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3-D Self-Assembling Leucine Zipper Hydrogel With Tunable Properties For Tissue Engineering

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Abstract

Peptide-based engineered hydrogel scaffolds present several advantages over traditional protein or polymeric hydrogels by imparting more robust control over hydrogel properties. In this manuscript, we report the synthesis and characterization of a leucine zipper (LZ) based self assembling hydrogel for use in tissue engineering applications. Although, LZ hydrogels possess several advantages, the stability of these hydrogels has always been elusive. In this study, we have standardized the procedure for creating a stable LZ hydrogel. Pore-size was tunable by altering the peptide concentration from 7% to 12% by weight. In order to create a microenvironment for cell adhesion, the LZ polypeptide was functionalized by the incorporation of the cell attachment RGD domain. *In vivo* implantation of the LZ scaffolds in a mouse model showed absence of foreign body reaction to the scaffold. *In vivo* experiments with human marrow stem cells (HMSCs) in immunocompromised mice showed the biological property of the hydrogel to promote cell attachment, proliferation and its ability to support neovascularization. Our results show for the first time, that it is possible to generate a functional and stable LZ scaffold that can be used *in vivo* for tissue engineering applications.

Keywords

Angiogenesis; Hydrogel; RGD peptide; Scaffold; Self assembly; Stem cell

1. Introduction

Tissue regenerative therapies require the synthesis of new biomaterials that are bioactive and are cell instructive. Ideally, scaffolds need to be porous to permit cell attachment, migration

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and enable diffusion of oxygen and flow of biological substances for maintaining metabolic cell activity and thereby provide a localized and sustained release of growth factors. Another important property is that the scaffold must possess a tunable degradation rate in order to match the rate of the new tissue that is being regenerated [1-4].

Native biomaterials such as collagen [5, 6], fibrin [7], chitosan [8] and silk [9] are derived from naturally occurring polymeric proteins. Recently, peptide-based biomaterials are receiving increasing attention for tissue engineering applications due to the versatility they offer over the control of several hydrogel properties ranging from stability and control of degradation rates to providing innovative ways to sequester and deliver growth factors [10]. Therefore, synthesis of peptide-based biomaterials has emerged as a high utility tool in tissue engineering applications to facilitate cell-matrix interactions [11-16]. The diversity of peptide modules available throughout the proteome broadens the horizon for peptide-engineered biomaterials. The native ECM is a hierarchically structured composite material that is able to furnish several cues for cellular proliferation and differentiation [6, 17, 18].

Complex protein-engineered scaffolds can be synthesized by designing scaffolds that self-assemble into specific hierarchical structures resembling the micro and nano properties of native ECM. Additionally, they can be functionalized with many of the cell instructive cues provided by native ECM creating a biomimetic environment. Molecular self-assembly, therefore, presents itself as a very attractive strategy to construct biomimetic materials [19, 20]. The DNA binding leucine zipper proteins contain a self-assembling LZ domain. Petka et al. first developed LZ based self-assembly in 1998 [21]. Since then there have been several studies with various modifications to the LZ motif to facilitate hydrogel formation [22-26]. The advantage of the LZ chimeric protein is that amino acid substitutions can be easily accomplished by genetic engineering. Therefore, peptides with different biological functions can be incorporated in the LZ protein backbone to create a functional chimeric protein with self-assembling properties [25]. This significantly increases the flexibility of LZ hydrogel for tissue engineering applications. However, the major drawback with the generic LZ hydrogel is its instability [27]. None of the published studies using the LZ motif show the development of a LZ scaffold that can support 3-D cell culture *in vivo* for an extended period of time.

In this manuscript we report the synthesis and characterization of a self-assembling LZ hydrogel which possess mechanical properties strong enough to use as a scaffold for tissue engineering applications. In designing this second-generation chimeric LZ protein we have introduced amino acid residues in the polypeptide to effectively cross-link the nanofibers into a high molecular weight polymer. Further, we have introduced a new processing protocol to increase the strength and stability of the scaffold. We then investigated the potential of this scaffold to favor cell attachment, proliferation and vascularization *in vivo*.

2. Materials and Methods

2.1 Cloning, Protein Expression and Purification of the LZ chimeric protein

The previous published leucine zipper peptide containing the six heptad repeat amino acid sequence was used (A1: SGELENE VAQLERE VRSLEDE AAELEQK VSRLKNE

IEDLKAE) [26]. This A1 sequence was modified by the introduction of a cysteine amino acid residue to generate A2 by site-directed mutagenesis kit (A2: CSGELENE VAQLERE VRSLEDE AAELEQK VSRLKNE IEDLKAE) in order to facilitate covalent binding by cysteine-cysteine cross-links. The site-directed mutation was done with the QuikChange kit (QIAGEN) according to manufacturer's protocol. Two A2 sequences were then separated by a spacer of two sets of 5 repeat of flexible peptides [C1: 5(AGAGAGPEG)]. An extra flexible tail containing another cysteine [C2: (AGAGAGPEG)₂C] was also incorporated to the C-terminal end of the polypeptide. The combination of the peptide domains A2-C1-C1-A2-C2 formed the LZ-Control polypeptide. This initial construct was then modified to incorporate the integrin-binding motif RGDS by incorporating this domain between the two C1 blocks (A2-C1-RGDSC1-A2-C2). This polypeptide was designated as LZ-RGDS (Fig 1). Both the final LZ-Control and LZ-RGDS chimeric protein constructs were verified by sequencing at the DNA Services Facility at University of Illinois at Chicago Research Resources Center. The final construct was then cloned into pQE9 vector (QIAGEN) and expressed recombinantly in *E. coli*.

For expression of the chimeric protein, a single bacterial colony was inoculated in 500mL LB broth containing 100 µg/mL ampicillin and 30 µg/mL kanamycin and grown in a 37°C shaker overnight. The culture was then transferred into larger 5L LB broth culture with 100 µg/mL ampicillin and 30 µg/mL kanamycin and incubated in a 37°C shaker until OD 600 was reached. Protein production was induced using 1.0ml of 1.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) added to the culture and grown for an additional period of 5 hours. The cells were then pelleted and stored at -80°C. The LZ chimeric protein purification was carried out using Ni-NTA Superflow resin (QIAGEN) following manufacturer's protocol. The elution buffer with LZ chimeric protein was extensively dialyzed for 5 days, lyophilized and stored at -20°C until use. The purified protein was then run on SDS-PAGE gel and stained with Coomassie blue. The size of the LZ chimeric protein was approximately 37 kDa and the yield was about 80 mg/L.

2.2. Preparation of the LZ hydrogel

LZ hydrogels were prepared by mixing specific ratios of the LZ-Control protein with LZRGDS at concentrations from 7% ~12% (w/v) by dissolving in 100 mM phosphate buffer. The pH was adjusted to 7.4. The self-assembled LZ hydrogel formed within 3 hours when incubated at 37°C. The hydrogel was then lyophilized and baked at 121°C for 12 hours for dehydrothermal treatment to facilitate further physical crosslinking and sterilization. Hydrogels were stored in sterile containers at room temperature.

2.3. Circular Dichroism (CD) spectroscopy

The CD spectrum was recorded using Jasco J-720 spectrometer at 25°C. 14 µM LZ-Control or LZ-RGDS solution was prepared using 100 mM phosphate buffer and the pH adjusted with either HCl or NaOH to 4, 7.4, or 11. The samples were heated to 50°C for 30 minutes and slowly cooled down to room temperature before CD measurements to allow any possible refolding. A quartz cuvette with path length of 0.1 cm was used and the measurement was taken at a resolution of 1 nm, bandwidth of 1 nm, and the wavelength

range was from 200-250 nm. Spectra were corrected by subtraction of the corresponding buffer readings. Triplicated scans were obtained for each sample.

2.4. Scanning electron microscopy (SEM)

The LZ hydrogels from 7% ~12% w/v were quenched in liquid nitrogen and then placed in a pre-cooled turbo freeze-drying chamber. The series of drying steps from -160°C to room temperature was performed in increments of 20°C/hour at 1×10^{-3} mmHg ultra low pressure. After drying, the samples were fractured and the exposed inner structure was mounted onto SEM grids. The samples were coated with 10nm osmium tetroxide and the imaging was performed using a Field Emission Hitachi S-4800-II SEM operating at 5keV.

2.5 Cryo-TEM

Cryo-TEM was performed using a Hitachi HD-2300A STEM. A 7% w/v LZ-Control hydrogel was prepared and applied directly on the TEM carbon-coated grid and excess water was blotted with a filter paper. The samples were quickly frozen by plunging into liquid ethane and were kept in liquid nitrogen before and during the cryo-TEM imaging.

2.6. *In vitro* degradation study

The *in vitro* degradation rate was determined by immersing the hydrogel in PBS (Gibco) containing 0.5% sodium azide (Fisher Scientific). The 7% w/v LZ-Control hydrogel was weighed, and placed in a tube containing excess amount of PBS and stirred at 37°C for a period of up to 30 days. The hydrogel was then removed and rinsed with distilled water for several times to remove the salts. After lyophilization, the hydrogel was weighed again. The weight loss (W_L) and remaining weight (RW) was calculated according to the equation below $W_L = \frac{W_0 - W_1}{W_0}$, where W_0 and W_1 represent the hydrogel weight before and after soaking in PBS respectively and Remaining weight $RW = 1 - W_L$.

2.7. Mechanical properties

2.7.1. Microscopic magnetic resonance elastography (μMRE)—μMRE experiments were performed on a vertical bore (54 mm diameter) Bruker DRX 11.74 T (500 MHz for protons) Avance MR Spectrometer (Oxford Instruments, Oxford, UK) equipped with the MRE electro-mechanical setup [28]. Briefly, 7%, 10% and 12% w/v LZ hydrogel samples (6 mm diameter discs) were embedded in 0.75% (w/v) agarose gel (SeaKem LE Agarose, Lonza, Rockland, ME USA, Catalog No: 50000) in MRE test tubes (I.D = 8.8 mm). Agarose gel was made with PBS to maintain osmotic and pH balance. The MRE tube was attached to a piezo-ceramic actuator. Axisymmetric mechanical harmonic shear wave excitation (frequencies from 1 kHz to 4 kHz) was applied to the entire test tube through the actuator driven by a power amplifier. The agarose gel was used to promote the transfer of shear wave motions within the samples. Shear motions were generated within the sample and were synchronized with the motion-encoding gradient of a gradient-echo based MRE pulse sequence to create shear wave images. The gradient echo based MRE pulse sequence was used with the following imaging parameters: pulse repetition time/echo time = 400/6 ms; flip angle = 30°; field of view = 10 mm; slice thickness = 0.5 mm; 1 slices; in-plane resolution = 78 μm × 78 μm; MSG strength = 120 G/cm. From the wave image data, the

shear stiffness was estimated using the local frequency estimation (LEF) inversion algorithm with directional filtering [29].

2.7.2. Oscillatory rheology—The dynamic viscoelastic properties of the hydrogel were obtained on a Bohlin Gemini rheometer (Malvern Instruments, Worcestershire, UK) with 8 mm/0° parallel plate geometry. Samples were prepared as described previously. 7%~12% w/v LZ hydrogel samples were fixed on a glass slide and placed on the rheometer sample stage. The upper plate was lowered until it was in uniform contact with the LZ hydrogel top surface. The corresponding gap distances were 1.3-1.6 mm. Dynamic time sweep experiments (DTS) were performed to monitor the storage (G') and loss (G'') modulus changes of the hydrogels with time. Frequency sweeps between 0.01 and 1 Hz at 0.1% strain at 25°C were performed. To prevent evaporation, a chamber cover was used to protect the samples during the analysis. All measurements were made in triplicates on 3 different preparations of LZ hydrogel.

2.8. *In vitro* cell culture—HMSCs isolated from the iliac crest of normal adult donors were obtained from the NIH –funded Tulane Cancer Research Centre [30]. These cells were cultured in alpha MEM (minimum essential medium) (Gibco) supplemented with 20% fetal bovine serum (Gibco), 200 mM L-glutamine (Gibco), 1x Antibiotic-Antimycotic cocktail (Gibco) and incubated in a humidified, 5% CO₂ atmosphere at 37°C. When required, HMSCs stably expressing the GFP gene was used.

2.8.1. 2-D and 3-D cell culture—For 2-D cell culture, a non-tissue culture 96 well plate was coated with different ratios of LZ-Control and LZ-RGDS proteins. Briefly, 5 μ M LZ protein solution with different ratios of LZ-Control and LZ-RGDS (100:0, 75:25, 50:50) or bovine serum albumin (BSA) in bicarbonate/carbonate buffer (100 mM) was used. 100 μ L of the mixture was placed in each well overnight at room temperature to allow protein adsorption on the plates. The next day plates were washed with PBS and HMSCs were seeded at concentrations of 1.5×10^4 cells/well suspended in 100 μ L media and cell growth media was used to cover the cells. Medium was changed every other day. HMSCs seeded on 96 well polystyrene tissue culture plates (TCPS) served as positive control.

For 3-D cell culture, 7% w/v LZ hydrogel containing different ratios of LZ-Control and LZ-RGDS were used. The freeze-dried hydrogel was first rehydrated by incubating with the culture media at 37°C, and 5% CO₂ for 1 hr before use. The 1% w/v PuraMatrix hydrogel (BD Biosciences) was prepared according to the manufacture's protocol and served as positive control. 1.5×10^4 HMSCs were seeded on to the preformed LZ hydrogel placed in 96 well plates and cultured for 3 weeks. The medium was changed every other day.

2.8.2. MTS cell proliferation assay—The MTS cell proliferation assay was carried out on both 2-D surface and within the 3-D hydrogel with HMSCs utilizing CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instructions. For 2-D cell proliferation assay, the cells were cultured for 1, 4, 7, 14, 21 days. At the end of the specified time points, 20 μ L of proliferation assay reagent was mixed with 100 μ L of culture media and added to each well. The plate was then placed in a 37°C incubator for 60 minutes and the absorbance measured at 490 nm using a microplate reader.

(BioTek Synergy 2). For 3-D cell proliferation assay, the cells were cultured for 1, 4, 7, 14 and 21 days. The samples were washed twice with PBS and 100 μ L of fresh media with 20 μ L of proliferation assay reagent were added to each well. After 90 minutes of incubation in a humidified 37°C chamber, the gels were removed and the absorbance was measured at 490 nm using a microplate reader (BioTek Synergy 2).

2.8.3. Time-lapse live cell imaging—The live HMSC images were captured using the Zeiss imaging system equipped with an incubator that maintains temperature and carbon dioxide levels. 1.5×10^4 of the GFP-HMSCs were seeded on the 7% w/v LZ-Control hydrogel. The LZ hydrogel was hydrated by the cell suspension. The images were captured every 15 minutes per frame via GFP signal for 16 hours after initial seeding. The playback of the time-lapse video is 15 frames per second.

2.8.4. Live-dead cell assay—Live-dead cell assay was carried out on HMSCs cultured in 3-D LZ matrix for upto 7 days. At 1 day, 4 days and 7 days post seeding, live and dead cells were analyzed in triplicate samples using the live-dead cell assay kit (Molecular Probes) as per the manufacturer's recommended protocol. The calcein AM and the ethidium homodimer concentration were 10 μ M each to indicate the live (green) and dead (red) cells respectively. The samples were imaged using a Zeiss Axio-Observer D1 fluorescent microscope equipped with the required filter sets. Five individual fields of view were imaged for each and the percentage of dead cells to live cells was calculated.

2.8.5. Actin staining—HMSCs were cultured for 7, 14 & 21 days within the LZ hydrogel and then rinsed three times with PBS, fixed with 4% paraformaldehyde for 4 hr and permeabilized with 0.5% Triton X-100 in PBS. The LZ hydrogel was incubated with Phalloidin-TRITC (1:1000, Sigma) in PBS overnight at 4°C. The gels were then washed 3 times with PBS to remove nonspecific bound reagent. They were then viewed and z-stack images were captured using a Zeiss 710 LSM Meta confocal microscope.

2.9. *In vivo* subcutaneous implantation of the LZ scaffold

7% w/v of LZ-Control and LZ-RGDS hydrogels were used *in vivo* subcutaneous implantation without cells. The hydrogels were implanted subcutaneously on the dorsal side of wild type C57BL6 mice (Charles River Laboratories) in the dehydrated form. After 1 and 2 weeks of implantation, the animals were sacrificed and the scaffolds were retrieved, fixed in 4% neutral buffered formalin, embedded and sectioned into 5 μ m thick sections for histological evaluation.

7% w/v of LZ-Control and LZ-RGDS hydrogel pre-seeded with HMSCs expressing GFP were used for *in vivo* subcutaneous implantation. Cell suspensions were prepared at concentrations of 5×10^5 cells/ 20 μ L in culture media. The dry scaffolds were hydrated using the cell suspension. The cell-impregnated hydrogel was then implanted subcutaneously on the dorsal side of athymic nude mice (Charles River Laboratories). 2 weeks post implantation, the animals were sacrificed and the scaffolds were retrieved, fixed in 4% neutral buffered formalin, embedded and sectioned into 5 μ m thick sections for histological

evaluation. All animal experiments were performed as per the protocol approved by the UIC animal care committee (Assurance number A-3460-01).

2.10. Histology and immunohistochemistry

All sections were deparaffinized in xylene, hydrated in graded ethanol solutions, and immunostained with peroxidase conjugated secondary antibodies or fluorescent probes according to published protocols.[18, 31] The following antibodies were used: rabbit anti-CD68 antibody (1/100, Abcam), rabbit anti-Fibronectin (FN) antibody (1/100, Sigma), rabbit anti-VEGF antibody (1/100, Santa Cruz Biotechnology), Mouse anti-Dentin Matrix Protein 1 (DMP1) antibody (1/2000, a gift from Dr. Chunlin Qin from Baylor College of Dentistry), Rabbit anti-Pigment epithelium-derived factor (PEDF) antibody (1/500, Millipore), Mouse anti-von Willebrand factor (vWF) antibody (1/100, Santa Cruz Biotechnology), rabbit anti-CD31 antibody (1/100, Abcam). Alizarin red staining to visualize calcium deposition was performed as per standard procedures. All fluorescently stained sections were imaged at the University of Illinois at Chicago Research Resource Center core imaging facility. Imaging was performed using a Zeiss LSM 710 confocal microscope equipped with Zen image analysis software and peroxidase stained sections were imaged using a Zeiss Axio-observer D1 microscope. All comparative fluorescence images were obtained using the same imaging conditions.

3. Results and discussion

3.1. Synthesis and secondary structure of chimeric LZ proteins

Both LZ-Control and LZ-RGDS were expressed from corresponding artificial genes in *E. coli*, purified via His-tag column, and analyzed by SDS-PAGE gel electrophoresis. The molecular size of the LZ chimeric protein was ~37 kDa and the yield was about 80 mg per liter. Circular dichroism spectroscopy was utilized to evaluate the folding of the chimeric LZ proteins. As expected, the molar ellipticity of the LZ-Control protein displayed minima at 208 and 222 nm implying that the alpha-helical structure as the predominant secondary structure at different pH (pH 4, pH 7.4 and pH 11) even in the presence of the disulfide bonds (Fig. 2A). However, the signal intensity was prominent at pH 4 and pH7.4, suggesting stronger electrostatic interactions are capable of facilitating self-assembly of the alpha-helical peptides. Similar CD spectra were obtained for LZ-RGDS in pH 7.4 (Fig. 2B), indicating that the chimeric LZ proteins retained the alpha-helical structure even after incorporation of the RGDS motif.

3.2. Analysis of the surface characteristics and morphology of the LZ hydrogel

The LZ hydrogel is stable and can be stored at room temperature for several months after lyophilization. Rehydration in PBS resulted in swelling of the LZ hydrogel to its original dimensions, and is flexible, soft and semi-transparent (Fig. 3A). SEM images in Fig. 3B, 3C, & 3D depict the 3-D porous nature of the LZ hydrogel. The pore size measured from the SEM images ranged from ~100 μm in 7% w/v, ~80 μm in 10% w/v, and ~20 μm in 12% w/v respectively. Interconnectivity of the pores was also observed. These results indicate that it is possible to control pore size by adjusting the composition of the hydrogel.

Cryo-TEM enables visualization of the LZ hydrogel network under cryogenic conditions, thereby preserving the *in situ* nanostructure. Dark areas in Fig. 3E represent self-assembled hydrogel matrix while the gray areas represent vitrified water. Cryo-TEM images of 7% LZ hydrogel showed the presence of dense interconnected fibrillar microstructures resulting from the self-assembly process.

3.3. LZ hydrogel mechanical properties

Hydrogels for tissue engineering applications must possess sufficient strength as cells sense and respond to their microenvironment [32, 33]. Therefore, we examined the material properties of the scaffolds using oscillatory shear rheology. Rheological studies conducted on 7, 10 & 12% w/v LZ hydrogels at frequency range from 0.01 to 1 Hz (Fig. 4A) showed that the G' and G'' increased with increasing concentrations of the hydrogel. The G' was greater than G'' by approximately an order of magnitude for all the samples studied and in fact the G' values were an order of magnitude higher than the previously reported values [34, 35]. For example, the average storage moduli (G') and loss moduli (G'') at 0.1 Hz were 2480 Pa and 203.2 Pa for 7% w/v, 4300 Pa and 302 Pa for 10% w/v, and 4390 Pa and 391.2 Pa for 12% w/v respectively. Additionally, there was no crossover point of G' and G'' at the frequencies used in this study. This behavior corroborates well with published reports of such hydrogels and demonstrates that the LZ hydrogel can assemble into a fairly rigid viscoelastic material with covalent crosslinking [36].

The loss tangent values represent the ratio between viscous and elastic properties and is the most sensitive indicator of various molecular motions within the material, $\tan \delta = G''/G'$. The loss tangent values were 0.082, 0.07, and 0.089 for 7%, 10% and 12% w/v LZ-Control hydrogel respectively. Similar $\tan \delta$ values with different concentrations of the hydrogel indicate a similar connectivity pattern. Further, a lower $\tan \delta$ value suggests a higher crosslink density [35]. However, the effects from microstructure stiffness and morphology may also change at the specific range of the oscillating frequency [37]. Thus, the results from the rheology experiments showed consistency between various concentrations of the LZ hydrogel including the oscillating frequency insensitivity and loss tangent values.

μ MRE was used to estimate the shear stiffness of the hydrogel at different concentrations (7%, 10% and 12% w/v) using different frequencies (1k, 2k, 3k and 4k Hz) as shown in Fig. 4B. In the frequency range, the 12% w/v hydrogel showed significantly higher shear stiffness compared to 7% and 10% w/v LZ hydrogel. The higher shear stiffness implies greater fibrillar density [38, 39] due to increasing number of crosslinks present with higher hydrogel concentrations. Thus, the data from both the rheological and μ MRE studies show that the mechanical strength is tunable by adjusting the LZ hydrogel concentration.

3.4. *In vitro* degradation rate of the LZ hydrogel

Directly related to the material stiffness property is the stability of the hydrogel. Degradation rate of the hydrogel was determined *in vitro*. Result presented in Fig. 5 shows that 7% w/v LZ hydrogel still retained 60% of its original weight at 30 days. We hypothesize that the stability of the hydrogel is due to the presence of the 3 cysteines engineered into the construct. The stability of the LZ hydrogel was further enhanced by the dehydrothermal

treatment that provided additional crosslinks [40, 41]. In its absence the hydrogel was unstable (data not shown). The most probable cause for degradation after four weeks could be due to the hydrolysis of the peptide bonds. Thus, the introduction of cysteines and the thermal treatment provided stability to the hydrogel.

3.5. Cell viability assays

Biomaterials can be designed to interact with cells by utilizing biomimetic principles [42, 43]. Therefore, the LZ protein with and without the RGDS motif was tested for its ability to present a microenvironment that would support cell attachment. The MTS assay was used for this purpose. The reason for the choice of this assay was to quantitate the presence of metabolically active cells. The color change observed during this assay is possible only if metabolically active cells are present. Therefore, the results from this experiment can be used to gauge the ability of the LZ protein mixtures to favor cellular attachment and maintain cell functionality. Results from the MTS assay (Fig. 6A) showed that HMSCs on LZ substrate containing varying amounts of RGDS ligand showed faster cellular attachment when compared to LZ-Control. Specifically, at the four hour time point, the results indicate significantly higher number of metabolically active cells were attached to the plates coated with 25 and 50 percent LZ RGDS. However, at all the time points, the number of cells attached to the plates were less than those in tissue culture plates. Thus the presence of RGDS elicited the desired cellular responses. Biomaterials can be designed to interact with cells by utilizing biomimetic principles [42, 43].

3.6. Mesenchymal stem cell proliferation

The LZ hydrogels were also tested for their ability to promote proliferation of HMSCs in 3-D culture conditions. 7% w/v hydrogel prepared with varying amounts of LZ-RGDS (LZ-control: LZ-RGDS = 100:0 or 75:25 or 50:50) were examined for proliferation at 4, 7, 10, 14 & 21 days post cell seeding (Fig. 6B). Interestingly, all conditions favored cell adhesion. At days 4 & 7 higher absorbance values were observed for a mixture containing higher amount of RGDS (LZ-Control: LZ-RGDS = 50:50). Even though the LZ-Control favored cell proliferation, it did so after 14 days. This suggests that the presence and density of the RGDS ligand might be the driving force for initial cell adhesion and proliferation into the hydrogel. Commercially, available PuraMatrix, used as a positive control. However, the LZ hydrogels proved to be significantly better in promoting viability and proliferation when compared to PuraMatrix indicating its superior characteristics in terms of cellular compatibility (Fig. 6B).

3.7. Cell viability within the LZ hydrogels

Fig. 7 shows the live-dead cell assay for up to 7 days after seeding HMSCs on the LZ hydrogel. Green staining in the images represents live cells and red staining represent dead cells. From the images it is clear that while the LZ-Control hydrogel promoted cell viability, the morphology of the cells were rounded at day 1 (Fig. 7A1). In contrast, cells on the higher RGDS containing scaffold (LZ-Control: LZ-RGDS = 50:50, Fig. 7A3) demonstrated good cell adhesion and had distinct spindle shaped morphology with long processes. Templates with lower amount of RGDS (LZ-Control: LZ-RGDS = 75:25) showed the least cell attachment (Fig. 7A2) suggesting that the density of the RGDS motif is important for initial

attachment. After 4 days, cells within all the LZ hydrogel types proliferated well and exhibited distinct 3-D cellular network (Fig. 7B1, 7B2 and 7B3), however templates with no or lower amount of RGDS showed higher number of dead cells at the same time point (comparing 7B1 and 7B2 with 7B3). From these results, we can conclude that although the presence of the RGD motif is beneficial for initial cell attachment, over a longer period of time, the cell viability is not greatly affected. On the other hand, because of reduced initial attachment, the total number of cells present in the hydrogel at a specific time point may be lesser in the LZ-Control hydrogels and the LZ hydrogels with reduced RGD motif (25%) Based on this data we opted to use LZ hydrogels with higher amount of RGDS for in-vivo experiments.

3.8. Cell migration in 3-D culture system

Next we investigated the migration of GFP-HMSCs during the proliferative phase in 3-D LZ-Control hydrogel for 7, 14 and 21 days. As the cell viability and proliferation of HMSCs were similar after 7 days in culture irrespective of the presence or absence of RGDS, we opted to use only the LZ-Control hydrogel for this experiment. At the end of each time point the HMSCs were stained with actin and observed with confocal microscopy. Results in Fig. 8 show cell viability and the color-coding scheme depicts the depth of cell migration (green represents the top and blue represents the bottom of the hydrogel). Examination of the cell morphology showed long elongated polarized cells with well-developed actin-fiber bundles. Results in Fig. 8A showed that by 1 week, cells from the surface had migrated within the gel. At 14 days (Fig. 8B) the cells had migrated to a depth of 20 μm as indicated by the green color. At 21 days (Fig. 8C) the cells still remained viable and migrated to $\sim 100 \mu\text{m}$ within the LZ hydrogel as indicated by the blue color. Limitations with the confocal microscopy technique prevented measuring distances greater than 100 μm . Time-lapse observation revealed that the cells migrated by extending processes towards other cells in the matrix (Supplementary video). Thus, HMSCs traveled long distances within the LZ-RGDS hydrogel due to a combination of favorable properties of the microenvironment.

3.9. Foreign body response to LZ hydrogel

The LZ hydrogels were implanted subcutaneously in wild type mice to observe if there was a host generated foreign body response to the hydrogels. The scaffolds were implanted for a period of 1 and 2 weeks and explant sections were examined by immunohistochemistry. CD68, a macrophage marker was used. Immunohistochemical analysis for CD68 positive cells showed no staining at one week (Fig. 9A1, 9B1) indicating the absence of foreign body reaction. After 2 weeks of implantation, positive staining for CD68 was observed at the periphery of the hydrogel in both the samples; however, they were not multi-nucleated macrophages (Fig. 9A3 and 9B3). Multi-nucleated macrophages are the hallmark of adverse foreign body response. The absence of such macrophages after 2 weeks of implantation indicated that the LZ scaffolds were not subjected to foreign body reaction *in vivo*. CD68 is a broad-spectrum macrophage marker. Therefore, the positive mono nucleated cells could be macrophage cells present in the blood.

3.10. Host cell response to LZ hydrogels

Hematoxylin staining in Fig. 9 shows that the LZ gels supported colonization by host cells. To analyze if the colonized host cells were able to secrete a matrix and promote vascularization, a series of markers was used for immunohistochemical analysis. Staining for fibronectin, the predominant ECM protein was particularly interesting. Intense staining of fibronectin in the ECM was observed with cells recruited to the LZ-RGDS scaffold (Fig. 10B1-10B4) when compared with the control (Fig. 10A1-10A4). Similar staining pattern was observed for DMP1 (dentin matrix protein 1) a regulatory protein in osteogenesis (Fig. 10B1-10B4). This suggested that the presence of RGDS was critical for the initial colonization and matrix production by host cells. In order to determine if the LZ scaffold created a microenvironment for neovascularization, staining for VEGF (vascular endothelial growth factor) was performed. Results indicated that more VEGF staining was predominant at 1 week and reduced amounts were observed at 2 weeks (Fig. 10C1-10C4). This staining pattern corroborated well with the levels of PEDF (Pigment-Epithelium Derived Factor). PEDF is one of the most potent anti-angiogenic factors and its expression increased at 2 weeks (Fig. 10D1-10D4). These results imply that the LZ scaffolds can provide an environment that can trigger favorable host cell response by enabling deposition of the host cell ECM and permitting host cell mediated vascularization.

3.11. *In vivo* response of LZ-scaffolds pre-seeded with HMSCs

The LZ hydrogels provided an excellent microenvironment for host cell colonization and proliferation without triggering foreign body response. Our next step was to ascertain if LZ scaffolds seeded with mesenchymal stem cells were able to support the survival and proliferation of these cells *in vivo*. Although we seeded cells transfected with GFP, after post processing and section, the inherent GFP fluorescence was lost. Therefore, the sections were stained using a GFP antibody to visualize the implanted cells. At the end of 2 weeks, staining for GFP showed the presence of GFP-positive cells within the scaffold (Fig. 11A1, 11A2). As before, the ECM synthesized by the HMSCs seeded on the LZ-RGDS showed predominant staining for fibronectin (Fig. 11B1, 11B2) and DMP1 (Fig. 11C1, 11C2). H&E staining of the tissues showed the presence of numerous RBCs indicating the presence of active blood flow within the scaffolds. Neovascularization was observed originating from the surface of the hydrogel to the interior by H&E staining (Fig. 11F1, 11F2). Higher vessel density was observed with LZ-RGDS scaffold (Fig. 11F2). Both VEGF and PEDF were expressed by the HMSCs in the control and the RGDS hydrogels indicating control over the process of angiogenesis. Presence of endothelial cells was further confirmed by observing the immunohistochemical localization of vWF (von Willebrand factor) (Fig. 11G1, 11G2) and CD31 markers for endothelial cells (Fig. 11H1, 11H2). Results from these experiments showed that the HMSCs seeded within the LZ-Control and LZRGDS scaffolds were able to proliferate and secrete an active ECM that favors vascularization.

4. Conclusions

In this study we have shown that leucine zipper, a self-assembling peptide can be utilized for the synthesis of a defined matrix for tissue engineering. Synthesis of the engineered leucine zipper domain can facilitate the formation of physical and covalent crosslinks in hydrogel

structures leading to the formation of interconnected pores of varying sizes and mechanical properties based on peptide concentration. This is the first report on the development of a stable LZ hydrogel with tunable properties. Increased cell adhesion, proliferation and migration of HMSCs within LZ-RGDS scaffolds suggest the potential use of this scaffold in various in-vivo tissue engineering applications. In-vivo experiments demonstrate that the LZ scaffolds do not trigger unfavorable foreign body response. This property is a significant advantage for tissue engineering applications. The unique ability of LZ scaffolds to permit neovascularization without the need for delivery of growth factors such as VEGF creates new opportunities in tissue regeneration. Overall, the *de novo* synthesized LZ chimeric protein had better rheological properties; tunable pore size, permitted dynamic cellular interaction, host cell colonization and actively permitted vascularization making it a biologically active scaffold for tissue engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic representation showing the peptide sequences used in the synthesis of the second generation chimeric leucine zipper protein with and without RGDS. The motif referred to in the figure represents the RGDS cell binding motif.

Figure 2. Secondary structural analyses of chimeric leucine zipper proteins by Circular Dichroism (CD).

(A) CD spectra of LZ-Control at pH=4 (●, 14 μ M), pH=7.4 (○, 14 μ M), pH=11 (▼, 14 μ M). (B) CD spectra of LZ-Control (●, 14 μ M), LZ-RGDS (○, 14 μ M) at pH7.4. Spectra were recorded at 25°C in 100mM phosphate buffer.

Figure 3. Microscopic Characterization of the LZ hydrogel

Image of the hydrated 7% w/v LZ-Control hydrogel (A). Examination of the microstructures using scanning electron microscopy (SEM) showing the microstructure of the fractured surface of lyophilized 7% w/v LZ-Control (B), 10% w/v LZ-Control hydrogel (C) and 12% w/v LZ-Control hydrogel (D) in 100mM phosphate buffer. Scale bar for B, C & D represents 100 μm .

(E) Is a representative Cryo-TEM image showing the micro structure of self-assembled 7% w/v LZ-Control hydrogel. Image shows interconnected dense network of the self-assembled

hydrogel matrix (dark areas) surrounded by vitreous water (light areas). Scale bar represents μm in (E).

Figure 4. Viscoelasticity measurements of LZ-Control hydrogel

(A) Graphical representation of the storage modulus G' (solid data points) and the relaxation modulus G'' (unfilled data points) with change in frequency. Together, the data represents the viscoelastic properties of the hydrogel. Note the increases in the G' and the G'' values with increase in the LZ concentration. (B) Shows changes in stiffness with frequency from magnetic resonance elastography. Shear stiffness was determined at all frequencies of vibration (1k, 2k, 3k and 4k Hz) with different concentrations of the LZ hydrogel (7%, 10%, 12% w/v). * represents statistical significance as determined by students t-test of the 12% hydrogel values with respect to both 10% and 7% hydrogels ($p, 0.05$)

Figure 5. Stability of the LZ-Control hydrogel

The graph represents drop in the weight (indicated as percentage of total weight) of the LZ-Control hydrogels immersed in PBS over time (in days). Results show that about 60% of the hydrogel weight still remained intact after 30 days indicating an approximate half-life of over a month.

Figure 6. Live-dead cell assay

The live-dead cell assay was performed on HMSCs seeded on 7% w/v LZ-Control (**Group A**); LZ-Control: LZ-RGDS 75:25 (**Group B**) or LZ Control: LZ-RGDS 50:50 (**Group C**) for 1, 4 and 7 days. “Green” staining represents live cells and “red” staining represents dead cells. Scale bar represents 50 μ m for all images.

Figure 7. Attachment and Proliferation of HMSCs cultured on 2-D coated surfaces and 3-D hydrogels

(A) Represents attachment of HMSCs in 2D protein coated surfaces shown as change in absorbance at 490 following treatment with MTS assay reagent. Cells grown in tissue culture plastic served as positive control. LZ-RGDS motifs containing combinations showed increase number attached cells. * Represents statistical significance ($p < 0.05$ using students t-test) with respect to LZ-Control. (B) Represents proliferation of HMSCs in 3D hydrogels containing the indicated combinations of LZ-Control and LZ-RGDS motifs. * Represents statistical significance ($p < 0.05$ using students t-test) with respect to PuraMatrix. Note that the LZ hydrogels behaved significantly better than PuraMatrix.

Figure 8. Z-stack confocal microscopy

3D reconstructed images of HMSC cultured on 7% w/v LZ-Control hydrogel after 7 days (A), 14 days (B), and 21 days (C) and stained with TRITC-phalloidin staining for actin. Color-coding represents the depth profile from the surface red to blue at a depth of 150 μm . Phalloidin stains actin filament and shows the cytoskeletal organization of HMSCs within the LZ hydrogels.

Figure 9. CD68 Immunohistochemistry

Images represent CD68 stained sections of LZ-Control (**A1, A2 and A3**) and LZ-RGDS (50:50) (**B1, B2 and B3**) scaffolds implanted subcutaneously in wild type mice for 1 week (**A1 and B1**) and 2 weeks (**A2, A3 and B2, B3**) respectively. Note the absence of CD68 positive cells in A1 and B1 indicating the absence of macrophages after 1 week. At 2 weeks macrophages were observed (**A2 and B2**) positive staining. The boxed areas in **A2** and **B2** are shown in higher magnification in **A3** and **B3** respectively. These images show the presence of macrophages and also absence of multi-nucleated foreign body giant cells indicating the absence of adverse foreign body reaction.

Figure 10. Immunofluorescence staining of scaffold sections from hydrogels implanted in wild type mice

Images are representative confocal micrographs showing the immunohistochemical localization of Fibronectin (A1, A2, A3 and A4), DMP1 (B1, B2, B3 and B4), VEGF (C1, C2, C3 and C4) and PEDF (D1, D2, D3 and D4) in LZ-Control (A1-D1, A3-D3) and 50:50 LZ-Control: LZRGDS (A2-D2, A4-D4) after 1 and 2 weeks of implantation subcutaneously in wild type mice. Scale bar represents 20μm for all images.

Figure 11. Immunofluorescence staining and histology of HMSC-seeded scaffolds implanted in immunocompromised mice

(A1-E1) represent immunohistochemical localization of GFP, fibronectin (FN), DMP1, VEGF and PEDF in sections of LZ-Control scaffolds implanted subcutaneously in immunocompromised mice for 2 weeks. (A2-E2) are representative images showing the same from 50:50 LZ-Control:LZ-RGDS scaffolds. (F1, F2) are representative images of H&E stained sections of the two types of implanted scaffolds containing HMSCs. Note arrows indicate the red blood cells. (G1, H1 and G2, H2) are representative images showing the immunohistochemical localization of vWF (G1, G2) and CD31 (H1, H2) respectively in the two types of scaffolds.