMA, generating POEGMA brushes presenting an average dry thickness of around 10 nm. POEGMA brushes uniformly covering the initiator-functionalized PCL 3D scaffolds were obtained by immersing the supports in the polymerization medium for 10 min. Following extensive rinsing, to remove unbound species and residual catalyst, the POEGMA brush-3D scaffolds were uniformly functionalized with BMP-2 and TGF-β3 (finally yielding BMP-2-POEGMA brush and TGF-β-POEGMA-brush 3D scaffolds) (Figure 1A). The choice of POEGMA as linker is related to its antifouling activity.[26–27] To confirm the presence of the GF on the surface, an antibody staining of BMP-2 (Figure 1B and C) was performed. The functionalized scaffolds showed a uniform presence of the GF, in contrast to the non-functionalized scaffolds (Figure 1D and Figure S1, Supporting Information). Whereas the adsorbed growth factors are washed away during sterilization and culture medium immersion prior to cell seeding, the growth factors linked to the nanobrushes, being covalently bound, remain attached to the scaffolds. This is one of the advantages of using covalent binding of GFs to a 3D scaffold. The GFs are available at the interface with the adhering cells. In addition, they are robustly linked to the support, in contrast to physisorbed proteins on similar, yet bare, polyester constructs. The amount of GFs attached on the PCL scaffolds and POEGMA brush-PCL scaffolds was quantified via ELISA on the supernatant solution used for the functionalization reaction. Being part of the same superfamily, BMP-2 (Figure 1D) and TGF-β3 (Figure S1, Supporting Information) displayed similar attachment, with a $99.94 \pm 0.02\%$ and a $99.67 \pm 0.04\%$ coupling efficiency for BMP-2 and TGF-β3 respectively, on the brush-PCL scaffolds. Despite a similar attachment could be measured on bare PCL scaffolds as well as on POEGMA brush-PCL scaffolds, it is to be noted that, after washing the scaffolds, in the first case GFs just adsorbed and desorbed on the scaffolds, whereas in the second the GFs were covalent bound to the brushes. Hence, for GFs covalently bound to the scaffolds, no release is expected.

To further confirm the activity of the growth factors bound to the surface, an ALP and GAG analysis was conducted on PCL scaffolds seeded with hMSCs and cultured for 10 days in basic medium (**Figure 2**). We chose specifically 10 days of culture to assess the effect of GF covalent binding on early cellular differentiation. hMSCs typically need a 7-day period of culture to adhere and form their own extracellular matrix on 3D plotted scaffolds.[28] As a negative control, POEGMA brush-PCL scaffolds did not show cells attached, due to their antifouling properties. Therefore, no ALP activity, cell number or GAG amount could be measured (data not shown).

As expected, scaffolds presenting POEGMA brush layers decorated with either BMP-2 or TGF-β3 stimulated the adhesion of hMSCs compared to unfunctionalized POEGMA brush-PCL scaffolds, confirming the already reported activity of these GFs as not only differentiation but also cell adhesive proteins.[29,30] The ALP activity was significantly higher both on a structural (per scaffold, Figure 2C) and on a cellular (per DNA, Figure 2E) level on functionalized PCL scaffolds with respect to bare PCL scaffolds. On the other hand, hMSCs cultured on TGF-β3 functionalized scaffolds did not show any difference in GAG synthesis, both at a structural and at a cellular level with respect to hMSCs cultured on PCL scaffolds (Figure 2D and F). Since the

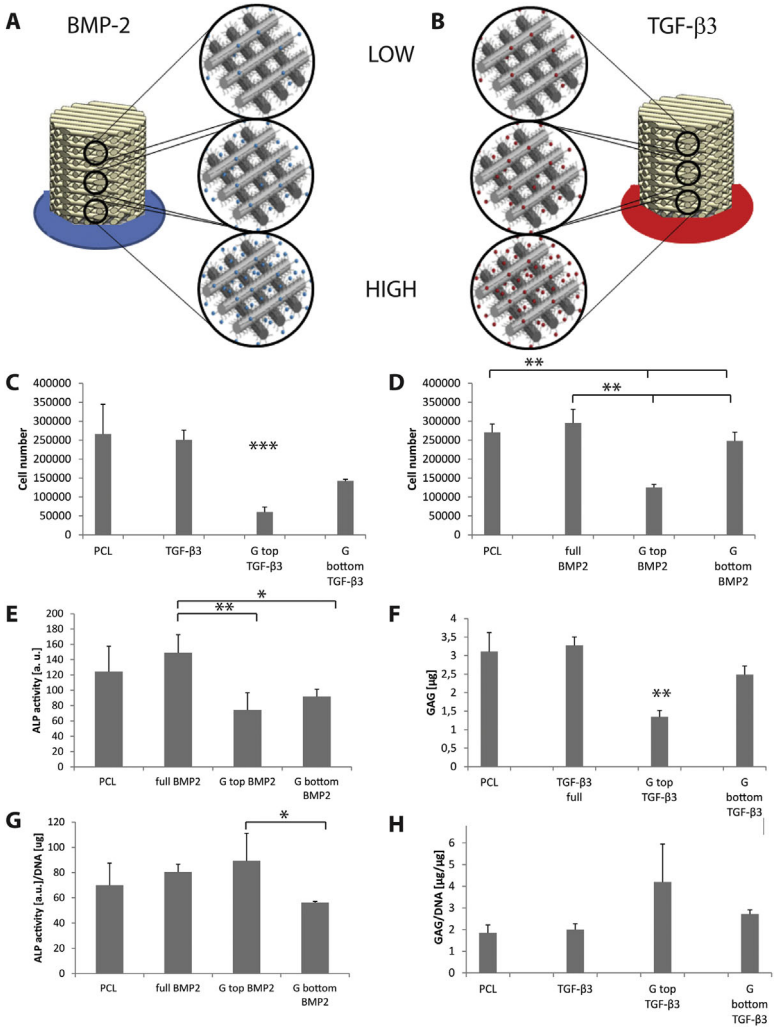

Figure 4. Gradient covalent binding of GFs on 3D scaffolds and biological characterization of hMSCs differentiation. Cartoon displaying the gradient functionalization with BMP-2 (A) and TGF-β3 (B). Plots displaying cell attachment in fully functionalized scaffolds, PCL scaffolds and bottom and top part of the gradient (C and D). G top BMP2 showed a significantly lower cell number with respect to all the other conditions (C). ALP expression (E) and ALP activity normalized by DNA amount (G) of hMSCs cultured on BMP-2 fully functionalized scaffolds, gradient scaffolds and PCL scaffolds. Plots showing GAG per scaffold (F) and normalized by DNA (H) of hMSCs cultured on PCL scaffolds, fully TGF-β3 functionalized scaffolds and top and bottom part of the gradient. *Statistical significance $p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

functionalized and non-functionalized scaffolds presented the same cell number, the normalization by DNA amount did not determined any significant variation.

To further test the activity of the GF-POEGMA brush-PCL scaffold, the expression of selected osteogenic and chondrogenic markers was assessed on the functionalized scaffolds, comparing bare PCL scaffolds and PCL scaffolds cultured in basic medium supplemented with the same GFs dispersed in the medium (not bound to the scaffold's surface) (**Figure 3**). Both GFs linked to the scaffolds surface were not only active, but outperformed the addition of their soluble form to the cell culture media. Runt-related transcription factor (Runx2) and osteocalcin (OCN) were significantly upregulated in scaffolds presenting BMP-2 on the surface compared to both the PCL scaffolds cultured in basic medium and basic medium supplemented with soluble BMP-2. Culturing hMSCs on PCL scaffolds in basic medium supplemented with soluble BMP-2 did not increase the expression of the early osteogenic marker runx2 and caused the downregulation of the late osteogenic marker OCN (Figure 3A). In literature, the effect of BMP-2 addition to cell culture media is controversially reported. Some studies indicated that continuous stimulation with BMP2 (0.1–800 ng mL^{-1}) affects the differentiation, but not the proliferation of hMSCs,[31,32] whereas other studies did not demonstrate an osteogenic effect of 100 ng mL^{-1} BMP2.[33,34] In particular Runx2 is an essential regulator of hMSCs differentiation into the osteogenic lineage.[35] In vitro studies indicated that Runx2 triggers the expression of major bone protein genes like collagen type I, osteopontin, bone sialoprotein, and osteocalcin during the early stage of osteoblast differentiation.[36] As expected, since runx2 was not upregulated, also OCN did not show any upregulation when BMP-2 was supplemented in

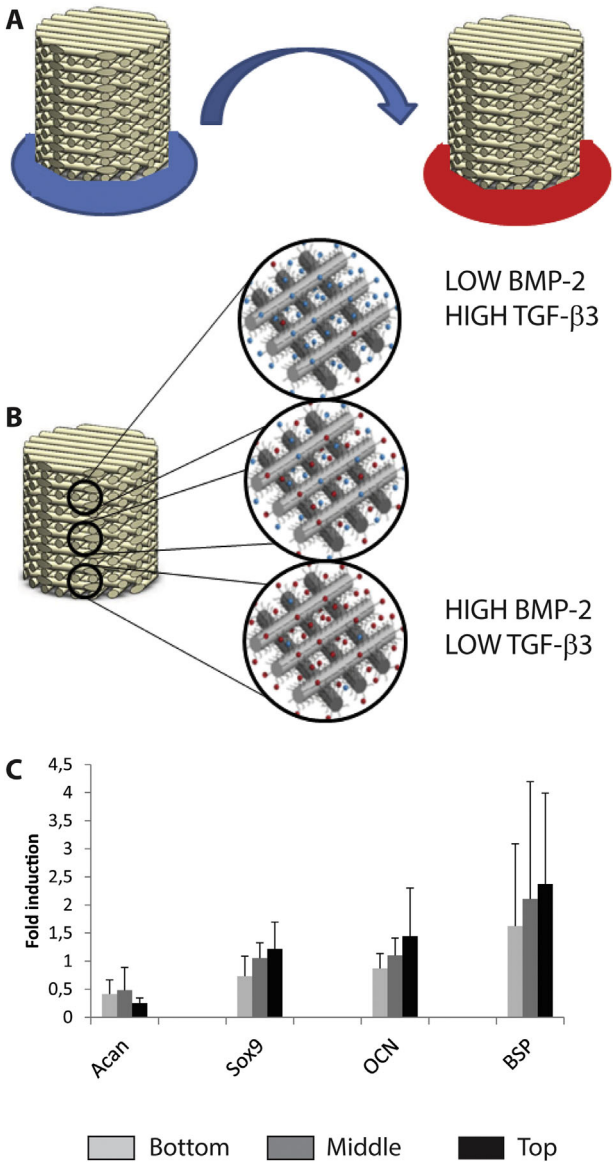

Figure 5. Double gradient covalent binding of GFs on 3D scaffolds and gene analysis of hMSCs differentiation. Cartoon displaying the double functionalization in a gradient fashion with BMP-2 and TGF-β3 (A) and the resulting disposition of the growth factors within the scaffold (B). Fold induction of chondrogenic markers (aggrecan, sox9) and osteogenic markers (osteocalcin, bone sialoprotein) in the different portion of the scaffolds.

culture medium. Yet, the use of immobilized BMP-2 resulted in early osteogenic differentiation, thus possibly indicating a more powerful route toward designing bioactive scaffolds to guide stem cell activity. Sox-9 and aggrecan levels were significantly higher on TGF-β3-POEGMA brush-PCL scaffolds compared to the PCL scaffolds cultured in basic medium alone or supplemented with soluble TGF-β3. The late marker collagen type 2 was not changed by the functionalization with TGF-β3 nor by the culture in medium supplemented with the growth factor (Figure 3B).

Cells in direct contact with the functionalized surface presenting the GFs were constantly stimulated to differentiate. Soluble factors dispersed in solution were not as effective as the linked proteins. This might have created a higher concentration of the linked GFs in the area where the cells were attached compared to soluble GFs used during the culture time. Conversely, the biological molecules linked to the surface determine a shorter differentiation time of 10 days due to the constant stimulus, whereas the differentiation of hMSCs treated with soluble factors occurs after 3 to 4 weeks at least.[37] Linking growth factors to the surface reduces the costs with a mono-dose treatment which prevents the continued infusion of expensive GFs during the culture time. hMSCs differentiation using GFs such as BMP-2 and TGF-β3 is widely described in 2D in vitro. The main limitation of 2D supplementation is the constant infusion of the GFs at concentration of 5–20 ng mL^{-1} in the culture media.[34,38,39] The use of GF supplemented cell culture media must be ensured for the whole culture time in order to prevent de-differentiation of hMSCs.[40,41] A few studies described the use of growth factors linked to materials' surface. The major disadvantage of these studies, however, is the use of highly concentrated growth factor solutions. For techniques such as inkjet printing, the preparation of the ink was based on 10 μg mL^{-1} GF-solution, as described by Miller et al.,[42] whereas Cabanas-Danés et al. used a BMP-6 solution of 20 μg mL^{-1},[43] which is 4 and 8 times higher, respectively, than the one described in this study.

3.2. Gradient Scaffold Functionalization

To further exploit the potential of covalently bound GFs, brush gradients were built in the 3D scaffolds as previously shown.[7] Briefly, GF gradients along the z axis (**Figure 4**A and B), were obtained by applying a porous paper sheet as a solution reservoir and allowing the protein medium to diffuse by capillary forces into the functionalizable POEGMA brush-PCL scaffolds. The lower fibers (G bottom), being in contact for longer time with the GF, presented a higher amount of the linked BMP-2 and TGF-β3, which decreased along the z axis. Additionally, scaffolds presenting a double gradient were produced (**Figure 5**A). The same strategy used to produce a single gradient was followed; as additional step, scaffolds were placed up-side down in contact with the porous paper sheet soaked with the second GF solution.

The effect of the BMP-2 and TGF-β3 gradient on the hMSCs residing in the different area of the scaffolds was determined again after 10 days in basic medium. Since the POEGMA brushes are anti-fouling, POEGMA brush-PCL scaffold displayed no cell attachment. PCL scaffolds as well as GF-POEGMA brush-PCL scaffold displayed the same cell number due to the activity of BMP-2 and TGF-β3 as adhesion proteins. Scaffolds presenting gradients of BMP-2 and TGF-β3 showed a significantly higher number of cells in the portion of the scaffold with the higher concentration of GF (bottom) with respect to the other half (top). This confirmed the presence of a gradient along the z axis. In the bottom part of the scaffolds, the concentration of GFs was higher, determining a greater number of anchoring point for the hMSCs. On the top half, the major part of the surface was covered predominantly by the antifouling

brushes and cells had less adhesion sites available (Figure 4A–D). Although full scaffolds displayed a significantly higher ALP activity compared to the bottom and top part of the gradient scaffolds, no major differences could be seen between the bottom and top part of the gradient scaffolds (Figure 4E). At a cellular level, when the ALP was normalized by DNA no differences could be seen among the PCL scaffolds, the BMP-2-POEGMA brush-PCL scaffolds, and the top part of the gradient scaffolds (Figure 4G). However, a significantly higher ALP activity was measured on the top part with respect to the bottom part. In order to further evaluate hMSCs osteogenic differentiation, G scaffolds were cut into three parts (bottom, mid, and top) and osteogenic markers were analyzed via PCR (Figure S2, Supporting Information). No major differences were seen between the different areas.

The same analysis was performed on TGF-β3 functionalized scaffolds. To assess chondrogenic differentiation, GAG analysis was performed. At a cellular level, the trend was similar to the one displayed by ALP activity: no statistically significant differences were displayed between functionalized and non-functionalized scaffolds and within the functionalized scaffolds between the top and the bottom part of the scaffolds (Figure 4H). As previously explained, this might be due to the low cell number present in the top part of the gradient and to the presence of anti-fouling brushes. When considering the total GAG per scaffold, a statistically significant difference in GAG production was measured in the lower and top part of the scaffolds (Figure 4G). Yet, no differences were observed between the bare PCL and fully functionalized scaffolds, indicating that further studies should aim at longer culturing time. The lack of chondrogenic differentiation might also be due to the culture for 10 days in basic medium. Bilgen et al. demonstrated that there is an interaction between the FBS, contained in the basic medium, and the TGF-β3 which can interfere with its activity.[44]

To limit the differences in cell number and to determine if the combined effect of the 2 GFs could have triggered the expression of osteogenic and chondrogenic genetic markers, a double gradient was generated (Figure 5A and B). After 10 days in basic medium, scaffolds were divided into three parts: bottom, mid, and top, corresponding to high, mid, and low BMP-2 concentration, respectively, and to low, mid, high TGF-β3 concentration, respectively (Figure 5C). Neither the chondrogenic markers nor the osteogenic ones were upregulated in any of the scaffolds' portion.

In this study, we presented a platform based on a continuous stimulation of cells through covalent binding of growth factors on the surface of 3D plotted scaffolds. Cell differentiation was enhanced on the fully functionalized scaffolds compared to conventional supplementation of soluble growth factors in the culture medium. This was probably due to a sufficient presence and concentration of growth factors throughout the whole surface. hMSCs adhering on the scaffolds were immediately stimulated in the BMP-2 driven osteogenic differentiation and TGF-β3 driven chondrogenic differentiation. We might speculate that cells in direct contact with the scaffold backbone, while differentiating, released bioactive molecules that re-generated within the pore a differentiating microenvironment, which stimulated cells not directly adhering to the scaffolds to start the osteogenic or chondrogenic differentiation process.

Despite we have shown that covalently bound GFs could successfully trigger hMSCs differentiation when homogeneously decorating 3D plotted scaffolds, the gradient constructs did not show any sign of cell differentiation. In the gradient structure the overall concentration of growth factors was significantly lower and probably not sufficient to trigger a cell–cell propagation of the differentiation process. Possible variables that should be taken into account in future studies comprise the growth factor concentration (here we used 10 times lower concentration in the gradient scaffolds compared to the homogenously treated scaffolds), different time points at which the culture is carried on, and a more gradual analysis of cellular differentiation by, for example, immunofluorescent histology. Several studies in literature presented scaffolds functionalized with growth factors as release platforms.[14,45–48] The concept of growth factor delivery presents several disadvantages, such as the need of scaffolds with high loading efficiency and a fine controlled release kinetic in order to reach the working concentration at the target site. Additionally, once released the growth factor has a short half-life in vivo,[49] thus needing a carrier or protector. Nanobrushes alongside being the linker chain between the surface of the scaffold and the growth factor provides protection and ensure its bio-activity. As shown in Figure 2, the BMP-2 and TGF-β3 functionalized scaffolds determined the upregulation of osteogenic and chondrogenic markers respectively, whereas the soluble factors brought to a downregulation of the same genes. Although release platforms can be designed to deliver a signal in a time specific and sequential manner,[47,50] they lack the ability to fine tune the presentation of the growth factors in a specific area of the scaffold. In osteochondral regeneration the position of BMP-2 and TGF-β3 within the scaffold is fundamental for providing the osteogenic stimulus in the part of the scaffold in contact with the subchondral bone, decreasing toward the joint surface, while the chondrogenic signal increasing in the same direction. The method presented in this work showed to be more effective and cheaper than the addition of soluble growth factors in solution for homogenously functionalized scaffolds. Further studies are needed to understand if such promising strategy is also beneficial in single and double gradient scaffolds.

Supporting Information

Supporting information is available from the Wiley Online Library or from the author.

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Conflict of Interest

Authors declare no commercial or financial conflicts of interest.

Keywords

additive manufacturing, growth factors, osteochondral, polymer brushes, scaffolds

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