Step-Gradient Composite Hydrogels for Local Drug Delivery and Directed Cell Migration

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and regulate cell migration. For example, Byambaa et al. described 3D-printed gelatin methacryloyl (GelMA)-based hydrogels to support gradient vasculogenesis and osteogenesis. Di Luca et al. reported 3D-printed poly(c-caprolactone) scaffold functionalized with bone morphogenetic protein-2 and transforming growth factor-β3 for osteochondral tissue regeneration. Further, Gurkan et al. reported the generation of a fibrocartilage microenvironment via 3D bioprinting of mesenchymal stem cells in another study, Cross et al. developed a GelMA and methacrylated kappa carrageenan-based gradient structure for interface TE. We reported the alginate-based step-gradient nanocomposite scaffolds for migration and subsequent enhanced osteogenic differentiation of human bone marrow-derived mesenchymal stem cells.

Recently, TE researchers using 3D printing have focused intensively on designing injectable biomaterials to replace invasive surgery with injectable (minimally invasive) implantation. Furthermore, injectable biomaterials hold the potential for achieving more precise implantation into poorly reachable tissue sites, and they can be designed to perform controlled local drug delivery. Such systems can provide site-specific, controlled, and prolonged drug/bioactive molecule delivery at a desired region of the tissue while limiting drug interactions and side effects at other (unintended) sites in the body because these sites will never see high doses of the drug. Therefore, injectable local drug delivery systems are suitable for reducing/eliminating inflammation/infection at the tissue implantation site, and they can also promote and direct cell migration via controlled site-specific release of bioactive molecules.

Despite recent advances in the field of TE, there are still an increasing number of demands from the biomedical field regarding how to improve biomaterials and develop ones with new properties. Still, few biomaterials can combine the required ECM properties (such as mechanical, biochemical and topographical gradients, 3D network, porosity, biocompatibility, biodegradability, injectability, and the ability to perform local drug delivery) into one biomaterial to generate multifunctional advanced tissue constructs.

In this context, we previously developed and reported on a algin and periodic mesoporous organosilica (PMO)-based 3D-printed bilayer nanocomposite scaffolds for controlled cell migration in the XZ plane of the 3D alginate[Alg]/PMO network. In this study, we used bifunctional nanomaterials (NMs) [3pmo-PMO-PDL], which we synthesized by functionalizing PMO with the anti-inflammatory and chemotherapeutic drug molecule dexamethasone (Dex) and the cell-adhesive bio-polymer poly-t-lysine (PDL). This construction allowed us to influence cell migration from the bottom of the scaffold to the top (XZ plane of the scaffold). The biodegradable PDLe-enhanced cell adhesion and, at the same time, controlled the release kinetics of Dex via pH and offered sustained release of Dex into the cellular environment. Thus, PDLe prevented the cells from interacting with high concentrations of free Dex, limiting Dex’s side effects on cell viability.

In this current contribution, we bring this strategy (controlled interaction of cells with PDL and Dex) into a 3D step-gradient composite hydrogel and scaffold to achieve enhanced beneficial effects of [3pmo-PMO-PDL] on improved healthy fibroblast cell viability, proliferation, and directed migration in the XY plane, whereas at the same time reducing cancer (Colo 818) cell viability and migration. Therefore, we mixed [3pmo-PMO-PDL] with cross-linkable Alg and GelMA polymer matrix to generate new injectable composite hydrogel precursors. Alg and gelatin-based polymers were used because they are biocompatible, nontoxic, nonimmunogenic, biodegradable, and can form gels. Algin is composed of guluronic acid and mannanuronic acid, and alginate hydrogels can form by ionic crosslinking via calcium ions, thus through noncovalent bonding. In contrast, GelMA and alginate hydrogels can form by ionic crosslinking via calcium ions, thus through noncovalent bonding. GelMA displays high elasticity with good cell adhesiveness due to Arg-Gly-Asp (RGD) sequences of the gelatin. The incorporation of GelMA into Alg improved the 3D printability of the composite hydrogel and improved cell viability in the 3D-printed structures. The prepared composite hydrogel precursors were used for the fabrication of a new 3D step-gradient composite hydrogel and scaffolds (freeze-dried form of hydrogels) made by 3D bioprinting. The Alg/ GelMA/[3pmo-PMO-PDL] ratio was changed during the 3D printing process to achieve biochemical, mechanical, and physical gradients in the 3D network of the composite hydrogel. The 3D-printed step-gradient composite hydrogel and its scaffold influenced the viability and migration of fibroblast and Colo 818 cells in the XY plane of the step-gradient composite hydrogel and scaffold. The [3pmo-PMO-PDL] allowed for sustained and pH-responsive controlled release of chemotherapeutic Dex within the 3D composite hydrogel network, which enhanced the viability and migration of fibroblasts and, at the same time, reduced the cancer cells’ (Colo 818) viability and migration.

2. Results and Discussion

2.1. Preparation of 3D-Printed Composite Hydrogels

To prepare step-gradient composite hydrogels, we prepared GelMA/Alg-based composite hydrogel precursors varying in GelMA, Alg, and [3pmo-PMO-PDL] concentration. First, GelMA (5% w/v)/Alg (7% w/v) and GelMA (10% w/v)/Alg (7% w/v) were prepared to obtain GA5 and GA10. Then, the previously synthesized and characterized [3pmo-PMO-PDL] (Figure 1a) was mixed with GA10 to obtain GA10-P [GelMA (10% (w/v))/Alg (7% (w/v))]/[3pmo-PMO-PDL (0.1% (w/v))].

GelMA, Alg, GA5, GA10, and GA10-P were first printed into hexagon structures (Video S1). 3D-printed structures were first exposed to visible light for covalent photocrosslinking of GelMA (except Alg sample) and then to calcium solution for ionic crosslinking of Alg (except GelMA sample) (Figure 1b–f). It is important to note that we also tried to crosslink hydrogels first and then print them into 3D hexagon structures (Video S2–S4, Supporting Information). GA10-P was injectable (Video S2, Supporting Information) but not printable (Video S3, Supporting Information), whereas GA5 was both injectable and printable (Video S4, Supporting Information). Therefore, in the next experiments, the hydrogel precursors were printed first and then crosslinked. The color of photoinitiator (red) in GelMA was changed to pale yellow after visible light that is the indication of crosslinking or in other words hydrogel formation like reported by Noshadi et al. The incorporation of GelMA into
Alg allowed us to improve the printability of the composite hydrogel, because the 3D printing of GelMA (Figure 1b) was not possible without visible-light crosslinking during printing and Alg (Figure 1c) resulted in 3D structures with poor shape fidelity. After freeze-drying the GA5, GA10, and GA10-P, we obtained 3D-printed scaffolds. We then analyzed the 3D-printed GA5, GA10, and GA10-P hydrogels and scaffolds for their morphological, rheological, mechanical, swelling, degradation, and porosity properties.

Scanning electron microscopy (SEM) images showed the porous 3D network (generated by freeze drying) of the GA5, GA10, and GA10-P scaffolds (Figure 1g–i). We did not observe any significant difference between the images. All samples (GA5, GA10, and GA10-P) have irregular pore sizes. However, some of the pores are in the range of 150–400 μm, thus they are suitable for efficient cell growth, migration, and nutrient flow. In contrast, the GA10-P hydrogels/scaffolds exhibited a higher porosity (%) (Table S1, Supporting Information), a higher swelling capacity (Table S2, Supporting Information), and less degradation (Table S3, Supporting Information) than the GA5 and GA10 materials due to the fortification of the GelMA/Alg network by DexPMO-PDL. The hydrophilic DexPMO-PDL promoted water diffusion, thus resulting in higher swelling. Furthermore, as these scaffolds showed higher swelling and porosity (%) but less degradation than the hydrogels, the scaffolds seem to have higher stability, making them potentially more suitable for long-term use than soft viscous hydrogels. In addition, the degradation of the hydrogels/scaffolds in the presence of cells was greater than without cells that may indicate the cell-mediated biodegradation of the hydrogels/scaffolds occurred.

The rheological tests for the GA5, GA10, and GA10-P hydrogels showed that the shear thinning and viscoelastic properties of GelMA/Alg hydrogels were improved by the incorporation of DexPMO-PDL (Figure 2a). The viscosities of the all samples (GA5, GA10, and GA10-P) decreased with increasing angular frequency, indicating shear-thinning behavior. All GelMA/Alg hydrogels showed a higher storage modulus (G') in comparison with the loss modulus (G'') over the range of angular frequencies, and these were also dependent on the DexPMO-PDL concentration. This result indicates that the GA5, GA10, and GA10-P have viscoelastic properties (Figure 2b). Furthermore, GA10-P and GA10 displayed a higher compressive modulus than GA5, indicating that the compressive mechanical properties of GelMA/Alg can be tuned by changing the ratio of GelMA, Alg and DexPMO-PDL (Figure 2c).

2.2. The Release Kinetics of Dex from GA10-P Hydrogel/Scaffolds and DexPMO-PDL

We determined the release kinetics of Dex from GA10-P at pH 7.4 and 6.0 (where this lower pH value is representative of a tumor and inflammatory tissue environment) to demonstrate the stimuli-responsive drug delivery from GA10-P. The release behavior of Dex from DexPMO-PDL in the GA10-P hydrogels and scaffolds was measured at different time intervals (e.g., from 3 min to 7 days) (Figure 3). The release profiles of Dex from GA10-P hydrogels/scaffolds at pH 7.4 and 6.0 show a sustained and extended release pattern (Figure 3) after 7 days of incubation. After 7 days of incubation, the Dex release from GA10-P hydrogels/scaffolds at pH 6.0 was 2 times higher than at pH 7.4, demonstrating the pH-responsive drug.
delivery ability of Dex in the GA10-P. In addition, after the same incubation period, we found that Dex release from the GA10-P scaffold was higher than that from the GA10-P hydrogel due to the higher swelling capacity of the scaffold (see Table S2, Supporting Information).

Hydrogels and porous nanoparticles have been intensively used for entrapment and delivery of bioactive molecules in a stimuli-responsive manner. In our system, all components of GA10-P are electrostatically interacted. The positively charged PDL can interact with the negatively charged PMO surfaces and carboxyl groups in Alg and GelMA. Furthermore, the degradation of GA10-P proceeded with time. Thus, Dex release from the PDL-coated PMO within the GelMA/Alg hydrogel was prolonged and pH dependent. As the electrostatic interaction between the charged components of GA10-P changed with pH, GA10-P provided a high dosage of the anti-inflammatory and chemotherapeutic drug Dex at pH 6, which is representative of a tumor and inflammatory tissue environment.

2.3. Cell Experiments in the GA5, GA10, and GA10-P Hydrogels

Cell experiments were first conducted to compare the effects of GelMA, Alg, and their composite (GA10) on cell viability and to demonstrate the beneficial effect of incorporating GelMA into Alg on cells. Therefore, GelMA, Alg, and GA10 were first interacted separately with healthy and malignant skin cells, namely healthy primary dermal fibroblast cells and malignant melanoma fibroblasts (Colo 818) for 1 and 7 days of incubation. Cell viability was measured using PrestoBlue, a resazurin-based metabolic...
We found more viable fibroblast and Colo 818 cells in GA10 than in GelMA and Alg in both incubation periods, which was more evident at 1 day of incubation than at 7 days. The results showed that the incorporation of GelMA into Alg (GA10) had a synergistic effect on cell viability. Therefore, the more detailed cell experiments were conducted with different GelMA/Alg-based composites that varied in the concentrations of GelMA, Alg, and Dex-PMO-PDL (GA5, GA10, and GA10-P).

GA5, GA10, and GA10-P materials were separately interacted with healthy primary dermal fibroblast cells and malignant melanoma fibroblast (Colo 818) cells. First, fibroblast or Colo 818 cells were homogeneously mixed separately with GA5, GA10, and GA10-P hydrogels and printed into a hexagon structure (called single hydrogel experiment). 3D-printed cell-laden GA5, GA10, and GA10-P hydrogels were first crosslinked with visible light and then with a calcium solution for covalent and ionic crosslinking, respectively. After different incubation periods (1 h, 1, 4, and 7 days), cell viability was measured using PrestoBlue, a resazurin-based metabolic assay (Figure 4). We observed a time-dependent increase in cell viability in each sample that was more significant in GA10-P than GA5 and GA10. When the number of viable healthy fibroblast cells had increased by 45% in GA10-P, that corresponded with an increase in only ≈13 and 31% in GA5 and GA10, respectively, from 1 h to 7 days of incubation. As these GA5 and GA10 values show a cell viability difference between GA5 and GA10 of 17%, this indicates that increasing the GelMA concentration in the composite hydrogel composition-enhanced cell viability. Moreover, the incorporation of Dex-PMO-PDL provided an additional benefit to cell proliferation.

In contrast to the viability results for healthy cells, the results for the malignant Colo 818 were significantly different (Figure 5a, Table S4A, Supporting Information). While GA5 provided almost the same amount of increase (≈13%) in the number of viable cells for fibroblast and Colo 818 cells over the 7-day incubation, GA10 (20%) and GA10-P (5%) resulted in much less viability of malignant cells than healthy fibroblast cells over the 7-day incubation. The difference between the number of viable healthy fibroblasts and malignant Colo 818 cells in GA10 and GA10-P was 11% (31% viability for healthy cells vs 20% for malignant cells) and 40% (45% viability for healthy cells vs 5% for malignant cells), respectively, showing that incorporating higher amounts of GelMA and Dex-PMO-PDL into the hydrogel composites reduced the malignant Colo 818 cells' proliferation rate compared with the healthy fibroblast cells. The first reason for the effect is the RGD unit in GelMA, as the RGD peptide motif has been explored as a drug candidate for cancer treatment.

![Figure 5.](image-url) The number of fibroblasts and Colo 818 cells ($\times 10^3$) in 3D-printed single GA5, GA10, and GA10-P hydrogels, b) in GradGA hydrogels where cells were mixed into all parts, c) in GradGA hydrogels where cells were mixed just into the GA5 part, and d) in GradGA hydrogels where cells were mixed just into the GA10-P part at different incubation times; values determined by the PrestoBlue assay. Number of repeated experiments ($N$) = 3; data show significant differences; ANOVA: $p < 0.01$ (**), $p < 0.001$ (***) ($a$ = significant difference between three different hydrogels for fibroblast or Colo 818 cells; $b$ = significant difference between fibroblast and Colo 818 cells in the same hydrogel).
RGD, a minimal integrin recognition sequence, can bind and thus inhibit tumor cells via their αvβ3 integrins, thereby inhibiting tumor angiogenesis.[46–48] The second reason for the effect is because on the Dex PMO-PDL particles, polylysine, and Dex have known inhibitory effects on cancer cells. Polylysine, a cationic biopolymer, was found to have antitumor and antitumor-angiogenic activity,[49,50] and has been found to penetrate into cancer cell membranes, causing cytotoxic effects and resulting in apoptotic cell death. Dex is also used as a chemotherapeutic drug because it can inhibit cancer cell growth and migration and it can induce apoptosis.[51–53] In addition, we demonstrated that the released amount of Dex from Dex PMO-PDL was higher at pH 6 (representative of a tumor tissue environment) than pH 7.4 (physiological condition), meaning that the local acidic environment of the malignant Colo 818 cells initiated the release of higher amounts of Dex from the pH-responsive Dex PMO-PDL, resulting in less viability for Colo 818 cells than for healthy fibroblast cells.

### 2.4. Cell Experiments in the 3D-Printed Step-Gradient Composite Hydrogels

In the light of these observations, we prepared step-gradient composite hydrogels to study directed cell migration. GA5, GA10, and GA10-P were mixed with fibroblast or Colo 818 cells and printed side by side into connected hexagonal structures (Figure 1j). The connected cell-laden GA5, GA10, and GA10-P were crosslinked and formed a step-gradient composite hydrogel (GradGA) (called gradient hydrogel experiment). After different incubation times (1 h, 1, 4, and 7 days), GradGA was divided into its components (GA5, GA10, and GA10-P) and cell viability was determined (Figure 5b, Table S4B, Supporting Information). Even though there was almost no difference between the results of previous single hydrogels (GA5, GA10, and GA10-P) and that of the gradient (GA5, GA10, and GA10-P parts of GradGA), we saw a small indication of fibroblast cell migration from GA5 toward GA10-P after 4 and 7 days of incubation. For example, when the GA5 and GA10 parts of GradGA resulted in a ~4×10 and 23% increase in the number of viable fibroblast cells from 1 h to 7 days of incubation, the GA10-P supported a ~74% increase. In the single hydrogel experiments, the resulting cell viability increases were 13, 31, and 45% in the single GA5, GA10, and GA10-P hydrogels, respectively. This difference (decrease in the GA5 and GA10 and increase in the GA10-P parts of GradGA) may indicate fibroblast cell migration from the GA5 part to the GA10-P part of the GradGA. Furthermore, the difference between the GA10-P and GA5/GA10 parts of the GradGA in fibroblast cell viability was 39%/21%. By contrast, only a very small difference was observed in the number of viable malignant Colo 818 cells in the GA5, GA10, and GA10-P parts of the GradGA, indicating the reducing effect of GelMA concentration and Dex PMO-PDL on malignant Colo 818 cell migration and viability.

To gain clearer results, in the next experiment, we only mixed GA5 with cells. The cell-laden GA5 and cell-free GA10 and GA10-P gels were printed and connected to each other to form GradGA (called cell migration experiment) (Figure 5c, Table S4C, Supporting Information). In this experimental setup, cells were only embedded into the GA5 parts of GradGA and no cells were found in GA10 and GA10-P after 1 h of incubation. However, after 7 days of incubation, the GA5, GA10, and GA10-P parts contained 68%/75%, 16%/14%, and 16%/11% of the total fibroblast/Colo 818 cells, respectively, in the GradGA. This demonstrates that the fibroblast/Colo 818 cell migration from GA5 toward GA10-P depended on GelMA concentration and Dex PMO-PDL amounts. When we compared fibroblast and Colo 818 cells, we saw that Dex PMO-PDL particularly reduced the malignant Colo 818 cell viability and migration, that resulted in less-migrated Colo 818 cells than fibroblast cells in the GradGA. The number of viable healthy fibroblast cells (3.9 × 10⁷) that had migrated to the GA10-P part of GradGA was 1.6 times higher than the number of Colo 818 cells that had migrated to the GA10-P part of GradGA (2.4 × 10⁶).

To support these results and to show that cells were not randomly migrated, we conducted reverse cell migration experiments. The cells were mixed with GA10-P and the cell-free GA5 and GA10 and the cell-laden GA10-P were printed to form GradGA (Figure 5d, Table S4D, Supporting Information). In this experimental setup, cells were embedded only in the GA10-P part of GradGA. Here, only 6%/93% and 12%/89% of the total fibroblast/Colo 818 cells in the GradGA were found in the GA5 and GA10, respectively, after 7 days of incubation period (Figure 5d, Table S4D, Supporting Information). Overall, 82%/89% of the fibroblast/Colo 818 cells remained in the GA10-P part of GradGA, showing that cell migration is not preferred toward parts of the GradGA that lack Dex PMO-PDL. These results were significantly different from the cell migration experiments (Figure 5c, Table S4C, Supporting Information) (the GA5, GA10, and GA10-P parts contained 68%/75%, 16%/14%, and 16%/11% of the total fibroblast/Colo 818 cells, respectively, in the GradGA). This means the cell migration can be triggered and that the direction of cell migration can be influenced by changing the concentration of GelMA and Dex PMO-PDL within the 3D GradGA materials. The migrated number of viable healthy fibroblast cells (1.9 × 10⁷) in GA5 was 2.7 times higher than that of Colo 818 cells (0.7 × 10⁷). Furthermore, we observed a 49 and 13% increase in the number of viable cells for fibroblast and Colo 818 cells, respectively, from 1 h to 7 days of incubation in the GA10-P part of GradGA. These results show that the migration and viability of malignant Colo 818 cells were reduced and influenced by Dex PMO-PDL in the GA10-P part of GradGA hydrogel due to the acidic pH-responsive release of chemotherapeutic drug molecules from Dex PMO-PDL.

The GradGA provides chemical, physical, and mechanical gradients in its 3D network. The change in the GelMA concentration from GA5 to GA10 and the incorporation of Dex PMO-PDL into the GA10 (GA10-P) resulted in a change in the concentrations of RGD, Dex, PDL, and PMO in the GradGA, whereby the concentration changes of RGD, Dex, and PDL provided chemical gradients, whereas PMO added physical gradients (topography). Furthermore, the increase in the GelMA/Alg and GelMA/Alg/PMO ratio from GA5 to GA10 and GA10 to GA10-P caused a mechanical gradient in the GradGA’s 3D network. Therefore, the observed results occurred because of the synergistic contribution of the chemical, mechanical, and physical gradients generated by GradGA’s different concentrations of GelMA and Dex PMO-PDL.
2.5. Cell Experiments in the 3D-Printed GradGA Scaffolds

The cell experiments were also conducted in 3D-printed GradGA scaffolds. 3D-printed GradGA scaffolds were obtained by freeze drying the 3D-printed GradGA hydrogels (Figure 6, Table S5, Supporting Information). The difference between cell seeding in the scaffolds and the hydrogels is that in the scaffolds, cells are seeded onto them after 3D printing and after the freeze-drying process. Thus, cells can be grown in preprepared biomaterials, which eliminates the mechanical stress applied to the cells during the printing process; this is important because such a mechanical stress may cause cell injury and influence cell viability. Furthermore, the scaffolds possess higher swelling (Table S2, Supporting Information), slow cell-mediated degradation (Table S3, Supporting Information), and higher porosity (Table S1, Supporting Information) than the hydrogels, pointing to the scaffolds’ stability and longer lifetime. One disadvantage of the scaffold method is that cells may not be homogeneously distributed into the 3D network of the scaffold as they are in the hydrogel approach, as in hydrogel experiments, the cells are homogeneously mixed first with hydrogels and then the cell-laden hydrogels are printed. Nonetheless, this may represent a trade-off to the potential mechanical stresses cells would face during hydrogel printing. Therefore, to investigate the difference in cell viability and migration between 3D-printed GradGA hydrogels and scaffolds, we performed further cell experiments using fibroblast and Colo 818 cells in 3D-printed GradGA scaffolds.

Our results showed no significant difference in cell viability when experiments were carried out in single/gradient hydrogels versus scaffolds. In addition, we observed that only a few more fibroblast cells migrated within the GradGA scaffolds than within the GradGA hydrogels (in cell migration and reverse cell migration experiments). However, Colo 818 cells showed an opposite trend in the GradGA scaffolds than the GradGA hydrogels in both the cell migration and reverse cell migration experiments. In the reverse cell migration experiment (Table S5D, Supporting Information), more Colo 818 cells had migrated toward GA5 in the GradGA scaffold than the hydrogel, whereas in the cell migration experiments (Table S5C, Supporting Information), fewer migrated Colo 818 cells were found in the GA10-P part of the GradGA scaffold than in the GradGA hydrogel. These results are likely because of the higher porosity of the GradGA scaffold than the GradGA hydrogel, allowing for better cell migration within the 3D GradGA scaffold network. Therefore, in the reverse cell migration experiments, more Colo 818 cells could escape from the GA10-P part of the GradGA scaffold and migrate toward the GA5. In the cell migration experiments, the migrated Colo 818 cells in the GA10-P part of the GradGA scaffold were able to migrate back into the GA10-GA5 part of the scaffold.
and GA5 parts. Overall, these results showed that in our study, the 3D printing of the cell-laden hydrogels did not reduce the cell viabilities, and the scaffold method did not result in reduced cell migration; in fact, it was slightly improved fibroblast cell migration in 3D GradGA scaffold, as a representative example, was also confirmed by fluorescence microscopy images (Figure S1, Supporting Information).

3. Conclusion

In this study, we described the preparation of a 3D step-gradient composite hydrogel and corresponding scaffold (GradGA) to study the impact of GradGA's composition on fibroblast and Colo 818 cells' viability and migration. GradGA was prepared by 3D printing of different composite hydrogel precursors in a side-by-side orientation and then by crosslinking. Composite hydrogels were generated using the various concentrations of GelMA, Alg, and multifunctional NMs (PMO-PDL). The multifunctional PMO-PDL allowed for sustained and pH-responsive controlled release of anti-inflammatory and chemotherapeutic drug molecules within the 3D network of GradGA, which improved cell viability, promoted cell migration, and resulted in a reduction in malignant cell (Colo 818) viability. GradGA possesses mechanical, biochemical, and topographical gradients, a 3D network, porosity, biocompatibility, biodegradability, 3D printability, and the capability for local drug delivery. Thus, GradGA has potential to mimic the ECM environment, to control directional cell migration, to promote the vascularization of implanted tissue constructs, to inhibit inflammation after implantation, and to be implanted in a minimally invasive manner.

4. Experimental Section

Materials: PDL (MW: 30,000–70,000), hexadecyltrimethyl-ammonium bromide (CTAB, 98%), 1,2-bis(trimethoxysilyl)-ethane (BTME, 96%), algic acid sodium salt powder, dexamethasone (≥98% high performance liquid chromatography (HPLC)), gelatin (from porcine skin), methacrylic anhydride (MA), N-vinylcaprolactam (VC), eosin Y, triethanolamine (TEA), and paraformaldehyde (PFA), were purchased from Sigma-Aldrich. Ethanol (absolute), ammonia solution (32%, pure), and hydrochloric acid (HCl) (32%, for analysis), were purchased from Merck. Dulbecco’s phosphate-buffered saline (DPBS) and cell medium (RPMI 1640) (HCl) (32%, for analysis), were purchased from Merck. Dulbecco’s phosphate-buffered saline (DPBS) and cell medium (RPMI 1640) were subsequently prepared (using Cellink HeartWare version 2.4.1) in a hexagon structure (Video S1, Supporting Information) (4 mm on each side and 1 mm in height), and crosslinked with visible light (450–550 nm) for 120 s using FocalSeal (Genzyme Biosurgical, Cambridge, MA), for covalent photocrosslinking of GelMA (except Alg sample) and then with a 225 μT CaCl2 solution for ionic crosslinking of Alg (except GelMA sample). Thus, the 3D-printed single GelMA, Alg, GelMA, and Alg10 and Alg10-P hydrogels were obtained. 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 GelMA, Alg, GelMA, and Alg10-P samples were frozen at −20 °C and then lyophilized with a freeze dryer, yielding the single GelMA, Alg, and Alg10-P scaffolds. Furthermore, we also tried to crosslink hydrogels first and then print them into 3D hexagonal structures (Video S2–S4, Supporting Information). GelMA was injectable (Video S2, Supporting Information) but not printable (Video S3, Supporting Information), whereas GelMA was both injectable and printable (Video S4, Supporting Information). Therefore, in the next experiments, the hydrogel precursors were printed first and then crosslinked.

Preparation of the 3D-Printed GradGA Scaffold: GelMA, Alg, and Alg10-P (100 μL each) were subsequently prepared (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 GradGA scaffold.

Cell Experiments in 3D-Printed Single (GelMA, Alg, GelMA, and Alg10-P) Hydrogels: The cells were carefully thawed and suspended in their specific medium (10% FBS + RPMI 1640). Then, the cells were mixed homogeneously within the single GelMA, Alg, and Alg10-P hydrogel precursors (≥20,000 cells per sample). The samples were 3D printed and then crosslinked with visible-light first and then with CaCl2. The formed hydrogels were covered with cell culture media (1 mL) and incubated for 1 h, 1, 4, and 7 days at 37 °C and 5% CO2. After the incubation periods, they were washed twice with PBS to remove nonadhered cells. These samples were transferred to new cell culture plates and treated with the PrestoBlue, a resazurin-based metabolic assay, to measure the cell viability.

Cell Experiments in 3D-Printed Gradient (GradGA) Hydrogels: The cells were carefully thawed and suspended in their specific medium (10% FBS + RPMI 1640). Then the cells were mixed homogeneously with GelMA, Alg, and Alg10-P (≥20,000 cells per sample). The cell-laden GelMA, Alg, and Alg10-P were 3D printed into the gradient GradGA constructs and then crosslinked with visible light first and then with CaCl2. The formed GradGA hydrogel constructs were covered with cell culture media (1 mL) and incubated for 1 h, 1, 4, and 7 days at 37 °C and 5% CO2. After the incubation periods, GradGA constructs were washed twice with PBS to remove nonadhered cells. Afterward, the GradGA constructs were split into their component parts (GelMA, Alg, and Alg10-P). These parts were transferred to a new cell culture plate and treated with the PrestoBlue assay to measure the cell viability.
Cell Migration Experiments in 3D-Printed Gradient (GradGA) Hydrogels: For the cell migration experiments, we used the aforementioned procedure, but cells were only mixed homogeneously into the G5 part of the GradGA hydrogel (~20,000 cells).

Reverse Cell Migration Experiments in 3D-Printed Gradient (GradGA) Hydrogels: For the reverse cell migration experiments, the aforementioned procedure was used, but here the cells were only mixed homogeneously within the GA10-P part of the GradGA hydrogel (~20,000 cells).

General Procedure for Cell Experiments in the Respective Scaffolds: The cell experiments in scaffolds were done using the respective 3D-printed Alg, GelMA, G5, GA10, and GA10-P, and GradGA scaffolds. The similar procedures performed in hydrogels experiments were used. The only difference is, in these experiments, cells were seeded homogeneously onto the 3D-printed scaffolds (~20,000 cells per scaffold).

Statistical Analysis: Each experiment was conducted in triplicate to calculate an average and a standard deviation. All the data were analyzed using a one-way ANOVA. The probability (p) value of p < 0.01 (***) and p < 0.001 (****) were used to show the significant differences. Microsoft Excel was used for statistical analysis.

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

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

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