**Ultrashort Peptide Bioinks Support Automated Printing of Large-Scale Constructs Assuring Long-Term Survival of Printed Tissue Constructs**

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**ABSTRACT:** We report about rationally designed ultrashort peptide bioinks, overcoming severe limitations in current bioprinting procedures. Bioprinting is increasingly relevant in tissue engineering, regenerative and personalized medicine due to its ability to fabricate complex tissue scaffolds through an automated deposition process. Printing stable large-scale constructs with high shape fidelity and enabling long-term cell survival are major challenges that most existing bioinks are unable to solve. Additionally, they require chemical or UV-cross-linking for the structure-solidifying process which compromises the encapsulated cells, resulting in restricted structure complexity and low cell viability. Using ultrashort peptide bioinks as ideal bodylike but synthetic material, we demonstrate an instant solidifying cell-embedding printing process via a sophisticated extrusion procedure under true physiological conditions and at cost-effective low bioink concentrations. Our printed large-scale cell constructs and the chondrogenic differentiation of printed mesenchymal stem cells point to the strong potential of the peptide bioinks for automated complex tissue fabrication.

**KEYWORDS:** Molecular self-assembly, ultrashort peptides, peptide bioinks, automated 3D bioprinting, postprinting differentiation.
bioinks for bioprinting applications (Figure 1A). Their purity and mass were confirmed by HPLC-HR-MS (Figures S1−S3). We observed that all three peptides were able to form transparent hydrogels at 0.1% w/v (1 mg/mL) in 1× PBS with a high water content and the shortest gelation time of 7 min for IIZK (Table 1, Figure S4). Remarkably, their minimum gelation concentrations (MGCs) are one of the lowest gelation concentrations for nonenzymatic supramolecular hydrogel at physiological conditions.13−16 These initial findings indicated that the three peptides could be promising candidates as bioinks.

Furthermore, we analyzed the intermolecular arrangement of all three peptides using 2D NMR (TOCSY and NOESY). By overlapping both TOCSY and NOESY spectra, we identified intermolecular cross-peaks that indicate the formation of antiparallel configuration (Figures S5−S7, Tables S2−S4).
some handed helical diameter. Notably, while both IIFK and IZZK show a left-negative stain TEM, we propose that the bundles are mostly supported by the values observed in AFM, cryo-TEM, and able to identify. Moreover, analyzing the negative-stain TEM images, we were with the measured height in AFM (Table S6) and with the S11, Table S7) of about 10 nm. This width matches quite well value at about 11 nm (Figure 1D, Figure S10, Table S6). Cryo-structures, possibly formed by several (Figure 1C). AFM images revealed the diversity of the assembled network structure was con-

To further analyze the self-assembly process, molecular dynamic (MD) simulations were conducted in water. For each peptide, assemblies of 2, 4, and 60 peptides were performed (Figures S13–S16, Tables S8 and S9, Videos 1–9). Simulation data of all three peptides confirmed the earlier proposed stepwise mechanism of self-assembly, starting from an antiparallel pair formation, formation of turnlike secondary structures, and finally condensation into long and stable fibers (Figure 2A). The assembly time of IIFK was longer than for IZZK and IIZK. IIFK formed relatively compact and ordered fibers, whereas IIZK and IZZK assembled into more loose fiber structures (Figure S17). We found that the distance between the Cha carbon atoms and their closest distance to the hydrogen atom in water (HW) or oxygen atom in water (OW) was longer than the distances of corresponding Phe carbon atoms. This suggests that Cha is less solvent-accessible than Phe, which increases the aggregation kinetics. This was confirmed by the 4-peptide cluster formation results via MD (Figure S18). Both short and longer peptide assemblies were maintained by hydrogen bond (H-bond) networks, where most of the hydrogen bonds were intermolecularly formed (Figures S19–S21). We provided evidence that aromatic π–π interactions are not crucial during the fiber formation of the peptides. The face-to-face distance between the closest side chains of the six-membered ring is >1 nm from each other (Figure S22). Since the nominal distance for aromatic π-stacking is <0.44 nm, this proves that the aromatic π-stacking is not the driving force of the assemblies. On the other hand, two pairs of Cha rings in IZZK were observed having face-to-face distance at less than 1 nm. This result indicates the possibility of IZZK forming Cha-Cha cross-strand interactions that can stabilize the assembled fibrils.

### Table 1. Overview of the Characteristics of the Tetrapeptide Bioinks

<table>
<thead>
<tr>
<th>Peptides</th>
<th>MGc, time secondarystructure</th>
<th>TEM</th>
<th>AFM</th>
<th>low conc</th>
<th>high conc</th>
<th>Cytocompatibility</th>
<th>3D Bioprinting</th>
<th>Printability and shape fidelity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIFK</td>
<td>1 mg/mL, &lt;2.5 h</td>
<td>majority β-turns</td>
<td>3.5 ± 0.3</td>
<td>3.2 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>108.2 ± 5.0</td>
<td>HDFn (up to 7 days)</td>
<td>1.0 cm cylinder (structures were preserved for at least 30 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>only bundles were observed (see main text)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>hBM-MSCs (up to 14 days)</td>
<td></td>
</tr>
<tr>
<td>IIZK</td>
<td>1 mg/mL, &lt;7 min</td>
<td>no single filaments, only bundles were observed</td>
<td>3.1 ± 0.3</td>
<td></td>
<td>6.3 ± 0.2</td>
<td>271.3 ± 35.1</td>
<td>HDFn (up to 7 days)</td>
<td>3.8 cm cylinder (structures were preserved for at least 30 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(see main text)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>hBM-MSCs (up to 14 days)</td>
<td></td>
</tr>
<tr>
<td>IZZK</td>
<td>1 mg/mL, &lt;30 min</td>
<td>no single filaments, only bundles were observed</td>
<td>3.0 ± 0.3</td>
<td></td>
<td>3.7 ± 0.2</td>
<td>314.9 ± 10.1</td>
<td>hBM-MSCs (up to 7 days)</td>
<td>4.0 cm cylinder (structures were preserved for at least 30 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(see main text)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>hBM-MSCs differentiate to chondrocytes (up to 30 days)</td>
<td></td>
</tr>
</tbody>
</table>

“MGc = Minimum gelation concentration, i.e., minimal concentration to form a soft gel. aOberved in ss-NMR, Raman, and FT-IR, also found in MD simulations. bObserved in negative staining TEM and AFM in air. cLow conc.: IIFK: 1 mg/mL eq 0.1 wt % = 1.76 mM; IIZK: 1 mg/mL eq 0.1 wt % = 1.65 mM. dHigh conc.: IIFK: 13 mg/mL eq 1.3 wt % = 23.1 mM; IIZK: 13 mg/mL eq 1.3 wt % = 22.9 mM; IZZK: 1 mg/mL eq 1.3 wt % = 1.76 mM.”
The mechanical stiffness of all peptide hydrogels was determined by the storage modulus ($G'$) (Figure 2B, Figure S23, and Table S10). We observed higher $G'$ values (stiffness) for Cha-containing peptides compared to the Phe-containing peptide. The mechanical stiffness of IZZK hydrogels increased up to 300.0 kPa at 21.45 mM (13 mg/mL), that is, significantly higher than earlier reported peptide hydrogels. We assume that the high stiffness of IZZK is due to Cha-Cha cross-strand interactions. The viscosities of all three peptide hydrogels were also in the range of 0.4–0.6 Pa·s, which is suitable for extrusion bioprinters (Figure 2C). To assess the cytocompatibility in 3D, human dermal fibroblasts (hDF) were cultured within the peptide hydrogels, followed by analyzing cell viability, metabolic activity, and morphology. The highly stretched and elongated cells showed well-defined actin fibers (Figure S24) and exhibited high cell viability and metabolic activity. In comparison to 2D cultures, an apparent change in cell morphology was observed, which was also earlier reported. To further examine cell compatibility, assessment of human bone marrow mesenchymal stem cells (BM-MSCs) and human dermal fibroblast cells (hDFn) (Figure 3A) in 3D cultures demonstrated strong cell viability and high proliferation rates up to 14 days, outperforming 2D and matrigel cultures (Figure 3A a,b, Figures S25 and S26, Videos 10–13). Sustained cell surface marker expression and trilineage differentiation proved that cellular properties were not compromised in 3D cultures (Figure S27). To act as an effective 3D cellular growth matrix, hydrogel scaffolds must support cell-matrix interaction and cellular invasion or migration. Compared to cell migration on plain surfaces (2D), cell migration within 3D matrices is a more complex process. Most synthetic hydrogels require functionalization with specific cues such as matrix metalloproteinases, MMPs, degradation sites and integrin-binding domains, in order to facilitate cell migration and invasion. We observed cell-matrix interaction and sustained migration ability of human BM-MSCs cultured in the peptide hydrogels that did not contain any additional functionalities (Figure 3A c,d). During 3D culturing, BM-MSCs retained their long bipolar morphology and were found to migrate out of a fibrin clot, invading the surrounding peptide hydrogel (Figure 3A d). Cells reside in vivo in 3D niches in which different environmental factors interact to support cellular function and fate. The extracellular matrix (ECM) provides key cues to cells. For instance, matrix elasticity was found to direct MSC differentiation without the need for additional induction factors. Similarly, when using pluripotent stem cells, differentiation toward mature functional cells is impaired if appropriate cell substrates are missing. The tunable stiffness of our designed peptide biomaterial, ranging from 3 up to 300 kPa, enables control by mechanical factors. This key feature can be exploited to study the effect of mechanical cues on cellular behavior and direct the differentiation of stem cells into specific lineages.
We further demonstrated the efficacy and potential of the peptide biomaterial as suitable 3D scaffold for other cellular systems. Mouse primary cortical neurons were cultured in 3D peptide hydrogels, and cell growth and cellular properties were assessed against poly-D-lysine (PDL) 2D cultures. Our data clearly show that the peptide hydrogels support viability and promote maturation of primary neurons and neurite outgrowth (Figure 3B a–d, Video 14). A significant increase in neurite length and number of neuronal branches for cells cultured in IIZK and IIFK hydrogels was observed when compared to PDL (Figure 3B d). Interestingly, the observed increase in neurite length and number of branches was higher in neurons cultured in IIZK when compared to IIFK. This could be attributed to the fact that IIZK has lower rigidity (G’ 6.52 ± 0.18 kPa) at the MGC when compared to IIFK (G’ 14.45 ± 1.37 kPa).
Our results confirm the findings of previously published studies where softer matrices were better at promoting neural sprouting, axonal growth, maturation, and differentiation.41−43 Our results give important insights into future developments of functional 3D neuronal models for the study of neurodegenerative disorders. Future studies will investigate gel degradation and remodeling capabilities.44

The cytocompatibility, instant gelation under favorable physiological conditions, and the tunable mechanical properties encouraged us to test the ultrashort peptides as printable bioinks. The printability was tested with our in-house developed robotic arm 3D bioprinter further optimized with a dual-coaxial extrusion nozzle (Figure 4A).45 Since the peptide self-assembly process can be modulated by external factors such as temperature and salt concentration,6,7,10 we considered these factors in the bioprinting system design and parameter optimization. The bioprinter’s extrusion unit housed separate inlets for the peptide solution, the cell solution in 1× PBS, and an additional PBS solution (>1×) (Figure 4A). The optimized dual-coaxial extrusion nozzle allowed a better gradual mixing of the three solutions involved, facilitating the printability.
233 instant gelation without developing cell clogs and backflow pressure. The optimized dual-coaxial extrusion nozzle consisted of three entry points and two merging points to enable thorough gelation and cell suspension. The first merging point allows the mixing of the peptide and PBS solutions while the second merging point, positioned close to the nozzle tip, incorporates the cell with the peptide bioink. This results in better gradual mixing of the three solutions involved, facilitating instant gelation without developing cell clogs and backflow pressure.

The peptide concentration was set to 13 mg/mL for printing, which was found to be most optimal for IIZK and IIZK. As for IIFK, this concentration produced the best possible results with the set parameters. We assessed the print...
quality by looking for gel consistency, filament stiffness, and 3D construct fidelity at increased heights. All peptide solutions were able to print 5 cm³ cubical and up to 4 cm in height cylindrical constructs that instantly solidified with good shape fidelity (Figure 4B, Figure S28, Video 15). In terms of gelation consistency, IZZK produced the most consistent gel, thus indicating a smooth gelation process. Mechanical stability was strongest with IZZK, while IIZK also showed very good stability with occasional ruptures. This was consistent with the reported stiffness data (Figure 2B). To assess resolution, we compared the width of the bioink strands of each peptide. IZZK had the finest resolution with a thickness of approximately 0.1 cm, whereas IIZK and IIFK had a thickness of approximately 0.2 cm (Figure S28). It was also observed that when the peptide concentration was increased to 15 mg/mL, the strand thickness of IIZK and IIFK became finer at around 1 mm due to higher gelation and water encapsulation. However, this in turn increased the possibility of clogging the nozzle so the concentration was optimized at 13 mg/mL.

A filament collapse test was conducted to assess the degree of filament sagging over different sizes of gaps (Figure 4C). Once printed, IZZK maintained its hold across the entire structure with no signs of sagging. IIZK was able to maintain its hold with slight sagging over the 0.8 and 1.6 cm gaps. On the other hand, IIFK maintained its hold over the 0.1, 0.2, and 0.4 cm gaps but sagged when extruded over the 0.8 cm gap. Overall, both IZZK and IIZK demonstrated superb printability by producing clearly defined structures and maintaining consistent layering and continuity of the filament. The inconsistency of IIFK at the used concentration could be because of its slightly higher viscosity as compared to the other peptides or due to its lower mechanical strength. It could also be due to other unmeasured factors, such as yield stress, which has been reported to affect printability for biomaterials with similar viscosities.46

Additional experiments were conducted to evaluate the shape fidelity of taller 3D constructs printed with IZZK and IIZK. Hollow cylindrical constructs of increasing heights were printed with both peptides (Figure 4B c, Figure S29). With IZZK, a construct of 4 cm height (200 layers) was printed without any deformation. The IIZK construct was able to achieve a height of 3.8 cm (180 layers) but with slight sagging. Initial attempts to print a similar structure with IIFK were difficult, however after adjusting the dispensing rate and arm speed, printing a 1.0 cm construct was possible (Figure 4B a).

The printability data of IIZK and IZZK indicates the promising potential of tetrameric peptides when compared to other materials, where a maximum height of 3.5 cm of a similar cylindrical construct was achieved.47 With the remarkable printing capability of the IZZK bioink, a human-like nose construct was successfully printed, hence further demonstrating the peptide’s printability for complex constructs and intrinsically detailed shapes (Figure 4B d).

We subsequently investigated the bioprintability of the newly developed peptide bioinks by printing different cell-laden 3D constructs. This included cuboids with 1.0 cm edges and 0.26 cm height, as well as cylinders with 1.0 cm diameter, 0.1–0.2 cm wall thickness, and 1.0 cm height. Immediately postprinting, a homogeneous cell distribution was observed, indicating the efficacy of our nozzle design in mixing cells with bioinks. The viability of cells was observed over multiple days by live/dead assays. Z-stack confocal images were taken to observe the viability in the entire 3D printed constructs. At day 0, a high percentage of cell viability was observed (>90%), which was preserved over a 24-day observation period (Figure S5A,B, Figure S30). This maintained cell viability rate was either better or comparable to other bioinks such as GelMA,46 alginate/nanocellulose, and kCA–nanosilicate.50

The novelty of our bioink stems from its instant gelation potential, eliminating the need for harmful cross-linking steps or holding gel resins as required by other bioinks.46,51,52 The instant gelation potential, in addition to the low viscosity of the proposed peptide bioinks, contributed to the observed high cell viability rate after bioprinting. The high cell viability rate confirms that the shear stress generated during the bioprinting process exerts a negligible effect on cell viability, further illuminating the potential of our bioprinting system.

Analysis of cell viability within deeper areas of the 3D printed cylinder structures revealed a high percentage of viable cells (Figure S31). However, higher cell density was observed on the surfaces of the structure, which might be due to enhanced nutrition supply on these surfaces. We observed a change in the visual appearance and also stiffness of the printed scaffolds between day 0 (translucent) and 30 (opaque, stiffer), indicating the deposition of ECM components by printed cells (Figure 4B e,f).

To better analyze the long-term biocompatibility of 3D bioprinted scaffolds in terms of 3D cell distribution, cell morphology, and cell-matrix interaction, cytoskeleton staining was performed. Using IIFK peptide bioink, a uniform distribution of cells was observed throughout the printed constructs (Figure 5A). While using IZZK, cells were found to be more aligned growing along the printed peptide threads. Additionally, in all peptide bioinks the cells were well spread, and the actin fibers were well-defined. Cells also retained a long bipolar spindle morphology with dense 3D multilayered networks, indicating cell–cell contact and cell–matrix interaction (Figure 5A, Videos 16–18). These results were similar to those obtained from manual 3D culture, pointing to the suitability of the peptide bioinks for 3D bioprinting even at high concentrations. SEM and TEM images of the printed peptide constructs (Figure 5C) and printed cell-laden constructs (Figure 5D), clearly demonstrate the alignment of fibers and the interaction between cells and the matrix. Additionally, TEM images revealed an alignment of printed cells with the bioink fibers (Figure 5D e,f). Suitable bioprintable scaffolds in which cells can grow, produce their own extracellular matrix, and mimic functional tissue is an essential requisite for regenerative medicine and tissue engineering. Using IZZK as bioink, we induced chondrogenic differentiation of hBM-MSCs constructs post-printing. After 4 weeks of differentiation induction, we characterized the printed constructs for differentiation and deposition of ECM components. Using immunofluorescent staining, we identified cartilage-specific molecules, including collagen II (Figure 5E). We observed a dramatic change in the appearance and texture/nature of the printed scaffold upon differentiation (Figure 5F a, Video 19). The differentiation was further confirmed by glucose amino glycan (GAG) formation which is an essential component of cartilage ECM using Alcian blue staining. Gene expression analysis of cartilage-related genes, including collagen II and aggrecan, demonstrated a high expression level and a significant difference in chondrogenic differentiated constructs compared to undifferentiated control (Figure 5F b). Additionally, collagen II was overexpressed expression in the printed constructs.
In summary, we have rationally designed three self-assembling tetrapiptides and have demonstrated their excellent tunable mechanical properties, making them highly suitable as robust bioinks for 3D bioprinting. Compared to other bioinks, these peptide bioinks overcome severe limitations in current bioprinting procedures, avoiding cell compromising abrasive conditions such as chemical treatments or UV-cross-linking during the printing process. The lysine-containing ultrashort peptides self-assemble into stable, rigid scaffolds with unprecedented stiffness of up to 300 kPa. They have the capability to be used as superior peptide bioinks because they are biocompatible, bodylike, but synthetic, and support an automated cell printing process. As a proof-of-concept, the peptide bioinks confirmed an instant solidifying cell-embedding 3D bioprinting process under physiological conditions and at a cost-effective low bioink concentration. We successfully created tissue scaffolds and showed that during the challenging printing process the cell-containing bioinks protected and maintained the integrity of the cellular constructs over several weeks. In addition, we successfully induced chondrogenic differentiation of bioprinted human mesenchymal stem cells, further indicating the strong potential of the peptide bioinks for automated complex tissue fabrication.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.0c04426.

Materials and Methods, Figure S1–S32, and Table S1–S11 (PDF)

Simulation on Assembly of 60 IIFK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 60 IIZK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 60 IZZK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 2 IIFK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 2 IIZK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 2 IZZK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 4 IIFK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 4 IIZK peptides in a period of 100 ns (AVI)

Simulation on Assembly of 4 IZZK peptides in a period of 100 ns (AVI)

Viability of hBM-MSCs cultured in IIFK at day 1 (AVI)

Viability of hBM-MSCs cultured in IIZK at day 4 (AVI)

Viability of hBM-MSCs cultured in IIZK at day 1 (AVI)

Viability of hBM-MSCs cultured in IIZK at day 4 (AVI)

Primary mouse cortical neurons cultured in IIFK at day 3 (AVI)

Demonstration of the printing process (AVI)

Z stack confocal microscopy images of cell-laden 3D printed structure using IIFK (AVI)

Z stack confocal microscopy images of cell-laden 3D printed structure using IIZK (AVI)

Z stack confocal microscopy images of cell-laden 3D printed structure using IZZK (AVI)

Demonstration of elasticity change of printed cell-laden construct after chondrogenic differentiation (AVI)

**Author Contributions**


**Author Contributions**

The peptides were synthesized and purified by H.H.S. and characterized by H.H.S., M.M., and A.H.E. R.G. did all of the MD simulations. *In vitro* cell studies were performed by D.A., S.Abdelrahman, and S.Alshehri, K.K., Z.K., and D.A. conducted.


