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Immobilized RGD concentration and Proteolytic Degradation Synergistically Enhance Vascular Sprouting within Hydrogel Scaffolds of Varying Modulus

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Abstract

Insufficient vascularization limits the volume and complexity of biomaterials-based tissue engineering approaches. The formation of new blood vessels (neovascularization) is regulated by a complex interplay of cellular interactions with biochemical and biophysical signals provided by the extracellular matrix (ECM) which necessitates the development of biomaterial approaches that enable systematic modulation in matrix properties. Poly(ethylene) glycol-based hydrogel scaffolds were engineered with a range of decoupled and combined variations in integrin-binding peptide (RGD) ligand concentration, elastic modulus and proteolytic degradation rate using free-radical polymerization chemistry. The modularity of this system enabled a full factorial experimental design to simultaneously investigate the individual and interaction effects of these matrix cues on vascular sprout formation in 3D culture. Enhancements in scaffold proteolytic degradation rate promoted significant increases in vascular sprout length and junction number while increases in modulus significantly and negatively impacted vascular sprouting. We also observed that individual variations in immobilized RGD concentration did not significantly impact 3D vascular sprouting. Our findings revealed a previously unidentified and optimized combination whereby increases in both immobilized RGD concentration and proteolytic degradation rate resulted in significant and synergistic enhancements in 3D vascular spouting. The above-mentioned findings would have been challenging to uncover using one-factor-at-time experimental analyses.

Keywords

Hydrogel; PEG; Angiogenesis; Cell adhesion; Matrix stiffness; Proteolytic degradation

1 Introduction

The inability to promote rapid, stable, and functional neovascularization (new blood vessel formation) within implantable scaffolds remains as a major obstacle to clinical translation of biomaterial-based strategies in tissue engineering. Oxygen and nutrient mass transfer limitations and inadequate removal of waste products restrict the volume of tissue that can be engineered to smaller than clinically relevant dimensions. Thus, successful engineering of metabolically demanding tissues of large volume requires establishment of an extensive and stable vascular network throughout the implant for support and maintenance of cell viability and promotion of functional tissue regeneration [1]. Neovascularization is regulated by a complex interplay of cellular interactions with biochemical and biophysical signals provided by the extracellular matrix (ECM), including diffusible and immobilized growth factors, cell adhesion ligands, as well as ECM mechanical and structural properties [2, 3]. Leveraging knowledge of key cell-ECM interactions of native vascularized tissues in the design of scaffolds will facilitate clinical translation of new biomaterials capable of promoting rapid neovascularization of damaged or diseased tissues.

Over the past several decades a variety of naturally-derived or synthetic hydrogel-based biomaterials have been developed to promote the formation of vascular networks through recruitment of endogenous host vasculature following scaffold implantation *in vivo*, or by implantation of pre-vascularized tissue constructs that inosculate with host vasculature [3]. For example, Garca and colleagues have engineered poly (ethylene) glycol (PEG) hydrogel scaffolds augmented with angiogenic growth factors in soluble or immobilized form to induce the growth of vasculature *in vivo* [4, 5]. In addition, 3D co-culture of endothelial progenitor cells (EPCs) and smooth muscle cells (SMCs) in protease-sensitive, cell adhesive PEG hydrogel scaffolds has been shown to promote *in vitro* microvessel formation which was dependent on the ratio of EPCs and SMCs [6]. In addition to growth factor supplementation or immobilization in matrices as well as co-culture of endothelial cells with supporting mural cells to prevent vessel regression, other matrix properties including immobilized cell adhesion concentration, stiffness and degradation rate play a critical role in scaffold neovascularization.

As compared to naturally-derived biomaterials, synthetic hydrogels enable more precise control of biochemical, mechanical, and physical properties critical to cell behavior and tissue regeneration. Using synthetic hydrogels, prior studies have demonstrated the critical importance of cell adhesion ligand concentration [7, 8], stiffness [9], or matrix susceptibility to proteolytic degradation [10] on vascularized tissue remodeling and synthesis. For example, biphasic angiogenic responses to variations in adhesion ligand concentration and integrin antagonists in 3D culture models suggest that neovascularization can be optimized by modulating adhesive ligand concentration [7, 11, 12]. Advances in mechanobiology have also significantly contributed to our understanding of how cells sense ECM mechanical properties, including matrix modulus through integrin-mediated adhesion and signaling highlighting the importance of cell adhesion ligand concentration and stiffness interactions on cell behavior [13, 14]. Recent *in vitro* findings demonstrate the dependence of 3D vasculogenesis on both adhesive ligand concentration and modulus [9]. While adhesion ligand concentration [7] and mechanical properties [15] have been implicated as critical

regulators of neovascularization, these cues dynamically change over the time course of scaffold degradation and vascularized tissue remodeling. ECM degradation results in release of ECM fragments and exposure of cryptic binding sites while matrix deposition provides new ligands for integrin ligation [16, 17]. Increases in scaffold stiffness have also been identified to contribute to increased matrix deposition [18], resulting in a positive feedback loop that may significantly impact dynamic properties of ECM surrounding angiogenic vessels. Finally, the importance of modulating proteolytic scaffold degradation on 3D vascular sprouting and endothelial network formation [19–23], as well as the influence of mechanical properties and susceptibility to proteolytic degradation on vascular morphogenesis have also been demonstrated [23]. The above-mentioned findings not only underscore the importance of cell adhesion, stiffness, and proteolytic matrix degradation on neovascularization, but also the complex dynamic interplay between these ECM cues on the process; however, the effects of these interactions on neovascularization responses are not clear.

Use of multifactorial experiments, where matrix properties and biochemical composition of synthetic scaffolds are varied individually and in controlled combinations, provides a promising route for optimization of biomaterials towards engineering vascularized tissues. High throughput screening techniques have been developed for this purpose [24, 25]. Extension of these types of analyses to multifactorial screening of 3D cell-scaffold interactions, however, has been proven challenging as variation in one matrix property often induces changes in another. In synthetic crosslinked hydrogel systems, co-polymerization of multiple co-monomers often induces compositional drift leading to alterations in hydrogel composition and matrix properties [26, 27].

To address this issue we utilized poly (ethylene glycol) (PEG) hydrogel scaffolds as a biomaterial platform to probe decoupled and synergistic variations in immobilized cell adhesion ligand concentration, elastic modulus and proteolytic scaffold degradation rate using polymerization strategies that enabled robust factorial analysis of these matrix cues on 3D vascular sprouting responses in vitro. Specifically, PEG scaffolds were engineered with decoupled and combined variations in immobilized concentration of RGD integrin binding peptides, elastic moduli within the range of soft tissues (0.8–4 kPa) [28], and rapid and slow proteolytic degradation rates by systematically variations in co-monomer composition and polymerization conditions (Figure1).

A statistical design of experiments (DOE) approach was applied to elucidate the effects of individual and combined variations of these extracellular matrix cues. A full factorial design with 18 combinations of immobilized RGD concentration, elastic modulus and degradation rate enabled statistical and significance comparisons of individual and two-factor interactions on vascular sprouting in 3D culture. This DOE approach provides systematic screening of the effects of multiple matrix properties and guidance in understanding fundamental principles in pro-angiogenic biomaterial design.

2 Methods

2.1 Materials

Dimethylformamide (DMF), O-benzotriazole- N,N,N', N' -tetra-methyluronium-hexafluoro-phosphate (HBTU), tri- fluoroacetic acid (TFA), Fmoc-amino acids, and the Wang resin were obtained from AAPPTec (Louisville, KY). N, N-Diisopropylethyl- amine (DIEA), thioanisole, triisopropylsilane (TIS), and diethyl ether were obtained from Fisher Scientific (Hanover Park, IL). Piperidine, phenol, N-vinylpyrrolidone (NVP), triethanolamine (TEA), and eosin Y were obtained from Sigma-Aldrich (St. Louis, MO). Acryl-PEG-SVA (MW= 3400 and 5000 g/mol) was obtained from Laysan Bio (Arab, AL)

2.2 Peptide Synthesis, Design, and Purification

MMP-sensitive peptide sequences containing either one or two protease-sensitive cleavage site repeats, GGVPMSS↓MRGGK (SSite, 1076.3g/mol) and GGVPMSS↓MRGDGVPMSS↓MRGGK(DSite, 2007.4 g/mol) (↓ denotes cleavage point), respectively, as well as the YRGDS cell adhesion peptide ligand were synthesized using solid-phase peptide synthesis with standard Fmoc chemistry on a Focus Xi automated peptide synthesizer (AAPPTec, Louisville, KY). Amino acid coupling was carried out on a Wang resin in the presence of DIEA and HBTU. The Fmoc group was deprotected with 20% piperidine in DMF. Peptides were cleaved from the resin and deprotected in a TFA cleavage cocktail (90% TFA, 2.5% TIS, 2.5% thioanisole, 2.5% (w/v) phenol, and 2.5% deionized water) for 2.5 h, precipitated in cold diethyl ether, and purified by reverse-phase high-performance liquid chromatography (HPLC). Peptide purity >95% was confirmed by ion trap time-of-flight (IT-TOF) mass spectroscopy. Purified peptides were lyophilized and stored at −20 °C until use.

2.3 Synthesis of Photopolymerizable Monofunctional and Difunctional PEG Macromers

Four types of photopolymerizable macromers were synthesized and used to create cell-adhesive and proteolytically degradable hydrogel scaffolds with tunable variations in modulus and or crosslink density, immobilized RGD concentration, and degradation kinetics. The synthesized macromers included: PEG diacrylate (PEGDA) crosslinkers of similar molecular weight containing (1) one (SSite) or (2) two (DSite) MMP-sensitive peptide cleavage sites between the terminal acrylate groups of PEGDA to modulate scaffold degradation kinetics without inducing variations in network crosslink density or elastic modulus, (3) an RGD-containing PEG monoacrylate macromer (RGD-PEGMA) to immobilize RGD cell adhesion ligands into the crosslinked network, and (4) a non-biofunctional PEG monoacrylate macromer (PEGMA) with molecular weight similar to RGD-PEGMA used as a surrogate during polymerization to modulate RGD concentration without inducing variations in scaffold degradation or modulus (Figure 1A). SSite and DSite PEGDA crosslinkers were synthesized by conjugation of the MMP-sensitive peptides to Acryl-PEG5000-SVA (Laysan Bio, Arab, AL, MW= 5 kDa) in a 2:1 PEG to peptide molar ratio. This reaction led to the formation of the SSite and DSite MMP-sensitive PEGDA difunctional cross-linkers (Acryl-PEG5000- GGVPMSS↓MRGGK-PEG5000-Acryl (MW ≈ 11 kDa) and Acryl-PEG5000- GGVPMSS↓MRGDGVPMSS↓MRGGK-PEG5000-Acryl (MW ≈ 12 kDa). The cell- adhesive peptide YRGDS was conjugated to Acrylate-PEG3400-SVA

in a 1:1 PEG to peptide molar yielding the RGD-PEGMA monofunctional macromer, Acryl-PEG3400-YRGDS (MW $\approx$ 4 kDa). A tyrosine residue (Y) was added to the RGD peptide sequence to enable quantification of the immobilized RGD concentration in the scaffold via radiolabeling with ^{125}I subsequent to gel formation as described below. All peptide conjugation reactions were carried out in 50 mM NaHCO_3 solution (pH 8.0) for 4 h while protected from light. Conjugated solutions were dialyzed for 24 h to removed unreacted reagents, lyophilized, and stored at -20°C until use. The non-biofunctional PEG monoacrylate macromer, PEGMA (MW = 5kDa), was synthesized through acrylation of monomethoxy-PEG (MW = 5 kDa) using a two-fold molar excess of acryloyl chloride relative to free hydroxyls in the presence of trimethylamine in anhydrous dichloromethane under argon overnight in the dark. The resulting product was separated from aqueous byproducts by the addition of 2M K_2CO_3 . The organic phase was collected in a separatory funnel and the final PEGMA solution was precipitated in ice-cold ether, filtered, dried under vacuum overnight, and stored at -20°C until use. Acrylation efficiency of PEGMA was confirmed to be $>95\%$ using ^1H NMR.

2.4 Hydrogel Formation

Hydrogel scaffolds were formed by free-radical photopolymerization of aqueous precursor of varying macromer composition following exposure to visible-light ($\lambda = 514\text{ nm}$) using an Argon Ion Laser (Coherent, Inc., Santa Clara, CA) at a laser flux of 100 mW/cm^2 . Scaffolds with decoupled and combined variations in elastic modulus, immobilized RGD concentration, and proteolytic degradation rate (fast vs. slow) were formed by varying macromer composition (SSite PEGDA, DSite PEGDA, PEGMA-RGD and PEGMA) in the precursor (Figure 1A). Variations in modulus were achieved by adjusting the precursor concentration of MMP-sensitive PEGDA crosslinkers (1.5, 2, and 2.5 mM) while maintaining the concentration of RGD-PEGMA (5 mM) constant. To achieve variations in immobilized RGD concentration without inducing changes in scaffold modulus and degradation kinetics, the molar ratio of RGD-PEGMA to PEGMA was adjusted while maintaining a constant total concentration (5 mM) of PEG monoacrylate (RGD-PEGMA and PEGMA) To create scaffolds with varying rates of proteolytic degradation without inducing changes in initial material properties and biochemical composition (i.e. prior to material degradation), precursor solutions were polymerized using either SSite PEGDA or DSite PEGDA crosslinking macromers of similar molecular weight and composition as described in our previously published studies [21]. Aqueous precursor consisted of 37 mM NVP (accelerator and comonomer), 7mM TEA (co-initiator) and 0.01 mM eosin Y (photosensitizer), with varying concentrations of SSite or DSite PEGDA, RGD-PEGMA, and PEG MA, as described above, dissolved in 1X phosphate-buffered saline (PBS, Fisher Scientific) with pH 7.4 adjusted by the addition of HCl. A volume of $100\text{ }\mu\text{L}$ of the precursor solution was placed into a well of a 96-well plate followed by photopolymerization. After crosslinking, hydrogels were rinsed with PBS and swollen to equilibrium for 48 hrs after which mechanical properties, degradation kinetics, and immobilized RGD concentration were quantified as described below.

2.5 Quantification of Immobilized RGD Concentration

Scaffold immobilized YRGDS concentration was quantified by tracking the radioactivity of radiolabeled (with ^{125}I) tyrosine residues (Y) prior to gel formation and subsequent to hydrogel formation as described in our previous studies. (Turturro2013b, He2018) Briefly, 5 mg of YRGDS was dissolved in reaction buffer (1XPBS, pH 6.5), combined with 1mCi sodium iodine (PerkinElmer Health Sciences) in the presence of iodination beads (Pierce) and allowed to react for 15 min. Radiolabeled RGD (^{125}I -YRGDS) was conjugated to Acryl-PEG3400-SVA (as described above) to generate a radiolabeled RGD-PEGMA macromer. To track the incorporation of immobilized YRGDS in hydrogel scaffolds, radiolabeled Acryl-PEG3400-YRGDS was added to the precursor in an amount not exceeding 20 wt % of the total peptide. Hydrogel scaffolds were swollen in DI water for 48 hrs with three water changes and scaffold radioactivity measured using a Multi-Wiper gamma counter (Laboratory Technologies, Inc. Elburn, IL). Hydrogel scaffolds were subsequently weighed in the fully-swollen state, frozen at -80°C , lyophilized, and the dry weight recorded. The immobilized RGD concentration in the scaffold, $[RGD]_{gel}$ was subsequently calculated using the following equation:

$$[RGD]_{gel} = \frac{R \times SA}{\frac{m_{swollen} - m_{dried}}{\rho_{water}} + \frac{m_{dried}}{\rho_{PEG}}} \quad (1)$$

where R is the measured radioactivity, SA is the specific activity (mole/Ci) that was measured and used to convert the measured radioactivity to moles of peptide, $m_{swollen}$ is the equilibrium the swollen weight of the hydrogel, m_{dried} is the weight of the hydrogel in the dried state, ρ_{water} is density of water at room temperature (0.997 g/ml) and ρ_{PEG} is the density of PEG (MW=10 kDa) at room temperature (1.3 g/ml).

2.6 Quantification of Hydrogel Degradation Kinetics

As we have demonstrated that the MMP-sensitive PEGDA scaffolds can be degraded by both mammalian MMP-9 and bacterial collagenase (Supplemental data Figure S1), we chose to use bacterial collagenase (Collagenase-IA from *Clostridium histolyticum*, Sigma-Aldrich) as a more economically feasible option to quantify hydrogel degradation kinetics and to discern differences in degradation rate due to matrix properties. After hydrogel equilibrium swelling was achieved, the degradation profiles of fully swollen MMP-sensitive PEGDA scaffolds were obtained gravimetrically by incubation with collagenase enzyme solution in 10 mM HBS with 1 mM CaCl_2 (pH 7.4) at a concentration of $0.1 \mu\text{g/mL}$ at 37°C . At pre-determined time points the swollen weight of hydrogels was recorded after which fresh enzyme solution was replenished at every time point until complete hydrogel degradation was achieved.

2.7 Quantification of Static and Dynamic Changes in Hydrogel Modulus

To quantify scaffold mechanical properties hydrogels were photopolymerized in between two parallel glass slides using a 1mm thick spacer. The initial elastic modulus (prior to material degradation and exposure to enzyme incubation) of equilibrium swollen hydrogels was quantified by uniaxial compression. In the case of uniaxial compression experiments,

hydrogels using a TA RSA3 mechanical tester (TA Instruments) controlled by TA Orchestrator software. Hydrogels were compressed at a rate of 0.5 mm/min and the elastic modulus was quantified from the slope of the linear region (<10% strain, $r^2 > 0.95$) of the stress-strain curve. Dynamic changes in mechanical properties, storage modulus (G'), during the time course of proteolytic degradation following exposure to collagenase enzyme were quantified using rheometric measurements. Fully swollen scaffolds were exposed to 0.1 $\mu\text{g/mL}$ at 37 ° C at varying times (1,2,3,4,24,48 and 72 hrs) and loaded on a TA Discovery Hybrid Rheometer (TA Instruments, Newcastle, DE) using a parallel plate geometry. The storage modulus of gels exposed to collagenase enzyme over the different durations was then measured at 0.5% strain and frequency of 1 rad/s. An apparent degradation constant, k_{app} , was extrapolated for SSite and DSite hydrogels of varying initial modulus (prior to degradation) using the following equation:

$$\frac{G'}{G'_0} = e^{-k_{app}t} \quad (2)$$

In equation 2, G' denotes the storage modulus, G'_0 denotes the initial storage modulus prior degradation and t denotes the time in hours.

2.8 3D Culture Model of Vascular Sprout Formation

The effects of scaffold mechanical and degradative properties and immobilized RGD concentration on neovascularization were evaluated using a 3D co-culture spheroid model of sprouting angiogenesis. Cell spheroids composed of 50% human umbilical vascular endothelial cells (HUVECs, Lonza) and 50% human umbilical arterial smooth muscle cells (HUASMCs, Lonza) were formed by suspending both cells types at 1:1 ratio (5000 total cells/well) in endothelial growth media (EGM, Cell Application, Inc) containing 0.24% (w/v) methyl cellulose in round-bottomed, low binding, 96-well plates and incubated overnight at 37 ° C and 5% CO₂. Spheroids were suspended in the precursor and subsequently encapsulated in scaffolds following photopolymerization and gel formation. Spheroid-embedded hydrogel scaffolds were cultured in EGM for 7 days with media changes every other day.

At day 7, hydrogel scaffolds were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, rinsed three times with PBS for 10 min, permeabilized with 1% Triton X-100 in PBS (PBST) for 1 hr, and blocked using 6% normal goat serum in PBST for 1 hr at room temperature and overnight at 4 ° C. Hydrogel scaffolds were then incubated with 25UI/ml Alexa Fluor 546-phalloidin in 2% normal goat serum PBST to stain F-actin overnight at 4 ° C. F-actin stained spheroids were imaged using PASCAL laser scanning confocal microscopy (Carl Zeiss MicroImaging, Inc.) to produce a series of z-stack images that could be flattened to obtain z-projected images. F-actin stained images were analyzed with sprout morphology plugin of ImageJ/Fiji to quantify total sprout length and total number of junctions. To determine the cell type contributing to 3D vascular sprouting and invasion in response to material properties, differential staining of ECs and SMCs was performed at day 7 following spheroid encapsulation in scaffolds. Hydrogels were incubated with fluorescent Ulex Europaeus Agglutinin I (UEAI) and mouse anti-human α -SMA

primary antibody (1:20 dilution) in 2% normal goat serum PBST overnight at 4 ° C to stain for ECs and SMCs, respectively. Subsequently, scaffolds were rinsed 3 times with PBST for 10 minutes and incubated with a secondary goat anti-mouse smooth muscle cell antibody for 4 hours at room temperature and overnight at 4 ° C. Differentially stained hydrogels were then rinsed 3 times with PBST for 10 minutes prior to confocal imaging.

2.9 Statistical Analysis

All data are presented as mean $\pm$ standard deviation. To determine statistical significance between groups, one-way ANOVA or two-way ANOVA was performed followed by a Tukey's HSD post-test for pairwise and multiple group comparisons (GraphPad Prism8). In all cases, p-values <0.05 were considered statistically significant.

A design of experiments (DOE) approach was used to assess and model vascular sprouting in response to matrix properties (immobilized RGD concentration, elastic modulus, degradation rate) as well as the interactions between these factors on vascular sprouting. A full factorial design with three levels of immobilized RGD concentration (0.7mM, 2.1 mM and 3.5 mM), three levels of elastic modulus (800Pa, 2kPa, 4kPa) and two levels of degradation rate (rapid and slow) was implemented (Figure1B and Table1). The response variables into the model included the total sprout length and total number of junctions. Coded factors were used so that the effect of each factor could be compared. The values of the continuous factors (immobilized RGD concentration and elastic modulus) in the experiments were normalized to range between -1 and 1 , and the degradation rate was coded as 0 for slow degradation and 1 for rapid degradation. The postulated model for each response variable, y , was described by the following equation:

$$y = a_1 + a_2x_1 + a_3x_2 + a_4x_3 + a_5x_1x_2 + a_6x_1x_3 + a_7x_2x_3 + \epsilon \quad (3)$$

Where a_1 represents the overall average, a_2 , a_3 and a_4 represent coefficients related to the individual or main effects of each factor including immobilized RGD concentration (x_1), elastic modulus (x_2) and degradation rate (x_3), respectively, a_5 , a_6 and a_7 account for the two factor synergistic interactions between RGD and modulus, RGD and degradation rate, and modulus and degradation rate, respectively, and; ϵ accounts for any variability not captured by the model. The value of coefficients extrapolated from the models represent the relative magnitude of the identified individual and synergistic effects. The positive or negative effects of each factor were represented in terms of positive or negative coefficients in the model. All numerical computational algorithms and routines were programmed in Matlab R2018b (Mathworks, Inc., Natick, MA, USA).

3 Results

3.1 Decoupling immobilized RGD concentration, elastic modulus and proteolytic degradation rate of hydrogel scaffolds

To determine the impact of hydrogel properties on *in vitro* 3D vascular sprouting responses, scaffolds were engineered with individual and combined variations in elastic modulus, immobilized RGD concentration and proteolytic degradation by controlled adjustments in polymerization conditions. The chosen ranges of scaffold properties investigated are

identified in Table 1 and were based on mechanical properties of soft tissues and RGD concentrations previously identified to support cell adhesion in 3D culture [9]. For each of the selected RGD concentrations and elastic modulus conditions, scaffolds were also designed to exhibit fast and slow proteolytic degradation to better elucidate the impact of dynamic changes in matrix properties on vascular sprouting. Hydrogel mechanical properties were modulated through variations in proteolytically degradable PEGDA crosslinker concentration ranging from 1.5mM, 2mM and 2.5mM which led to formation of scaffolds of low (818 ± 123 Pa), intermediate (2079 ± 154 Pa), and high (3981 ± 160 Pa) elastic modulus, respectively (Figure 2A). Independent adjustments in scaffold degradation rate were achieved by polymerizing precursors that consisted of degradable PEGDA macromers of similar molecular weight and composition, but with either one (SSite) or two (DSite) repeats of peptides susceptible to proteolytic cleavage between the terminal acrylate groups of the macromer. This approach was used to induce changes in proteolytic degradation without producing variations in initial (prior to material degradation) crosslink density, elastic modulus, and immobilized RGD concentration of scaffolds. The data in Figure 2A confirm that polymerization of SSite and DSite PEGDA crosslinkers at a given macromer concentration does not alter scaffold modulus, as demonstrated in our previously published studies [21], which enables tuning of degradation kinetics over the range of mechanical properties investigated (Table 1). Decoupled variations in immobilized RGD concentration and modulus and/or degradation were achieved through the addition of a PEG monoacrylate surrogate (PEGMA) in the precursor. Specifically, the total monoacrylate PEG prepolymer concentration consisted of PEGMA and PEGMA-RGD and was kept constant at 5mM while the PEGMA-RGD concentration was varied from 1mM, 3mM, and 5mM. The data in Figure 2B indicate that adjustments in the PEGMA-RGD concentration using this approach does not induce variations in hydrogel elastic modulus/crosslink density.

Finally, to ensure that variations in scaffold mechanical properties did not induce changes in immobilized RGD concentration when adjusting the PEGDA and/or PEGMA precursor concentration, scaffolds with elastic moduli ranging from 0.8–4kPa (Figure 2A) were polymerized with fixed PEGMA-RGD concentration (5mM). As shown in Figure 3A, the percentage of RGD incorporated in gels of varying modulus remained constant at 80%. Interestingly, significant increases in hydrogel volume were noted with the addition of PEGMA (1mM and 2mM) in the precursor (Figure 3B). According to equation 1, increases in gel volume decrease the immobilized RGD concentration in the scaffold. As shown in Figure 3C, the addition of PEGMA in the precursor leads to significant decreases in immobilized RGD concentration compared to RGD gels synthesized in the absence of PEGMA. These results indicate that modulation of elastic modulus achieved through alteration in PEGDA concentration (1.5 – 2.5mM) does not induce changes in immobilized RGD concentration while increases in PEGMA concentration reduces the concentration of immobilized RGD.

3.2 Protease-sensitivity modulates dynamic changes in scaffold mechanical properties

For both SSite and DSite hydrogels, increases in crosslinker concentration led to significant increases in scaffold modulus (Figure 2 A) and hydrogel degradation time (Figure 4A). Furthermore, DSite hydrogels exhibited more rapid degradation relative to SSite hydrogels

over the range of moduli investigated. DSite hydrogels of low, intermediate, and high modulus underwent complete degradation at 4, 7.5, and 25 hrs, respectively, while SSite hydrogels of low, intermediate, and high modulus completely degraded by 26, 48, and 57 hrs, respectively. It should be noted that quantification of degradation kinetics using gravimetric measurements in hydrogel wet weight does not directly correlate with the extent of degradation as the gel network imbibes additional solvent during proteolysis due to time-dependent decreases in crosslink density. This contributes to the observed increases in gel weight in the initial phase of degradation followed by decreases in wet weight as the network begins to collapse during the final phase of degradation.

To further characterize scaffold degradation kinetics, time-dependent changes in hydrogel storage modulus (G') were quantified using rheometry. SSite and DSite gels of varying modulus were incubated in collagenase solution at pre-determined times up to 72 hours followed by measurements in storage modulus. The storage modulus of SSite hydrogels remained relatively constant following short collagenase incubation times (1–4 hrs) and decreased exponentially with further increases in enzyme incubation time (24–72 hrs) (Figure 4B). Conversely, DSite gels exhibited more dramatic and exponential decreases in modulus following short collagenase incubation times (1–4 hrs). Although SSite and DSite hydrogels of varying initial storage modulus (G'_0) appeared to exhibit variable dynamic reduction in G' similar rates of reduction in normalized modulus values were observed for both degradable gel types (Figure 4C). To further illustrate differences in time-dependent changes in modulus, an apparent degradation constant, k_{app} , was extrapolated from the rheological data of SSite and DSite hydrogels shown in Figure 4 B using equation 2 as described above. The k_{app} values were found to be similar among SSite or DSite hydrogels regardless of G'_0 (Table 2). Comparisons of k_{app} values, as well as the rheological data in Figure 4 C, reveals that DSite scaffolds exhibit significant increases in the rate of modulus reduction relative to SSite hydrogels. Furthermore, DSite hydrogels exhibit a 16-fold higher value in k_{app} relative to SSite hydrogels. This is expected as k_{app} is representative of the intrinsic susceptibility to degradation, or the sensitivity of the peptide substrate to cleavage by collagenase enzyme.

3.3 Analysis of 3D Vascular Sprouting as a function of matrix properties using DOE Analysis

The independent and combined effects of matrix mechanical properties, cell adhesiveness and proteolytic degradation rate on vascular sprouting responses were evaluated using a 3D spheroid co-culture (HUVECs and HUASMCs) model of sprouting angiogenesis [29] in combination with DOE. Sprouting of encapsulated spheroids was monitored over 7 days culture period using microscopy at days 1, 3, 5, and 7 (supplemental Figure S3) for which spheroid sprout diameter was measured at each time point (supplemental Figure S4) as a quantitative assessment of dynamics in vascular invasion. We found that the trends of the effect of each matrix property investigated on sprouting were consistent at various the various time points. The most pronounced effects occurred at the 7 day time point which was therefore chosen for further quantitative analysis of the individual and combinatorial effects of matrix cues on vascular sprouting. To determine the cell type leading the invasion and sensing the material properties manipulated ECs and SMCs embedded in the hydrogels

after 7 days were differentially labeled with UEA I (for labeling ECs) and α -SMA primary and secondary antibodies (for labeling SMCs). Confocal images of differentially labeled cell sprouting indicate that the majority of vascular sprouts are composed of ECs (stained red) while SMCs (stained green) are mainly located at the core of the spheroid with a minimal portion of SMC vascular sprouts located adjacent to the EC sprouts (Figure 5).

To visualize the extent of 3D sprouting within hydrogel scaffolds, 2D projections of fluorescent z-stack confocal images of spheroids stained with F-actin marker (phalloidin) were obtained after a 7 day culture period as illustrated in Figures 6 and 7. Within SSite hydrogels, the extent of vascular sprouting appeared to be dependent on scaffold modulus with more extensive vascular sprout formation occurring in scaffolds of low modulus relative to scaffolds of intermediate or high modulus. (Figure 6) SSite scaffolds of high elastic modulus exhibited minimal sprouting regardless of immobilized RGD concentration (Figure 6). Thus, in the case of the slower degrading SSite scaffolds, modulus, but not immobilized RGD concentration, appears to be the prominent factor influencing the extent of 3D vascular sprouting. Alternatively, in the case of the more rapidly degrading DSite scaffolds, the extent of vascular sprout formation appears to be dependent on RGD concentration (Figure 7). DSite scaffolds exhibited extensive vascular sprouting for all conditions investigated. Exceptions to these findings included for scaffolds of low adhesion ligand concentration and intermediate and high modulus (Figure 7).

To further elucidate the impact of matrix properties on *in vitro* neovascularization, the extent of sprouting parameters was quantified in terms of total sprout length and total number of junctions. As demonstrated in the analysis of the data in Figure 8, vascular sprouting was dependent on scaffold modulus but not on immobilized RGD in scaffolds exhibiting slow proteolytic degradation (SSite scaffolds). SSite low modulus hydrogels exhibited a 2–3 fold increase in sprout length and junction number relative to scaffolds of intermediate modulus and a 2–3 fold increase in sprout length and 100-fold increase in junction number compared to SSite high modulus scaffolds (Figure 8A and 8D). In contrast, cell adhesion ligand concentration did not significantly impact total sprout length or number of junctions within SSite hydrogels except in the case of low modulus where increases in immobilized RGD concentration led to increases in vascular sprout length (Figure 8A). In contrast, increases in immobilized RGD concentration within DSite hydrogels significantly increased total sprout length and junction number in a manner that was not found to be dependent on scaffold modulus (Figure 8B and 8E). Specifically, DSite hydrogels of intermediate and high modulus and high immobilized RGD concentration exhibited 2-fold and 10-fold increases in sprout length and junction number relative to DSite hydrogels of intermediate and low adhesiveness, respectively. In general, vascular sprouting was not dependent on the initial stiffness of the matrix in DSite hydrogels. An exception to this trend was observed in the case of low RGD concentration for which decreases in modulus led to significantly increased sprout length and junction number. Finally, to illustrate the influence of proteolytic scaffold degradation kinetics on vascular sprouting parameters, differences in total sprout length and junction number among DSite and SSite hydrogels of varying ranges of immobilized RGD and modulus were also quantified (Figure 8C and 8F). The data illustrate that DSite hydrogels promote significant increases in sprout length and number of junctions compared to SSite scaffolds except in the case of low immobilized RGD concentration. Moreover, the

observed differences in vascular sprouting between rapid and slower matrix degradation were found to be dependent on cell adhesion ligand concentration across all modulus values investigated. As negative controls, spheroids encapsulated in non-degradable cell-adhesive RGD containing scaffolds or protease-sensitive, non-adhesive (lacking immobilized adhesion ligands) exhibited no sprouting after 7 day culture demonstrating the need for functionalization of PEG hydrogels with cell adhesive and protease-sensitive peptides for support of 3D vascular cell invasion (Supplemental Figure S2).

These findings are also summarized in Figure 9 where the data in 9A and 9C depict the individual measurements of total sprout length and total junctions, respectively, for each scaffold condition investigated as circles of diameter proportional to the magnitude of the measurement. Figures 9B and 9D illustrate the average measurements of total sprout length and total junctions across all conditions investigated as surface plots. Based on the data in Figure 9 it is evident that the more rapidly degrading DSite scaffolds require increases in immobilized RGD concentration to achieve enhancements in total sprout length and number of junctions, while in the case of the slower degrading SSite scaffolds vascular sprouting parameters are not dependent on immobilized RGD but significantly impacted by modulus. Overall, DSite hydrogels appear to promote higher sprout length and number of junctions relative to SSite hydrogels, except in the case of low immobilized RGD concentration.

Using a full factorial DOE analysis, a regression model was postulated to evaluate individual and two-factor interactions of modulus, proteolytic degradation and immobilized RGD concentration on vascular sprouting in 3D culture. The coefficients extrapolated using the regression model are presented in equation 3 and represent single effects and two-factor interactions on total sprout length (Figure 10A) and junction number (Figure 10B). Positive coefficients indicate that the single factor or two-factor interactions positively impact vascular sprouting parameters, while negative coefficients signify a negative effect on vascular sprouting. The values of the coefficients presented in Figure 10 represent the magnitude of the corresponding effect(s). Enhancements in proteolytic degradation were found to significantly and positively influence total sprout length and junction number while increases in modulus significantly but negatively impacted vascular sprouting. The only two-factor interaction identified to be statistically significant as well as the most prominent among all investigated, was between immobilized RGD concentration and proteolytic degradation rate, with increases in both leading to enhanced vascular sprouting in 3D culture.

4 Discussion

In this study, we utilized a statistical DOE methodology and full factorial experimental analysis to determine the individual and combined effects of matrix stiffness, proteolytic degradation and immobilized RGD cell adhesion ligand concentration on vascular sprouting in 3D culture using PEG-based hydrogels as a tunable biomaterial platform. The main advantage of application of the DOE analysis in the present study is that it enabled screening of the interactions of multiple matrix cues which would otherwise only reveal effects of individual as opposed to factor interactions. The majority of studies in engineering biomaterials for vascularized tissue formation have investigated the effect of one or two

matrix cues at a time without considering potential interactions of the investigated factors on vascular responses [7, 9–11, 19–22]. In this study, application of modular synthetic scaffolds in combination with DOE allowed us to identify the strong synergistic impact of immobilized RGD concentration and proteolytic scaffold degradation rate leading to optimal vascular sprouting responses in 3D culture over a range of matrix properties investigated (Table 1). To the best of our knowledge, this is the first time that statistical DOE has been used to study the interplay between modulus, proteolytic degradation and adhesion ligand concentration on neovascularization. These matrix cues form the fundamental basis for engineering functional tissue replacements that require a specific stiffness, composition of integrin-binding peptide ligands and degradation rates to promote cellular differentiation and vascularized matrix remodeling.

Decoupled variations in matrix properties have been demonstrated using various synthetic materials [30, 31], which is crucial for multifactorial screening of matrix cues on cell behavior because every factor incorporated into statistical design models is an independent variable. Using a full factorial statistical DOE, we created a series of hydrogel scaffolds with 18 different combinations of immobilized RGD concentration, elastic modulus and proteolytic degradation rate to probe their effects on neovascularization (Figure 1). We utilized a 3D co-culture (endothelial and smooth muscle cell) model of sprouting angiogenesis to evaluate the angiogenic responses to matrix properties [29]. The results of vascular sprouting (Figure 6 and 7) and plots of surface responses (Figure 9) demonstrated that more rapidly degrading DSite hydrogels enhance vascular sprouting in an immobilized RGD dependent manner. In the case of slower degrading SSite hydrogels, enhancements in vascular sprouting were found to respond to increases in immobilized RGD concentration only in hydrogels of low modulus (Figure 8A and 8D). Studies have demonstrated biphasic angiogenic responses to changes in adhesive ligand concentration or integrin antagonists, suggesting that intermediate levels of matrix adhesivity result in optimal 3D angiogenic responses [7, 11, 12]. The concentration range of immobilized RGD investigated in our presented study appears to lie within the rising side of the vascular sprouting response curve. Interestingly, enhancements in sprouting in response to RGD concentration were significantly reduced when the degradation of the scaffold switched from rapid (DSite) to slow (SSite). The strong interactions between increased degradation rate and immobilized RGD concentration was also confirmed using the DOE model based on the coefficients obtained using the regression models. Predicted coefficients representing RGD concentration and degradation rate interactions on total vascular sprout length and junction number were found to be statistically significant (Figure 10). Interestingly, the coefficient representing the individual effect of RGD concentration was not found to significantly impact 3D vascular sprouting over the range of mechanical and degradative properties investigated. This finding is contradictory to prior literature findings identifying adhesive ligand concentration to be among the most prominent factors impacting neovascularization responses [7]. The underlying mechanism of the interaction between RGD concentration and scaffold degradation in the present study is not entirely clear and warrants further investigation. Mechanistic studies have shown that RGD matrix concentration is directly related to the extent of integrin-RGD interactions leading to formation of focal adhesion complexes that initiate a cascade of downstream signaling to regulate different aspects of

cellular behavior during neovascularization [32]. However, matrices with restricted (slow) degradation limit the formation of focal adhesions [33]. We hypothesize that rapid proteolytic degradation enhances cell-mediated matrix degradation which results in increased formation of focal adhesions thereby enabling cells to more readily respond to RGD concentration changes in their ECM environment; however, more detailed investigation is required to confirm this hypothesis by exploring the impact of dynamic changes of RGD integrin binding peptides and focal adhesions on vascular sprouting as will be explored in future studies.

DOE analysis also revealed that increases in scaffold modulus leads to decreases in total sprout length and number of junctions independent of immobilized RGD concentration or proteolytic degradation rate. This observation agrees with prior studies indicating that increases in matrix stiffness impedes neovascularization responses [34–36]. Other studies have demonstrated biphasic neovascularization responses to matrix stiffness with intermediate stiffnesses being optimal [9, 37, 38]. These optimal stiffness values, however, lie within the range of 500 – 1000Pa, which fall on the low end of the range of modulus values investigated in our study. Cells sense matrix stiffness through establishment of focal adhesions suggesting potential interactions of matrix modulus and cell adhesion ligand concentration [13, 14, 39]. In our study, however, statistical DOE analysis revealed no significant interactions between modulus and immobilized RGD concentration on vascular cell responses (Figure 10). Enhancements in 3D tubulogenesis can be achieved in an RGD and modulus dependent manner [9]. For example, total tubule length was enhanced with increases in RGD concentration in hydrogels of intermediate modulus (1kPa) but not in scaffolds possessing high (3k Pa) or low (300 Pa) modulus values. Our present study also reveals that increases in RGD concentration lead to enhancements in sprout length in SSite hydrogels of low modulus (800 Pa); however, the effect of RGD concentration becomes insignificant with further increases in scaffold modulus (Figure 8A). DOE analysis revealed that the interaction between modulus and immobilized RGD was insignificant relative to the other individual and combined factor interactions investigated. We speculate that the interaction effect between modulus and adhesion is dependent on proteolytic degradation rate and a potential three-way interaction between degradation rate, immobilized RGD concentration and modulus on vascular sprouting. This analysis will require further increases in sample sizes to ensure sufficient statistical power for inclusion of a three-way interaction term in the regression model which will be explored in future studies.

Among the matrix cues investigated in this study, proteolytic scaffold degradation rate was found to be the most prominent factor contributing to enhancements in vascular sprouting. In Figure 8, increases in total sprout length and total number of sprouts are observed within more rapidly degradable DSite hydrogels. This observation also agrees with prior literature [11, 19–22]. Our studies and others have shown that matrix stiffness dynamically changes over time due to cell-mediated degradation and the rate of degradation is tuned based on the proteolytic sensitivity of the hydrogel scaffold (Figure 4). Furthermore, the importance of scaffold degradation on vascular morphogenesis has been previously demonstrated by controlling the protease-mediated degradation rate through the incorporation of distinct peptide sequences with different proteolytic susceptibility to cleavage [19, 23]. MMP-sensitive peptide sequences with wide ranges of enzymatic sensitivity have been identified

based on combinatorial screening of peptide libraries. Incorporation of these distinct peptide sequences may further contribute to desired modulation of scaffold degradation required for functional tissue regeneration [19, 22, 40]. As these identified peptide sequences can be degraded by multiple MMPs at various rates depending on enzyme type and amount secreted by different cell types, identification of the protease-sensitive peptide combinations required to optimize vascularized tissue remodeling *in vivo* becomes challenging [22]. To ensure that hydrogels are degraded by the same types of cellular enzymes at varying degradation rates, we chose to utilize an alternative approach to modulate protease-mediated degradation. This was achieved by using crosslinkers of similar molecular weight which contained one or two repeats of the same peptide substrate (VPMS↓MRGG), previously identified to exhibit high sensitivity to MMP-1, MMP-2, and MMP-9 [19], between network crosslinks. Using this approach, we engineered hydrogel scaffolds with rapid and slow proteolytic degradation and decoupled and combined variations in modulus and immobilized RGD concentration. The differences in degradation kinetics were evaluated based on dynamic gravimetric measurements in hydrogel wet weight in collagenase incubation (Figure 4A) and quantification of storage modulus using rheometric measurements over the course of degradation (Figure 4B and 4C). Rheological degradation kinetics demonstrated exponential decreases in storage modulus (Figure 4B and 4C), similar to degradation profiles achieved with photodegradable hydrogels upon exposure to irradiation [41, 42]. Using network degradation models whereby the storage modulus (G') scales with hydrogel crosslink density (ρ_x), allowed normalized storage modulus $\frac{G'}{G'_0}$ to be equivalent to the normalized

hydrogel crosslink density ($\frac{\rho_x}{\rho_{x^0}}$). Thus, the kinetic profiles of normalized storage modulus ($\frac{G'}{G'_0}$) among scaffolds of similar degradation rate but varying initial modulus and

immobilized RGD fall on the same curve, while the profiles become significantly different as the proteolytic degradation varies (SSite vs. DSite hydrogels) (Figure 4D and 4E). DSite hydrogels were found to exhibit a 16-fold higher apparent degradation constant (k_{app}) value relative to SSite hydrogels suggesting that proteolytic degradation rate or sensitivity can be modulated independent of variations in initial scaffold modulus using our presented approach for tuning scaffold degradation.

Finally, *in vitro* vascular sprouting in slow degrading SSite scaffolds was found to be dependent on modulus while the negative effect of increasing modulus on sprout formation diminished in rapidly degrading DSite hydrogels. Although we anticipated a degree of interaction between initial modulus and degradability on vascular sprouting, DOE analysis revealed that modulus and degradation rate were found to individually, but not synergistically influence total sprout length and total junction number (Figure 10). The positive effect of enhanced degradation in DSite hydrogels on vascular sprouting may overshadow the negative effect of increasing modulus on vascular sprout formation in DSite hydrogels.

5 Conclusion

In this study, PEG hydrogel scaffolds were engineered with tunable and decoupled variations in immobilized RGD concentration, modulus and proteolytic degradation to investigate the individual and combined effect of these matrix properties on vascular sprouting in 3D culture. Using 3D culture studies and a full factorial DOE approach we demonstrated that an optimal combination of matrix cues, specifically, increases in immobilized RGD concentration and proteolytic degradation rate of PEG scaffolds synergistically enhanced 3D vascular sprouting. The presented factorial design approach using PEG hydrogels with tunable matrix properties can be tailored to investigate the cell-matrix interactions for various tissue engineering applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Schematic representation of approach used to engineer hydrogels with decoupled and combined variations in immobilized RGD concentration, elastic modulus and proteolytic degradation rate for testing the impact of these matrix properties on vascular sprouting in 3D culture. (A) Photopolymerizable macromers synthesized for tuning hydrogel properties during crosslinking. (B) DOE approach used in full factorial design to determine individual and combined effects of input variables (immobilized RGD concentration, modulus and degradation rate) on vascular sprouting response. (C) Synthesis and characterization of hydrogel properties with individual and combined variations in matrix properties and biochemical composition. (D) Screening of matrix properties on 3D *in vitro* neovascularization responses

Fig. 2. Mechanical characterization of hydrogel scaffolds. Elastic modulus as a function of (A) Single Site or Double Site PEGDA concentration and (B) PEGMA-RGD concentration. Asterisk (*) indicates statistical significance (n=4, p<0.05)

Fig. 3. Quantification of radiolabeled RGD in hydrogel scaffolds. (A) RGD incorporation (%), (B) gel volume, and (C) immobilized RGD concentration as a function of PEGDA and PEGMA concentration. (D) immobilized RGD concentration as function of PEGMA-RGD concentration. Asterisk (*) indicates statistical significance ($n=4$, $p<0.05$)

Fig. 4.

Characterization of degradation kinetics of SSite and DSite hydrogel scaffolds of varying initial modulus. (A) Degradation profiles of scaffolds based on gravimetric measurements in wet weight over time in collagenase incubation. (B) Time-dependent changes in (B) storage modulus (G') and (C) normalized shear storage modulus (G'/G'_0) of SSite and DSite hydrogels after collagenase incubation. Insets in (B) and (C) represent the dynamics in G' and G'/G'_0 for DSite hydrogels, respectively (n=4).

Fig. 5. Confocal images of differentially labeled spheroid sprouting. Endothelial cells were stained with UEA I (red) and smooth muscle cells were stained with anti α -SMA antibody (green). Images A, B, C were taken at 10x magnification, and images D, E, F were taken at 20x magnification.

Fig. 6.

Vascular sprout invasion in SSite hydrogel scaffolds with variations in immobilized RGD cell adhesion ligand concentration and modulus. Representative 2D projections of fluorescent z-stack confocal images of sprouting spheroids photo encapsulated in hydrogel scaffolds with low modulus and (A) low adhesion ligand, (B) intermediate adhesion and (C) high adhesion, in scaffolds with intermediate modulus and (D) low adhesion, (E) intermediate adhesion and (F) high adhesion and scaffolds with high modulus and (G) low adhesion, (H) intermediate adhesion and (I) high adhesion.

Fig. 7.

Vascular sprout invasion in DSites hydrogel scaffolds with variations in immobilized RGD cell adhesion ligand concentration and modulus. Representative 2D projections of fluorescent z-stack confocal images of sprouting spheroids photo encapsulated in hydrogel scaffolds with low modulus and (A) low adhesion ligand, (B) intermediate adhesion and (C) high adhesion, in scaffolds with intermediate modulus and (D) low adhesion, (E) intermediate adhesion and (F) high adhesion and scaffolds with high modulus and (G) low adhesion, (H) intermediate adhesion and (I) high adhesion.

Fig. 8.

Effect of modulus, cell adhesion and degradation rate on vascular sprout invasion. Total sprout length and total number of junctions of SSite (A,D) and DSite (B, E) hydrogel scaffolds. Differences between DSite and SSite scaffolds in terms of total sprout length (C) and total number of junctions (F). *, #, and \$ indicate statistical significance among variations in immobilized RGD concentration, modulus, and proteolytic degradation (SSite vs. DSite gels) ($p < 0.05$, $n = 4$).

Fig. 9.

Quantification of vascular sprout invasion. Individual measurements of total sprout length (A) and total junctions (C) of each condition indicated as circles with diameter proportional to the magnitude of the measurement. Surface plots of average measurements in total sprout length (B) and total junctions (D) across all conditions investigated ($n=4$).

Fig. 10.

Analysis of individual and two-factor interactions on vascular responses (A) total sprout length and (B) total number of junctions. Regression model coefficients are presented by the green circles and the 95% confidence intervals by blue and red bars). X•Y denotes two factor interactions and * denotes statistical significance ($n=4$, $p<0.05$).

Table 1

List of experimental hydrogel conditions investigated in vitro and in full factorial design of the DOE model.

Group	Immobilized RGD Concentration	Young's Modulus	Degradation Rate
1	Low (0.7 mM)	Low (800 Pa)	Slow (SSite)
2	Intermediate (2.1 mM)	Low (800 Pa)	Slow (SSite)
3	High (3.5 mM)	Low (800 Pa)	Slow (SSite)
4	Low (0.7 mM)	Intermediate (2 kPa)	Slow (SSite)
5	Intermediate (2.1 mM)	Intermediate (2 kPa)	Slow (SSite)
6	High (3.5 mM)	Intermediate (2 kPa)	Slow (SSite)
7	Low (0.7 mM)	High (4 kPa)	Slow (SSite)
8	Intermediate (2.1 mM)	High (4 kPa)	Slow (SSite)
9	High (3.5 mM)	High (4 kPa)	Slow (SSite)
10	Low (0.7 mM)	Low (800 Pa)	Rapid (DSite)
11	Intermediate (2.1 mM)	Low (800 Pa)	Rapid (DSite)
12	High (3.5 mM)	Low (800 Pa)	Rapid (DSite)
13	Low (0.7 mM)	Intermediate (2 kPa)	Rapid (DSite)
14	Intermediate (2.1 mM)	Intermediate (2 kPa)	Rapid (DSite)
15	High (3.5 mM)	Intermediate (2 kPa)	Rapid (DSite)
16	Low (0.7 mM)	High (4 kPa)	Rapid (DSite)
17	Intermediate (2.1 mM)	High (4 kPa)	Rapid (DSite)
18	High (3.5 mM)	High (4 kPa)	Rapid (DSite)

Table 2

Apparent degradation constants (k_{app}) extrapolated from rheological degradation kinetics (n=4).

$k_{app} (\times 10^{-3} hr^{-1})$	Low Modulus	Intermediate Modulus	High Modulus
SSite	18.77±0.94	17.81±2.29	18.21±0.94
DSite	289.48±17	301.63±6.72	298.85±5.578