Dynamic control of hydrogel crosslinking via sortase-mediated reversible transpeptidation

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A B S T R A C T

Cell-laden hydrogels whose crosslinking density can be dynamically and reversibly tuned are highly sought-after for studying pathophysiological cellular fate processes, including embryogenesis, fibrosis, and tumorigenesis. Special efforts have focused on controlling network crosslinking in poly(ethylene glycol) (PEG) based hydrogels to evaluate the impact of matrix mechanics on cell proliferation, morphogenesis, and differentiation. In this study, we sought to design dynamic PEG-peptide hydrogels that permit cyclic/reversible stiffening and softening. This was achieved by utilizing reversible enzymatic reactions that afford specificity, biorthogonality, and predictable reaction kinetics. To that end, we prepared PEG-peptide conjugates to enable sortase A (SrtA) induced tunable hydrogel crosslinking independent of macromer contents. Uniquely, these hydrogels can be completely degraded by the same enzymatic reactions and the degradation rate can be tuned from hours to days. We further synthesized SrtA-sensitive peptide linker (i.e., KCLPRTGCK) for crosslinking with 8-arm PEG-norbornene (PEG8NB) via thiol-norbornene photocrosslinking. These hydrogels afford diverse softening paradigms through control of network structures during crosslinking or by adjusting enzymatic parameters during on-demand softening. Importantly, user-controlled hydrogel softening promoted spreading of human mesenchymal stem cells (hMSCs) in 3D. Finally, we designed a bis-cysteine-bearing linear peptide flanked with SrtA substrates at the peptide’s N- and C-termini (i.e., NH2-GGGCKGGGKCLPRTG-CONH2) to enable cyclic/reversible hydrogel stiffening/softening. We show that matrix stiffening and softening play a crucial role in growth and chemoresistance in pancreatic cancer cells. These results represent the first dynamic hydrogel platform that affords cyclic gel stiffening/softening based on reversible enzymatic reactions. More importantly, the chemical motifs that affords such reversible crosslinking were built-in on the linear peptide crosslinker without any post-synthesis modification.

Statement of Significance

Cell-laden ‘dynamic’ hydrogels are typically designed to enable externally stimulated stiffening or softening of the hydrogel network. However, no enzymatic reaction has been used to reversibly control matrix crosslinking. The application of SrtA-mediated transpeptidation in crosslinking and post-gelation modification of biomimetic hydrogels is innovative because of the specificity of the reaction and reversible tunability of crosslinking kinetics. While SrtA has been previously used to crosslink and fully degrade hydrogels, matrix softening and reversible stiffening of cell-laden hydrogels has not been reported. By designing simple peptide substrates, this unique enzymatic reaction can be employed to form a primary network, to gradually soften hydrogels, or to reversibly stiffen hydrogels. As a result, this dynamic hydrogel platform can be used to answer important matrix-related biological questions that are otherwise difficult to address.

1. Introduction

Stiffening and softening of extracellular matrix (ECM) contribute to many biological processes including cancer progression, stem cell differentiation, and tissue fibrosis [1–3]. Specifically,
mechanical properties of ECM affect changes in cellular morphologies [4,5], protein expression and secretion [6], and stem cell differentiation potentials [7,8]. For example, matrix stiffening has been shown to promote aggressive phenotypes, epithelial-to-mesenchymal transition (EMT), and chemo-resistance in pancreatic ductal adenocarcinoma (PDAC) [1,9]. On the molecular level, prior studies have established a causal link between matrix stiffness and nuclear localization of yes-associated protein (YAP). In particular, Rice and colleagues reported that increasing matrix stiffness (~1 kPa to ~4 kPa) lead to an increase in YAP nuclear localization in three different pancreatic cancer cell lines [9]. Nguyen and colleagues identified that higher matrix moduli (~3 kPa) promoted metastatic potential of PDAC cell lines [10]. On the other hand, matrix softening was shown to reduce sensitivity of myeloid leukemia subtypes to chemotherapeutics [11]. ECM biophysical stimuli also affect morphology and differentiation potentials of human mesenchymal stem cells (hMSCs). Discher and colleagues demonstrated that culturing hMSCs on soft (0.1–1 kPa) substrates yielded cellular morphology akin to neurogenic lineage, whereas stiffer substrate (25–40 kPa) promoted osteogenic differentiation [12].

Cell fate processes have been increasingly studied using cell-laden three-dimensional (3D) hydrogels. However, hydrogels with static mechanical properties may not fully recapitulate the dynamic nature of tissue mechanics [13–20] and few 3D culture materials permit user-initiated control over matrix physiochemical properties [21–23]. Recent efforts on ‘dynamic’ hydrogels have allowed researchers to control matrix stiffening and/or softening patterns that mimic the ever-changing ECM microenvironment. ‘Stiffening’ hydrogels are often designed to permit secondary crosslinking upon exposure to external stimuli such as light, temperature, pH, or enzymes. On the other hand, hydrolytic [24–26] or enzymatic cleavage [27,28] are typically utilized to soften matrices without user intervention. User-controlled matrix softening can be achieved through the addition of photoresponsive linkers [29]. For example, Anseth and colleagues developed elegant photodegradable hydrogels that permit the investigation of local matrix softening on cell behaviors [29], Rosales et al. [14,19], Zheng et al. [15], and Lee et al. [18] fabricated hydrogels with photoswitchable azobenzene moieties that afford reversible control of stiffness. Reversible tuning of matrix stiffness is achieved upon irradiation with the appropriate wavelength of light. In another example, controlling diol and boronic acid reactivity through azobenzene cis–trans transition was implemented to dynamically stiffen hydrogels [16]. Our group has also utilized supramolecular host–guest interactions to reversibly stiffen and soften hydrogels [17].

The above examples highlight the importance of creating a hydrogel network with reversible stiffening/softening capability. To this end, enzymatic reactions can afford substrate specificity and mild/predictable reaction kinetics [23,30–36]. Our lab has previously utilized mushroom tyrosinase (MT) to dynamically stiffen hydrogels containing either tyrosine residues [23,36] or 4-hydroxyphenyl acetic acid (HPA) [35]. We have also reported the design of PEG-peptide hydrogels that were sensitive to both MT and sortase A (SrtA). The hydrogels were susceptible to orthogonal and enzymatic control of crosslinking and on-demand stiffening [36]. While MT-mediated tyrosine dimerization affords efficient stiffening regimes, this enzymatic reaction is limited by the availability of dissolved molecular oxygen, the irreversibility of the di-tyrosine products, and unexpected oxidation of tyrosine-rich proteins. These disadvantages could be circumvented by using SrtA, a cysteine transpeptidase highly useful in site-specific protein labeling [37–41], peptide cyclization [42–46], and antibody modification [47–51]. Mechanistically, SrtA cleaves the amide bond between threonine and glycine residues of the LPXTG (where X is any amino acid except proline) sequence, resulting in a thiol-acyl intermediate that is subsequently resolved by an oligoglycine substrate (G₄₅). The end product of this enzymatic reaction is a new peptide sequence LPXTG(G₄₅) (Fig. 1A) that itself serves as a substrate for further SrtA transpeptidation. Of note, this reaction is also susceptible to hydrolysis, resulting in the formation of an unreactive pendant LPRT-OH [52]. Nonetheless, this biorthogonal enzymatic reaction can be utilized to crosslink, degrade, and modify hydrogels by incorporating the peptide substrates into the superstructure of the hydrogels. Specifically, the LPXTG and G₄₅ peptides can be tethered to macromers using either Michael-type addition [53,54] or thiol-norbornene click chemistry [36]. Upon addition of SrtA, the peptides are ligated to create new crosslinks. Broguire et al. recently reported the use of SrtA to crosslink hyaluronic acid-based hydrogels for tissue engineering applications [53]. In another example, Griffith and colleagues incorporated SrtA-labile LPXTG substrate as part of the linkers in hydrogels formed by Michael-type addition [54]. Addition of SrtA and soluble glycine led to breakage of the LPXTG peptide, eventually resulted in degradation of the hydrogels.

In this work, we attempt to expand the utility of SrtA-mediated transpeptidation to: (1) control hydrogel crosslinking and degradation; (2) permit controllable degrees of hydrogel softening; and (3) enable reversible stiffening and softening of PEG-peptide hydrogels. We used a hepta-mutant variant of SrtA for its increased catalytic activity and calcium-independency [39,55,56]. We designed simple linear peptide linkers containing SrtA-specific sequences to afford rapid and controllable crosslinking of PEG-peptide hydrogels. These gels can be dissolved/degraded within hours to days. We also used a SrtA-sensitive peptide crosslinker to afford controllable softening and the matrix was leveraged to control spreading of hMSCs in 3D. Finally, we designed a linear bis-cysteine peptide crosslinker whose N- and C-terminus was flanked with the two SrtA substrates to demonstrate cyclic/reversible hydrogel stiffening and softening. Most importantly, this dynamic cell culture platform was exploited for studying stiffness-dependent growth and chemo-resistance in pancreatic cancer cells (PCCs).

2. Materials & methods

2.1. Materials

Eight-arm PEG-OH (20 kDa) was acquired from JenKem Technology, USA. 5-Norbornene-2-carboxylic acid, N,N-dicyclocycexyldiiododiibodi (DCC), 4-(dimethylamino)pyridine (DMAP), dimethyl phenylphosphonite, 2,4,6-trimethylbenzoyl chloride, 2-butanone, lithium bromide, diethyl ether, and Isopropyl-2-thialactobopyranoside (IPTG) were obtained from Sigma-Aldrich. Fmoc-protected amino acids N,N,N,N-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorphosphate (HBTU), and hydroxybenzotriazole (HOBt) were purchased from Anaspec. BL21 Escherichia coli (E. coli) was purchased from New England Biolabs. Kanamycin sulfate was obtained from IBI Scientific. Lysogeny broth, Lennox formulation, agar and broth were obtained from DOT Scientific. Gencatidine was obtained from TSZ CHEM. Mammalian cell Live/Dead staining kit was obtained from Life Technologies Corp. All other chemicals were obtained from Fisher Scientific unless otherwise noted.

2.2. Macromer and peptide synthesis

Eight arm PEG-norbornene (PEG8NB, ~95% substitution) and photoinitiator lithium ary phosphinate (LAP) were synthesized as described previously [36,57]. Peptides were synthesized in an automated microwave-assisted peptide synthesizer (Liberty 1, CEM). Crude peptides bound to resin were reacted with a solution
containing 95% trifluoroacetic acid (TFA), 2.5% ddH2O, 2.5% triisopropylsilane (TIS), and 5% (w/v) phenol for 3 h at room temperature for cleavage. Immediately following cleavage, the peptides were precipitated in cold ethyl ether. Dried, crude peptides were purified using High-Performance Liquid Chromatography (HPLC, Flexar System, Perkin Elmer). Purified peptides were lyophilized and their masses confirmed via mass spectrometry (Agilent Technologies). All peptides were stored at \(-20^\circ\text{C}\) prior to use.

2.3. Heptamutant SrtA expression and purification

Heptamutant SrtA (P94R, E105K, E108Q, D160N, D165A, K190E, K196T) was expressed and purified as described previously\[36,58]\). Competent BL21 E. coli were transformed with pet30b-7M SrtA plasmid (a gift from Hidde Ploegh. Addgene plasmid #51141) and grown on an LB-agar selection plate containing kanamycin (30\(\mu\text{g/mL}\)). Individual colonies were inoculated in 10 mL of LB broth supplemented with kanamycin (30\(\mu\text{g/mL}\)). After overnight shaking (220 rpm, 37\(^\circ\text{C}\)), the cultures were diluted 100-fold in LB media supplemented with kanamycin and placed on an orbital shaker (220 rpm at 37\(^\circ\text{C}\)), and the optical density at 600 nm (OD\(_{600}\)) was monitored. SrtA expression was induced at OD\(_{600}\) = 0.4–0.6 by adding IPTG (400 \(\mu\text{M}\)) to the cultures and shaking for 3 h at 37\(^\circ\text{C}\) and 220 rpm. Following induction, the cell pellets were isolated by centrifugation (8000 rpm, 15 min) and stored at \(-80^\circ\text{C}\) prior to lysis. Cell lysis was performed by suspending the pellets in lysis buffer (20 mM Tris, 50 mM NaCl, 0.2 mg/mL lysozyme, 1x Halt EDTA-free protease inhibitor cocktail, and DNase I). Following a 30-minute incubation at 4\(^\circ\text{C}\), the suspended pellets were subject to sonication (2 cycles of 3 min with 30% duty cycle, 20% amplitude followed by a 3-minute cool-down period). Lysates were clarified by centrifugation (10,000 \(\times\) g, 20 min, 4\(^\circ\text{C}\)) and purified via His60 Ni Superflow resin and columns per manufacturer’s protocol. Purified SrtA (\(-40 \mu\text{g/mL}\) of culture) was concentrated with Ultra-15 Centrifugal Filter Units (3 kDa MWCO, Amicon) and desalted with Zeba Spin desalting columns (7 kDa MWCO, Thermo Fisher Scientific). The enzyme in PBS (pH = 7.4) was aliquoted, flash frozen, and stored at \(-80^\circ\text{C}\) Stock concentrations of
SrtA were obtained by Ellman’s assay measuring the free sulphydryl on SrtA. Typical concentrations of SrtA stock aliquots were ~4 mM.

2.4. Sortase A-mediated hydrogel crosslinking and degradation

PEG-peptide conjugates (i.e., PEG-GGGG and PEG-LPRTG) were prepared by reacting free norbornene groups of PEG8NB with cysteine-bearing peptides via thiol-norbornene photoclick chemistry. GGGGC or LPRTGC peptides were separately dissolved in PBS (pH = 7.4) prior to quantification of their concentrations with Ellman’s assay. To a round bottom flask, PEG8NB, peptides (1.5-fold excess to norbornene moieties), and photoinitiator LAP (5 mM) were added. The conjugation of peptide to PEG8NB was initiated upon exposure to light (Omnicure s1000, 365 nm, 40 mW/cm²) for 2 min. Additional LAP was added at 15 min to improve conjugation efficiency. The PEG-peptide conjugates were dialyzed against ddH₂O for 2 days to remove residual peptides and LAP. Using TNBSA assay, we determined the amine group concentration of the PEG-GGGG conjugate (~2.66 mM/10% of PEG-GGG). Note that the PEG-conjugate peptides can also be prepared by Michael-type reaction between cysteine-bearing peptides and multi-arm PEG-vinylsulfone or PEG-maleimide. It is also worth noting that the cysteine residue at the active site of SrtA is not reactive towards norbornene groups as the thiol-norbornene reaction only occurs under radical-mediated light exposure. The purified conjugates were lyophilized and stored at −20 °C prior to use.

To initiate gelation, PEG-peptide conjugates were mixed in the presence of SrtA and immediately injected between glass slides separated by 1 mm Teflon spacers. After 10 min of gelation, the hydrogels were transferred to a 24-well plate containing PBS. The hydrogels were washed three times with PBS (30 min per wash) to remove residual SrtA and incubated at 37 °C for 16 h prior to degradation studies. After SrtA-mediated gelation, the initial masses (W₀) of the hydrogels were measured, followed by placing the hydrogels in buffer solution containing specified concentrations of: (1) SrtA with soluble glycinamide for transpeptidation-mediated degradation, or (2) SrtA alone for hydrolytic degradation. At specified time points, the hydrogels were removed from the degradation buffers, blotted dry, weighed (W₁), and returned to solution. This process was repeated until the hydrogels were fully degraded. For the hydrolytic degradation, fresh SrtA-containing buffer was exchanged after 24 h of incubation. The results were presented as: Mass loss (%) = 100% × (W₁ − W₀)/W₀.

2.5. Thiol-norbornene photopolymerization of SrtA-responsive hydrogels

For experiments related to tuning matrix stiffening and softening, hydrogels were fabricated by reacting PEG8NB and bis-cysteine containing peptides (e.g., KC-LPRTG-KC, KC-LPRTA-KC, or GGG-CGGGCGCG-LPRTG) via thiol-norbornene photoclick chemistry. Briefly, PEG8NB and peptides were mixed at a stoichiometric ratio of thiol to norbornene along with photoinitiator LAP (1 mM). The precursor solution with pre-defined compositions was injected into a 1 mL syringe with cut-open tip. Cell-laden gels were cast upon exposure to 365 nm light (5 mW/cm²) for 2 min and placed in a 24-well plate with fresh media. For the hMSC study, 3.5 wt% PEG-peptide gels were crosslinked with: (1) MMP-labile crosslinks (KCPQGCK, KCLPRTACK, or GGG-CGGGGC-LPRTG), and LAP (1 mM). The solution was mixed and pipetted into a 1 mL syringe with cut-open tip. Swollen hydrogels were incubated with solutions containing specified concentrations of SrtA and oligoglycine substrate (GGGGC, glycinamide) for 4 h on day 7 post-encapsulation. Gels were subsequently washed with fresh media for 4 h to remove SrtA and glycinamide. Live/dead staining and confocal microscopy imaging were performed on day 1, 7, and 14 post-encapsulation to assess cell viability and morphology. At least three z-stacked images per gel (10 slices, 100 μm thick) were taken. F-actin and DAPI staining and confocal imaging were performed on day 14 post-encapsulation for assessing cytoskeletal structure and cell morphology. Circularity and average cell area values were measured using ImageJ software and the F-actin/DAPI stained confocal images. Circularity is quantified as $4\pi (A_{\text{area}}) / (P_{\text{perimeter}})$ and the area was quantified using the “Analyze Particles” function in ImageJ.

2.6. Sortase A-mediated hydrogel softening & stiffening

Controlled softening of PEG-peptide hydrogels was achieved by incubating the swollen gels in buffer solution containing specified concentrations of SrtA and oligoglycine substrate (GGGGC, glycinamide, etc.). Storage moduli of the gels were measured before softening and at specified time points during, and after softening using oscillatory rheometry in strain sweep mode (8 mm parallel plate geometry, 0.1% to 5% strain at 1 Hz frequency).

Swollen hydrogels were incubated with solutions containing either SrtA or SrtA with soluble glycinamide to induce stiffening or softening, respectively. After each incubation step, SrtA or SrtA with soluble glycinamide buffers were replaced with phosphate-buffered saline solution (PBS, pH = 7.4) to remove residual SrtA. Storage moduli were measured prior to and after stiffening or softening. The gap sizes were reduced from 750 to 500 μm after softening due to observed volumetric shrinkage or swelling.

2.7. Cell culture and encapsulation

Human mesenchymal stem cells were maintained in low glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 ng/mL basic fibroblast growth factor (bFGF), and 1x-penicillin streptomycin. Briefly bFGF was supplemented in the media at 1 ng/mL as the concentration has been shown to maintain viability and proliferation of hMSCs during in vitro culture [59]. Media was refreshed every 2–3 days. PDAC cell line COLO-357 were maintained in high glucose DMEM supplemented with 10% FBS and 1x-penicillin streptomycin. For encapsulation, hMSCs and COLO-357 cells were rinsed with Dulbecco’s phosphate-buffered saline (DPBS), trypsinized, counted via hemocytometry, and resuspended at specified cell density in DPBS. The cells were mixed with sterile-filtered (0.2 μm) pre-polymer solutions containing PEG8NB, peptide crosslinkers (KCLPRTGCK, KCLPRTRACK, or GGG-CGGGGC-LPRTG), and LAP (1 mM). The solution was mixed and pipetted into a 1 mL syringe with cut-open tip. Swollen hydrogels were incubated with solutions containing specified concentrations of SrtA and oligoglycine substrate (GGGGC, glycinamide) for 4 h on day 7 post-encapsulation. Gels were subsequently washed with fresh media for 4 h to remove SrtA and glycinamide. Live/dead staining and confocal microscopy imaging were performed on day 1, 7, and 14 post-encapsulation to assess cell viability and morphology. At least three z-stacked images per gel (10 slices, 100 μm thick) were taken. F-actin and DAPI staining and confocal imaging were performed on day 14 post-encapsulation for assessing cytoskeletal structure and cell morphology. Circularity and average cell area values were measured using ImageJ software and the F-actin/DAPI stained confocal images. Circularity is quantified as $4\pi (A_{\text{area}}) / (P_{\text{perimeter}})$ and the area was quantified using the “Analyze Particles” function in ImageJ.

2.9. Reversible stiffening of COLO-357-laden hydrogels and related assays

COLO-357-laden hydrogels (2.5 wt% PEG8NB) were incubated in media for 7 days prior to matrix stiffening. Static controls include soft (2.5 wt% PEG8NB) and stiff (3.5 wt% PEG8NB) hydrogels that were not responsive to SrtA treatment. To induce matrix
stiffening, hydrogels were placed in media supplemented with 50 µM of SrtA and incubated for 4 h. On day 14 post-encapsulation, the hydrogels were placed in media with 50 µM of SrtA and 15 mM of soluble glycine substrate to induce softening. Live/dead staining and confocal microscopy imaging were performed on day 1, 7, 14, and 21 to assess cell viability and spheroid morphology. The spheroid diameters were measured using ImageJ software.

For the gemicitabine treatment study, COLO-357 cells were allowed to grow into spheroids for 4 days prior to SrtA-induced stiffening (50 µM, 4 h). On day 7, hydrogels were treated with SrtA (50 µM) and glycinate (15 mM) to induce softening. On day 10, these ‘softened’ cell-laden hydrogels were treated with gemicitabine (1 µM) for 3 additional days prior to live/dead staining and imaging on day 13. Control groups include cell-laden hydrogels that only went through stiffening (i.e., Stiffened group), non-dynamic soft (i.e., Soft group, G’ ~ 1 kPa), and non-dynamic stiff (i.e., Stiff group, G’ ~ 3 kPa). Metabolic activity was assessed via AlamarBlue reagent before and after gemicitabine treatment. Viability and spheroid morphology were monitored via live/dead staining and confocal imaging as described above.

2.10. Statistical analysis

A two-way analysis of variance (ANOVA) with a Bonferroni post-hoc test to evaluate significance between experimental groups. For spheroid diameter experiments, two-way ANOVA was performed on the average diameter between three values from independent experiments with three time points (i.e., day 7, day 14, and day 21). Single, double, and triple asterisks represent p < 0.05, 0.01, 0.001, respectively. All experiments were completed independently thrice. Quantitative results are presented as mean ± SEM.

3. Results

3.1. SrtA-mediated crosslinking of PEG-peptide hydrogels

In order to exploit SrtA-initiated transpeptidation (Fig. 1A) for crosslinking of PEG-peptide hydrogels, we conjugated PEG8NB macromers with cysteine-bearing SrtA substrates (i.e., NH2-GGGGC-NH2 and NH2-CLPRG-NH2) via thiol-norbornene photoclick chemistry. Notably, the substitution of the peptide conjugate was approximately 70% as measured by TNBSA assay. In addition to the inherent limitation on peptide conjugation efficiency, it is also possible that some peptides were ‘damaged’ during the conjugation process. However, we showed that the resulting PEG-peptide conjugates were still functional and could be used to crosslink hydrogels using SrtA-initiated transpeptidation (Fig. 1B). At a fixed total PEG-peptide macromer content (6 wt%), gelation speed could be independently tuned from 10 min with 300 µM SrtA to 90 min with 50 µM SrtA (Fig. 1C).

3.2. SrtA-mediated degradation of SrtA-crosslinked PEG-peptide hydrogels

The reversibility of SrtA-mediated transpeptidation affords control not only in hydrogel crosslinking, but also in temporal degradation of PEG-peptide hydrogels. Specifically, hydrogels were degraded on-demand through addition of SrtA and soluble glycine substrates (e.g., glycinate), which induce transpeptidation-based degradation (Fig. 1A). As concentrations of SrtA or glycinate increased, gel degradation occurred significantly faster (Fig. 2A and B), as supported by the initial swelling (i.e., negative loss of mass) and then rapid mass loss of the gels. Complete degradation was achieved in approximately 5, 7, and 9 h using 50, 25, and 10 µM of SrtA, respectively. At a fixed SrtA concentration (e.g., 25 µM), hydrogels treated with 16 or 48 mM of glycinate (i.e., 1- and 3-fold to crosslinker concentration in hydrogels) degraded completely in 7 or 5 h, respectively.

Incubation of hydrogels with SrtA alone permits slow hydrolytic degradation as water can react with the thioacyl intermediate to yield LPRT-OH (Fig. 1A) [52]. Compared with transpeptidation-mediated degradation, hydrolytic degradation occurs more slowly and has a nearly linear mass loss profiles (Fig. 2C). The hydrogels degraded in approximately 50 to 58 h when using only 50 to 25 µM of SrtA (i.e., without glycinate), respectively.

3.3. SrtA-mediated softening of thiol-norbornene PEG-peptide hydrogels

SrtA-initiated transpeptidation provides a high degree of control in crosslinking PEG-peptide conjugates into hydrogels. However, it is challenging to use this enzymatic reaction and hydrogel design to achieve controlled gel softening owing to the erosion of gel surface and mass loss upon encountering the enzyme. We hypothesized that a different PEG-peptide hydrogel architecture could prevent gel mass loss while permitting SrtA-mediated on-demand softening. To achieve this, we synthesized bis-cysteine peptides sensitive (KCLPRTGCK, “G” peptide) or insensitive (KCLPRTACK, “A” peptide) to SrtA-mediated transpeptidation. Cysteine residues were added to afford thiol-norbornene photo-click gelation between macromer PEG8NB with “G” and/or “A” peptides, whereas lysine residues were added to improve the solubility of the peptides. Upon incubation in a solution containing SrtA and an oligoglycine substrate, only SrtA-sensitive linkages (i.e., G peptide) underwent cleavage (Fig. 3A). Varying the concentrations of A and G crosslinkers within 3 wt% PEG8NB-peptide hydrogels resulted in tunable degrees of SrtA-induced gel softening (Fig. 3B). Specifically, A:G ratios of 100%:0%, 75%:25%, and 50%:50% respectively yielded 0.15, 0.75, or 1 kPa, respectively and 0.75-fold moduli decrease compared with the initial modulus (G’0). Next, we investigated the role of SrtA concentration on softening time (3 wt% PEG, A:G ratio = 50%:50%). Above 25 µM SrtA, there was no notable difference in softening time, but hydrogels incubated with only 10 µM SrtA were degraded in a slower rate (Fig. 3C). Furthermore, hydrogels formed with 2.5, 3, or 3.5 wt% of PEG showed increasing initial moduli (~0.75, ~2.5 or ~3 kPa, respectively), which were softened upon SrtA treatment to 0.15, 0.75, or 1 kPa, respectively (Fig. 3D). In addition, hydrogels incubated in SrtA/oligoglycine substrate solution for 0.5, 2.5, and 4 h softened to approximately 0.75, 0.5, and 0.2-fold of the initial modulus (Fig. 3E).

While hydrogels crosslinked with SrtA-sensitive peptide (i.e., LPRTG) permitted controlled softening, attempts to re-stiffen these softened gels yielded disappointing results. Specifically, the hydrogels could only be re-stiffened to about 20% of the original gel modulus (data not shown). This was likely due to the difficulty for the infused SrtA to ‘reach’ the ‘broken’ peptides simultaneously in a highly swollen hydrogel.

3.4. SrtA-mediated softening of hMSC-laden PEG-peptide hydrogels

The SrtA-induced softening hydrogels were used as a cell culture platform to evaluate the effect of temporal matrix softening on hMSCs spreading. In this study, hMSCs were encapsulated in hydrogels formed by 3.25 wt% PEG8NB with MMP-labile crosslinks (KCGPOQ/IWGQCK, for cell-mediated local degradation) and ‘G’ peptide linkage for SrtA-mediated softening (‘A’ peptide was used as control). Note that all cell-laden hydrogels also contained 1 mM CRGDS for promoting cell adhesion. Furthermore, peptide crosslinkers were mixed at 40% SrtA-responsive ‘G’ peptide (or SrtA
Fig. 2. Effect of (A) SrtA and (B) glycaminde concentration on the transpeptidation degradation of PEG-peptide hydrogels (6 wt% PEG-peptide, R_{GGGG:LPRTG} = 2 with 48 mM glycaminde and 50 μM SrtA for (A) and (B), respectively. (C) Effect of SrtA concentration on the hydrolytic degradation of PEG-peptide hydrogels (6 wt%, R_{GGGG:LPRTG} = 2).

Fig. 3. (A) Schematic of SrtA-mediated peptide cleavage. (B) Effect of alanine:glycine composition on softening of PEG8NB-A-G gels (3.0 wt% PEGNB, 25 μM SrtA, & 12 mM GGGGC). (C) Effect of SrtA concentration on softening of PEG8NB-A-G gels (50%A:50%G, 3.0 wt%, 12 mM GGGGC). (D) Effect of initial PEG8NB concentration on softening of PEG8NB-A-G gels (50%A:50%G, 25 μM SrtA, & 12 mM GGGGC). (E) Effect of treatment time on softening (50%A:50%G, 3.0 wt%, 12 mM, 25 μM SrtA, and 12 mM GGGGC).
insensitive ‘A’ peptide) and 60% MMP-sensitive peptide linker. Live/dead and F-actin/DAPI staining results showed that cells in both groups displayed mostly non-spreading morphology prior to SrtA-induced softening on day 7 (Fig. 4A). Note that a higher initial gel stiffness \( \left( G_0 \sim 4 \text{ kPa} \right) \) was used to demonstrate the effect of SrtA-mediated on-demand gel softening on cell fate processes. The high initial gel stiffness may be the reason causing noticeable number of dead cells on Day 1. By day 14, cells within the on-demand softened hydrogels adopted long and thin protrusions compared with cells encapsulated within statically stiff hydrogels. Semi-quantitative analysis of cell spreading revealed a significant reduction in circularity (Fig. 4B) and increase in cell area (Fig. 4C) in cells experienced on-demand softening.

3.5. Reversible stiffening of PEG-peptide hydrogels

Owing to its unique reversibility in peptide conjugation, we hypothesized that SrtA can be used to create hydrogels with dynamically and reversibly tunable stiffness. We designed a linear peptide flanked with the two SrtA substrates (GGG-CGGGC-LPRTG) for crosslinking into hydrogels via thiol-norbornene photopolymerization. The N-terminal GGG and C-terminal LPRTG sequences became pendant motifs permitting SrtA-mediated secondary crosslinking and degradation, thus leading to cyclic/reversible hydrogel stiffening and softening (Fig. 5A). To optimize SrtA-mediated matrix stiffening, PEG-peptide hydrogels (2.5 wt%) were incubated with varying time and concentrations of SrtA. A 1.75- to 2.5-fold increase in gel modulus \( \left( G' \sim 1 \text{ kPa} \right) \) were observed upon 3 h of incubation with 25 \( \mu \text{M} \) of SrtA (Fig. 5B). Furthermore, 2- to 4-fold increases in moduli were observed when 10 to 50 \( \mu \text{M} \) of SrtA were added for 4 h (Fig. 5C).

The stiffened hydrogels could be further softened by incubating the hydrogels with SrtA alone or with SrtA and soluble glycine substrate. As shown in Fig. 5D, moduli of the stiffened gels \( \left( G' \sim 3 \text{ kPa} \right) \) were decreased 0.8 to 0.5-fold with increasing concentrations of glycinamide, where maximum softening was achieved with 15 mM of soluble glycinamide and 25 \( \mu \text{M} \) of SrtA. SrtA-mediated hydrolytic degradation could also be utilized to soften hydrogels at a slower rate (Fig. 5E). For example, hydrogels incubated with SrtA alone (25 \( \mu \text{M} \)) were softened to approximately 0.4-fold of the initial gel modulus. Finally, multiple cycles of reversible gel stiffening/softening could be achieved by incubating hydrogels alternatively with SrtA (for stiffening) and SrtA and soluble glycineamide (for softening) (Fig. 6).

3.6. Effect of stiffening and softening on COLO-357 viability and spheroid formation

To demonstrate the feasibility of using SrtA-mediated reversible stiffening hydrogels as a platform for investigating cell fate pro-

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Fig. 4. (A) Representative confocal images of encapsulated hMSCs in statically stiff and softened hydrogels. At least three z-stacked images per gel (10 slices, 100 \( \mu \text{m} \) thick) were taken. (Scale: 200 \( \mu \text{m} \)). (B) Circularity and (C) average cell area measurements of hMSCs in the statically stiff and softened hydrogels on day 14 post-encapsulation.
cesses, COLO-357 cells were encapsulated in SrtA-sensitive hydrogels and exposed to stiffening and softening conditions. Cell-laden soft (2.5 wt% PEG8NB, G₀/C₂₄ 1 kPa) and stiff (3.5 wt% PEG8NB, G₀/C₂₄ 3 kPa) hydrogels were utilized as controls (i.e., without dynamic stiffening). Dynamically-stiffened PEG-peptide hydrogels (2.5 wt% PEG8NB) formed with pendant SrtA substrates (i.e., GGG-CGGGC-LPRTG) were stiffened on day 7 and softened on day 14 (Fig. 7A). Per the confocal images, both the initial thiol-norbornene gel crosslinking and SrtA-mediated stiffening/softening were cytocompatible as indicated by the high cell viability (Fig. 7B). We quantified diameters of the cell spheroids and found that when cultured in ‘statically soft’ hydrogels, the spheroid sizes grew steadily from 40 μm to 51 μm over 21 days (Fig. 7C, Table 1). On the other hand, the growth was significantly hindered in cells maintained in ‘statically stiff’ hydrogels (Fig. 7D, Table 1). In the dynamic hydrogel group (i.e., Soft-Stiff-Soft), the growth of spheroids was initially delayed following stiffening (from D7 to D14). Subsequent softening of the hydrogels allowed the cell spheroids to grow again (Fig. 7E, Table 1). The patterns of spheroid size changes in the ‘Soft-Stiff-Soft’ hydrogels demonstrate the effect of dynamic matrix stiffening/softening on PCC growth.

3.7. Effect of matrix stiffening on the chemoresistance of encapsulated COLO-357 cells

Fig. 8 shows the effect of cyclic matrix stiffening/softening on growth of COLO-357 cells under gemcitabine treatment (Fig. 8A). Live/dead staining results show that gemcitabine treatment only caused significant cell death in non-dynamic soft hydrogels.
In the dynamic ‘stiffened’ group, which mimics the effect of drug treatment in a stiffened stromal tissue, gemcitabine treatment caused limited amount of cell death (Fig. 8D). Finally, in the dynamic ‘softened’ group, which mimics the effect of softening a stiffened stromal tissue, gemcitabine treatment only led to some cell death (Fig. 8E) when compared with that in the non-dynamic soft hydrogels (Fig. 8B). AlamarBlue assay results mirrored the results in the live/dead staining images where only cells encapsulated in soft or softened hydrogels exhibited reduced metabolic activity upon gemcitabine addition, whereas cells in the stiffen or stiffened hydrogels were non-responsive to gemcitabine treatment (Fig. 8F).

Fig. 7. (A) Timeline of alternate stiffening and softening of COLO-357-laden hydrogels. Hydrogels were stiffened on day 7 and softened on day 14. Time of confocal imaging is indicated by the open arrows. All imaging was completed prior to enzyme treatments. (B) Representative confocal images of encapsulated COLO-357 cells in statically soft, stiff, and reversibly stiffened hydrogels. At least three z-stacked images per gel (10 slices, 100 μm thick) were taken. (Scale: 200 μm). Histogram of spheroids diameters for (C) non-dynamic soft, (D) non-dynamic stiff, and (E) reversibly stiffened and softened hydrogels (i.e., soft-stiff-soft).
4. Discussion

Investigating the role of biophysical and biochemical cues on cell fate processes is highly challenging owing to the complex, heterogeneous, and dynamically changing nature of the ECM. Commercially available 3D culture platforms such as Matrigel afford convenience, but lack well-defined or tunable properties. Additionally, static 3D hydrogels do not provide spatiotemporal control of matrix properties critical for guiding cell fate processes. Accumulating evidence suggests that matrix stiffness plays an integral role in cell mechanobiology. Therefore, designing ‘four-dimensional’ cell culture platforms are necessary for studying these phenomena. Our lab has utilized enzymatic strategies to dynamically control hydrogel crosslinking [23,35,36]. We have also utilized SrtA-mediated transpeptidation to facilitate crosslinking of PEG-peptide hydrogels [36]. While previous work has demonstrated the utility of SrtA in hydrogel crosslinking, degradation, and ligand immobilization [36,53,54,60,61], this unique reversible enzymatic reaction has not been explored for dynamically and reversibly modulating hydrogel crosslinking density.

In this work, thiol-norbornene photoclick chemistry was used to prepare the PEG-peptide conjugates needed for investigating SrtA-induced hydrogel crosslinking (Fig. 1). A functional group substitution of ~70% was obtained for the PEG-GGGG conjugate. This could be a result of peptide damage caused by extended UV irradiation and/or simply an inherent limitation in conjugation efficiency. If desired, these PEG-peptide conjugates may also be prepared using Michael-type addition between cysteine-bearing peptides and PEG-vinylsulfone or PEG-maleimide. Nevertheless, we were able to use these PEG-peptide conjugates to form hydrogels with tunable gelation time (from minutes to hours) by simply adjusting enzyme concentration in the precursor solution. A relatively high concentrations of SrtA were required for rapid crosslinking of hydrogels in this study compared with previous results [53]. While this may be caused by lower substitution (~70%) of the PEG-peptide substrate, macromer functionality (e.g., 8 in this study, compared with tens to hundreds in modified hyaluronic acid) may also be the reason of a reduced crosslinking efficiency. In addition to controlling network crosslinking, the reversible SrtA-mediated transpeptidation was also exploited to

Table 1
Summary of average spheroid diameters in Fig. 7.

<table>
<thead>
<tr>
<th>Day</th>
<th>Soft (μm)</th>
<th>Soft-Stiff-Soft (μm)</th>
<th>Stiff (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>40.08 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.76 ± 0.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.07 ± 0.84</td>
</tr>
<tr>
<td>14</td>
<td>48.45 ± 3.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.60 ± 1.84&lt;sup&gt;d&lt;/sup&gt;</td>
<td>33.85 ± 0.35</td>
</tr>
<tr>
<td>21</td>
<td>51.36 ± 2.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.89 ± 1.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.41 ± 0.51</td>
</tr>
</tbody>
</table>

Note: Superscripts “a” and “d” represent p < 0.05 and p < 0.01, respectively. Subscripts “b” and “c” denote p < 0.01.

Fig. 8. (A) Timeline of gel stiffening (at D4) and softening (at D7), as well as three-day gemcitabine treatment (D10-D13). All imaging was completed prior to enzyme or drug treatments. (B-E) Representative live/dead images of cell-laden non-dynamic soft (B), non-dynamic stiff (C), stiffened (D), and softened (E) hydrogels with and without gemcitabine treatment. At least three z-stacked images per gel (10 slices, 100 μm thick) were taken. (Scale: 200 μm). (F) Metabolic activity of encapsulated cells pre- (at D10) and post-gemcitabine treatment (at D13). Data represent Mean ± SEM (n = 3, *p < 0.05, **p < 0.01).
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