Published in final edited form as:

Biomaterials. 2018 December ; 185: 86–96. doi:10.1016/j.biomaterials.2018.09.003.

A self-healing hydrogel as an injectable instructive carrier for cellular morphogenesis

Zhao Wei^a and Sharon Gerecht^{a,b,*}

^aDepartment of Chemical and Biomolecular Engineering, The Institute for NanoBioTechnology Physical-Sciences Oncology Center, Johns Hopkins University, Baltimore, MD 21218, USA

^bDepartment of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA

Abstract

Transplantation of progenitor cells can accelerate tissue healing and regenerative processes. Nonetheless, direct cell delivery fails to support survival of transplanted cells or long-term treatment of vascular related diseases due to compromised vasculature and tissue conditions. Using injectable hydrogels that cross-link *in situ*, could protect cells *in vivo*, but their sol-gel transition is time-dependent and difficult to precisely control. Hydrogels with self-healing properties are proposed to address these limitations, yet current self-healing hydrogels lack bio-functionality, hindering the morphogenesis of delivered cells into a tissue structure. Here we establish a gelatin (Gtn)-based self-healing hydrogel cross-linked by oxidized dextran (Odex) as an injectable carrier for delivery of endothelial progenitors. The dynamic imine cross-links between Gtn and Odex confer the self-healing ability to the Gtn-I-Odex hydrogels following syringe injection. The self-healing Gtn-I-Odex not only protects the progenitors from injected shear force but it also allows controllable spatial/temporal placement of the cells. Moreover, owing to the cell-adhesive and proteolytic sites of Gtn, the Gtn-I-Odex hydrogels support complex vascular network formation from the endothelial progenitors, both *in vitro* and *in vivo*. This is the first report of injectable, self-healing hydrogels with biological properties promoting vascular morphogenesis, which holds great promise for accelerating the success of regenerative therapies.

Keywords

Self-healing; Hydrogels; Cell delivery; Endothelial progenitor cells; Vasculogenesis

1. Introduction

Stem cell delivery therapy can potentially improve treatment for many disorders. These include vascular-related ischemic injuries, caused by infarction, stroke, arterial diseases and other disorders, most of which depend on rapid vascularization to increase potency [1–3].

*Corresponding author. Department of Chemical and Biomolecular Engineering, The Institute for NanoBioTechnology, Physical-Sciences Oncology Center, Johns Hopkins University, Baltimore, MD 21218, USA. gerecht@jhu.edu (S. Gerecht).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2018.09.003>.

The establishment of an adequate vasculature from delivered stem cells will support nutrient and oxygen transport, as well as removal of metabolic wastes, for tissue survival and growth [4,5]. Strategies to generate therapeutic vascular networks have included the delivery of healthy endothelial progenitor cells (EPCs) such as endothelial colony-forming cells (ECFCs), to promote vascular regeneration [6–8]. As a subtype of EPCs, ECFCs are characterized by robust proliferative potential in formation of de novo blood vessels both *in vitro* and *in vivo* [9–11]. However, most direct delivery attempts fail to demonstrate high survival of transplanted EPCs or long-term treatment due to the poor tissue conditions around the injured/disease regions [12–15].

A highly hydrated hydrogel, with physicochemical properties similar to natural extracellular matrix, can provide a suitable 3D scaffold to embed cells for delivery [15–17]. Over the past decades, injectable hydrogels were extensively applied to deliver cells into localized lesion sites, via minimally invasive approaches [18–21]. The *in situ* cross-linking process of injectable hydrogels can occur either during or after injecting hydrogel/cells precursor solutions. As a result, the relationship between injection and sol-gel transition is time sensitive and difficult to precisely control. This in turn may lead to cell loss and material dispersion by slower gelation, or syringe clog by faster gelation [22,23]. To address these shortcomings, self-healing hydrogels, which can spontaneously restore their integrity of structures and functionalities after damages, have been explored as novel vehicles for cell delivery [24–27]. The dynamic cross-links in self-healing hydrogels allow them to flow during injection but self-heal rapidly after cessation of extruded stress [28–31]. The use of self-healing hydrogels as cell carriers not only avoids the gelation timing issues but can also provide mechanical protection for delivered cells from the shear damage during injection, as well as maintain the retention and integrity of the implant *in vivo* [32–35].

More recently, several self-healing hydrogels were developed as injectable vehicles for cell delivery [36–38]. For example, Burdick's group developed a self-healing hyaluronic acid hydrogel using dynamic host-guest interactions for 3T3 fibroblasts and mesenchymal stem cells [39,40]. Chen et al. synthesized self-healing hydrogels via dynamic Schiff base reaction between oxidized sodium alginate and N-carboxyethyl chitosan, for delivery of neural stem cells [41]. In addition, there are series of polysaccharide-based self-healing hydrogels reported for culturing or delivering a wide range of cell types, including fibroblasts, HeLa cells, chondrocytes, marrow stem cells, and so on [26,32,36,42,43]. Nonetheless, despite the high cell viability of these self-healing hydrogels, most cells encapsulated in them are observed as invariably rounded shapes without cell-cell communications, even following a long culture period. This severely impedes the therapeutic effects once delivered. These outcomes may be attributed to the lack of bio-functionality in the networks of current self-healing hydrogels, which is critical in cell-material interactions, cell responses and morphogenesis [44–48]. Most recently, Hsu's group developed a composite self-healing hydrogel cross-linked by chitosan and formylbenzoic-functioned poly (ethylene glycol) [76]. The fibrin was introduced into the network to provide bio-functional sites for inducing blood capillary formation. However, the utilization of this hydrogel as an injectable/self-healing cell carrier was not studied. Our work here focuses on developing injectable/self-healing cell carrier hydrogels with bio-functionality and suitable moduli for EPCs delivery using subcutaneous model.

Studies have shown that the cell adhesion motif sequence Arg-Gly-Asp-Ser, RGD regulates vacuole and consequent lumen formation of endothelial cells, as the first crucial step in vascular tubulogenesis [44,45]. Cell-mediated matrix metalloproteinases (MMPs) then facilitates matrix remodeling and cell migration, which are crucial to the subsequent steps of morphogenesis and vessel growth [46–48]. We hypothesized that hydrogel carriers with self-healing capabilities and bio-functionality for vascular morphogenesis, including cell adhesion and degradation sites, would enhance the therapeutic impact of ECFC delivery. Using this approach, we also tested whether a short pre-culture period of encapsulated ECFCs would further enhance delivery success.

We developed a facilely prepared gelatin-based self-healing hydrogel system, cross-linked by the reversible imine cross-links formed by Schiff base reaction of the amino groups on gelatin (Gtn) and the aldehyde groups on oxidized dextran (Odex), denoted as Gtn-I-Odex (“I” means “linked-by”). Gelatin and dextran were chosen as the backbone and cross-linker respectively, to generate the hydrogel networks based on their biocompatibility and water solubility [49,50]. Specifically, the bio-functional cell adhesive RGD and MMP-sensitive degradable peptide sites are available on gelatin chains, achieving the basic requirements for vascular morphogenesis of ECFCs [51–53]. Moreover, the imine bond belongs to dynamic covalent bonds, used in preparation of numerous self-healing biomaterials, which can offer an intrinsic dynamic equilibrium of bond association and dissociation in polymer networks under physiological conditions (37 °C, pH 7.4) [54,55]. In addition, the only byproduct of this cross-linking reaction is water, minimizing the cytotoxicity of the hydrogel. Therefore, the self-healing Gtn-I-Odex hydrogel holds promise for the delivery of ECFCs and accelerating vascular reconstruction in injured or diseased tissue.

2. Materials and methods

Materials: Gelatin (Gtn, type A from porcine skin, < 300 bloom), Dextran (Dex, M_n = 110,000), sodium (meta) periodate (> 99.0%), acrylic acid (99%), Chitosan (75–85% deacetylated, M_n = 50,000–190,000 Da), collagenase type IV were purchased from Sigma-Aldrich. All other chemicals were analytical grade and used without further purification. Endothelial growth media-2 (EGM-2) was purchased from Lonza and used for ECFCs culture. Dulbecco’s phosphate-buffered saline (DPBS), fetal bovine serum (FBS) and trypsin were all purchased from Gibco, Invitrogen. Dialysis membrane (molecular cutoff = 3500 Da) was purchased from Spectrum Laboratories.

2.1. Synthesis of odex

The synthesis of Odex was based on a reported method, with a slight modification [58]. Dextran (5.0 g, 0.03 mol) was dissolved in 250 mL distilled water, and then sodium periodate (5.0 g, 0.023 mol) was added. The solution was magnetically stirred in the dark for 3.5 h. This reaction was terminated by adding ethylene glycol (3.0 mL) and stirring for an additional 1 h. The mixture was then dialyzed (MWCO 3500) against distilled water for 3 days, with the water changed every day, followed by lyophilization to obtain Odex. The oxidation percentage of Odex was 51.8%, which was determined by quantifying the number of aldehyde groups in the polymer using *tert*-butylcarbazate (*t*-BC) [59]. In brief, the

carbazates of *t*-BC (excess amount) reacted with aldehyde groups of Odex to form carbazones, then the unreacted *t*-BC was quantified by adding trinitrobenzene sulfonic acid (TNBS) and measuring the resulting colored trinitrophenyl derivative at 334 nm using a spectrophotometer. Aqueous *t*-BC solutions can be used as standards to determine the unreacted carbazates in experimental samples. The oxidized degree (OD) of 19.3% and 78.0% Odex were obtained by reacting with 2 g (0.0094 mol) and 8 g (0.037 mol) sodium periodate respectively following the same protocol mentioned above.

2.2. Cytocompatibility of odex

The cytotoxicity of Odex was investigated by wst-1 assay according to the manufacturer's instructions. In brief, 2×10^4 cells were cultured in 100 mL EGM-2 media in each well of 96-well plate and in the presence of different concentrations of Odex (0.2–0.8 wt%) for 24 h, followed by adding 10 μ L of wst-1 mixture to each well. After placement in an incubator for 2 h, we measured the absorbance of each sample using a microplate reader at a wavelength of 450 nm. The cell viability was determined as a percentage of control cells.

2.3. Preparation of the Gtn-I-odex hydrogel

For hydrogel formation, the EGM-2 media of Gtn was mixed with Odex EGM-2 media solution at different molar ratios (*R*) of 0.75, 1.0 and 2.0, respectively. The total weight concentration of Gtn was kept as 6 wt%. The mixture was shaken uniformly by vortex and then placed in 37 °C water bath. After several minutes, the viscosity of the solution generally decreased and homogeneous Gtn-I-Odex hydrogels would eventually be obtained.

2.4. Measurement of gelation time

The gelation time of Gtn-I-Odex hydrogels was determined through vial inversion method [73]. The precursor solutions of Gtn and Odex were mixed gently to carry on the Schiff base reaction at 37 °C. The gelation time was measured by inverting the mixture solution and observing no flow for more than 5 min. All the gelling times were determined in triplicate samples for each group.

2.5. Rheological measurements

The storage moduli (G') of the Gtn-I-Odex hydrogel, with various *R* of 0.75, 1.0 and 2.0, were tested by a rheometer equipped with an 8 mm parallel plate at 37 °C. All hydrogel samples were prepared as discs 8 mm in diameter. The angular frequency was swept from 0.1 to 100 rad s⁻¹ at a fixed strain of 0.1%. Moreover, the Gtn-I-Odex hydrogel of *R* = 1, with or without ECFCs, were also tested by rheology at different time points along with the increased incubating time.

2.6. Examination of degradation rate

The *in vitro* degradation of the Gtn-I-Odex hydrogels, with a various *R* of 0.75, 1.0, and 2.0, were determined by weighing the samples at different time points. The hydrogels (W_0) were prepared in microtubes (100 μ L) and subsequently immersed in additional 500 μ L EGM-2 media at 37 °C. The media was removed completely by pipette at every predetermined time point. To confirm the media had been completely removed, the microtubes were inverted and

confirmed for no excessive media along the tube wall for > 1 min. The weight of each sample (W_t) was then measured. Fresh media was added to the microtubes after each weighing. The degradation rate was calculated by $(W_t/W_0) \times 100\%$. Similarly, we also tested the hydrogel degradation of $R = 1.0$ by the same method, except for adding different concentrations of collagenase from 0.001% to 0.05% in each of the additional media.

2.7. Injection experiments

(1) A disc-like Gtn-I-Odex hydrogel ($R = 1.0$) was put into a syringe and extruded through 22-gauge needles into a PDMS mold containing three tiny round hollows. After incubation in 37 °C for 5 min, the newly formed tiny hydrogel disc were taken from the mold and placed in a vial filled with PBS (pH 7.4). The stability of the self-healed hydrogels were evaluated by strongly shaking and inverting the vials. The same injecting test was also carried out on the self-healing N-carboxyethyl chitosan (CEC)-I-Odex hydrogels. (2) The Gtn hydrogel, CEC-I-Odex hydrogel and Gtn-I-Odex hydrogel were prepared and then directly injected into 37 °C PBS to observe their stability and cargo retention.

2.8. Rheological recovery test

The strain amplitude sweep of the Gtn-I-Odex hydrogel disc was measured at a fixed angular frequency (0.1 rad s^{-1}) at 37 °C. Amplitude oscillatory strains were switched from a small strain ($\gamma = 0.1\%$) to large strain ($\gamma = 2200\%$). Once the hydrogel network collapsed, the strain was switched back and fixed at $\gamma = 0.1\%$ after waiting for 1 min of healing time. This strain lasted for 300 s. In addition, the alternate step strain sweep of the Gtn-I-Odex hydrogel discs were measured at a fixed angular frequency (0.1 rad s^{-1}) at 37 °C. Amplitude oscillatory strains were switched from a small strain ($\gamma = 0.1\%$) to a subsequent large strain ($\gamma = 2200\%$) and back to a small strain ($\gamma = 0.1\%$). Each strain was induced for 200 s.

2.9. Encapsulation of ECFCs in Gtn-I-odex hydrogel

The ECFCs were cultured as previously described on a plate coated with Collagen type I [74]. The Gtn and Odex were separately dissolved in the prepared EGM-2 media and then mixed uniformly. The Gtn concentration was 6 wt% and the R was kept at 1. The ECFC pellet was then mixed with the Gtn and Odex media to obtain a cell suspension at a concentration of $4 \times 10^6 \text{ cell mL}^{-1}$. The cell mixture was placed in the 96-well plate of 50 μL and put into incubator for 30 min of gelation, before adding additional 200 μL EGM-2 media on them. The media was replaced every 24 h. The ECFC morphologies were observed by optical microscopy (phase-contrast) and confocal microscopy (LSM 510 Meta, Carl Zeiss). For confocal, the ECFC-loaded hydrogels were fixed in 2% paraformaldehyde for 20 min at room temperature. After washing three times using PBS, they were permeabilized in 0.1% TritonX-100 for 20 min and then blocked with 10% BSA solution for 1 h. The hydrogel samples were then incubated with phalloidin and DAPI to visualize the cytoplasm and nuclei, respectively.

2.10. Cell viability of post-injection

The ECFCs delivered within EGM-2, 6 wt% non-cross-linked Gtn and Gtn-I-Odex hydrogel were injected through a 22-gauge needle into the 96-well plate at an uniform flow rate of

1000 $\mu\text{L}/\text{min}$ set up by the extruder equipment. After 2 h, the cell viability of different injection methods were evaluated by a live/dead kit (Invitrogen). We treated each well with a mixture of 100 μL of 2 mM calcein AM and 4 mM of ethidium homodimer-1 (EthD-1) and placed them in incubator for 30 min. The stained samples were washed with PBS and counted with fluorescence microscopy. The viscosities of EGM-2 media, non-cross-linked Gtn media (6 wt%) and Gtn-I-Odex hydrogels were tested by a rheometer equipped with an 25 mm parallel plate. All the samples are roughly considered as Newtonian fluid. The shear rate was kept constant at 308 s^{-1} which was determined using the equation of $4Q/\pi r^3$ [77], where “ Q ” is the flow rate of 1000 $\mu\text{L}/\text{min}$ and “ r ” is the internal radius of the 22-gauge needle (0.41 mm).

2.11. Dissolved oxygen (DO) measurement

The DO levels were measured non-invasively throughout the Gtn-I-Odex hydrogels using commercially available sensor patches [75]. The polymer precursor solutions, with or without ECFCs ($4 \times 10^6 \text{ mL}^{-1}$), were added on top of the sensors, which were pre-immobilized in each well of 96-well plate. After gelation, 200 μL media was added in each well and the plate was placed in culture incubators for DO data recording.

2.12. In vivo subcutaneous injection of ECFC-loaded Gtn-I-odex hydrogel

The ECFC-loaded Gtn-I-Odex hydrogels (4 million cells per mL) were subcutaneously injected into nude mice (7–8 week-old females) to study the *in vivo* vasculogenesis. For this, two ECFC-loaded Gtn-I-Odex hydrogels were prepared and cultured *in vitro* for 0 h and 4 h respectively. Thereafter, they were put into a syringe and subcutaneously injected into both sides of mice backs (100 μL on each side). At each time point (12 h and 3 days), the mice were killed and the hydrogels were removed with surrounding tissue, which were fixed in 3.7% paraformaldehyde and proceeded to histological analysis. Collagen hydrogels (type I Collagen; Corning; 2.5 mg/mL) were set as the positive control for the ECFC morphogenesis *in vivo*. Collagen gels were formed as previously described [78]. The ECFC-loaded collagen hydrogels (4 million cells per mL; 100 μL) were generated and cultured for 0 h and 4 h *in vitro* prior to subcutaneous transplantation followed by skin suturing on both sides of the backs of nude mice. At each time point (12 h and 3 days), the mice were sacrificed and the hydrogels were removed with surrounding tissue and fixed in 3.7% paraformaldehyde and proceeded to histological analysis. The ECFC-loaded CEC-I-Odex hydrogels were set as negative control. The gelled ECFC-loaded CEC-I-Odex hydrogels (1.0 wt% CEC; 4 million cells per mL; 100 μL) were directly injected subcutaneously on both sides of the back of nude mice (0 h of *in vitro* culture). At each time point (12 h and 3 days), the mice were sacrificed and the hydrogels were removed with surrounding tissue and were fixed in 3.7% paraformaldehyde and proceeded to histological analysis. All the explants were then dehydrated in graded ethanol (80–100%), embedded in paraffin, serially sectioned using a microtome and stained with CD31. The animal study was performed using a protocol (RA15A152 and RA14A186) approved by The Johns Hopkins University Institutional Animal Care and Use Committee.

3. Results and discussion

3.1. Design and preparation of Gtn-I-odex hydrogel

The overall strategy to develop and study self-healing Gtn-I-Odex hydrogels for vascular morphogenesis is shown in Fig. 1A–D. The Gtn polymer chains would be cross-linked by Odex, through dynamic imine bonds between the functional groups of amino on Gtn and aldehyde on Odex, to generate the ECFC-loaded Gtn-I-Odex hydrogels prior to culture and/or injection (Fig. 1A–B). To meet the necessary nutritional needs for ECFC cultivation and injection, the Gtn-I-Odex hydrogels are directly prepared in endothelial growth media-2 (EGM-2) under physiological conditions. Based on other's studies and our own [56,57], we postulate that the encapsulated ECFCs could progress through all stages of vascular morphogenesis in the Gtn-I-Odex hydrogels, increasing their regenerative capacity. The process starts when ECFCs form individual vacuoles that merge and coalesce into a larger structure of lumens (Fig. 1Ci–ii). This is followed by sprouting and continuation of the process toward comprehensive multicellular networks, with patent lumenized structures (Fig. 1Ciii–iv). The completely polymerized ECFC-loaded Gtn-I-Odex hydrogel, either immediately after gelation or following a short-term *in vitro* culture, can be squeezed through a syringe for subcutaneous injection (Fig. 1D). The injected fragments of the hydrogel would then self-heal and be located at the target site. By taking advantage of the self-healing feature of the Gtn-I-Odex hydrogels, injection timing can be tuned to allow the ECFCs to adapt to the hydrogel environment in a short-term *in vitro* culture period with enough nutrients in culture media, which in turn will induce morphogenesis via vacuoles formation (Fig. 1Ci–D). Injection following this short-term *in vitro* culture could potentially improve delivery success and effectiveness.

The synthesis and fabrication of Gtn-I-Odex follows several steps. First, the multi-aldehyde groups are introduced on dextran chains via periodate-oxidation according to the procedures in the literature [58,59] to obtain the Odex with different oxidized degree (*OD*) of 19.3%, 51.8% and 78% by adjusting reacted amount of sodium periodate. The *in situ* formation of Gtn-I-Odex hydrogel is achieved by homogeneously mixing Gtn and Odex endothelial growth media, EGM-2, under physiological conditions with a fixed Gtn concentration ($C_g = 6.0$ wt%). To investigate the Gtn-I-Odex gelling kinetics and hydrogel properties with different cross-linking densities, a series of Gtn-I-Odex hydrogels were synthesized by increasing the molar ratio of the reactive groups between Odex and Gtn ($R = \text{M-CHO}:\text{M-NH}_2$) from 0.75, 1.0 to 2.0. For Odex with *OD* of 51.8%, the maximum concentration of Odex for gelation will be close to 0.7 wt% when *R* is 2.0. The cytotoxicity of Odex was then examined as the aldehyde modification has the potential to induce toxicity in the hydrogels. As shown in Fig. S1, no obvious viability decrease was observed even when the concentration of Odex reached up to 0.8 wt%, which is over the highest concentration of Odex needed for gelation, thus demonstrating the cytocompatibility of the Gtn-I-Odex hydrogels.

The gelation times of the Gtn-I-Odex hydrogels were determined by phase transition through the vial inversion method (Fig. 1E). The fluidity of the Gtn and Odex mixture slowly decreased with mixing time. Gelation time was found to be dependent on the molar ratio of

functional groups of the two components in this system, which can be accelerated by increasing R . By using Odex with OD of 51.8% as cross-links, the gelling process took the longest time of 70 min for the lowest R of 0.75, which gradually decreased to 25 and 12 min, when R was increased to 1.0 and 2.0 respectively. There was no hydrogel formation when $R < 0.5$, most likely due to a lack of required cross-links to leap the gelling threshold, which is defined as the minimum concentration of Odex for achieving the gelation process. These results indicate that the higher concentration of the Odex cross-linker in the hydrogel networks improves the efficiency of Schiff base cross-linking reaction. However, when R was kept as 1, the gelation time of hydrogels cross-linked with different OD of Odex had no significant difference due to the constant reactive concentration and ratio of functional groups of Schiff base reaction (Fig. S2A).

To evaluate the mechanical strength of the Gtn-I-Odex hydrogels, oscillatory frequency sweeps of the hydrogels with various R were conducted at 37 °C by rheology measurements (Fig. 1F). By using Odex with OD of 51.8% as cross-links, the storage modulus (G') of the hydrogel increased stepwise, from 33.0 ± 6.5 to 175.3 ± 24.5 Pa with R varying from 0.75 to 2.0, which was attributed to the increased amount of cross-links. When R was kept as 1, the G' of Gtn-I-Odex hydrogel cross-linked with Odex of 19.3% OD (139.8 ± 13.1 Pa) was significantly higher than samples with OD of 51.8% (102.2 ± 15.0 Pa) and 78% (88.4 ± 18.4 Pa). This is because more Odex with the lowest OD (19.3%) was added to the system to achieve the constant ratio of reactive groups ($R = 1$), resulting in an increased dextran polymer concentration and increased modulus of the system (Fig. S2B). Based on our former study, the G' of the hydrogel scaffolds used for vasculo-genesis should be adjusted approximately $\sim 10^2$ Pa [52,53,60], and thus, these Gtn-I-Odex hydrogels with tunable mechanical properties could be used as cell scaffolds to support vessel formation.

The Gtn-I-Odex hydrogels are also self-degradable through hydrolysis due to the reversibility of Schiff base reaction [61,62]. The degradation behaviors of the Gtn-I-Odex hydrogels with various R were investigated under physiological conditions by immersion in EGM-2 media (Fig. 1G). Generally, the hydrogels swelled in the EGM-2 media for the first few days and then gradually degraded by hydrolysis. By using Odex with OD of 51.8% as cross-links, the sample of $R = 0.75$ was degraded completely after 7 days, while the $R = 1.0$ sample remained 76.5% after 2 weeks. No obvious weight loss was observed with the highest R of 2.0, indicating that excess cross-linkers slow down or even prevent the network collapse. Another important parameter for Gtn-I-Odex hydrogel is its proteolytic degradability, which facilitates cell migration and matrix remodeling. We also examined the protease-sensitive degradation of Gtn-I-Odex hydrogels (Fig. 1H). The hydrogel samples of $R = 1.0$, incubated with increased collagenase concentrations from 0.001 to 0.05%, degraded rapidly within 24 h. Degradation rates were accelerated along with the increased concentrations of collagenase, demonstrating that the proteolytic degradability of Gtn is retained in Gtn-I-Odex hydrogels. Ultimately, we selected the Gtn-I-Odex sample of $OD = 51.8\%$ and $R = 1.0$ to evaluate the following self-healing properties, ECFC morphogenesis and *in vivo* tests because it incorporated the theoretically complete reaction of amino groups and aldehyde groups in two components, as well as best matching the desirable mechanical properties ($\sim 10^2$ Pa) for vascular formation.

3.2. Self-healing performance and injectability of the Gtn-I-odex hydrogel

To assess the self-healing of the Gtn-I-Odex hydrogels ($R = 1.0$), we fabricated three cylindrical samples: one with red dye (rhodamine B), one transparent and one with blue dye (methylene blue; Fig. 2Ai). The three individual samples of the hydrogels were subsequently assembled and combined into one hydrogel cylinder with stacked colors (Fig. 2Aii). After incubating for 20 min at 37 °C without any external intervention, the boundaries between different colored pieces were obscured and the three tiny hydrogel samples merged into an integrated one (Fig. 2Aiii). A simple mechanical test was performed to demonstrate the success of the self-healing by using tweezers to lift up and constantly stretch the self-healed hydrogel. No splitting was observed, illustrating that the healed interfaces were strong enough to bear a tensile force (Fig. Aiii, see video 1 in SI).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2018.09.003>

The Gtn-I-Odex hydrogels can also be injected after gelation. The gel fragments squeezed through syringes self-heal, restoring the hydrogel's integrity at the target site. The injectability of self-healing hydrogels has been demonstrated to confer more uniform distribution of loaded cells along with controllable locations of delivery *in vivo*, as well as faster mechanical recovery of the hydrogels after injection [28,29]. To evaluate the injectability of Gtn-I-Odex hydrogels, we loaded a hydrogel disc, stained with a red dye, into a 22-gauge syringe and then injected it into three tiny disc-like PDMS molds (Fig. 2Bi–iii). The extruded hydrogel fragments were fully filled into the molds and incubated at 37 °C for 5 min for self-healing without any other interventions. The three tiny self-healed hydrogel discs were easily taken out and maintained their integrity even after being immersed in a vial filled with PBS (pH 7.4) (Fig. 2B iv–v). No dispersion was observed by strongly shaking the vials. These results indicate that one of the mechanisms behind self-healing Gtn-I-Odex networks is the dynamic chemical bond re-formation instead of simply adhesive interactions [26]. Moreover, the Gtn-I-Odex hydrogels cross-linked by Odex with different *OD* of 19.3% and 78% are also self-healing after injection (Fig. S2C).

The self-healing Gtn-I-Odex hydrogels are also expected to retain their cargos and shapes after injection into an aqueous environment. To assess cargo retention and further confirm the self-healing mechanism behind Gtn-I-Odex hydrogels, we injected red dyed Gtn-I-Odex hydrogels into PBS (pH 7.4) at 37 °C. The red dye was used for the cargo model, facilitating visualization of its retention. As first control, we used a Gtn hydrogel that gels at room temperature (25 °C) yet will dissolve quickly at body temperature (37 °C) [63,64]. This behavior is well-known from Gtn and is due to the weak physical interactions between Gtn polymer chains, such as hydrophobic entanglement, hydrogen bonds and π - π stacking, etc. [65,66]. We prepared the 6 wt% Gtn gel and injected it into the 37 °C PBS. The extrusion maintained a hydrogel state at the beginning of the injection but ultimately dissolved due to the high temperature of PBS, and the red dye leaked out rapidly (Fig. 2Ci, see video 2 in SI). For another control, we used a popularly reported polysaccharides-based self-healing hydrogels system that cross-linked through the dynamic imine bonds, the cross-linking manner of which is the same as Gtn-I-Odex hydrogels [32,41]. We synthesized this polysaccharides-based hydrogel by using N-carboxyethyl chitosan (CEC) (instead of Gtn)

and Odex, which can also exhibit perfect self-healing capabilities (Fig. S3). However, we found that squeezing the CEC-I-Odex hydrogel through a needle, immediately broke it into numerous tiny hydrogel beads that fully dispersed throughout the PBS, although the red dye still remained in the hydrogel beads (Fig. 2Cii, see video 3 in SI). In contrast to these controls, hydrogel integrity with no dispersion of the extruded hydrogel or leak of dye was observed after injection of Gtn-I-Odex hydrogel into PBS (Fig. 2Ciii, see video 4 in SI), demonstrating its retention capacity of payload and material integrity for use in aqueous injection. These results suggest that both the physical interactions between Gtn chains and dynamic imine cross-links are indispensable contributors to the excellent injectability and cargo retention of Gtn-I-Odex hydrogels.

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2018.09.003>

Rheological measurements were conducted to further confirm the mechanical recovery of Gtn-I-Odex hydrogels after self-healing (Fig. 2D). The strain amplitude sweep was first loaded on the disk-shaped Gtn-I-Odex hydrogels under 37 °C at a fixed frequency of 0.1 rad s⁻¹. The G' and loss modulus (G'') curve overlapped and subsequently reversed along with the increase of strain, indicating the network collapse of the hydrogel. However, as expected, the G' and G'' quickly restored their initial values after unloading the large amplitude oscillatory (2200%) and fixing the strain at 0.1%. The results manifest that self-healing Gtn-I-Odex hydrogels can effectively repair and prevent potential shear damages to their internal structures that are induced by the surrounding tissues. Thereafter, oscillatory shear strains of 0.1% and 2200% were alternately loaded on the Gtn-I-Odex hydrogel for multiple cycles, and each strain was maintained for 200 s (Fig. S4). The G' and G'' of Gtn-I-Odex hydrogel repeatedly recovered to its original value each time after removing the large amplitude strain, demonstrating the reversibility and stability of self-healing response of the Gtn-I-Odex hydrogels.

3.3. Vascular morphogenesis of ECFCs within Gtn-I-odex hydrogel

Previously, our group demonstrated that ECFCs could follow a specific morphogenetic process to form functional vascular networks within various bio-functional hydrogel networks [52,56,67]. Here, we postulate that the self-healing Gtn-I-Odex hydrogel, with cell adhesive/degradable peptide motifs and appropriate mechanical properties, will provide a suitable 3D environment for vasculogenesis. To test this, we examined the progress of ECFCs network formation within a Gtn-I-Odex hydrogel. As shown in Fig. 3, the encapsulated ECFCs undergo all stages of vascular morphogenesis in Gtn-I-Odex hydrogels. We clearly found vacuole formation occurring in many cells and some of these vacuoles coalesced with neighboring cells into a larger structure of lumens, within 4–6 h of encapsulation (Fig. 3Ai, iii). The corresponding vacuole and lumen formation could also be visualized by vacuole stain (Fig. 3Aii, iv). Subsequently, sprouting and branching of the ECFCs were observed as tubulogenesis progressed (Fig. 3Av). We noted that the hydrogel matrix began to degrade alongside cell elongation. After 24 h and 48 h of ECFCs encapsulation, we observed the formation of extensive and complex vascular networks throughout the hydrogels (Fig. 3Avi, vii). Moreover, the patent lumen structures are easily

detected by 3D z-stack confocal analysis, suggesting mature vascular tube formation in the Gtn-I-Odex hydrogels (Fig. 3Bi–ii).

We also analyzed the cell-material interactions between ECFCs and Gtn-I-Odex hydrogels by measuring the mechanical loss of no cell and cell-loaded hydrogels with increased culture periods (Fig. 3C). We found that the G' of ECFCs-loaded Gtn-I-Odex hydrogel significantly decreased as tubulogenesis progressed from 12 h of encapsulation, which agreed well with the timing of sprouting and branching. On the other hand, hydrogels that did not contain cells also showed little reduction in mechanics, due to the hydrolysis of the hydrogel networks. The tubulogenesis of ECFCs was complete after one day, resulting in complex vascular networks. This is considerably quicker than observed in other synthetic hydrogels [53,56,57], and could be attributed to hydrolytic degradation or, more likely, to the dynamic cross-links in the hydrogels, which were previously shown to directly promote cell elongation and migration [68,69].

To test the effects of different matrix mechanical properties on vascular network formation, the ECFCs were encapsulated in a series of Gtn-I-Odex hydrogels with various R . As shown in Figs. S5A–C, the tubulogenesis of the encapsulated ECFCs occurred in the $R = 0.75$ sample (33.0 ± 6.5 Pa) and the vascular network formation within 24 h, which was similar to the $R = 1.0$ hydrogel (102.2 ± 15.0 Pa). However, after 48 h of culture, the hydrogel with $R = 0.75$ was almost fully degraded due of its low cross-linking density, leading to a collapse of the hydrogel structure. All the encapsulated cells clustered and fell to the bottom of the petri-dish (Fig. S5D). Conversely, only a few sprouting and branching events could be observed in ECFCs in the $R = 2.0$ sample (175.3 ± 24.5 Pa) after culturing for 48 h (Fig. S5D).

To obtain a more comprehensive analysis of the kinetics of vascular formation, we used 3D image analysis to quantify the tube length and volume of the Gtn-I-Odex hydrogels with different moduli [57] (Fig. 4A). After 24 h in culture, the mean tube length of the ECFCs encapsulated in $R = 0.75$ and $R = 1.0$ samples ranges from 8 to 2319 μm and 8–1865 μm respectively, with no significant difference. Both of these have significantly longer tubes than $R = 2.0$ samples, ranging from 8 to 383 μm (Fig. 4B). The total combined tube length of the $R = 0.75$ and $R = 1.0$ hydrogels are more than two fold longer than $R = 2.0$ samples (Fig. 4C). In addition, the total 3D volume covered by ECFC networks in $R = 0.75$ samples is $8.1 \times 10^5 \mu\text{m}^3$, triple the volume of ECFC networks in $R = 2.0$ samples ($2.4 \times 10^5 \mu\text{m}^3$), but similar to the case of $R = 1.0$ samples ($6.2 \times 10^5 \mu\text{m}^3$) (Fig. 4D). After continuous culture for 48 h, the length of tubes in $R = 1.0$ hydrogel ranges from 8 to 1952 μm , compared with 8–745 μm in $R = 2.0$ condition (Fig. 4E). Moreover, the corresponding total tube length and volume of $R = 1.0$ samples were more than $3 \times$ larger than $R = 2.0$ hydrogels (Fig. 4F–G). Overall, our data demonstrate that ECFCs are responding to lower mechanical modulus, which enables tubulogenesis and subsequent network formation in the Gtn-I-Odex hydrogel. In contrast, the hydro-gels with high mechanical properties severely restrict and hinder the morphology changes of the encapsulated ECFCs.

3.4. Cell protection and injectability of ECFC-loaded Gtn-I-odex hydrogel

Several previous studies reported that cell viability is largely reduced after exposure to extrusion through a needle without scaffold protection during direct injecting cell suspension *in vitro* or *in vivo* [70–72]. Cell membranes can easily be damaged by the shear stress of injection [34]. To evaluate the effects of Gtn-I-Odex hydrogel, as an injectable carrier on cell viability, ECFCs were loaded in the hydrogel and then extruded through a 22-gauge needle into a 96-well plate at the uniform flow rate of 1000 $\mu\text{L}/\text{min}$ set by the extruder equipment (Fig. 5A). After a few minutes in an incubator, the extruded hydrogel fragments self-healed into an integrated hydrogel mass located at the bottom of the culture well. For controls, ECFCs were suspended in culture media only or non-cross-linked Gtn media solutions before injection, both of which showed a significant percentage of cell death when compared with their non-injection control (Fig. S6). We also found that ~60% of cells were alive in the media-only samples post injection, while cells delivered with 6 wt% Gtn had a higher viability of ~75% (Fig. 5B). This may be attributed to the higher viscosity of Gtn solutions protecting cells during injection (Fig. S7). In contrast, the cells delivered with Gtn-I-Odex hydrogels had similar high cell viability (~82%) compared with non-injection controls, indicating the protection offered by self-healing Gtn-I-Odex hydrogels. Moreover, post-injection, the ECFCs encapsulated in the Gtn-I-Odex hydrogel were distributed homogeneously throughout the 3D hydrogel. The cells delivered with media or Gtn media all fell to the bottom of the culture dish immediately, forming a dense cell layer. These data suggest that the rapid self-healing rate of Gtn-I-Odex hydrogel is sufficient to maintain structural frameworks for supporting cells in 3D distribution at the target site.

After 24 h culture of the injected ECFC-loaded Gtn-I-Odex hydrogel, we observed vascular tube formation, verifying the maintenance of cellular functions following the injection and self-healing process (Fig. 5Ci, ii). Moreover, to improve on delivery safety and efficiency *in vivo*, cells should have enough time and nutrition to pre-adapt to the 3D environment *in vitro*, which also motivated us to confirm the normal morphologies and functions of the encapsulated ECFCs before injection. Analysis of *DO* revealed that oxygen was significantly consumed by ECFCs within the first 4 h after encapsulation (Fig. S8). This signifies that the most active cellular functions were taking place at this time period, which also matches the time frame of vacuoles formation stage of ECFCs observed above. However, injecting the hydrogels after 24 h in culture will impose shear forces that significantly damage the sprout cells and branched vascular network (Fig. S9), preventing the further morphogenesis of the ECFCs and ultimately compromising the neovascularization therapeutic impact. As a result, the ECFC-loaded hydrogel was also injected after 4 h of culture. As shown in Fig. 5Ciii and iv, the tubulogenesis of encapsulated ECFCs still normally occurred on day 1 as expected, which could potentially improve the effectiveness of the delivery treatment.

3.5. In vivo vasculogenic effect of ECFC-loaded Gtn-I-odex hydrogel

To determine the impact of our newly established delivery system on vascular regeneration *in vivo*, the ECFCs-loaded Gtn-I-Odex hydro-gels were injected subcutaneously on both sides of the backs of 7–8 week old, immune deficient, nude mice. The encapsulated ECFCs were either directly injected (0 h culture) or cultured for 4 h *in vitro* prior to implantation

(denoted here as 0 h samples and 4 h samples). For positive control, we transplanted the collagen hydrogels with bio-functionality but no self-healing capabilities. The ECFC-loaded collagen hydrogels were also cultured *in vitro* for 0 h and 4 h prior to implantation. For negative control, the completely gelled ECFC-loaded CEC-I-Odex (1.0 wt% CEC) hydrogels with self-healing properties but no bio-functionality, were directly injected subcutaneously (ie with 0 h of *in vitro* culture). It should be noted that as the ECFC-loaded CEC-I-Odex hydrogels culture *in vitro* for 4 h could not self-heal following injection, since the injected hydrogel fragments turned into individual gel beads in the aqueous media or PBS, which cannot self-heal into an integrity (Fig. 2Cii, see video 3 in SI). Nonetheless, the injected ECFC-loaded Gtn-I-Odex hydrogel fragments can self-heal *in vivo* into an integrated gel after extraction (Fig. 6A–B). To evaluate the presence of vascular structures, histological examination was conducted by CD31 staining. After 12 h, numerous CD31⁺ cells were observed uniformly distributed throughout the hydrogel section of the 0 h sample (Fig. 6A) while fewer CD31⁺ cells were found in the 4 h construct (Fig. 6B, K). This could be due to the vacuole's formation stage of the 4 h sample of ECFCs, which are more sensitive and vulnerable to the extruded pressure during injection, leading to the cell loss. After 3 days, lumenized vessels were observed in both 0 h and 4 h constructs (Fig. 6C–D). In collagen gels, CD31⁺ cells were observed in both 0 h and 4 h constructs with no apparent difference (Fig. 6 E–F, K) with lumenized vasculature on day 3 ((Fig. 6 G–H)). There were only a few CD31⁺ cells observed in the negative non-biofunctional CEC-I-Odex hydrogels and no vessels were found after day 3 (Fig. 6I–J). Quantification of the microvessel density revealed no significant differences between the 0 h (~32.4 vessels mm⁻²) and 4 h (~25.8 vessels mm⁻²) samples in Gtn-I-Odex hydrogels, as well as collagen hydrogel controls (Fig. 6L). However, when examining vessel size, no significant difference was observed in the collagen gels between 0 h and 4 h, while in the Gtn-I-Odex hydrogels, more mature and larger vessels (> 50 μm²) were observed in the 4 h injected hydrogels than in the 0 h samples (Fig. 6 M–N). This suggests that allowing vacuole formation *in vitro* prior to injection, may facilitate rapid vasculogenesis *in vivo*. Taken together, these results show that ECFC-loaded self-healing Gtn-I-Odex hydrogels are able to undergo rapid vasculogenesis *in vivo*, and can be further utilized to initiate ECFC morphogenesis before delivery.

4. Conclusion

The present study developed instructive self-healing hydrogels with bio-functional RGD and proteolytic peptide sites in their networks, allowing complex cellular functions to occur. This inexpensive Gtn-I-Odex hydrogel can be facilely utilized as an ECFC delivery vehicle, with excellent self-healing and injectability, for treatment of vasculature injuries and related diseases. We demonstrated the suitability of Gtn-I-Odex hydrogels to support complex vascular network formation of ECFCs, both *in vitro* and *in vivo*. To our knowledge, this is the first report of injectable self-healing hydrogels with biological activities for promoting cellular morphogenesis. Based on these findings, bio-functional Gtn-I-Odex hydrogels with self-healing features can be further adapted for injecting other types of cells or patient-specific cell lines and a wide range of future cell delivery treatments that require healthy vascular beds, such as cardiac ischemia or diabetic diseases.

4.1. Data availability

All the data needed to reproduce the work performed and evaluate the conclusions made are presented in the paper and/or the Supplemental Materials. Additional raw/processed data forms part of an ongoing study and may be requested from the authors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Christine Schutzman for editing the manuscript. This work was supported by the shared resources from the Sydney Kimmel Cancer Center, Johns Hopkins University (P30 CA006973), the fellowship from the Maryland Stem Cell Fund (MSCRF-3928) (to Z.W), the NCI Physical Sciences-Oncology Center (U54CA210173) and the President's Frontier Award (both to S.G).

References

- [1]. Segers VF, Lee RT, Stem-cell therapy for cardiac disease, *Nature* 451 (2008) 937. [PubMed: 18288183]
- [2]. Rafii S, Lyden D, Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration, *Nat. Med* 9 (2003) 702. [PubMed: 12778169]
- [3]. Perin EC, López J, Methods of stem cell delivery in cardiac diseases, *Nat. Rev. Cardiol* 3 (2006) S110.
- [4]. Cheng L, Huang Z, Zhou W, Wu Q, Donnola S, Liu JK, Fang X, Sloan AE, Mao Y, Lathia JD, Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth, *Cell* 153 (2013) 139–152. [PubMed: 23540695]
- [5]. Lloyd-Griffith C, McFadden TM, Duffy GP, Unger RE, Kirkpatrick CJ, O'Brien FJ, The pre-vascularisation of a collagen-chondroitin sulphate scaffold using human amniotic fluid-derived stem cells to enhance and stabilise endothelial cell-mediated vessel formation, *Acta Biomater.* 26 (2015) 263–273. [PubMed: 26300337]
- [6]. Masuda H, Kawamoto A, Ii M, Asahara T, Endothelial Progenitor Cells for Vascular Medicine, *Regenerative Medicine for Peripheral Artery Disease*, 2016, pp. 71–90.
- [7]. Silva EA, Kim E-S, Kong HJ, Mooney DJ, Material-based deployment enhances efficacy of endothelial progenitor cells, *Proc. Natl. Acad. Sci. Unit. States Am* 105 (2008) 14347–14352.
- [8]. Urbich C, Dimmeler S, Endothelial progenitor cells: characterization and role in vascular biology, *Circ. Res* 95 (2004) 343–353. [PubMed: 15321944]
- [9]. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA, Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals, *Blood* 109 (2007) 1801–1809. [PubMed: 17053059]
- [10]. Blatchley MR, Gerecht S, Acellular implantable and injectable hydrogels for vascular regeneration, *Biomed. Mater* 10 (2015) 034001. [PubMed: 25775039]
- [11]. Park KM, Gerecht S, Harnessing developmental processes for vascular engineering and regeneration, *Development* 141 (2014) 2760–2769. [PubMed: 25005471]
- [12]. Kawamoto A, Tkebuchava T, Yamaguchi J-I, Nishimura H, Yoon Y-S, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia, *Circulation* 107 (2003) 461–468. [PubMed: 12551872]
- [13]. Fujita J, Itabashi Y, Seki T, Tohyama S, Tamura Y, Sano M, Fukuda K, Myocardial cell sheet therapy and cardiac function, *Am. J. Physiol. Heart Circ. Physiol* 303 (2012) 1169–1182.
- [14]. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T, Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization, *Proc. Natl. Acad. Sci. Unit. States Am* 97 (2000) 3422–3427.

- [15]. Burdick JA, Mauck RL, Gerecht S, To serve and protect: hydrogels to improve stem cell-based therapies, *Cell Stem Cell* 18 (2016) 13–15. [PubMed: 26748751]
- [16]. Toh WS, Loh XJ, Advances in hydrogel delivery systems for tissue regeneration, *Mater. Sci. Eng. C* 45 (2014) 690–697.
- [17]. Yuan X, Wei Y, Villasante A, Ng JJ, Arkonac DE, Chao P.-h.G., Vunjak-Novakovic G, Stem cell delivery in tissue-specific hydrogel enabled meniscal repair in an orthotopic rat model, *Biomaterials* 132 (2017) 59–71. [PubMed: 28407495]
- [18]. Young SA, Sherman SE, Cooper TT, Brown C, Anjum F, Hess DA, Flynn LE, Amsden BG, Mechanically Resilient Injectable Scaffolds for Intramuscular Stem Cell Delivery and Cytokine Release, *Biomaterials* 159 (2018) 146–160. [PubMed: 29324306]
- [19]. Gil MS, Thambi T, Phan VG, Kim SH, Lee DS, Injectable hydrogel-incorporated cancer cell-specific cisplatin releasing nanogels for targeted drug delivery, *J. Mater. Chem. B* 5 (2017) 7140–7152.
- [20]. Dong Y, Rodrigues M, Li X, Kwon SH, Kosaric N, Khong S, Gao Y, Wang W, Gurtner GC, Injectable and tunable gelatin hydrogels enhance stem cell retention and improve cutaneous wound healing, *Adv. Funct. Mater* 27 (2017) 1606619.
- [21]. Cai L, Dewi RE, Heilshorn SC, Injectable hydrogels with in situ double network formation enhance retention of transplanted stem cells, *Adv. Funct. Mater* 25 (2015) 1344–1351. [PubMed: 26273242]
- [22]. Donaghue IE, Tator CH, Shoichet MS, Sustained delivery of bioactive neurotrophin-3 to the injured spinal cord, *Biomaterials Science* 3 (2015) 65–72. [PubMed: 26214190]
- [23]. Martens TP, Godier AF, Parks JJ, Wan LQ, Koeckert MS, Eng GM, Hudson BI, Sherman W, Vunjak-Novakovic G, Percutaneous cell delivery into the heart using hydrogels polymerizing in situ, *Cell Transplant.* 18 (2009) 297–304. [PubMed: 19558778]
- [24]. Wei Z, Yang JH, Zhou J, Xu F, Zrínyi M, Dussault PH, Osada Y, Chen YM, Self-healing gels based on constitutional dynamic chemistry and their potential applications, *Chem. Soc. Rev* 43 (2014) 8114–8131. [PubMed: 25144925]
- [25]. Wei Z, Lewis DM, Xu Y, Gerecht S, Dual cross-linked biofunctional and self-healing networks to generate user-defined modular gradient hydrogel constructs, *Adv. Healthcare Mater.* 6 (2017) 1700523.
- [26]. Wei Z, Yang JH, Liu ZQ, Xu F, Zhou JX, Zrínyi M, Osada Y, Chen YM, Novel biocompatible polysaccharide-based self-healing hydrogel, *Adv. Funct. Mater* 25 (2015) 1352–1359.
- [27]. Wei Z, Yang JH, Du XJ, Xu F, Zrínyi M, Osada Y, Li F, Chen YM, Dextran-based self-healing hydrogels formed by reversible diels-alder reaction under physiological conditions, *Macromol. Rapid Commun* 34 (2013) 1464–1470. [PubMed: 23929621]
- [28]. Appel EA, del Barrio J, Loh XJ, Scherman OA, Supramolecular polymeric hydrogels, *Chem. Soc. Rev* 41 (2012) 6195–6214. [PubMed: 22890548]
- [29]. Guvendiren M, Lu HD, Burdick JA, Shear-thinning hydrogels for biomedical applications, *Soft Matter* 8 (2012) 260–272.
- [30]. Thakur A, Jaiswal MK, Peak CW, Carrow JK, Gentry J, Dolatshahi-Pirouz A, Gaharwar AK, Injectable shear-thinning nanoengineered hydrogels for stem cell delivery, *Nanoscale* 8 (2016) 12362–12372. [PubMed: 27270567]
- [31]. Highley CB, Rodell CB, Burdick JA, Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels, *Adv. Mater* 27 (2015) 5075–5079. [PubMed: 26177925]
- [32]. Lü S, Gao C, Xu X, Bai X, Duan H, Gao N, Feng C, Xiong Y, Liu M, Injectable and self-healing carbohydrate-based hydrogel for cell encapsulation, *ACS Appl. Mater. Interfaces* 7 (2015) 13029–13037. [PubMed: 26016388]
- [33]. Yang B, Zhang Y, Zhang X, Tao L, Li S, Wei Y, Facilely prepared inexpensive and biocompatible self-healing hydrogel: a new injectable cell therapy Carrier, *Polym. Chem* 3 (2012) 3235–3238.
- [34]. Aguado BA, Mulyasmita W, Su J, Lampe KJ, Heilshorn SC, Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers, *Tissue Eng.* 18 (2011) 806–815.
- [35]. Yu L, Ding J, Injectable hydrogels as unique biomedical materials, *Chem. Soc. Rev* 37 (2008) 1473–1481. [PubMed: 18648673]

- [36]. Dong R, Zhao X, Guo B, Ma PX, Self-healing conductive injectable hydrogels with antibacterial activity as cell delivery Carrier for cardiac cell therapy, *ACS Appl. Mater. Interfaces* 8 (2016) 17138–17150. [PubMed: 27311127]
- [37]. Wang Y, Adokoh CK, Narain R, Recent development and biomedical applications of self-healing hydrogels, *Expet Opin. Drug Deliv.* 15 (2018) 77–91.
- [38]. Chen Y, Wang W, Wu D, Nagao M, Hall DG, Thundat T, Narain R, Injectable Self-healing Zwitterionic Hydrogels Based on Dynamic Benzoxaborole-sugar Interactions with Tunable Mechanical Properties, *Biomacromolecules*, 2018.
- [39]. Ouyang L, Highley CB, Rodell CB, Sun W, Burdick JA, 3D printing of shear-thinning hyaluronic acid hydrogels with secondary cross-linking, *ACS Biomater. Sci. Eng* 2 (2016) 1743–1751.
- [40]. Rodell CB, Dusaj NN, Highley CB, Burdick JA, Injectable and cytocompatible tough double-network hydrogels through tandem supramolecular and covalent crosslinking, *Adv. Mater* 28 (2016) 8419–8424. [PubMed: 27479881]
- [41]. Wei Z, Zhao J, Chen YM, Zhang P, Zhang Q, Self-healing polysaccharide-based hydrogels as injectable carriers for neural stem cells, *Sci. Rep* 6 (2016) 37841. [PubMed: 27897217]
- [42]. Hou S, Wang X, Park S, Jin X, Ma PX, Rapid self-integrating, injectable hydrogel for tissue complex regeneration, *Advanced Healthcare Materials* 4 (2015) 1491–1495. [PubMed: 25946414]
- [43]. Chang G, Chen Y, Li Y, Li S, Huang F, Shen Y, Xie A, Self-healable hydrogel on tumor cell as drug delivery system for localized and effective therapy, *Carbohydr. Polym* 122 (2015) 336–342. [PubMed: 25817677]
- [44]. Bayless KJ, Salazar R, Davis GE, RGD-dependent vacuolation and lumen formation observed during endothelial cell morphogenesis in three-dimensional fibrin matrices involves the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins, *Am. J. Pathol* 156 (2000) 1673–1683. [PubMed: 10793078]
- [45]. DeForest CA, Polizzotti BD, Anseth KS, Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments, *Nat. Mater* 8 (2009) 659. [PubMed: 19543279]
- [46]. Stratman AN, Saunders WB, Sacharidou A, Koh W, Fisher KE, Zawieja DC, Davis MJ, Davis GE, Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices, *Blood* 114 (2009) 237–247. [PubMed: 19339693]
- [47]. Sacharidou A, Koh W, Stratman AN, Mayo AM, Fisher KE, Davis GE, Endothelial lumen signaling complexes control 3D matrix-specific tubulogenesis through interdependent Cdc42-and MT1-MMP-mediated events, *Blood* 115 (2010) 5259–5269. [PubMed: 20215637]
- [48]. Chen YM, Sun L, Yang SA, Shi L, Zheng WJ, Wei Z, Hu C, Self-healing and photoluminescent carboxymethyl cellulose-based hydrogels, *Eur. Polym. J* 94 (2017) 501–510.
- [49]. Kang H-W, Tabata Y, Ikada Y, Fabrication of porous gelatin scaffolds for tissue engineering, *Biomaterials* 20 (1999) 1339–1344. [PubMed: 10403052]
- [50]. Bergqvist D, Dextran and haemostasis. A review, *Acta Chir. Scand* 148 (1982) 633–640. [PubMed: 6189321]
- [51]. Davis GE, Senger DR, Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization, *Circ. Res* 97 (2005) 1093–1107. [PubMed: 16306453]
- [52]. Park KM, Gerecht S, Hypoxia-inducible hydrogels, *Nat. Commun* 5 (2014) 4075. [PubMed: 24909742]
- [53]. Park KM, Blatchley MR, Gerecht S, The design of dextran-based hypoxia-inducible hydrogels via in situ oxygen-consuming reaction, *Macromol. Rapid Commun* 35 (2014) 1968–1975. [PubMed: 25303104]
- [54]. Kathan M, Kovařík P, Jurissek C, Senf A, Dallmann A, Thünemann AF, Hecht S, Control of imine exchange kinetics with photoswitches to modulate self-healing in polysiloxane networks by light illumination, *Angew. Chem. Int. Ed* 55 (2016) 13882–13886.
- [55]. Chao A, Negulescu I, Zhang D, Dynamic covalent polymer networks based on degenerative imine bond exchange: tuning the malleability and self-healing properties by solvent, *Macromolecules* 49 (2016) 6277–6284.

- [56]. Hanjaya-Putra D, Bose V, Shen Y-I, Yee J, Khetan S, Fox-Talbot K, Steenbergen C, Burdick JA, Gerecht S, Controlled activation of morphogenesis to generate a functional human microvasculature in a synthetic matrix, *Blood* 118 (2011) 804–815. [PubMed: 21527523]
- [57]. Chan XY, Black R, Dickerman K, Federico J, Lévesque M, Mumm J, Gerecht S, Three-dimensional vascular network assembly from diabetic patient-derived induced pluripotent stem Cells Significance, *Arterioscler. Thromb. Vasc. Biol* 35 (2015) 2677–2685. [PubMed: 26449749]
- [58]. Weng L, Romanov A, Rooney J, Chen W, Non-cytotoxic, in situ gelable hydrogels composed of N-carboxyethyl chitosan and oxidized dextran, *Biomaterials* 29 (2008) 3905–3913. [PubMed: 18639926]
- [59]. Maia J, Ferreira L, Carvalho R, Ramos MA, Gil MH, Synthesis and characterization of new injectable and degradable dextran-based hydrogels, *Polymer* 46 (2005) 9604–9614.
- [60]. Shen Y-I, Abaci HE, Krupski Y, Weng L-C, Burdick JA, Gerecht S, Hyaluronic acid hydrogel stiffness and oxygen tension affect cancer cell fate and endothelial sprouting, *Biomaterials Science* 2 (2014) 655–665. [PubMed: 24748963]
- [61]. Cordes E, Jencks W, On the mechanism of Schiff base formation and hydrolysis, *J. Am. Chem. Soc* 84 (1962) 832–837.
- [62]. Cordes EH, Jencks WP, The mechanism of hydrolysis of Schiff bases derived from aliphatic amines, *J. Am. Chem. Soc* 85 (1963) 2843–2848.
- [63]. Djabourov M, Leblond J, Papon P, Gelation of aqueous gelatin solutions. II. Rheology of the sol-gel transition, *J. Phys* 49 (1988) 333–343.
- [64]. Dumas J, Bacri J-C, New method of viscosity measurement near the gelatin sol-gel transition, *J. Phys., Lett* 41 (1980) 279–282.
- [65]. Zhang Y, Ouyang H, Lim CT, Ramakrishna S, Huang ZM, Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds, *J. Biomed. Mater. Res. B Appl. Biomater* 72 (2005) 156–165. [PubMed: 15389493]
- [66]. Gómez-Guillén M, Turnay J, Fernández-Díaz M, Ulmo N, Lizarbe M, Montero P, Structural and physical properties of gelatin extracted from different marine species: a comparative study, *Food Hydrocolloids* 16 (2002) 25–34.
- [67]. Kusuma S, Shen Y-I, Hanjaya-Putra D, Mali P, Cheng L, Gerecht S, Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix, *Proc. Natl. Acad. Sci. Unit. States Am* 110 (2013) 12601–12606.
- [68]. Madl CM, LeSavage BL, Dewi RE, Dinh CB, Stowers RS, Khariton M, Lampe KJ, Nguyen D, Chaudhuri O, Enejder A, Maintenance of neural progenitor cell stemness in 3D hydrogels requires matrix remodelling, *Nat. Mater* 16 (2017) 1233. [PubMed: 29115291]
- [69]. Wang H, Heilshorn SC, Adaptable hydrogel networks with reversible linkages for tissue engineering, *Adv. Mater* 27 (2015) 3717–3736. [PubMed: 25989348]
- [70]. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE, Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies, *J. Mol. Cell. Cardiol* 33 (2001) 907–921. [PubMed: 11343414]
- [71]. Kong HJ, Smith MK, Mooney DJ, Designing alginate hydrogels to maintain viability of immobilized cells, *Biomaterials* 24 (2003) 4023–4029. [PubMed: 12834597]
- [72]. Li W, Ma N, Ong LL, Nesselmann C, Klopsch C, Ladilov Y, Furlani D, Piechaczek C, Moebius JM, Lützwow K, Bcl-2 engineered MSCs inhibited apoptosis and improved heart function, *Stem Cell* 25 (2007) 2118–2127.
- [73]. Park KM, Ko KS, Joong YK, Shin H, Park KD, In situ cross-linkable gelatin–poly (ethylene glycol)–tyramine hydrogel via enzyme-mediated reaction for tissue regenerative medicine, *J. Mater. Chem* 21 (2011) 13180–13187.
- [74]. Kusuma S, Zhao S, Gerecht S, The extracellular matrix is a novel attribute of endothelial progenitors and of hypoxic mature endothelial cells, *Faseb. J* 26 (2012) 4925–4936. [PubMed: 22919069]
- [75]. Abaci HE, Devendra R, Smith Q, Gerecht S, Drazer G, Design and development of microbioreactors for long-term cell culture in controlled oxygen microenvironments, *Biomed. Microdevices* 14 (2012) 145–152. [PubMed: 21947550]

- [76]. Hsieh F-Y, Tao L, Wei Y, Hsu S, A novel biodegradable self-healing hydrogel to induce blood capillary formation, *NPG Asia Mater.* 9 (2017) e363.
- [77]. Jain VK, Ranganatha C, Muralidhar K, Evaluation of rheological properties of medium for AFM process, *Mach. Sci. Technol* 5 (2001) 151–170.
- [78]. Kusuma S, Macklin B, Gerecht S, Derivation and network formation of vascular cells from human pluripotent stem cells, *Meth. Mol. Biol* 1202 (2014) 1–9.

Fig. 1. Schematic displaying the strategy of the self-healing ECFC-loaded Gtn-I-Odex hydrogels and the hydrogel properties.

(A) The ECFCs are suspended in the mixture of Gtn and Odex in media solution. (B) The Gtn-I-Odex hydrogels are formed by the dynamic imine cross-links between aldehyde groups (from Odex) with the amino groups (from Gtn) under physiological conditions. (C) Schematic of vascular morphogenesis of ECFCs at each stage during cultured in Gtn-I-Odex hydrogel. (i) The encapsulated ECFCs undergo vacuole formation at the initial step; (ii) vacuolated ECFCs subsequently merge with the neighboring cells to form lumens. (iii, iv) The progressive tubulogenesis of ECFCs then occurs through both sprouting and branching of the cells to form complex vascular networks. (D) The ECFC-loaded self-healing Gtn-I-Odex hydrogel can be loaded into a syringe and injected subcutaneously at the back of nude mice at different *in vitro* culture time points of 0 h and 4 h. (E) The gelation time of the Gtn-I-Odex hydrogels with various ratio of reactive groups of aldehyde and amino ($R = 0.75, 1.0$ and 2.0) (F) The G' of the Gtn-I-Odex hydrogels with various R . *-symbol indicated the significant differences ($p < 0.05$). (G) The degradation behaviors of Gtn-I-Odex hydrogels with various R immersed in the ECFC culture media for 2 weeks in 37°C incubator. (H) Effect of collagenase concentration on degradability of Gtn-I-Odex hydrogel with constant $R = 1.0$.

Fig. 2. Self-healing performance and injectable capability of Gtn-l-Odex hydrogel ($R = 1.0$). (A) (i) Three cylindrical Gtn-l-Odex hydrogel are prepared, a red dyed (using rhodamine B), original transparent and blue dyed (using methylene blue). (ii) The three hydrogels are placed together in 37 °C for 20 min without any external intervention. (iii) The three pieces of hydrogels are fully self-healed into an integrated hydrogel and are stretchable by tweezers. (B) (i, ii) A disk-like shaped Gtn-l-Odex hydrogel is loaded into a 22-gauge syringe, (iii) and injected into three tiny PDMS disc mold. (iv, v) After healing for 5 min in 37 °C, the tiny self-healed hydrogel discs are taken out and immersed in PBS (pH 7.4). (C) Three different hydrogels are injected into 37 °C PBS (pH 7.4) respectively, including (i) Gtn hydrogel, (ii) CEC-l-Odex hydrogel, and (iii) Gtn-l-Odex hydrogel. (D) Strain sweep (left) and rheology recovery (right) tests for Gtn-l-Odex hydrogels in 37 °C at a fixed frequency of 0.1 rad s⁻¹. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Vascular network growth and complexity of encapsulated ECFCs in Gtn-I-Odex hydrogels.

(A) (i, iii) Many of the encapsulated ECFCs contain vacuoles, some of which coalesced into lumen after 4–6 h in culture as visualized using LM images. (ii, iv) Corresponding vacuoles and lumen formation were visualized using vacuole stain. Red: FM 4–64 FX; Blue: nuclei. (v) Sprouting and branching, followed by (vi, vii) vascular networks formation within 48 h of encapsulation. Scale bars are 100 μ m. The upper right subpanels are high magnifications of relevant black boxes, scale bars are 20 μ m. (B) Confocal image of 48 h microvascular networks (i) and the orthogonal view of luminal structures (indicated with asterisks) (ii). Phalloidin (green) and nuclei (blue). Scale bars are 100 μ m. (C) The G' of Gtn-I-Odex hydrogels and ECFC-loaded hydrogels decrease along the culture period. *symbol indicates the significant differences ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. Three-dimensional analysis of ECFC networks in Gtn-I-Odex hydrogels with various R . (A) Representative 24 h and 48 h confocal images of hydrogels with $R = 0.75$, 1.0 and 2.0 encapsulated ECFCs analyzed with Imaris Filament Tracer. Phalloidin in green. Scale bars are 100 μm . Multiple aspects of the analysis are compared and presented: (B, E) mean tube length, (C, F) total tube length, and (D, G) total tube volume for culturing 24 h and 48 h respectively. ($*p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. Cell viability and morphology of encapsulated ECFCs after injection.

(A) The extruder equipment is set up with $1000 \mu\text{L min}^{-1}$ flow rate of injection. (B) The ECFC viability within EGM-2 media, non-cross-linked Gtn (6 wt%), or Gtn-I-Odex hydrogel with and without ejection through syringe. ($*p < 0.05$). (C) Tubulogenesis of ECFCs after injection at 0 h and 4 h of *in vitro* culture. Vacuoles (iii) and vascular tube (ii and iv) are indicated by arrows. Scale bars are 100 μm .

Fig. 6. *In vivo* vasculogenesis tests.

(A) The self-healing ECFC-loaded Gtn-I-Odex hydrogels are directly injected subcutaneously to both sides of the backs of nude mice. The implants are retrieved after 12 h *in vivo*. (B) The ECFC-loaded Gtn-I-Odex hydrogels are incubated *in vitro* for 4 h before injection *in vivo* for 12 h. Subpanels i and ii with high magnification of correlated boxes in panel A and B show the CD31⁺ cells (indicated by arrows). (C) The implants of 0 h sample are retrieved after 3 days *in vivo*. (D) The implants of 4 h sample are retrieved after 3 days *in vivo*. Subpanels iii and iv are high magnification of correlated boxes showing the positive endothelial lining and microvessels (indicated by arrowheads), respectively. (E, G) The ECFC-loaded collagen controls are directly transplanted and sutured *in vivo* for 12 h and 3 days. Subpanels i and iii are high magnification of correlated boxes showing CD31⁺ cells and microvessels (indicated by arrows and arrowheads). (F, H) The ECFC-loaded collagen hydrogels are incubated *in vitro* for 4 h before transplanted *in vivo* for 12 h and 3 days.

Subpanels ii and iv are high magnification of correlated boxes showing CD31⁺ cells and microvessels (indicated by arrows and arrowheads). (I, J) The ECFC-loaded CEC-I-Odex hydrogel controls are directly injected *in vivo* for 12 h and 3 days. Subpanels i and ii are high magnification of correlated boxes showing CD31⁺ cells and microvessels (indicated by arrows and arrowheads). All the scale bars are 100 μ m. Quantification of the density of (K) CD31⁺ cells at different injected time points for retrieving at 12 h and (L) the vessels on day 3. (M) Quantification shows vessel area distributions of different injected points in collagen controls for retrieving on day 3. (N) Quantification shows vessel area distributions of different injected points in Gtn-I-Odex hydrogels for retrieving on day 3. *symbol indicates the significant differences ($p < 0.05$).