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Dynamic peptide-folding mediated biofunctionalization and modulation of hydrogels for 4D bioprinting

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Abstract
Hydrogels are used in a wide range of biomedical applications, including three-dimensional (3D) cell culture, cell therapy and bioprinting. To enable processing using advanced additive fabrication techniques and to mimic the dynamic nature of the extracellular matrix (ECM), the properties of the hydrogels must be possible to tailor and change over time with high precision. The design of hydrogels that are both structurally and functionally dynamic, while providing necessary mechanical support is challenging using conventional synthesis techniques. Here, we show a modular and 3D printable hydrogel system that combines a robust but tunable covalent bioorthogonal cross-linking strategy with specific peptide-folding mediated interactions for dynamic modulation of cross-linking and functionalization. The hyaluronan-based hydrogels were covalently cross-linked by strain-promoted alkyne-azide cycloaddition using multi-arm poly(ethylene glycol). In addition, a de novo designed helix-loop-helix peptide was conjugated to the hyaluronan backbone to enable specific peptide-folding modulation of cross-linking density and kinetics, and hydrogel functionality. An array of complementary peptides with different functionalities was developed and used as a toolbox for supramolecular tuning of cell-hydrogel interactions and for controlling enzyme-mediated biomineralization processes. The modular peptide system enabled dynamic modifications of the properties of 3D printed structures, demonstrating a novel route for design of more sophisticated bioinks for four-dimensional bioprinting.

1. Introduction

Hydrogels are water-swollen polymer networks that can mimic mechanical, structural and chemical properties of the extracellular matrix (ECM) [1]. ECM-mimicking hydrogels are used for both fundamental studies of cell-ECM interactions [2] and for numerous biomedical applications [3], such as tissue engineering [4], three-dimensional (3D) cell culture [5], and cell-based therapies [6, 7]. ECM-mimicking hydrogels further facilitates the development of human ex vivo tissue and disease models [8], including organs-on-chips [9], for cancer research [10], drug screening [11], and toxicology [12]. This can reduce both the need for animal models as well as time and costs in drug development [13]. Progress is also boosted by the rapid development of 3D bioprinting technologies that enables fabrication of more complex tissue mimetic constructs, comprising of multiple cell types organized in spatially defined structures [14, 15]. The improvements in 3D cell culture strategies and 3D bioprinting put new requirements on hydrogels, both with respect to ECM mimicking capabilities and processability [16, 17].

A large number of different biomolecules and synthetic polymers, such as collagen [18] and collagen
mimetic peptides [19], cellulose [20], alginate [21], hyaluronan [3], and poly(ethylene glycol) (PEG) [22], are commonly used as components in ECM-mimicking hydrogels. The polymer networks can be cross-linked by either covalent bonds or by non-covalent supramolecular interactions [23]. The latter strategy results in supramolecular hydrogels that can self-assemble under benign conditions, and show shear thinning and self-healing properties [24], which facilitates both bioprinting and cell encapsulation. In particular, alginate hydrogels, crosslinked by Ca$^{2+}$, have been widely used in this context [25, 26]. Other supramolecular hydrogel systems based on host-guest recognition [27], oligonucleotide-hybridization [28], peptide-folding [29], and protein-ligand interactions [30], have also been explored. However, because of the reversibility of the interactions involved in the cross-linking [31], supramolecular hydrogels tend to dissociate over time, which can complicate long-term cell culture experiments and result in poor shape fidelity during bioprinting. Covalently cross-linked hydrogels, on the other hand, are typically more robust and can demonstrate a wider range of viscoelastic properties [32]. Unfortunately, many conventional strategies for covalent cross-linking suffer from problems with cross-reactivity with cell surface receptors, or require precursors, initiators or catalysts that are cytotoxic or result in the generation of harmful by-products, which can have a negative impact on cell viability [23, 33]. Development of bioorthogonal chemistries [34], such as strain-promoted alkyne-azole 1,3-dipolar cycloaddition (SPAAC) [35], have in recent years emerged as promising techniques for covalent cross-linking of hydrogels that circumvent these issues. SPAAC does not require any additional reagents or catalysts to be initiated and show no or limited cross-reactivity with biomolecules, which facilitate cell encapsulation [36, 37]. Irrespectively, covalently cross-linked hydrogels can be difficult to bioprint and often fail to mimic the dynamic nature of the ECM [17]. The native ECM is not static but constantly subject to change as a result of specific cell-matrix interactions and interaction of various endogenous biomolecular cues, including association and dissociation of growth factors and other proteins to ECM components [38]. The integration of time as a parameter in hydrogel design allows for controlling changes in shape or function of the materials [39]. The possibility to dynamically modify the properties of hydrogels can enable fundamental studies of cell-matrix interactions with temporal resolution [40], and is critical for development of stimuli-responsive structures and four-dimensional (4D) bioprinting strategies [41, 42].

In the current work, we have developed a novel strategy for 4D bioink development based on a modular peptide-polymer hybrid hydrogel system that combines the structural and mechanical robustness of bioorthogonal covalent cross-linking with specific supramolecular interactions that enable dynamic tuning of both mechanical properties and the biochemical functionality of the hydrogel. The hyaluronan (HA) and PEG-based hydrogel polymer network was covalently cross-linked by SPAAC by modifying HA with a bicyclo[6.1.0]nonyne (BCN) to enable bioorthogonal covalent cross-linking using an eight-armed PEG with terminating azides (p(NH)$_3$) (figure 1(a)) [37]. In addition, a helix-loop-helix polypeptide (JR2EK), designed to fold into a helix-loop-helix motif and dimerize into four-helix bundles, was also conjugated to the HA backbone [43]. The peptides facilitate dynamic modulation of multiple properties via specific peptide-folding mediated interactions, including supramolecular cross-linking, tuning of cell-hydrogel interactions, and specific recruitment of active enzymes. In addition, the combined possibility to control the cross-linking kinetics and density by exploiting the temperature-sensitive SPAAC reaction [37] and peptide-dimerization, enabled bioprinting of intricate 3D structures using the freeform reversible embedding of suspended hydrogels (FRESH) technique [44]. The homodimerization and folding of the conjugated peptides further allowed for changes to the cross-linking density of the hydrogels, and thus the size and mechanical properties of the bioprinted structures. Peptide heterodimerization [45], on the other hand, was utilized to dynamically sequester alkaline phosphatase, triggering mineralization of the bioprinted hydrogels.

Combining bioorthogonal covalent cross-linking with dynamic and specific peptide-folding mediated supramolecular functionalization strategies can enable fabrication of modular ECM-mimicking hydrogels that can be tailored and dynamically modified for a wide range of applications, including 4D bioprinting, and facilitate fundamental studies of both cell- and protein-matrix interactions.

2. Materials and methods

**General:** Eight-armed poly(ethylene glycol), Mw 10 kDa, (supporting figure S1(b) (available online at stacks.iop.org/BF/12/035031/mmedia)), denoted in the paper as p(NH)$_3$ was acquired from Creative PEGWorks (USA). Hyaluronan (Mw 100–150 kDa) was acquired from Lifecore Biomedical (USA). All other chemicals were purchased from Sigma Aldrich unless otherwise noted and used without further purification.

**Peptide synthesis:** All amino acids were bought from Iris biotech GmbH. All peptides were synthesized using standard fluoroethylmethoxycarbonyl (Fmoc) protected amino acids (4 equiv) with O-(7-benzotriazole-1-yl)-1, 3, 3-tetramethyluronium tetra-fluoroborate (TBTU, 4 equiv) as activator and diisopropylamine (DIPEA, 8 equiv)
as base. Removal of the Fmoc groups were accomplished by treatment with piperidine (20% in DMF, v/v). The peptides JR2K and JR2EK-Az were synthesized as described earlier [43]. JR2 KK (H2N-NAADLKKAIKALKHLKAGPKDAAQILKQ AFKAFKRAO-COOH) was synthesized on an Fmoc-Gly-Wang resin in 0.1 mmol scale using a Quartet automated peptide synthesizer from Protein Technologies, Inc. The peptides were synthesized with an allylcarboxycarbonyl (Alloc) protected Lys residue in position 22 to allow for site specific functionalization. Orthogonal deprotection was performed using tetakis(triphenylphosphine) palladium(0) ((Pd(PPh3)4) dissolved in chloroform: acetic acid: morpholine (85:10.5, v/v/v) in an N2 atmosphere for 2 h. The deprotected peptides were washed sequentially with diethylthiocarbamic acid (20 mM in DMF) and DIPEA (30 mM in DMF) follow by DMF, DCM and finally desiccated. JR2EK was functionalized with 3-azidopropionic acid as described previously [43]. JR2KK (0.005 mmol) was reacted with Biotin NHS ester (0.05 mmol, Sigma Aldrich), Cyanine3 (Cy3) NHS ester (0.005 mmol, Lumiprobe) or Cyanine5 (Cy5) NHS ester (0.005 mmol, Lumiprobe) and DIPEA (0.02 mmol) in dry DCM overnight to afford JR2KK-Biotin, JR2KK-Cy3 and JR2KK-Cy5. After post-synthesis modifications the N-terminal Fmoc group was removed and the peptides were cleaved. JR2KK-cRGD (0.1 mmol) was synthesized by treating the Fmoc deprotected N-terminal in JR2KK twice with Boc2O (1 mmol) and DIPEA (1 mmol) in DCM for 1 h prior to orthogonal deprotection. After orthogonal deprotection of the Alloc group on Lys22 the peptide was sequencially reacted with Fmoc-L-Glu-OAll, Fmoc-D-Phe-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-Gly-OH and Fmoc-L-Asp(tBu)-OH, Fmoc-D-Ala-NH2. All couplings were performed twice with a fourfold excess of amino acids and TBTU and an eightfold excess of DIPEA in DMF for 2 h. The OAll group on Glu was removed by treating the resin twice with Pd(PPh3)4 using the same protocol as above. After Fmoc removal the branched peptide was cyclized by first incubating the resin in HOBr (1 M in DMF) for 30 min and then reacting it with PyBOP (0.5 mmol), HOBt (0.5 mmol) and DIPEA (1 mmol) overnight. All peptides were cleaved from their solid support using a mixture of trifluoroacetic acid (TFA), triisopropylsilane and water (95:2.5:2.5, v/v/v) for 2 h before being filtered, concentrated and precipitated twice in cold diethylether. The crude peptides were purified on a reversed phase column (XBridge Phenyl, Waters) with an aqueous gradient of acetonitril containing 0.1% TFA. Purity was confirmed by analytical HPLC using a reversed phase column (XBridge Phenyl, Waters) with an aqueous gradient of acetonitril containing 0.1% TFA (figure S12(a)). Mass identity of all peptides and post synthetic modifications were confirmed by MALDI-ToF MS (Applied Biosystems) running in linear positive mode using o-cyano-4-hydroxycinnamic acid as matrix (figure S12(b)).

Synthesis of HA-BCN and HA-BCN/JR2EK: The polymers HA-BCN and HA-BCN/JR2EK were synthesized as described previously [43]. In short, to a solution of HA (200 mg, Mw 100–150 kDa, Lifecore Biomedical) in MES buffer (20 ml, 100 mM, pH 7), BCN-NH2 (50 mg, 0.15 mmol, Sigma Aldrich) was dissolved, followed by EDC (118 mg, 0.6 mmol) and HOBr (42 mg, 0.3 mmol). The reaction was allowed to proceed for 24 h in room temperature and the crude product was then exhaustively dialyzed (MW cutoff 12–14 kDa, Spectra/Por RC, Spectrum Laboratories) to afford HA-BCN with a degree of modification of 7% based on 1H-NMR (figure S2). HA-BCN/JR2EK was prepared by allowing JR2EK-Az (61 mg, 0.01 mmol) to react with HA-BCN (100 mg) dissolved in MES buffer (10 ml, 100 mM, pH 7) for 24 h in room temperature. The crude product was then exhaustively dialyzed (MW cutoff 12–14 kDa, Spectra/Por RC, Spectrum Laboratories) to afford HA-BCN/JR2EK with a degree of modification of 4% based on 1H-NMR signal originating from the aromatic residue's histidine and phenylalanine (figure S2).

Formation of hydrogels: HA-BCN/JR2EK or HA-BCN was mixed with p(N3)s at a volume ratio of 5:1 HA-BCN/JR2EK:p(N3)s (estimated BCN:N3 ~1:2) at 1.5–2.5% (w/v) total polymer concentration and added on top of a hydrophobic piece of parafilm. Except for the gelation experiments and if nothing else is stated, all hydrogels were allowed to gel at 20°C for 24 h prior further experiments.

Oscillatory rheology measurements: The rheological properties of the hydrogels were assessed with a Discovery HR-2 rheometer from TA instruments. Gelation kinetics studies were conducted with a 20 mm 1° cone geometry at 1% strain and 1 Hz oscillatory frequency. Samples were prepared in situ on the rheometer by mixing all components and quickly lowering the geometry. The gelation point was defined as G’ = G”. A solvent trap was used for all gelation time studies. Fully formed hydrogels were examined with (1) frequency sweeps at fixed strain of 1%, and (2) amplitude sweeps at fixed oscillatory frequency of 1 Hz using an 8 mm plate geometry. To adjust for any variability of thickness between the hydrogels, the axial force was set to be within 0.2–0.4 N for all measurements. All samples were run in at least duplicates.

Swelling ratio: Preformed hydrogels (2.5% (w/v)) were incubated in excess Bis-Tris buffer (30 mM) at pH 7, pH 4 or with Zn2+ (10 mM) to swell. After 24 h, the swelled hydrogel was weighted (Ww) and sequentially dried to acquire the dry weight (Wd). The swelling ratio was determined by equation (1):

\[
\frac{W_w - W_d}{W_d} \quad (1)
\]

All samples were run in triplicates.
Post-modification with JR2KK-Cy5: Preformed hydrogels (2.5% (w/v)) were added to 1 ml PBS (10 mM PO₄⁻, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) to swell for an additional 24 h. Subsequently, the swollen hydrogels were transferred to 1 ml PBS with JR2KK-Cy5 (1 µM) and allowed to incubate. After 24 h the solution was collected, and absorption spectra were recorded to estimate the amount of JR2KK-Cy5 that had been taken up by the hydrogels.

Post-modification with JR2KK-Biotin: ALP-streptavidin: Preformed hydrogels (2.5% (w/v)) were added to Bis-Tris-buffer (30 mM, pH 7.0) to swell. After 24 h, the hydrogels were transferred to 1 ml Bis-Tris containing 1 µM JR2KK-Biotin (or control with 1 µM JR2K). After 2 h, the hydrogels were extensively washed (with 1 ml Bis-Tris, 3 × 5 min incubation) to remove any unbound peptides. Sequentially, 1 ml Bis-Tris with ~200 nM ALP-streptavidin were added to the hydrogels and incubated for 10 min. After extensive washing washed (with 1 ml Bis-Tris, 3 × 5 min incubation) the hydrogels were transferred to a phosphate-containing buffer (BCIP-buffer or Calcification-buffer, see below).

BCIP experiment: Hydrogels pre-modified with ALP (see above) were added to BCIP-buffer (5-Bromo-4-chloro-3-indolyl phosphate, ~0.7 mM in Bis-Tris pH 7.0). After 2 h the hydrogels were transferred to Bis-Tris-buffer to quench the reaction and left for 24 h prior analysis. The chromogenic response was measured with a microplate photometer (CLARIOstar Plus from BMG Labtech) at 600 nm.

Bionaneralization: Hydrogels pre-modified with ALP (see above) were added to calcification-buffer (50 mM Calcium glycerophosphate and 30 mM Bis-Tris, pH 7.4). The calcification process was monitored over time with an optical microscope (Nikon Eclipse TI) and compared against a sample incubated with JR2K instead of JR2KK-Biotin. Z-stacks (22 images, 50 µm steps) were focus-stacked using Adobe Photoshop CC (20.0.3 Release). At day 5 the calcified hydrogels were prepared for SEM-analysis. The samples were chemically dried in increasing concentration of EtOH (50%, 70%, 80%, 90%, 100%, 10 min each) and finally twice in hexamethyldisilazane (10 min each). The samples were left in a desicator overnight prior to SEM-analysis. The SEM-analysis was conducted with LEO 1550 Gemini from Zeiss operated at a voltage of 5 kV. An elemental analysis of the samples was performed with energy dispersive x-ray spectroscopy (EDS) (X-Max EDS System Detector, Oxford Instruments) using Aztec 3.1 (Oxford Instruments). The operating voltage for EDS analysis was set to 20 kV.

Cell culture: Human hepatocyte carcinoma cells (HepG2) were acquired from ATCC and expanded in cell culture medium consisting of Dulbecco’s Modified Eagle Medium (DMEM, VWR) with 10% fetal bovine serum (FBS, Sigma–Aldrich), 1% Penicillin-Streptomycin (Sigma–Aldrich) and 1% non-essential amino acids (Sigma–Aldrich). Human pluripotent stem cell (hPSC) derived cardiac bodies were prepared at the Hannover Medical School, Hannover, Germany (Dr. Robert Zweigerdt’s lab) as previously described [46–48], and cultured in RPMI1640 (LifeTechnologies) supplemented with B27 minus insulin (LifeTechnologies). Dry powders of HA-BCN/JR2EK and p(N₃)₈ were UV-sterilized (60 000 J cm⁻²) and subsequently dissolves in DMEM when used for HepG2 cells or RPMI1640 when used for cardiac bodies. Prior to encapsulation of HepG2, the cells were dislodged via trypsinization and pelleted via centrifugation. Cells were resuspended in cell culture medium and counted. 25 000 cells were suspended in a 2.5% (w/v) p(N₃)₈-solution (5 µl) that sequentially were added to 2.5% (w/v) HA-BCN/JR2EK (25 µl). After thorough mixing and 5 min incubation, the hydrogel-mixture with encapsulated cells was added to a custom mold (glass plates with a separation of 4.7 mm) and incubated (37 °C, 5% CO₂) for 1 h. Subsequently, the hydrogels were removed from the mold and placed in fresh DMEM in a 96-well plate to allow the hydrogel to swell and for further incubation. Cell viability was assessed using a Live/Dead® Viability/Cytotoxicity Kit (Thermo Fisher). Samples were imaged with a confocal microscope (Zeiss LSM 700). Human neuroblastoma cells (SH-SY5Y) were acquired from ATCC and expanded in cell culture media consisting of Dulbecco’s Modified Eagle Medium (DMEM) and Ham’s F-12 Nutritional Mixture (F-12, VWR) in a 1:1 ratio, with 10% fetal bovine serum (FBS, Sigma–Aldrich), 1% Penicillin-Streptomycin (Sigma–Aldrich) and 1% non-essential amino acids (Sigma–Aldrich). Dry powders of HA-BCN/JR2EK and p(N₃)₈ were UV-sterilized (60 000 J cm⁻²) and subsequently dissolved in DMEM/F12 1:1 before allowing to swell overnight in cell media. The cell media was aspirated and peptides of JR2K or JR2KK-cRGD were added to the surfaces. PBS was added as a negative control. These were incubated for four hours (37 °C, 5% CO₂) and then washed very gently with PBS. Cells (SH-SY5Y) were added to the surface of the hydrogel, 40 000 in 100 µl of cell media, and the plate placed in the incubator (37 °C, 5% CO₂). Cell viability was assessed using a Live/Dead® Viability/Cytotoxicity Kit (Thermo Fisher) and using Alamar blue (Thermo Fisher) as a measurement of the reducing power of live cells. Cells were imaged with a confocal microscope (Zeiss LSM 780).

Gradient fabrication: Hydrogels consisting of HA-BCN/JR2EK and p(N₃)₈ (volume ratio of 5:1) were formed within the central chamber of µ-slide Chemotaxis channel slide (ibidi GmbH, Gräfelfing,
Germany) by incubating for 1 h at 37 °C. PBS was added to both reservoirs to allow the hydrogel to swell and to equilibrate overnight. The PBS was gently aspirated and then replaced with the corresponding Cy3(3/5) fluorophore conjugated JR2KK peptide. Imaging was carried out using a Nikon Ti-Eclipse fluorescence microscope. The excitation wavelength was 550–570 nm and 630–650 nm for Cy3 and Cy5 respectively. The emission was recorded over the interval 580–640 nm and 660–695 nm for Cy3 and Cy5, respectively.

3D bioprinting: 3D printing was conducted using the freeform reversible embedding of suspended hydrogels (FRESH) technique [44]. A slurry of gelatin was made by dissolving 4.5% (w/v) gelatin in 30 mM Bis-Tris buffer pH 7 in the presence or absence of 10 mM ZnCl₂ and incubated at 4 °C for 12 h to solidify. The the jar was topped up with buffer and the gel was vigorously mixed for 120 s using a kitchen blender. The resulting slurry was transferred to 50 ml centrifuge tubes and the gel particles were pelleted by centrifugation at 1500 rpm for 4 min at 4 °C. The pellet was washed with cold (4 °C) buffer (30 mM Bis-Tris buffer pH 7 ± 10 mM ZnCl₂). This process was repeated five times to completely remove any dissolved gelatin from the solution. The bioinks were prepared by mixing p(N₃)₈ (25 mg ml⁻¹) and HA-BCN/JR2EK (25 mg ml⁻¹) at a 5:1 volume ratio and incubated on ice for 5 min and then transferred to a 3 ml syringe affixed to a 27-gauge blunt end needle. The syringe was loaded into a Bio X bioprinter from Cellink (Gothenburg, Sweden). The printing head and the bed temperature were set to 8 °C. The structures were printed inside the gelatin slurry using 5 kPa pressure and 5 mm s⁻¹ printing speed. After printing, the structures were incubated at room temperature in the slurry for 1 h. Then structures were harvested by dissolving the bath by heating to 37 °C and washed in buffer. Human dermal fibroblasts (0.25% (w/v)) were mixed at room temperature in high glucose DMEM with 10% fetal bovine serum and 1% Penicillin-Streptomycin. The cell-p(N₃)₈ suspension (8 µl) was mixed with 40 µl of HA-BCN/JR2EK and incubated (37 °C, 5% CO2) for 5 min to initiate the cross-linking. The fibroblast containing hydrogel was printed on glass coverslips using a 27-gauge blunt end needle with a printer speed of 4 mm s⁻¹ and a pressure ranging from 1 to 5 kPa. The printed structures were incubated at 37 °C and 5% CO₂ for 20 min prior to addition of 3 ml of high glucose DMEM with 10% fetal bovine serum and 1% Penicillin-Streptomycin and incubated for 18 h. Media was removed and cell viability was investigated using Live/Dead® Viability/Cytotoxicity Kit (Thermo Fisher). The cells were imaged with a widefield microscope (Zeiss Axiovert) with a Zeiss HRm camera and a 20x/0.4 objective.

Statistics: Data presented are shown as mean ± S.D. For statistical analysis paired sample t-test using Origin version 9.0.0 was used. In graphs, * indicates a statically difference of P < 0.05 and ** indicates a statically difference of P < 0.01.

3. Results and discussion

3.1. Hydrogel fabrication

To allow for bioorthogonal SPAAC reaction with azide-moieties, HA was first modified with BCN groups (HA-BCN) using carbodiimide chemistry (supporting figure S1) as previously described by us [37, 43]. The degree of modification was estimated as ~7% based on ¹H-NMR signals originating from the BCN group (supporting figure S2). The azide-modified polypeptide JR2EK(N₃)₈ was then conjugated to HA-BCN yielding HA-BCN/JR2EK (figure S1, supporting information). The azide moiety in JR2EK(N₃)₈ was included in the loop-region of the polypeptide to minimize the influence of conjugation on dimerization and folding. The design of the polypeptide JR2EK was based on the helix-loop-helix polypeptide JR2EC, and has previously been explored for self-assembly of HA-based hydrogels [43]. The degree of polypeptide conjugation to HA (~4%) was estimated based on the ¹H-NMR signals originating from histidine and phenylalanine residues in the polypeptide (supporting figure S2). Roughly half of the initial BCN groups were thus left unreacted and could be used for covalent cross-linking of HA-BCN/JR2EK using the SPAAC reaction. Covalent cross-linking was achieved by mixing HA-BCN/JR2EK with p(N₃)₈, resulting in the formation of a fully transparent hydrogel HA-BCN/JR2EK-p(N₃)₈ (figure 1(b)).

To determine a suitable polymer ratio between HA-BCN/JR2EK and p(N₃)₈, the two components were mixed at different ratios and the change in viscoelastic properties caused by gelation was monitored with oscillatory rheology. A HA-BCN/JR2EK-p(N₃)₈ polymer ratio of 5:1 (with 2.5% w/v polymer stock-solutions) was found to generate hydrogels with the most rapid gelation kinetics and highest storage modulus (G’) after 2 h (supporting figure S3). The 5:1 polymer ratio was thus used in all future experiments. Furthermore, using this ratio, different total polymer concentrations (1.5–2.5% (w/v)) were mixed at room temperature (~20 °C) and the rheological response was evaluated. The G’ after 2 h ranged from 2 to 150 Pa (figure 1(g), table 1). The gelation point, i.e. when G’ = G”, occurred within 20 min after mixing the components at concentrations of 2.0–2.5% (w/v). When lowering the concentration to 1.5% (w/v) the gelation was slower, and the gelation point was reached after approximately 100 min. No further increase in G’ was seen after 24 h incubation, with G’ values ranging from 200 to 500 Pa (figure 1(c), table 1, supporting figure S4). The mechanical properties of the hydrogels were thus in a biologically relevant range for mimicking soft tissues [49].
Figure 1. (a) Schematic representation of the bioorthogonal cross-linked hydrogels. Upon mixing of HA-BCN/JR2EK and p(N3)8, the BCN and azide groups react via a strain-promoted alkyne-azide 1,3-dipolar cycloaddition to form a stable 1,2,3-triazole. (b) Photograph of a HA-BCN/JR2EK-p(N3)8 hydrogel (2.5% (w/v)). (c) Frequency sweeps at 1% strain of hydrogels after 24 h of gelation. Rate of change in storage (G’) and loss modulus (G”) during the bioorthogonal cross-linking of HA-BCN/JR2EK with p(N3)8, showing the difference in gelation kinetics during the initial 60 min of gelation at 20 and 37 °C for (d) 2.5% (w/v), (e) 2.0% (w/v), and (f) 1.5% (w/v). (g) Rheological assessment of gelation kinetics, showing an increase in storage modulus during the initial 2 h of gelation at 20 °C.

Table 1. Storage modulus (G’) of the HA-BCN/JR2EK-p(N3)8 hydrogels at different time points and temperatures, and gelation time at 20 °C and 37 °C.

| Concentration (w/v) | 2 h at 20 °C (G’ (Pa)) | 60 min at 37 °C (G’ (Pa)) | 24 h at 20 °C (G’ (Pa)) | Gelation time (min) 20 °C | Gelation time (min) 37 °C |
|---------------------|-------------------------|---------------------------|-------------------------|---------------------------|---------------------------|
| 2.5 %               | 146 ± 43                | 260 ± 27                  | 490 ± 88                | 15                        | 3                         |
| 2.0 %               | 69 ± 16                 | 150 ± 44                  | 370 ± 36                | 19                        | 10                        |
| 1.5 %               | 2.5 ± 0.3               | 18 ± 4                    | 190 ± 86                | 97                        | 25                        |

3.2. Cell encapsulation

The possibility to control the viscosity and gelation kinetics of hydrogels is critical for both 3D cell encapsulation as well as 3D bioprinting [50]. Although the gelation time for the HA-BCN/JR2EK-p(N3)8 hydrogels was fairly rapid (20–100 min) for the concentrations tested, it was not as fast as many supramolecular hydrogels, which can reach their maximum G’ within seconds [29, 51]. Other SPAAC-based hydrogels have also shown a more rapid gelation [34, 50], although they typically have a higher degree of BCN and azide modification. However, by increasing the temperature to 37 °C the gelation rate could be greatly enhanced (figures 1(d)–(e), table 1). At 2.5% (w/v) and 37 °C the gelation point was reached within 3 min, and at 2.0% (w/v) it was reached within 10 min. At 1.5% (w/v) the gelation time was reduced to 25 min, as compared to 97 min at 20 °C. Likely, this is a result of the faster diffusion of the polymers and polymer segments at elevated temperatures, thus increasing the collision frequency and the probability that a BCN and an azide moiety will align correctly to react. To confirm that it was possible to encapsulate cells in 3D, and that the hydrogel components were cytotocompatible, human hepatocyte carcinoma cells (HepG2) and human pluripotent stem cell (hPSC) derived cardiac spheroids (cardiac bodies, CBs) were encapsulated and cultured in the hydrogels. 3D cell culture of both HepG2 and CBs are of interest for in vitro drug screening and toxicology testing [52]. It has previously been shown that HepG2 express improved liver-like properties when cultured in 3D [12]. In addition, HA is abundant in the ECM of the heart where it is involved in cardiac development during embryogenesis and healing after myocardial infarction, for example [53]. To encapsulate the cells in the hydrogel, the cells were premixed with the hydrogel components (2.5% (w/v)) and allowed to cross-link for 1 h at 37 °C, and then submerged in cell culture medium. The HepG2 cells showed high viability in the hydrogels and an increase in cell density and formation of spheroids occurred over a time course of seven days (supporting figures S5(a) and (b)). We have previously seen that HepG2 cells tend to form...
spheroids in HA-based hydrogels lacking cell adhesion motifs [37]. The diameter of the spheroids at day 7 was in the range of 80 µm, a size that is still sufficient for effective oxygen diffusion into the center of the spheroid [54]. Furthermore, the viability of the HepG2 remained high after 7 d and very few dead cells could be seen in, or close to, the spheroids (supporting figures S5(c)–(f)). The CBs showed high viability with characteristic beating several days after encapsulation in the hydrogels (figures 2(a)–(c), supporting movie 1). Initially separated CBs were able to self-assemble into larger structures during the time course of days and synchronize their beating. The bioorthogonal SPAAC reaction thus allows for encapsulation and 3D culture of both HepG2 cells and CBs, promoting high cell viability and maintaining characteristic cell functions.

3.3. Supramolecular cross-linking
In addition to the bioorthogonal covalent cross-linking of the hydrogels, the JR2EK polypeptide conjugated to the HA can provide supramolecular cross-links that can assist in the formation and contribute to the final viscoelastic properties of the hydrogel (figure 3(a)). JR2EK is designed to fold into a helix-loop-helix motif and homodimerize into four-helix bundles at acidic pH (pH < 6) or in the presence of Zn\(^{2+}\) [55]. The isoelectric point (pI) of JR2EK is 4.5 and protonation of Glu residues at the dimer interface readily promote homodimerization in a pH range close to the pI. To assess the impact of polypeptide folding and dimerization on the HA-BCN/JR2EK-p(N\(_3\))\(_8\) hydrogel properties, the gelation kinetics was examined at both acidic pH (pH 4.5) and after addition of Zn\(^{2+}\) (10 mM) to HA-BCN/JR2EK and p(N\(_3\))\(_8\) (2.5% (w/v), 20 °C). As expected, the addition of Zn\(^{2+}\) gave an instant increase in G' from ~1 to ~100 Pa (figure 3(b), table 2). This is in sharp contrast to the gradual and slower increase in G' seen when relying on the SPAAC cross-linking alone. Furthermore, the gelation kinetics and initial storage modulus was similar to fully supramolecular hydrogels obtained by the Zn\(^{2+}\)-triggered and folding-dependent self-assembly of JR2EK when conjugated to HA [43]. After the initial rapid supramolecular peptide-mediated cross-linking of the hydrogels, a slower increase in G' over time indicates the formation of additional covalent cross-links. Interestingly, the combined covalent and supramolecular cross-linking resulted in hydrogels with a significantly higher loss modulus (G") as compared to hydrogels that were only covalently cross-linked (table 2). The higher G" implies that the resulting hydrogels can dissipate more energy as heat, or reform when subjected to stress and strain, making them less brittle as compared to covalently cross-linked hydrogels. A similar response could be seen when triggering peptide dimerization by reducing the pH to 4.5, albeit with a less dramatic initial increase in G' (figure 3(c), table 2). Both strategies for triggering peptide homodimerization resulted in a distinct hydrogel deswelling (figure 3(d)) and a decrease in swelling ratio (figure 3(e)) as a result of the increase in cross-linking density.

In control experiments using hydrogels based on HA-BCN, i.e. HA without conjugated JR2EK, no significant change in the viscoelastic properties upon addition of Zn\(^{2+}\) or in acidic pH were observed (supporting figure S6). Hence, the results imply that it is possible to simultaneously cross-link the hydrogel by both covalent and supramolecular interactions. The supramolecular cross-links, however, appear to form on a much shorter timescale than the covalent bonds, providing efficient means to instantly tune the viscoelastic properties and the kinetics of hydrogel formation. Moreover, by combining robust covalent interactions that can provide elasticity with weaker and more dynamic supramolecular cross-links, it is possible to create hydrogels with a wider repertoire of mechanical properties.
Figure 3. (a) Schematic representation of the combined bioorthogonal covalent and supramolecular cross-linking of the hydrogels (b) Gelation time experiment showing the initial 2 h of gelation with and without 10 mM Zn\(^{2+}\) (c) Gelation time experiment showing the initial 2 h of gelation at pH 4.5 and at pH 7 (d) Photographs of hydrogels after subjected to pH 7, pH 4 and 10 mM Zn\(^{2+}\), respectively, and (e) corresponding swelling ratios.

Table 2. Storage (G') and loss (G'') modulus of the HA-BCN/JR2EK-p(N\(_3\))\(_8\) hydrogels at different time points and under different conditions.

| Condition          | 0 min, 20 °C | 120 min, 20 °C |
|--------------------|--------------|----------------|
|                    | G' (Pa)      | G'' (Pa)       |
| pH 7, 0 mM Zn\(^{2+}\) | 0.5 ± 0.2    | 0.7 ± 0.1      |
| pH 7, 10 mM Zn\(^{2+}\) | 89 ± 1.9     | 45 ± 4         |
| pH 4.5             | 12 ± 2.4     | 19 ± 1.5       |

3.4. Dynamic hydrogel functionalization

In addition to the possibility to tune the degree and rate of cross-linking, the JR2EK-polypeptide could also be used to dynamically modify the chemical composition of the formed hydrogels (figure 4(a)). As a proof of concept, a fluorophore labeled complementary helix-loop-helix polypeptide (JR2KK-Cy5, supporting figure S1(d)) was synthesized. JR2KK is designed to fold and heterodimerize with JR2EK to form an antiparallel four-helix bundle [45, 56]. Hydrogels were incubated in PBS buffer (10 mM PO\(_4^{3-}\), 137 mM NaCl, and 2.7 mM KCl, pH 7.4) for 24 h prior to incubation with JR2KK-Cy5 (1 \(\mu\)M, in PBS) for an additional 24 h. Buffer was then collected, and absorption spectra were recorded to estimate the amount of JR2KK-Cy5 adsorbed in the hydrogels. Although a certain amount of JR2KK-Cy5 was found to adsorb nonspecifically to the control hydrogel, the possibility to form heterodimers resulted in a significantly larger uptake of JR2KK-Cy5 (figure 4(b)). Furthermore, already after one hour of incubation, a distinct color change could be noticed for the JR2EK-functionalyzed hydrogels that could not be seen in the JR2EK-lacking control (supporting figure S7). In addition, after seven days of incubation in PBS, the polypeptide-functionalized hydrogel still maintained its distinct teal color caused by the associated Cy5-functionalized peptides, whereas the control was almost transparent (figure 4(c)).

The high loading and retention capacity of JR2KK-Cy5 in the HA-BCN/JR2EK-p(N\(_3\))\(_8\) hydrogels strongly indicate the possibility to use heterodimerization to dynamically change the composition of the hydrogel post-synthesis. Moreover, because of the relatively high local concentration of conjugated peptides (JR2EK) in the hydrogel, the dimerization with the complementary peptide JR2KK was diffusion limited, which facilitated fabrication of well-defined linear 3D gradients of peptide heterodimers. Gradients were fabricated using a microfluidic device (ibidi\textsuperscript{\textregistered} - µ-slide Chemotaxis) comprising a linear channel, 1 mm wide, with openings on each side exposing the hydrogel to liquid in two larger reservoirs (figure 4(d)). HA-BCN/JR2EK-p(N\(_3\))\(_8\) hydrogels were injected into the channel and after 1 h incubation at 37 °C to allow the hydrogel to cross-link, complementary peptides
labeled with Cy3 (JR2KK-Cy3) and Cy5 (JR2KK-Cy5) in the loop were added to the left and right reservoir, respectively. The diffusion of the peptides into the hydrogel and subsequent dimerization with conjugated JR2EK resulted in formation of almost linear gradients of the peptides that could be visualized using fluorescence imaging (figure 4(e)). Previous attempts to produce long-term stable gradients of immobilized biomolecules in 3D hydrogels typically rely on photopatterning using UV light [57, 58], which requires more complex fabrication techniques. Here, the slope of the gradient and the degree of functionalization could be controlled by simply changing peptide concentration and diffusion time (figure 4(f)).

The rapid association of the complementary peptide to conjugated JR2EK further enabled dynamic surface functionalization of hydrogels, which can be used for tuning of cell adhesion and spreading of cells cultured on the hydrogels. Seeding of SH-SY5Y neuroblastoma cells on the HA-BCN/JR2EK-p(N₃)₈ hydrogels resulted in cell aggregation due to the lack of cell-surface adhesion sites (figure 5). In contrast, surfaces modified with the complementary peptide JR2KK, with and without a cyclic RGD (cRGD) peptide motif in the loop region (JR2KK-cRGD), showed a substantial improvement in cell spreading and cell viability. In addition to the specific integrin binding to the cRGD peptide, the high lysine-content in JR2KK likely contributed to promoting cell adhesion.

3.5. Biomineralization
To further explore the potential to dynamically introduce new functionalities in the hydrogels using specific peptide-mediated interactions, we modified the complementary peptide with a biotin moiety in the loop (JR2KK-Biotin, supporting figure S1(d)). When associated to the HA-BCN/JR2EK-p(N₃)₈ hydrogels, this peptide could recruit avidin-modified proteins, which opens up for a plethora of functionalization possibilities. The enzyme alkaline phosphatase (ALP) catalyzes the dephosphorylation of several organic orthophosphates and is central for the mineralization of bone [59]. For functionalization of the hydrogels with ALP, the hydrogels were first incubated with JR2KK-Biotin, and subsequently exposed to streptavidin-modified ALP (figure 6(a)). To verify that ALP was adsorbed in the hydrogel, 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) was added to the buffer. BCIP is a chromogenic probe for ALP activity, and dephosphorylation of BCIP result in formation of a visible blue precipitate of 5,5′-dibromo-4,4′-dichloro-indigo white. Already after 2 h incubation, a visible and distinct color difference could be seen between the samples with JR2KK-Biotin and the control containing JR2K (figures 6(b)–(c)). The JR2KK-Biotin hydrogels had turned teal, whereas the control remained colorless. The presence of JR2KK-Biotin was hence crucial for recruiting and sequestering ALP to the hydrogel. The role of ALP in biomineralization relies on the ability of the enzyme...
Figure 5. Supramolecular tuning of cell-hydrogel interactions using SH-SY5Y neuroblastoma cells. (a) Cells were seeded on (i) the HA-BCN/JR2EK-p(Nis) hydrogel without any surface modification, (ii) hydrogels exposed to the complementary peptide JR2KK, and on (iii) hydrogels modified with the complementary peptide JR2KK-cRGD with a cyclic RGD peptide in the loop region. (b)–(d) Corresponding Live/Dead staining of the SH-SY5Y cells seeded on top of the hydrogels, imaged after one day, three days and seven days post-seeding. Scalebar = 100 µm.

Figure 6. (a) Schematic representation of the heterodimerization-mediated attachment of JR2KK-Biotin and ALP-streptavidin to the hydrogel. (b) Photograph of hydrogels after 2 h incubation with BCIP. (c) Absorbance at 600 nm, showing that negligible amount of BCIP has been converted into its chromogenic product on control-hydrogels as compared to JR2KK-Biotin containing hydrogels. (d) Focus-stacked micrograph of ALP-containing hydrogel after 2 d of incubation in calcification-buffer, indicating formation of mineral deposition (dark spots in micrograph). (e) Photograph of hydrogels after 5 d incubation in calcification-buffer, showing the visual difference between the hydrogels. (f) Scanning electron micrograph showing formation of mineral deposition on the ALP-containing hydrogel. Scale bar = 50 µm. (g) Energy-dispersive x-ray spectra of ALP-containing hydrogel after calcification, confirming high presence of calcium and phosphor in the hydrogel structure. (h) Storage modulus (G’) of non-mineralized (JR2K) control hydrogel and hydrogels after ALP-mediated biomineralization (JR2KK-biotin).

to generate inorganic phosphate in the presence of Ca²⁺, for example by addition of calcium glycerylphosphate (CaGP) [60]. When CaGP was added to the ALP-loaded hydrogels, mineral depositions were clearly seen after 24 h incubation, and continued to accumulate over time (supporting figure S8(a)). In addition, microscope images further indicated that mineral-like structures were formed in the hydrogel (figure 6(d), supporting figure S8(c)). SEM images clearly showed deposition of mineral in the hydrogel.
Figure 7. (a) 3D model of the buckyball structure that was bioprinted using the HA-BCN/JR2EK-p(N3)8 hydrogels. (b) The resulting bioprinted structure after incubation with the complementary Cy5-labeled peptide JR2KK-Cy5. (c) The same structure when illuminated using collimated white light to excite the Cy5 in the associated peptide. (d) 3D model of the grid structure that was used for biomineralization. (e) The bioprinted structure, stained with alcian blue to facilitate imaging. (f) A distinct mineral deposition within the bioprinted structures was observed after introducing the biotin-labeled complementary peptide and subsequent incubation with ALP-streptavidin in the presence of CaP.

with an element composition of Ca (20.6 ± 0.2 Wt%) and P (12.0 ± 0.1 Wt%) confirmed by EDS analysis. The ratio of Ca to P (Ca/P) corresponds to 1.7, which is close to the stoichiometric Ca/P ratio in hydroxyapatite (1.67) (figures 6(f)–(g)). No mineral deposition was seen in control hydrogels modified with JR2K and subsequent incubation with ALP-streptavidin (figure 6(e), supporting figure S8(b)), confirming that the specific peptide-mediated interaction between conjugated JR2EK and JR2KK-Biotin was required to trigger the ALP catalyzed biomineralization of the hydrogels. The stiffness of the hydrogels increased dramatically after biomineralization, resulting in a G’ that was about one order of magnitude higher at 1 Hz than the non-mineralized hydrogel (figure 6(h), supporting figure S9). Biomineralization of hydrogels have previously been widely investigated for bone tissue engineering for repair of bone defects [61, 62]. The possibilities to promote a high local concentration of ALP in the HA-BCN/JR2EK-p(N3)8 hydrogels using defined peptide-interactions represent a novel and efficient method for biomimetic mineralization.

3.6. 3D and 4D bioprinting
Because of the tunable cross-linking kinetics of the HA-BCN/JR2EK-p(N3)8 hydrogels they can be processed into complex structures using additive biofabrication techniques. 3D printing of the hydrogels was conducted on a Cellink Bio X. using the freeform reversible embedding of suspended hydrogels (FRESH) technique [44]. To demonstrate the possibility to print elaborate architectures requiring high shape fidelity, a buckyball structure comprising 44 layers and with a smallest feature size of 0.6 mm was designed and printed (figures 7(a) and (b)). A thermoreversible gelatin slurry was used as a temporary support for the soft hydrogels during printing to improve fidelity. Printing was conducted at room temperature and the gelatin slurry was removed by heating to 37 °C. The HA-BCN/JR2EK-p(N3)8 hydrogels could be printed both in the absence and presence of Zn2+ (supporting movie S2), although the latter resulted in significantly more robust structures due to the additional contribution from the supramolecular interactions to the cross-linking process (supporting movie S3 and movie S4). The subsequent removal of the Zn2+ by addition of EDTA resulted in an instant swelling of the printed structures by more than 20% (supporting figures S10(a) and (b)), due to the reduced cross-linking density caused by the dissociation of the peptide homodimers. Peptide dimerization and dissociation can thus be used to change the morphology of the printed structures. Longer incubation time with Zn2+ after printing resulted in reduced swelling after removal of the Zn2+, indicating that the supramolecular cross-linking facilitated the SPAAC-mediated covalent cross-linking of the hydrogels (supporting figures
In addition, fibroblasts encapsulated in the hydrogels showed high viability after printing (supporting figure S11).

To further investigate the possibility to modify the composition and function of the hydrogels after removal of the support hydrogel, the peptide JR2KK-Cy5 was added to the buffer and allowed to heterodimerize with the complementary and conjugated peptide JR2EK in the hydrogels. The specific association and gradual accumulation of dye-labeled JR2KK-Cy5 gave the printed hydrogel structures a teal color under ambient light and a bright red fluorescent emission when exciting the fluorophore (figures 7(a)–(c)). When instead introducing the biotin-labeled complementary peptide JR2KK-Biotin, the possibility to bind ALP-streptavidin added to the buffer resulted in a distinct biominalerization within the printed structures in the presence of CaGP (figures 7(d)–(f)). This possibility to modify the properties of biomaterials with explicit molecular and temporal control can facilitate fabrication of more complex biosfunctional materials and pave the way for development of advanced 4D bioprinting strategies.

4. Conclusions

In conclusion, a polypeptide-functionalized hyaluronic acid (HA) and poly(ethylene glycol) (PEG) based hydrogel, HA-BCN/JR2EK-p(N18), have been developed that can be cross-linked both covalently and non-covalently using a bioorthogonal SPAAC click reaction and by optical activation. The gels can be cross-linked both covalently and non-covalently using a bioorthogonal SPAAC click reaction and by optical activation. The gels can facilitate fabrication of more complex biosfunctional materials and pave the way for development of advanced 4D bioprinting strategies.

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cell spreading and viability of neural cells cultured on the hydrogels. Incorporating a biotin moiety in the loop (JR2KK-Biotin) provided means to bind streptavidin-functionalized molecules to the hydrogels. Streptavidin-labeled alkaline phosphatase (ALP) was bound and enzymatically active after allowing JR2KK-Biotin to first associate in the hydrogel, resulting in biominalerization of the hydrogels in the presence of CaGP. Furthermore, the hydrogels could be 3D printed using the freeform reversible embedding of suspended hydrogels (FRESH) technique. The possibility to use both peptide homo- and heterodimerization to modulate the properties of the printed structures was demonstrated and show that this modular toolbox can be utilized to dynamically engineer the properties of complex soft material architectures. In addition, this strategy is not limited to the peptides used here. A large number of different well-defined de novo designed peptides with tunable self-assembly properties are available [63, 64], which can provide an almost infinite number of possibilities to tailor materials properties. The possibility to modify the properties of 3D printed biomaterials with temporal control using highly defined molecular interactions can facilitate development of versatile bioinks for 4D bioprinting that can open new opportunities to create responsive and dynamic tissue-mimetic constructs for a wide range of biomedical applications.
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