3D printed step-gradient composite hydrogels for directed migration and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells

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Abstract
With the aid of a 3D printing technique, we create a 3D printed step-gradient (nano)composite scaffold (GradS). GradS is generated by spatially connecting different (nano)composite hydrogel precursors varying in their concentrations of GelMA, alginate, and functional nanomaterials (DexPMO-PDL = dexamethasone (Dex)- and poly-d-lysine (PDL)-functionalized periodic mesoporous organosilicas (PMO)]. GradS not only allows for improving the viability of human bone marrow-derived mesenchymal stem cells (hBMMSC), but GradS is also applied to direct the migration of hBM MSC toward the higher concentration of GelMA and nanomaterials (NMs) within the 3D gradient porous hydrogel network. Furthermore, we demonstrate that the osteogenic differentiation capacity of hBM MSC is promoted by an increase in the content of GelMA and DexPMO-PDL; increasing concentrations of GelMA and DexPMO-PDL within the GradS regulates the migration and subsequent osteogenic differentiation of hBM MSC within the 3D gradient hydrogel network.

KEYWORDS
3D printing, directed cell migration, local biomolecule delivery, osteogenic stem cell differentiation, step-gradient composite hydrogel

1 | INTRODUCTION

Bone is a complex and hierarchical tissue in which the extracellular matrix (ECM) is mineralized and primarily contains nano-hydroxyapatite and collagen. In bone tissue engineering (BTE), several attempts have been made to generate artificial bone constructs to replace the autograft and allograft treatment and to enhance bone repair and regeneration. Composite biomaterials, such as polymers, hydrogels, ceramics and metals, are suitable bone substitutes and can mimic the natural functions of bone due to their biocompatible and physical characteristics.

However, engineering bone tissue presents some challenges, mainly based on bone tissue’s gradual variation of several biological, mechanical, and structural features. Another challenge is to control the migration of stem cells, which have an inherent ability to migrate. Uncontrolled migration of stem cells can cause to pathological situations such as cancer and inflammatory diseases. Therefore, besides promoting stem cell differentiation, it is crucial to regulate stem cell migration in order to achieve...
their maximum capacity for differentiation and regeneration. Another remaining challenge in BTE is designing 3D structures, namely scaffolds, to support cell adhesion, growth, and differentiation and to mimic 3D porous architecture and intrinsic properties of natural bone tissues or organs.[10–13]

Therefore, BTE has focused on the use additive manufacturing (AM) or 3D printing techniques to generate 3D gradient scaffolds with fully interconnected pores and tunable pore sizes.[14–18] 3D printing also allows for good control on the spatial arrangement of components with different biophysicalchemical features in the 3D scaffold network. By controlling the arrangement of the different components, one can generate mechanical and chemical gradients within the 3D scaffold network, which is crucial for promoting cell adhesion, migration and differentiation during tissue regeneration. For example, Byambaa et al. described 3D printed GelMA-based hydrogels with a VEGF gradient to support gradient vasculogenesis and osteogenesis.[17] Di Luca et al. reported 3D scaffolds made with poly(ε-caprolactone) (PCL) functionalized with gradients of bone morphogenetic protein-2 and transforming growth factor-β3 for osteochondral tissue regeneration.[16] Di Luca et al. also described 3D printed scaffolds with porosity gradients for osteochondral tissue regeneration.[15]

Nowadays, nanomaterial-incorporated composite hydrogels (nanocomposite hydrogels) have gained attention as promising bioinks for 3D printing applications, in particular for bone tissue engineering due to their mechanically strong nature and their ability to mimic ECM nano/micro topography, and because they can gradually deliver desired bioactive agents that promote tissue regeneration and regulate cell migration.[18–25] For example, recently, hydroxyapatite- and whitlockite nanoparticle-loaded 3D scaffolds were reported for osteogenic differentiation of mesenchymal stem cells. In another study, Thakur et al. described NC hydrogels composed of nanohydroxyapatite and gelatin methacryloyl, which supported the osteogenic differentiation of seeded pre-osteoblast cells.[24] Gelatin methacryloyl- and Laponite-based nanocomposite hydrogels have been described by Paul et al. for the differentiation of hBM MSC into the osteogenic lineage in the absence of osteoinductive factors.[21]

Nanocomposite hydrogels are generated by incorporating different silica-, carbon-, or metal-based nanomaterials into the hydrogel's matrix.[26,27] Nanomaterials not only improve the mechanical, chemical and biological properties of hydrogels, but, depending on the type of nanomaterial, they can add new properties to the final composite system, such as electrical conductivity, pH, optical and magnetic responsiveness, bioactive molecule delivery, and so on.[26,27] It is of particular interest in bone tissue regeneration to use silica and derivatives, such as bioactive glass and mesoporous silica, for nanocomposite hydrogel preparation due to the mechanical and biocompatible properties of silica and its nanometer-scale preparation, which presents osteoconductive and osteoinductive properties and thus, can mimic bone tissue. In addition, the surface of silica-based nanomaterials can be easily functionalized covalently or non-covalently with bioactive molecules. This is beneficial for biochemically induced cell behavior, such as differentiation, adhesion, proliferation, and migration. Silica-based nanomaterials have recently been utilized for the differentiation of bone-forming osteoblasts.[28–31]

Naturally derived polymers such as alginate and gelatin-based polymers are widely used and versatile biomaterials for (nano)composite hydrogel preparation, and they are gaining importance in bone tissue engineering applications due to their biocompatibility as well as their non-toxic, non-immunogenic, biodegradable, and gel-forming properties.[4] While alginate forms a hydrogel by ionic crosslinking via calcium ions; thus, through non-covalent bonding, gelatin-based polymers such as gelatin methacryloyl (GelMA)[32–34] form hydrogels via photo-crosslinking through covalent bonding. GelMA hydrogels show good cell adhesiveness due to Arg-Gly-Asp (RGD) sequences of the gelatin,[35] while alginate hydrogels have weak binding properties to cells.[36] Furthermore, both show poor biodegradability and poor mechanical strength and toughness, but these can be improved by incorporating nanomaterials into the polymeric network of their hydrogel compositions. Various studied have examined alginate and GelMA-based biomaterials or composite biomaterials, such as silicate nanoplatelet-loaded GelMA or bioglass or biosilica-encapsulated alginate hydrogels, for bone tissue regeneration.[16,36–40]

Despite recent advances in the field of BTE, current research has still not combined many ECM properties (such as mechanical, chemical, porous and topographical gradients, 3D network, porosity, biocompatibility and biodegradability) into one biomaterial at the nanoscale to synergistically enhance osteogenic differentiation of human bone marrow derived mesenchymal stem cells (hBM MSC). In addition, to the best of our knowledge, no studies have linked stem cell migration with osteogenic differentiation, as regulated by 3D printed step-gradient composite hydrogels that possess mechanical, chemical, and topographical gradients. As such, no study has yet combined silica-based porous bifunctional nanomaterials, alginate, and GelMA into one gradient biomaterial and investigated their synergistic and/or additive effects on migration and osteoblast differentiation of hBM MSC in a 3D network.
In this contribution, we describe mechanically, biochemically, and topographically graded biocompatible and biodegradable 3D (nano)composite scaffolds generated by a 3D printing technique to mimic the gradient and nanoscale topographical structure of bone tissue and to regulate hBM MSC migration and to promote osteogenic differentiation of hBM MSC. Furthermore, we studied the behavior of hBM MSC within the different compartments of the gradient scaffolds to investigate the synergetic impact of different gradients on osteogenic differentiation of hBM MSC and on ECM mineralization.

We prepared our (nano)composite hydrogels by mixing GelMA, alginate, and functional periodic mesoporous organosilica (DexPMO-PDL) in different ratios. The prepared (nano)composite hydrogels were used for fabricating a new 3D step-gradient (nano)composite scaffold made by 3D bioprinting. Notably, we previously reported the preparation of similar 3D printed step-gradient (nano)composite hydrogels and scaffolds made of GelMA, alginate, and (DexPMO-PDL). In the previous study, the step-gradient systems were used to enhance the viability and migration of fibroblast cells and, at the same time, to reduce the cancer cells’ (Colo 818) viability and migration.[41] In the current study, the new 3D step-gradient (nano)composite scaffold was applied to improve the viability and the osteogenic differentiation of hBM MSC. Furthermore, this system allowed us to control the migration of hBM MSC within the 3D gradient porous hydrogel network.

2 | RESULTS AND DISCUSSION

2.1 | Preparation and 3D printing of GA5, GA10, and GA10-P

The external and internal surfaces of periodic mesoporous organosilicas (PMO) were functionalized with poly-d-lysine (PDL) and dexamethasone (Dex), respectively, as we have previously described and characterized,[42] to obtain bifunctional PMO (DexPMO-PDL) (Figure 1A). PDL and Dex were used in this study due to their beneficial effects on cells. PDL, a biodegradable antibacterial polymer,[43] can enhance cell adhesion and proliferation and inhibit biofilm formation. Dex is a bone-growth steroid...
used as an anti-inflammatory drug and can also regulate cellular proliferation.\textsuperscript{[42,44]} Further, Dex shows a dose-dependent beneficial impact on the differentiation of MSC into osteogenic lineages.\textsuperscript{[45]} Therefore, we expected that the biodegradable PDL would enhance cell adhesion and, at the same time, control the release kinetics of Dex from the PMO. Thus, we created $^{\text{Dex}}$PMO-PDL with the goal of achieving controlled and prolonged administration of Dex within the hydrogel scaffold’s 3D network, and we also aimed to achieve enhanced hBMMSC differentiation into osteoblasts.

Subsequently, we prepared three different GelMA/Alg-based composite hydrogels varying in GelMA, Alg, and $^{\text{Dex}}$PMO-PDL concentration (GA5, GA10, and GA10-P). GA5 was prepared by mixing GelMA (5\% w/v) and Alg (7\% w/v), while GA10 was composed of GelMA (10\% w/v) and Alg (7\% w/v). In order to prepare GA10-P, $^{\text{Dex}}$PMO-PDL (0.2\% w/v) was mixed with GA10.

The prepared GA5, GA10, and GA10-P were printed into hexagon structures with different infill density using a 3D printer. 3D printed hydrogel structures were crosslinked by visible light for covalent photo-crosslinking of GelMA and by using a calcium solution for ionic crosslinking of Alg (Figure 1B-J). The decreased pore size can be attributed to the collapse of underlying hydrogel strand (Figure 1E-G). The 3D printed GA5, GA10, and GA10-P scaffolds were obtained after freeze-drying the respective hydrogels. The prepared $^{\text{Dex}}$PMO-PDL, GA5, GA10, and GA10-P hydrogels/scaffolds were analyzed as we have described previously (see the supporting information).\textsuperscript{[42]} SEM images showed that the 3D printed GA5, GA10, and GA10-P scaffolds had similar interconnected porous networks with irregular pore sizes (Figure 1K-M). The GA10-P scaffolds showed a higher porosity (\%), a higher swelling capacity (Table S2), less degradation (Table S3), and a higher viscosity, compressive, storage ($G'$) and loss ($G''$) modulus and higher resistance to stretching (Figure 2) than the GA5 and GA10 scaffolds due to the enforcement of the GelMA/Alg network by hydrophilic $^{\text{Dex}}$PMO-PDL. The mechanism behind this reinforcement can be attributed to the positively charged PDL on the surface of PMO, which can interact electrostatically and/or form hydrogel bonds with the carboxyl-groups of GelMA and Alg. The slow degradation of GA10-P in the presence of cells further confirmed that the incorporation of $^{\text{Dex}}$PMO-PDL reinforced the GelMA/Alg hydrogel network and delayed the enzymatic degradation process.

2.2 The release kinetics of Dex from GA10-P and $^{\text{Dex}}$PMO-PDL

We examined the release kinetics of Dex from GA10-P and $^{\text{Dex}}$PMO-PDL at pH 7.4 and 6.0 (where this lower pH value reflects the pH in a tumor or an inflammatory tissue environment)\textsuperscript{[39–42]} at different time periods (e.g., from 3 min to 7 days) (Figure 3). Our results showed that the Dex release from the GA10-P scaffold at pH 7.4 and 6.0 was prolonged and pH dependent. After 7 days of incubation, the amount of Dex released from GA10-P scaffold at pH 6.0 was 2 times higher than at pH 7.4, demonstrating GA10-P’s ability to perform pH-responsive drug delivery.
This result likely occurred because the negatively charged PMO surface was coated with the positively charged PDL, making this interaction pH dependent. In addition, GA10-P showed time-dependent degradation. When comparing the release kinetics of Dex from GA10-P with Dex release from DexPMO-PDL in PBS, we found that the incorporation of DexPMO-PDL into GA10 slowed the release of Dex at pH 7.4 and 6.0.

2.3 | The impact of 3D printed single GA5, GA10, and GA10-P scaffolds on the viability of hBM MSC

We first performed cell experiments on 3D printed non-connected (single) GA5, GA10, and GA10-P scaffolds. The 3D printed non-connected (single) GA5, GA10, and GA10-P scaffolds were separately interacted with hBM MSC for different incubation periods (1 hour, 1 day, 4 days and 7 days) to determine the hBM MSC viability in the respective scaffolds (Figure 4A, Table S4A). Our results showed an increase in hBM MSC viability in each scaffold from 1 hour to 7 days of incubation time. This increase was greater in the GA10-P scaffolds than the GA10 and GA5 scaffolds. For example, we found ~32% and 18% more viable hBM MSC in GA10-P than GA5 and GA10, respectively, indicating that increasing the GelMA and DexPMO-PDL concentration in the composite hydrogel composition enhanced hBM MSC viability. The reason might be because of the cell-adhesive RGD and PDL units in GelMA and on PMO, respectively.

The higher GelMA and DexPMO-PDL concentrations in GA10 and GA10-P than GA5 and GA10, respectively, resulted in more cell-adhesive scaffolds. In addition, the increase in the GelMA concentration and the incorporation of DexPMO-PDL into the hydrogel network produced mechanically stronger scaffolds (GA10-P and GA10). Therefore, we considered that the chemical and mechanical enhancement of the hydrogel network generated by using different concentrations of GelMA and DexPMO-PDL influenced the hBM MSC viability.

The nuclei of cells in the single GA5, GA10, and GA10-P scaffolds after 7 days of incubation were stained with DAPI (4′,6-diamidino-2-phenylindole) to show the difference between the scaffolds with respect to the adhered number of cells. Fluorescence microscopy images (Figure 5) showed that the number of adhered cells in the GA10-P scaffold was higher than in the GA10 and GA5 scaffolds, and the number of adhered cells was higher in GA10 than GA5. These results indicate that increasing the cell-adhesive RGD and PDL units in GelMA and on PMO, respectively, increased the number of adhered cells within the scaffolds.

2.4 | Cell experiments in the 3D printed gradient scaffolds

The prepared GA5, GA10, and GA10-P hydrogels were subsequently printed side-by-side in a horizontal (XY) orientation and then connected, forming one hydrogel, namely the gradient hydrogel (Figure 1N). The 3D printed gradient hydrogel structure was then crosslinked by visible light and by a calcium solution and freeze-dried, yielding the gradient scaffold (GradS). As such, GradS was composed of three sections: GA5, GA10, and GA10-P. Subsequently, cell experiments were performed on GradS, where identical amounts of hBM MSC were added into each section (GA5, GA10, and GA10-P sections) of the GradS. After different incubation times (1 hour, 1 day, 4 days, and 7 days), the different GradS sections (GA5, GA10, and GA10-P parts) were separated, and we determined hBM MSC viability in each section (Figure 4B, Table S4B). We observed a small difference between the results of previous non-connected (single) hydrogels (GA5, GA10, and GA10-P) and those for the GradS sections (GA5, GA10, and GA10-P parts). We found ~43% and 22% more hBM MSC in GA10-P than in GA5 and GA10, respectively (at 7 days). Additionally, the number of hBM MSC in the GA5 part of GradS slightly decreased over time, while the number of cells in GradS’s GA10-P part increased over time. This difference may indicate hBM MSC migration from the GA5 part to the GA10-P part of the GradS.

Therefore, in the following experiment we only seeded hBM MSC into the GA5 section of the GradS. We called this the “cell migration experiment” (Figure 4C, Table S4C). After 1 hour incubation time, 100% of the hBM MSC were found in the GA5 part of GradS. However, after longer incubation periods, we also observed hBM MSC in the GA10 and GA10-P parts of the GradS. After 7 days of incubation, 68%, 15%, and 17% of the total hBM MSC in the GradS were found in the GA5, GA10, and GA10-P parts,
F I G U R E  4  A, Stem cells were separately interacted with GA5, GA10, or GA10-P scaffolds. B, Stem cells were added into all parts of the GradS scaffold. C, Stem cells were only added into the GA5 part of GradS scaffold. D, Stem cells were only added into the GA10-P part of GradS scaffold. Cell experiments were done at different incubation times; values determined by the WST-assay. Number of repeated experiments (N) = 3, data show significant differences; ANOVA: \( P < 0.01 (**), P < 0.001 (***) \); a = significant difference between two different groups (between GA5 and GA10 or GA10 and GA10-P); b = significant difference between three different groups (between GA5, GA10, and GA10-P).

respectively, indicating that hBM MSC migrated from the GA5 part toward GA10-P part of the GradS. Since GA10 and GA10-P have higher GelMa and \(^{\text{Dex}}\)PMO-PDL concentrations than GA5, this migration depended on an increase in the GelMA concentration and on the incorporation of \(^{\text{Dex}}\)PMO-PDL into the hydrogel network.

To gain clearer results and to demonstrate that cells did not randomly migrate to the empty sections of the GradS, we performed a “reverse cell migration experiment” (Figure 4D, Table S4D). Here, hBM MSC were only seeded into the GA10-P section of the GradS. Similar to the “cell migration experiment”, we did not observe any cells in the empty parts of the GradS after 1 hour of incubation. However, after 7 days of incubation, 7%, 13%, and 80% of the total hBM MSC in the GradS were found in the GA5, GA10, and GA10-P parts, respectively. These results were significantly different from the cell migration experiments, and they show that the direction of hBM MSC migration was not random. Indeed, the hBM MSC migration was influenced by the concentration of GelMA and \(^{\text{Dex}}\)PMO-PDL within

F I G U R E  5  Fluorescence microscopy images (20x magnification) of stem cells in single scaffolds (A) GA5, (B) GA10, and (C) GA10-P after 7 days (the red line shows the scale bar of ~20 µm) (Blue: DAPI stained cell nuclei)
FIGURE 6  Fluorescence microscopy images (20x magnification) of stem cells in the different sections of the GradS after different incubation periods. A, In this experiment, cells were added only into the GA5 section of the GradS scaffolds. Therefore, cells were only found in GA5 after 1 hour incubation. However, migrated cells were found in the GA10 and GA10-P sections of the GradS scaffold after different incubation periods (1 day, 4 days, and 7 days). B, In this experiment, cells were added only into the GA10-P section of GradS scaffolds. Therefore, cells were only found in the GA10-P part after 1 hour incubation. However, migrated cells were found in the GA10 and GA5 sections of the GradS scaffold after different incubation periods (1 day, 4 days, and 7 days). (red line shows the scale bar of ∼40 µm) (blue: DAPI stained cell nuclei)

the 3D GradS. Since GA5 has less GelMA and DexPMO-PDL than both GA10 and GA10-P, very few cells migrated from GA10-P toward GA5. As such, fewer migrated cells were found in GA5 (7%) in the “reverse cell migration experiment” (Figure 4D, Table S4D) than were found in GA10-P (17%) in the “cell migration experiment” (Figure 4C, Table S4C). The small number of cells that were found in GA5 likely arrived there due to cellular diffusion within the GradS scaffold’s 3D network.

The hBM MSC migration was also monitored by fluorescence microscopy (Figure 6). The fluorescence microscopy images confirm the GelMA and DexPMO-PDL concentration-dependent hBM MSC migration within the 3D network of the GradS.

Important parameters that influence the adhesion, viability, and the migration rate of cells within a 3D scaffold network are the type and density of adhesive ligands on a scaffold as well as the surface roughness, porosity, and mechanical properties of the scaffold. To promote cell adhesion or migration on scaffolds, many studies have used integrin-binding ligands, such as the RGD peptide sequence or polylysine, a cell-adhesive polymer. Besides using cell-adhesive ligands, the surface roughness and/or mechanical stiffness of a biomaterial can be altered by using nanoparticles, which can also enhance cell adhesion, spreading and migration due to an increase in the substrate’s surface area of the substrate surface. Furthermore, scaffolds with pore sizes in the range of 150-400 µm have been shown to be optimal for efficient cell growth and migration.

Our GradS 3D network offers chemical (RGD, PDL), physical (pores, surface roughness), and mechanical gradients. As such, the observed cell viability and migration we found on the GradS likely occurred via the synergetic contribution of the chemical, mechanical and physical gradients generated by GradS’s different concentrations of GelMA and DexPMO-PDL.

2.5 The hBM MSC differentiation toward an osteogenic lineage in the 3D printed gradient scaffolds

We also investigated the impact of GA5, GA10, GA10-P, and GradS on hBM MSC differentiation toward an osteogenic lineage. The hBM MSC were added into GA5,
The osteogenic differentiation potential of hBM MSC inside GA5, GA10, GA10-P, and GradS were analyzed by incubating the cell-containing scaffolds in osteogenic differentiation medium for 7 and 14 days. Osteogenic differentiation capacity was determined by alizarin red and BCIP/NBT colorimetric staining (Figure 7). The quantity of Ca\(^{2+}\) deposition was obtained using a Quantichrom Calcium Assay Kit (Figure 8).

Alizarin red staining allowed for qualitative comparison of bone-like calcium deposition in the GA5, GA10, GA10-P, and GradS scaffolds, as the alizarin red dye forms a reddish chelation complex with calcium, indicating the presence of osteogenic mineralization. BCIP/NBT allowed for the colorimetric detection of alkaline phosphatase activity. This system is based on the hydrolysis of BCIP by alkaline phosphatase and the reduction of NBT, producing a deep purple reaction product. We observed a proportional increase in the alizarin red- and BCIP/NBT-stained areas with an increase in the GelMA and DexPMO-PDL concentration in the GA5, GA10, GA10-P, and GradS. This indicates that the osteogenic differentiation capacity of hBM MSC was promoted by an increase in the content of RGD and DexPMO-PDL within the hydrogel network.

This result was further quantitatively supported by determining the calcium content in the GA5, GA10, GA10-P, and GradS. The amount of calcium in GA10-P was 25%
and 12% more than that in the GA5 and GA10, respectively, after 7 days of incubation (Figure 8B). The calcium content in the GA5, GA10, GA10-P, and GradS increased with time, indicating an increase in the number of differentiated hBM MSC in the osteogenic lineage.

In addition, we also determined the osteogenic differentiation of hBM MSC in correlation with the hBM MSC migration in GradS. When we seeded cells into the GA5 part of the GradS, we observed an increase in \( \text{Ca}^{2+} \) deposition in the GA5 and GA10-P parts of the GradS due to the migration of hBM MSC toward GA10-P (Figure 8C). We found \( \sim 38% \), \( 30% \), and \( 32% \) of the total calcium content in the GA5, GA10, and GA10-P parts of the GradS, respectively (after 14 days). For the experiment in the other direction, when we seeded the cells into the GA10-P part of the GradS, we found \( 20% \), \( 31% \), and \( 48% \) of the total calcium content in the GA5, GA10, and GA10-P parts of the GradS, respectively, after 14 days of incubation (Figure 8D). These results show that the migration and subsequent osteogenic differentiation of hBM MSC in our 3D printed gradient scaffold depend on the concentration of GelMA and \( \text{DeP} \text{MO-PDL} \).

In the next step, we used normal growth medium, not supplemented with the osteoinductive agent dexamethasone, as this media should not trigger the osteogenic differentiation of hBM MSC. This experiment was done to investigate the effect of Dex release from the DexPMO-PDL of GA10-P on the osteogenic differentiation of hBM MSC. The hBM MSC were added into GA5, GA10, GA10-P, and in either the GA5 or the GA10-P part of the GradS. For analysis, we used the alizarin red staining and a QuantiChrom Calcium Assay Kit (Figure 9). Greater calcium deposition and calcium content were achieved in GA10-P than in the GA5 and GA10 scaffolds. The production of mineralized matrix and the calcium content increased with time. These results show that Dex release from GA10-P scaffolds can trigger and prolong the osteogenic differentiation of hBM MSC in GradS, as the growth medium was not supplemented with Dex. (Figure 9). The hBM MSC migration in scaffolds and without Dex-supplemented growth medium was also monitored by fluorescence microscopy (Figure S2). The fluorescence microscopy images confirm the hBM MSC migration within the GradS’s 3D network.
3 | CONCLUSION

In this study, we prepared and tested a 3D printed step-gradient (nano)composite scaffold (GradS). GradS, composed of different concentrations of GelMA, Alg, and Dex-PMO-PDL, was used to influence the migration and osteogenic differentiation of hBM MSC. The cell experiments demonstrated that the viable number of hBM MSC in the GradS increased as the content of GelMA and Dex-PMO-PDL increased. This effect was used to control the migration of hBM MSC toward the GradS section possessing a higher concentration of GelMA and Dex-PMO-PDL. We also determined how the different GradS sections affected hBM MSC differentiation toward an osteogenic lineage. The osteogenic differentiation potential of hBM MSC within the GradS was analyzed by alizarin red and BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) colorimetric staining. We also quantified the amount of Ca$^{2+}$ deposition in the GradS using a QuantiChrom Calcium Assay Kit. Our results showed that the alizarin red- and BCIP/NBT-stained areas and the Ca$^{2+}$ content increased as the concentration of GelMA and Dex-PMO-PDL increased in the GradS. Finally, cell experiments were done using normal growth medium, not supplemented with osteoinductive agent dexamethasone, to show the effect of Dex release from the Dex-PMO-PDL on the osteogenic differentiation of hBM MSC. The results showed that Dex release from GA10-P scaffold can trigger and prolong the osteogenic differentiation of hBM MSC in GradS when Dex is absent from the differentiation growth medium. In the long term, we propose that such gradient biomaterials generated using different functional nanomaterials may find particular applications in the field of interface tissue engineering.

4 | MATERIALS AND METHODS

4.1 | Materials

Poly-D-lysine (MW:30,000-70,000), hexadecyltrimethylammonium bromide (CTAB, 98%), 1,2-bis(trimethoxysilyl)-ethane (BTME, 96%), alginic acid
sodium salt powder, dexamethasone (≥98% HPLC), gelatin (from porcine skin), trypsin, methacrylic anhydride (MA), N-vinylcaprolactam (VC), eosin Y, triethanolamine (TEA), paraformaldehyde (PFA), fibroblast growth factor 2 (FGF-2), ascorbic acid, β-glycerophosphate, and cell proliferation reagent WST-1 solution were purchased from Sigma-Aldrich. Ethanol (absolute), ammonia solution (32%, pure) and hydrochloric acid (HCl) (32%, for analysis), were purchased from Merck. Hoechst 33342 (Hst) was obtained from Invitrogen, and Phalloidine Alexa Fluor 488 was also purchased from Invitrogen. Dulbecco’s phosphate buffered saline (DPBS) and cell medium (DMEM) supplemented with 1% (v/v) penicillin/streptomycin, and 10% (v/v) fetal bovine serum (FBS) were obtained from Biochrom, Germany. Transforming growth factor-β1 human (TGF-β1) was purchased from Thermo Fisher Scientific, Germany. Bone Marrow-Derived Mesenchymal Stem Cells (hBM MSC) were obtained from ATCC.

4.2 Preparation of GelMA

Photo-crosslinkable GelMA was synthesized as described previously. Gelatin (10 g) was dissolved in DPBS (10% w/v) at 60°C. Then, methacrylic anhydride (8 mL) was added to the mixture and kept on a stirrer for 2 hours at 50°C in order to modify the lysine group on the gelatin chains. The solution was transferred into dialysis membrane tubes (with 12-14 kDa) and kept in distilled water for 7 days at 50°C to remove unreacted methacrylic anhydride. After 7 days, the solution was filtered with a vacuum filter (110 mm pore size) and then lyophilized to achieve a dried GelMA foam. The methacrylation of gelatin was determined using H-NMR spectroscopy. H-NMR of gelatin and GelMA was obtained in deuterium oxide (Figure 1). For the quantification of the degree of methacrylation (DM) by 1H-NMR, the spectra were normalized to the phenylalanine signal (7.2-7.4 ppm), which shows the concentration of gelatin. Then, the lysine methylene signals (2.9-3.0 ppm) of gelatin and GelMA spectra were integrated to obtain the areas (A). The DM of the GelMA was calculated using the following equation.

\[
DM(\%) = \left(1 - \frac{A_{\text{lysinemethylene of GelMA}}}{A_{\text{lysinemethylene of gelatin}}} \right) \times 100
\]

\[
DM(\%) = 97\%
\]

4.3 Preparation of GA5, GA10, GA10-P hydrogels

A GelMA stock solution was first prepared with GelMA (50 mg mL⁻¹) in phosphate-buffered saline (PBS) (10 mL) mixed with eosin Y in PBS (0.1 mM) as a photoinitiator, TEA as a co-initiator (1.5 w/v%), and VC as a co-monomer (1.0 w/v%) and kept at 80°C for 10 min in order to dissolve GelMA. To achieve GA5, the above solution was mixed with alginate (70 mg mL⁻¹), which was dissolved in the solution homogeneously. Another GelMA stock solution was prepared with GelMA (100 mg mL⁻¹) in PBS (10 mL) mixed well with eosin Y (0.1 mM), TEA (1.33 w/v%) and VC (1.0 w/v%) and kept at 80°C for 10 min to dissolve GelMA. Then, to achieve GA10, this GelMA stock solution was mixed with alginate (70 mg mL⁻¹), which was dissolved in the solution homogeneously. To prepare GA10-P hydrogels, 2.0 mg mL⁻¹ DexPMO-PDL particles were mixed with GA10 and, after 20 min of sonication, alginate (70 mg mL⁻¹) was homogeneously mixed into this suspension.

4.4 3D printing of GA5, GA10, GA10-P scaffolds

GA5, GA10, GA10-P hydrogels (100 μL each) were printed (using Cellink HeartWare version 2.4.1) in a hexagon structure (4 mm on each side and 2 mm in height), and crosslinked with visible light (450-550 nm) for 120 seconds using FocalSeal (Genzyme Biosurgical, Cambridge, MA), for covalent photo-crosslinking of GelMA and then with a 22.5 M CaCl₂ solution for ionic crosslinking of Alg. The syringe temperature and printing plate temperature were set at 37°C. A needle with an inner diameter of 0.41 mm was used for printing, and the speed of the syringe was 20 mm s⁻¹. For preparing the scaffolds, the samples were frozen at -20°C and then lyophilized with a freeze dryer, yielding the GA5, GA10, GA10-P scaffolds.

4.5 3D printing of GradS scaffold

GA5, GA10, and GA10-P hydrogels (100 μL each) were subsequently printed (using Cellink HeartWare version 2.4.1) with a syringe in a horizontal (XY) orientation to yield the GradGA hydrogel. The syringe temperature and printing plate temperature were set at 37°C. A needle with an inner diameter of 0.41 mm was used for printing, and the speed of the syringe was 20 mm s⁻¹. The final construct was crosslinked immediately with visible light and a CaCl₂ solution; then to prepare the scaffold, samples were frozen at -20°C and then lyophilized with a freeze dryer, yielding the GradS scaffold.

4.6 General procedure for cell experiments in 3D printed (GA5, GA10, GA10-P) scaffolds

The hBM MSC were carefully thawed and suspended in their specific medium (10% FBS + 1% Pen./Strep. + 10 ng
mL\(^{-1}\) FGF-2 + DMEM). Then, the cells were seeded homogeneously onto the GA5, GA10, and GA10-P scaffolds (~20,000 cells per scaffold, 3 x scaffolds were used for each cell experiments). The samples were covered with cell culture media (1 mL) and incubated for 1 hour, 1 day, 4 days and 7 days at 37°C and 5% CO\(_2\). After the incubation periods, scaffolds were washed twice with PBS to remove non-adhered cells. These samples were transferred to new cell culture plate and treated with the WST assay to measure the cell viability. WST assay was also used to quantitatively determine the number of cells. We prepared the calibration curve using different concentrations of viable cells that interacted with WST-assay. The relationship achieved from the calibration curve was used to determine the number of viable cells in the scaffolds.

4.7 General procedure for cell experiments in 3D printed gradient (GradS) scaffolds

The cells were carefully thawed and suspended in their specific medium (10% FBS + 1% Pen./Strep. + 10 ng mL\(^{-1}\) FGF-2 + DMEM). Then, the cells were seeded homogeneously onto each part of the GradS scaffold (~20,000 cells per scaffold). The GradS was covered with cell culture media (1 mL) and incubated for 1 hour, 1 day, 4 days and 7 days at 37°C and 5% CO\(_2\). After the incubation periods, the scaffold was washed twice with PBS to remove non-adhered cells. Afterwards, the GradS was split into its parts (GA5, GA10, GA10-P). These parts were transferred to a new cell culture plate and treated with the WST assay to measure the cell viability.

4.8 General procedure for cell migration and reverse cell migration experiments in 3D printed gradient (GradS) scaffolds

For the cell migration experiments the above procedure was used, but the cells were only seeded homogeneously onto the GA5 part of the GradS scaffold (~20,000 cells). For the reverse cell migration experiments the above procedure was used, but here the cells were only seeded homogeneously onto the GA10-P part of the GradS scaffold (~20,000 cells).

4.9 Osteogenic differentiation capacity of hBM MSC inside the printed construct

The cells were carefully thawed and suspended in their specific medium (10% FBS, 1% Pen./Strep. + 50 µg mL\(^{-1}\) ascorbic acid + 10 mM β-glycerophosphate + 10 nm dexamethasone + DMEM). Then, the cells were seeded homogeneously onto single GA5, GA10, and GA10-P scaffolds and different parts of the GradS scaffold (~20,000 cells per scaffold).

4.9.1 Alizarin red staining

The osteogenic differentiation of the hBM MSC cells cultured within the scaffolds was determined by alizarin red staining. After incubation for 7 and 14 days. The scaffolds were washed twice with PBS to remove non-adhered cells and transferred to a new cell culture plate. The cells in the scaffolds were fixed with 4% paraformaldehyde for 10 min then stained with 2% alizarin red S for 10 min in darkness at room temperature. Afterwards the non-specific stain was removed four times with Milli-Q. Undifferentiated hBM MSC are slightly purple, whereas MSC-derived osteoblasts stain red.

4.9.2 Calcium content determination

The mineralization of the hBM MSC cells cultured within the scaffolds was determined by Quanti Chrom Calcium Assay Kit. After incubation for 7 and 14 days, the scaffolds were washed twice with PBS to remove non-adhered cells and transferred to a new cell culture plate. 1 mL of 0.6 M HCl was added to the mineralized matrix. After 4 hours, a 5 µL of the sample supernatant was transferred to 96-well plate. Then, 95 µL of the working solution (mixture of reagent A and B with the same ratio) was added to each sample. The samples were kept for 3 min. The absorbance was measured at the wavelength of 612 nm.

4.9.3 BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) colorimetric staining

Differentiated osteoblasts show very high AP activity, whereas undifferentiated hBM MSC show weak alkaline phosphatase (AP) activity. AP can be detected using BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) as a substrate after incubation for 7 and 14 days, by fixing cells with fixed with 4% paraformaldehyde for 60-90 seconds. Then, cells were stained with adding enough BCIP/NBT substrate solution to cover the sample for 10 min in darkness at room temperature. Afterwards the non-specific stain was removed twice with PBS. Cells stain blue-violet when AP is present.
4.10 | Osteogenic differentiation capacity of the hBM MSC when the normal growth medium, not supplemented with osteoinductive agent dexamethasone was used

The cells were carefully thawed and suspended in their specific medium (10% FBS, 1% Pen./Strep. + 50 μg mL⁻¹ ascorbic acid + 10 mM β-glycerophosphate + DMEM). Then, the cells were seeded homogeneously onto single GA5, GA10, and GA10-P scaffolds and different parts of the GradS scaffold (~20,000 cells per scaffold). After the incubation periods, the scaffolds were washed twice with PBS to remove non-adhered cells and transferred to a new cell culture plate. The osteogenic differentiation capacity of the hBM MSC cells cultured within the scaffolds was determined by alizarin red staining. The mineralization of the hBM MSC cells cultured within the scaffolds was determined by QuantiChrom Calcium Assay Kit.

4.11 | Characterization

The morphologies of the scaffolds were investigated using a Zeiss 1540 EsB dual beam focused ion beam/field emission SEM. Zeta potential measurements and dynamic light scattering (DLS) were measured by Malvern Zetasizer Nano Series. The morphologies of cells in hydrogels were determined using Nikon ECLIPSE Ts2R fluorescence microscopy. Inkredible 3D bioprinter (CELLINK) was used to print all scaffolds into computer-designed 3D structures. Anton Paar (Modular Compact Rheometer) was used for rheological analyses. A ZwickRoell (type 066590) material testing machine was used to determine the compression and tensile stress/strain curves and compression modulus of the samples. The number of repeated experiments (N) was 3. The ANOVA test was used for statistical analyses.

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DATA AVAILABILITY STATEMENT

Research data are not shared.

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