Dynamic PEG–Peptide Hydrogels via Visible Light and FMN-Induced Tyrosine Dimerization

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1. Introduction

Hydrogels with tunable physicochemical properties have been extensively utilized as 3D cell culture platforms.\(^1\) To mimic a cellular microenvironment, hydrogels are commonly immobilized with biochemical motifs, including cell adhesive peptides (e.g., fibronectin-derived peptide sequence, RGD) or proteoglycans derived from extracellular matrix (ECM) (e.g., heparin sulfate proteoglycan, hyaluronan, etc.).\(^2\) These signaling motifs are crucial for guiding complex cell–matrix interactions, as well as for promoting tissue morphogenesis within the otherwise inert matrix. On the other hand, mechanical properties of native tissue play an indispensable role in stem cell differentiation and cancer progression.\(^3–8\) In order to investigate the effect of matrix mechanics on cell fate processes in 3D, cells are typically encapsulated in gels with varying but static or degrading mechanical property. However, recent studies have revealed the critical influence of spatial-temporal (i.e., dynamic) matrix stiffening in tissue mechanics on cell behaviors.\(^7\) Unfortunately, conventional cell culture devices cannot be readily adapted to study the influence of dynamic matrix properties on cell fate processes.

Photoresponsive materials are ideal candidates for designing dynamic cell-laden hydrogels. The most common approach is to perform cell encapsulation using photoresponsive materials that are compatible with secondary crosslinking. For example, Burdick and co-workers prepared photoresponsive hydrogel from acrylated hyaluronic acid (AHA) and bis-cysteine-bearing matrix metalloprotease (MMP) sensitive peptides.\(^9,10\) The hydrogel was partially crosslinked through Michael-type addition between acrylate and thiol motifs, leaving additional acrylates available for photoinitiator (I-2959) and UV-light-mediated in situ gel stiffening. In another example, Fiedler et al. and Mabry et al. designed dynamic hydrogels through repeated photopolymerization of cell-laden hydrogels.\(^11,12\) Photopolymerized hydrogels were submerged in buffer solution containing additional PEG macromers and photoinitiator (lithium phenyl-2,4,6-trimethylbenzoylphosphinate, LAP). The macromer/initiator imbibing gels were polymerized again under UV light exposure to create “double network,” which exhibited higher degree of crosslinking and gel stiffness. These gels could also be regionally patterned with bioactive peptides through utilizing a photomask.\(^11\) While the use of I-2959 or LAP enables gel crosslinking and stiffening, these cleavage-type photoinitiators generate highly reactive radicals upon absorbing light at the UV spectrum (e.g., 365 nm).\(^13\) LAP does absorb light at the visible light wavelength (405 nm);\(^14\) however, high concentration (e.g., \(\approx 6 \times 10^{-3} \text{m}\)) is generally required to achieve significant degree of additional crosslinking owing to its low molar absorbability at the visible light wavelengths.\(^15\)
Noncleavage type (Type II) photoinitiators or photosensitizers, such as eosin-Y (EY),\textsuperscript{16,17} rose bengal (RB),\textsuperscript{18} riboflavin (RF),\textsuperscript{19} and flavin mononucleotide (FMN)\textsuperscript{20} can also be used to initiate polymerization under visible light (400–700 nm) exposure.\textsuperscript{21} A particularly attractive type II photoinitiator is RF (i.e., vitamin B2) and its natural derivatives, including flavin adenine dinucleotide (FAD) and FMN. Flavins are natural photosensitizers that are commonly used as food additives and have high molar absorbability at the UVA (370 nm) and visible light spectra (440 and 480 nm).\textsuperscript{22} FMN has also been used to crosslink copolymers with tyrosinamide side chains, a mechanism similar to the Tyr-Tyr (di-tyrosine or bi-tyrosine) crosslinks found in some proteins.\textsuperscript{20} Di-tyrosine crosslinking is also an important method for hydrogel crosslinking, including using flavins as the photoinitiators.\textsuperscript{23–26} Finally, compared to RF, FMN has much higher solubility in water (92 mg mL\textsuperscript{-1} for FMN vs 0.045 mg mL\textsuperscript{-1} for RF), making it an ideal choice for preparing photoresponsive hydrogels.\textsuperscript{27}

Current photoresponsive dynamic hydrogels have been successfully used to answer important biological questions, such as the effect of changing matrix mechanics on activation of human mesenchymal stem cells,\textsuperscript{28,29} hepatic stellate cells,\textsuperscript{4,15} and valvular interstitial cells.\textsuperscript{12} However, the preparation of these dynamic hydrogels usually requires multiple steps of chemical synthesis and purification that may not be friendly to many biomedical laboratories. Here, we introduced a simple photoresponsive dynamic PEG–peptide hydrogel system via visible light and FMN-initiated tyrosine dimerization if the peptide crosslinkers contained at least one tyrosine residue. We fabricated the primary hydrogel using PEG8NB (Figure 1A) and bis-cysteine peptide linkers (e.g., CYGGGYC, Figure 1B) via LAP initiated thiol–norbornene photopolymerization.\textsuperscript{30} Following the initial thiol–norbornene gel crosslinking, soluble FMN was added in the hydrogel via diffusion to permit visible light-induced tyrosine dimerization, which led to on-demand hydrogel stiffening. The degree of stiffening could be readily tuned through adjusting dosage of visible light exposure and FMN concentration, as well as the amount of tyrosine residues built-in in the sequence of the peptide crosslinkers. In addition to investigating the conditions and parameters for dynamic gel crosslinking, we also demonstrate spatiotemporally tunable stiffening scenarios and post-gelation conjugation/patterning of biochemical motifs. Finally, we evaluated in vitro cytocompatibility of the visible light-mediated stiffening process and utilized this platform to study the impact of matrix stiffening on responses of pancreatic cancer cells (PCCs) and cancer-associated fibroblasts (CAFs). Since all components used in this study (i.e., PEG macromers, peptides, LAP, FMN) as well as the visible light source (a cold halogen microscope light) can be acquired commercially, this dynamic hydrogel system should be beneficial to many biomedical laboratories interested in studying cell biology in a dynamically tunable microenvironment.

2. Results and Discussion

2.1. Dynamic Hydrogel Stiffening via Visible Light and FMN-Initiated Tyrosine Dimerization

FMN has been recently used as a visible light photoinitiator in thiol–ene polymerization of PEG-based hydrogels.\textsuperscript{27} In the current contribution, we discovered that visible light and FMN-initiated photochemistry could be used as a cytocompatible mechanism for tuning crosslinking density of PEG–peptide hydrogels if the peptide crosslinkers contained at least one tyrosine residue. We fabricated the primary hydrogel using PEG8NB (Figure 1A) and bis-cysteine peptide linkers (e.g., CYGGGYC, Figure 1B) via LAP initiated thiol–norbornene photopolymerization.\textsuperscript{30} In principle, tyrosine residues on the peptide crosslinker were susceptible to visible light and FMN-mediated dimerization, leading to increased crosslinking density of the PEG–peptide hydrogel (Figure 1C). Following
thiol–norbornene photopolymerization, soft hydrogels ($G' = 1–1.2$ kPa) were incubated in buffer solution containing $1 \times 10^{-3}$ m FMN for 30 min to allow its equilibration in the gel. We determined that 30 min incubation was sufficient for FMN (MW: 456.3 g mol$^{-1}$; $D_0 = 4.8 \times 10^{-10}$ m$^2$ s$^{-1}$)[31] to reach equilibrium in the hydrogel based on a model developed previously.[5] First, we fabricated hydrogels with different crosslinking densities and measured their respective swelling ratios ($Q$, Figure S1A, Supporting Information) and water content (Figure S1B, Supporting Information). The data were used to calculate hydrogel mesh sizes ($\xi$, Figure S1C, Supporting Information)[32] for deriving FMN diffusivity in hydrogels using the Lustig–Peppas relationship.[33] For example, it was determined that for stiff hydrogels with shear moduli of 5 kPa, FMN diffusivity was reduced to $4.54 \times 10^{-10}$ m$^2$ s$^{-1}$, or 95% of $D_0$. The small reduction of FMN diffusivity in stiff hydrogel was reasonable due to its small molecular size. We utilized this diffusivity to estimate the time needed for FMN to reach 99% of that in the solution. As shown in Figure S1D (Supporting Information), 30 min of incubation was sufficient for the center of the gel to reach $C_{FMN}/C_0 = 0.99$. Experimentally, hydrogel color changed from transparent to bright yellow (color of FMN) after 30 min incubation in $1 \times 10^{-3}$ m FMN solution (Figure 2A). After visible-light exposure for 2 min, the gels were transferred to phosphate buffered saline (PBS) for 30 min to allow for the removal of residual soluble FMN. At which time, the gel returned to transparent, suggesting that FMN was not retained in the post-stiffened hydrogels. The initially soft ($G' = 1$ kPa) hydrogels were effectively stiffened to $\approx 2.3$ kPa after the visible light and FMN-initiated stiffening process (Figure S2, Supporting Information) and the stiffened hydrogels remained elastic as demonstrated by the strain-independent moduli. It is worth noting that, regardless of shear moduli (i.e., 1 or 2.3 kPa), all hydrogels (even the stiffened ones) described in this study exhibited high water contents (above 95.8%, Figure S1B, Supporting Information) owing to the highly swollen nature of these hydrogels. Furthermore, all components used in this study were commercially available, including the linear peptides crosslinkers that can be designed to exhibit other functionality (e.g., protease sensitive sequence). While thiol–norbornene photopolymerization was used here to prepare the primary PEG–peptide hydrogel network, this stiffening strategy can be applied to other step-growth hydrogels, such as those prepared by multiarm PEG–vinylsulfone/PEG–maleimide via Michael-type addition, or by other commercially available click-based macromers. After establishing the feasibility of FMN-induced photostiffening, we adjusted several parameters to identify a range of conditions suitable for inducing stiffening to a pathophysiological relevant degree ($G' = 4–6$ kPa).[34,35] When the gels were incubated in buffer containing $0.1 \times 10^{-3}$ m FMN, no significant stiffening was observed (Figure 2B) under $3$ mW cm$^{-2}$ of visible light (440 nm) exposure for 2 min. When FMN concentration was increased to $0.5 \times 10^{-3}$ m, the same light exposure led to almost two-fold of stiffening (i.e., $G'$ from $\approx 1.2$ to $\approx 2.3$ kPa, Figure 2B). Further increasing FMN concentration to $1 \times 10^{-3}$ m did not improve the degree of stiffening. Next, we prepared gels by PEG8NB and peptide crosslinker with 0, 1, 2, or 3 tyrosine residues (i.e., CGGGC, CGGYGGC, CYGGYGC, and KCYGGGYGYYCK). Under a stoichiometric ratio of thiol to norbornene, the use of these peptides led to different amount of tyrosine residues immobilized in the hydrogels (i.e., 0, 5, 10, 15 $\times 10^{-3}$ m, respectively). As expected, gels crosslinked by peptide without tyrosine (i.e., CCGGC) were not susceptible to FMN and visible light-induced stiffening ($0 \times 10^{-3}$ m group in Figure 2C). On the other hand, gels crosslinked with increasing tyrosine residues (i.e., $5 \times 10^{-3}$ m CGGYGGC, $10 \times 10^{-3}$ m CYGGYGC, or $15 \times 10^{-3}$ m KCYGGGYGYYCK) exhibited increased gel moduli (from 1 kPa to 1.9, 2.2, and 2.5 kPa, respectively, Figure 2C). In principle, it is possible to further increase the degree of stiffening by inserting more Tyr residues in the peptide crosslinkers.

Figure 2. Effect of FMN and tyrosine residues on hydrogel stiffening. A) Images of soft hydrogel (2.5 wt% PEG8NB, $5 \times 10^{-3}$ m CYGGYGC) formed by thiol–norbornene photopolymerization (left), gel after incubation with $1 \times 10^{-3}$ m FMN for 30 min (middle), and gel after stiffening and additional incubation in PBS for 30 min to remove soluble FMN (right). B) Shear moduli of hydrogels before and after FMN and visible light-mediated stiffening at different FMN concentrations. C) Shear moduli of hydrogels formed with different contents of tyrosine residues in the peptide crosslinker (i.e., 0Y, 1Y, 2Y, and 3Y peptides). Data were presented as mean ± SEM ($n = 3$). P-values were calculated using one-way ANOVA with Bonferroni correction (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).
2.2. Investigating Mechanism of FMN and Visible Light-Mediated Di-Tyrosine Crosslinking

Different from cleavage-type photoinitiators that generate radicals via photolysis, polymerization initiated by non-cleavage type photoinitiators was based on electron transfer (Figure 3A).[35] In the case of FMN, its ground state is excited to short half-life singlet state (\(1^{1}\text{FMN}^*\), 5 ns) upon visible light exposure, and then to triplet state (\(1^{3}\text{FMN}^*\), \(t_{1/2} = 15 \mu s\)) with longer half-life through intersystem crossing (ITC).[36,37] Triplet state FMN (\(1^{3}\text{FMN}^*\)) is a strong oxidant capable of generating tyrosyl radical from tyrosine in Type 1 reaction (Figure 3A).[37] The redox state of \(1^{3}\text{FMN}^*\), flavin-hydroquinone (FMNH\(_2\)) could further react with dissolved O\(_2\), regenerating FMN and producing hydrogen peroxide (H\(_2\)O\(_2\)). On the other hand, in Type 2 reaction, \(1^{3}\text{FMN}^*\) generates tyrosyl radical by means of singlet oxygen (\(1^{1}\text{O}_2\)) excitation without producing hydrogen peroxide (Figure 3A). We reasoned that gel stiffening observed in Figure 2 was a result of FMN-initiated tyrosyl radicals combination, leading to di-tyrosine crosslinking. We monitored contents of dissolved O\(_2\) and reactive by-product H\(_2\)O\(_2\) during the photoreaction. After 2 min of visible light exposure in the presence of \(15 \times 10^{-3}\) M soluble tyrosine, dissolved O\(_2\) decreased from 20 to 18% (Figure 3B). No oxygen reduction was detected in the absence of tyrosine. Under the same light irradiation conditions, H\(_2\)O\(_2\) concentration increased from 0 to \(0.16 \times 10^{-3}\) M in the presence of \(15 \times 10^{-3}\) M soluble tyrosine (Figure 3C). Note that even in the absence of tyrosine, FMN and visible light-mediated photooxidation still produced H\(_2\)O\(_2\), albeit in lower amounts. The O\(_2\) consumption and H\(_2\)O\(_2\) production profiles were indicative of Type 1 reaction mechanism. Additional experiments revealed that, in the presence of soluble tyrosine, FMN and visible light exposure led to significant increase of sample absorbance at 314 nm, which was indicative of di-tyrosine formation (Figure 3D). These results were consistent with literature regarding the effect of visible light and FMN on tyrosine.[38]

2.3. Cytotoxicity of FMN and Visible-Light Mediated Di-Tyrosine Crosslinking

Prior to applying the FMN-mediated reaction to stiffening cell-laden hydrogels, we evaluated cytotoxicity of this photochemistry using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Specifically, COLO-357 cells, a PCC line, were incubated with soluble tyrosine (\(15 \times 10^{-3}\) M) in 2D well plate to test effect of the additional tyrosines on cell viability (Figure S3A, Supporting Information). Additionally, cytotoxicity of FMN and visible light-mediated reaction was also evaluated by incubating cells with \(15 \times 10^{-3}\) M tyrosine and \(1 \times 10^{-3}\) M FMN, followed by exposure of visible light with different duration (i.e., 1–5 min) at 3 mW cm\(^{-2}\) (Figure S3B, Supporting Information) or with various intensities (i.e., 1.5, 3, 6 mW cm\(^{-2}\)) for 2 min (Figure S3B, Supporting Information). Results indicated that cell viability was above 90% for all conditions, suggesting that the parameters used in this study did not cause noticeable cell death. This is not surprising as under these conditions, we only expect a slight elevation of H\(_2\)O\(_2\) production (e.g., \(\approx 0.15 \times 10^{-3}\) M after 2 min light exposure. Figure 3C) and a mild reduction in dissolved oxygen (e.g., \(\approx 2\%\) reduction after 2 min light exposure, Figure 3B). Combining the results of dynamic hydrogel stiffening (Figure 2) and cytotoxicity of FMN photochemistry (Figure S3, Supporting Information), we have identified a range of reaction conditions suitable for future cell studies (see Sections 2.4 and 2.5).

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**Figure 3.** Investigation of FMN and visible light-induced photochemistry. A) Potential mechanisms of FMN-induced tyrosyl radical formation. B) Oxygen consumption in the absence or presence of \(15 \times 10^{-3}\) M tyrosine (Tyr). C) Hydrogen peroxide (H\(_2\)O\(_2\)) production in the absence or presence of \(15 \times 10^{-3}\) M Tyr. D) Solution absorbance at 314 nm as a function of light exposure time. All photoreactions were conducted in the presence of \(1 \times 10^{-3}\) M FMN with visible light exposure (440 nm, 3 mW cm\(^{-2}\)). Data were presented as mean ± SEM (n = 3).
2.4. Physicochemical Patterning of Hydrogel Network

We next demonstrated that the degree of dynamic stiffening could be modulated by controlling the duration and intensity of light exposure without changing formulation of gel precursors or concentration of FMN. Specifically, PEG8NB–peptide hydrogels initially crosslinked with shear moduli of 1 kPa were incubated with $1 \times 10^{-3}$ M FMN for 30 min, followed by visible light (3 mW cm$^{-2}$) irradiation for 0.5–7 min or with intensity from 0.6 to 3 mW cm$^{-2}$ (for 3 min). Bulk gel moduli were characterized before and after the secondary visible light treatment. As shown in Figure 4A, longer duration of light exposure led to higher magnitude of gel stiffening ($\approx$ 1.7–2.5-fold). Similar degrees of stiffening were achieved via adjusting light intensity (Figure 4B). In addition to changes in matrix mechanics, spatially distributed biochemical cues in ECM also regulate cell behavior. To exploit the potential of FMN-mediated photochemistry on presenting biochemical cues spatiotemporally, we designed a proof-of-concept experiment where controlled amounts of 5(6)-carboxy-X-rhodamine-tyramide (5(6)-ROX-tyramide) were patterned as strips in hydrogels via tuning the duration of light exposure through a photomask (Figure 4C). The patterned hydrogels were imaged by confocal microscopy. As shown in Figure 4C, higher 5(6)-ROX fluorescence was detected in strips with longer visible light exposure. By analyzing fluorescent intensity semiquantitatively with Image J (Figure 4D), we showed that the 5(6)-ROX patterned gels exhibited regions of higher intensities correlated proportionally to visible light dosage (i.e., 3 and 5 min). Similar to the patterning of 5(6)-ROX-tyramide, future work may focus on visible light and FMN-based immobilization or patterning of tyrosine-containing peptides/proteins for providing desired biochemical signaling to the cells.

2.5. Effect of Dynamic Matrix Stiffening on PCC Behavior

To study the influence of the temporal matrix stiffening on cell fate processes, we evaluated formation of multicell spheroids
and expression of selected genes at the mRNA level in COLO-357 cells grown in modularly crosslinked and dynamically stiffened hydrogels. We first prepared two groups of cell-laden gels (i.e., PEG8NB crosslinked by CGGCC or KYGGYGYCK peptide) with similar initial stiffness \( (G’ = 1 \text{ kPa}) \). All cell-laden gels were treated with soluble FMN \( (1 \times 10^{-3} \text{ M}) \) and visible light \( (3 \text{ mW cm}^{-2}) \) for 3 min 1 day post-encapsulation. Note that only gels crosslinked by tyrosine-containing peptide could be dynamically stiffened and the stiffened gels exhibited decreased swelling compared to the nonstiffened and soft gel (Figure S4, Supporting Information). However, these gels still exhibited high water content \( (=95.8\%, \text{ Figure S1C, Supporting Information}) \) owing to the highly swollen nature of the PEG-based hydrogels. After two weeks of culture, the encapsulated single cells grew into PCC spheroids in both soft and stiffened gels. However, spheroids formed in gels susceptible to FMN-mediated stiffening were smaller than those formed in the non-dynamic soft hydrogels (Figure 5A). We quantified spheroid size from the live/dead stained images and found that average diameter of PCC spheroids on day 2 was around 18 \( \mu \text{m} \) in both conditions (Figure 5B). The differences in spheroid size were statistically significant on day 7 and 14. After two weeks of culture, the average spheroid size in soft gels was \( \approx 39 \mu \text{m} \) and that in the stiffened gels was only \( \approx 28 \mu \text{m} \). Additionally, cell metabolic activity was consistently and significantly lower in dynamically stiffened matrices (i.e., day 7 and 14, Figure 5C). These phenomena could be attributed to slower cell growth imposed by the strain of a tighter hydrogel network, a result similar to our previous findings.[39]

In addition to cell morphology, we also examined the effect of matrix stiffening on mRNA expression of matrix metallo-proteinases-2 (MMP-2), MMP-7, and sonic hedgehog (SHH). These genes were selected as they were found to be significantly upregulated in cells undergoing enzyme-mediated dynamic matrix stiffening.[40] In the current study, we show similar trends of upregulation at the mRNA levels (Figure 5D). Furthermore, we noticed the expression of hypoxia inducible factor 1-alpha (HIF-1\( \alpha \)) was also significantly increased in cells experiencing dynamic gel stiffening. HIF-1\( \alpha \) was stabilized in cells maintained under hypoxia and has been suggested as a target for cancer therapy.[41,42] Since oxygen reduction during hydrogel stiffening was mild (Figure 3B), the upregulation of HIF-1\( \alpha \) was not likely due to hypoxia in the stiffened gels. Instead, HIF-1\( \alpha \) upregulation could be due to increased cellular stress in a stiffened matrix. The upregulation of MMP-2 and MMP-7 suggested that cancer cells were potentially primed to migration/invasion in a stiffened matrix due to enhanced matrix remodeling by these proteases. While future studies are necessary to test these hypotheses, the experimental results presented here collectively highlight the significant impact of a dynamic stiffening microenvironment on cancer cell fate.

2.6. Effect of Dynamic Stiffening/Patterning on Cancer-Associated Fibroblasts

CAFs are a group of activated stromal cells supporting the growth and metastasis of PCCs. While the importance of CAFs in PDAC progression has been recognized, the effects of biophysical cues on their activation have not been fully defined. Utilizing FMN/visible light dynamic hydrogel system, we further examined the effect of matrix stiffening on activation of CAFs. We seeded pancreatic CAFs on top of the initially soft hydrogel (i.e., \( G’ = 1 \text{ kPa} \)) and regionally stiffening half of the gel through a photomask 1 d after cell seeding. Based on the degree of cell spreading, there appeared to be a distinguishable soft/stiff boundary. On the stiffened region (i.e., right side of the gel), CAFs adopted spreading morphology, likely due to an increased intracellular tension resulted from the higher matrix stiffness. On the other hand, cells grew into smaller clusters in the nonstiffened gel (i.e., left side of the gel, Figure 6A).

Increased tissue stiffness during pathophysiological processes can lead to nuclear translocation of yes-associated protein (YAP) in stromal cells.[28–43] Activation of YAP-related signaling pathways triggers EMT in various cancer cell types, including colorectal, breast, hepatic, and pancreatic origins.[34,46–50] To gain insight into stiffness-regulated YAP localization in pancreatic CAFs, we performed immunostaining of F-actin, YAP, and nuclei in cells cultured on top of regionally stiffened hydrogel. We found that cells adhered on the visible light and FMN-stiffened area of the gel exhibited more nuclear translocation of YAP, as compared to cells attached on the nonstiffened and soft gel (Figure 6B). It is important to note that while hydrogel moduli measured by a rheometer might not describe the local moduli sensed by the cells especially when they could actively remodel their surroundings, one can reasonably expect a positive correlation between bulk gel moduli and local moduli sensed by the cells. Regarding the homogeneity of the gel network, we believe that the dynamic hydrogels described in this work would exhibit higher level of homogeneity compared to the conventional chain-growth methacrylate-based stiffening strategy. This could be attributed to the orthogonal reactions of both the primary thiol–ene network crosslinking[51] and the secondary stiffening induced by di-tyrosine crosslinking. Collectively, these results established a causation link between matrix stiffness and activation of pancreatic CAFs. This contribution presents a visible-light-driven dynamic hydrogel system that is highly beneficial for studying tumor–ECM interaction in a more physiological culture condition.

3. Conclusion

In summary, we have developed a visible light-initiated photoresponsive hydrogel system using synthetically simple macromers and FMN, a vitamin B2 derivative, as the photoinitiator. The simple design of tyrosine-bearing peptide crosslinkers permits tunable degree of photoresponsive stiffening and ligand immobilization without complicated macromer modification. We identified parameters critical for tuning gel stiffening and showed that the stiffening/patterning process was highly compatible. Additionally, we evaluated the effect of dynamic matrix stiffening on inhibition of PCC growth in 3D and on potential activation of cancer-associated fibroblasts. Future work will focus on using this dynamic hydrogel platform for studying cell–cell interactions and durotaxis of PCCs under dynamically tunable matrix properties.
Figure 5. Effect of hydrogel stiffening on in vitro culture of COLO-357 cells. A) Cell spheroid formation in soft (nonstiffened) and stiffened hydrogels. Cell-laden hydrogels were stained with live/dead staining kit and imaged with confocal microscopy. B) Diameters and C) metabolic activity of cell spheroids formed in the dynamically stiffened hydrogels \((n = 100)\). D) mRNA expression on day 14. Housekeeping gene: GAPDH \((2^{−\Delta\Delta C_T} \text{method})\). Data were presented as mean ± SEM \((n = 3)\). \(P\)-values were calculated using one-way ANOVA with Bonferroni correction (\(*p < 0.05\), \(**p < 0.01\), and \(***p < 0.001\)).
4. Experimental Section

Materials: Hydroxyl-terminated eight-arm PEG (20 kDa) and 5-norbornene-2-carboxylic acid were obtained from JenKem Technology, USA and Sigma-Aldrich, respectively. Reagents and Fmoc-capping amino acids for solid-phase peptide synthesis were acquired from Anaspec or ChemPep. All other reagents for chemical synthesis were purchased from Sigma-Aldrich or Thermo Fisher unless noted otherwise.

Macromers and Peptide Synthesis: While all macromers, peptides, and LAP photoinitiator can be purchased from commercial vendors, these materials were synthesized using established protocols. PEG8NB and LAP were synthesized as described elsewhere.[13,30] Bis-cysteine bearing peptides with varied amounts of tyrosines, including CGGC (0Y), CGGC (1Y), CYGGY (2Y), and KCYGGY (3Y) were synthesized via standard Fmoc coupling chemistry with an automated microwave-assisted peptide synthesizer (Liberty 1, CEM). Crude products were cleaved in trifluoroacetic acid (TFA) cleavage cocktail and purified by reverse phase high-performance liquid chromatography (HPLC) to obtain final products with over 90% purity. All peptides were characterized by mass spectrometry (MS). 5(6)-Carboxy-X-rhodamine-tyramide

Figure 6. Effect of dynamic stiffening on pancreatic stromal cells (KPC/CAFs). A) Morphology and viability of CAFs cultured on a regionally stiffened (1 × 10^{-3} M FMN, 2 min of visible light at 3 mW cm^{-2}) PEG-peptide gel (2.5 wt% PEG8NB, 5 × 10^{-3} M 3Y peptide). B) Immunostaining of F-actin, YAP, and cell nuclei (counterstained with DAPI).
was synthesized from 5(6)-ROX and L-tyrosine using carbodiimide chemistry. Briefly, 0.01 mole of 5(6)-ROX was activated by 5 molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) in 1.5 mL solvent composed of three parts DMF and two parts dH2O. After 30 min of activation, fivefold excess (0.05 mole) of l-tyrosine was added and the reaction was allowed to proceed for 24 h. The final product was purified by column chromatography using silica gel as stationary phase and mobile phase composed of one part ethyl acetate and nine parts hexane.

Hydrogel Fabrication and Characterization: Gels were prepared by conjugating norborneone moieties of PEG8NB and terminal cysteine moieties of the peptide crosslinker through thiol–norborneone photopolymerization. Briefly, gel precursors (45 µL per sample, 1 × 10−3 μL LAP) were deposited between two glass slides separated by spacers (1 mm). The samples were exposed to 365 nm light (5 mW cm−2) for 2 min. Following gelation, hydrogels were incubated in PBS at 37 °C for 24 h prior to characterization or the stiffening process. Stoichiometric ratio of thiol to norborneone was maintained at 1 to afford the highest degree of crosslinking within the initial gel network. Shear moduli of the hydrogels were characterized in strain-sweep mode (0.1–5% strain) and oscillation pre- and poststiffened gels, respectively. Shear modulus measurements were performed in strain-sweep mode (0.1–5% strain) and oscillation process. Prediction of Time Needed for FMN Diffusion into Hydrogel: The time-scale of FMN diffusion into a disc-shaped hydrogel was estimated by Fick’s second law of diffusion using mesh size-dependent diffusivity (i.e., Lustig–Peppas estimation of solute diffusivity in a highly swollen hydrogel).[31] Hydrogel mesh size was calculated from experimentally obtained mass swelling ratio.[32] Correlations between hydrogel shear modulus and mesh size were established and this information was used to determine diffusivity of FMN in hydrogel. Gel swelling ratio (Q, Equation (1)) and water content (Equation (2)) were calculated according to the following equations

$$Q = \frac{M_{gel}}{M_{polymer}}$$

(1)

$$\text{Water content}(\%) = 100\% \times \frac{M_{gel} - M_{polymer}}{M_{gel}}$$

(2)

Here, $M_{gel}$ is the mass of swollen hydrogel and $M_{polymer}$ is the mass of dried gel/polymer.

Detection of Dissolved Oxygen and Hydrogen Peroxide Produced during Photoreaction: The contents of dissolved oxygen and hydrogen peroxide produced during visible light exposure (3 mW cm−2, 5 min) were measured in the presence of L-tyrosine (15 × 10−3 M) and FMN (1 × 10−1 M) in PBS. Mixtures were deposited in a 24-well plate. Oxygen contents in solution were detected with a dipping-type oxygen microsensor (MicroX4, PreSens) which was extended to about 2 mm above the bottom of well plate. To quantify H2O2 production during FMN-mediated photoreaction, 10 µL of the mixture solutions were collected periodically and quantified with a Quantichrom Peroxide Assay Kit (BioAssay Systems).

FMN and Visible Light-Mediated In Situ Gel Stiffening and Molecular Patterning: Process of hydrogels stiffening was initiated from incubating the hydrogels in FMN solution for 30 min at 37 °C. Next, gels were transferred to the top of glass slide and exposure to visible light (480 nm, 3 mW cm−2) for 2 min. The stiffened hydrogels were returned to PBS and incubated for a day prior to second measurement of the shear moduli. For proof-of-concept spatial molecular patterning, hydrogels were fabricated in a rectangular silicon mold (5 (L) × 2 (W) × 1 (H) mm) using 2.5 wt% PEG8NB, 5 × 10−3 M peptide crosslinkers (KCYGGYGGYCK), and 1 × 10−3 μL LAP. After overnight swelling in DPBS, gels were submerged in buffer solution containing 1 × 10−3 M FMN and 0.03 × 10−3 M 5(6)-ROX tyramine for an hour. Next, gel was exposed to strips of visible light (440 nm, 30 mW cm−2) for 1, 3, and 5 min through a photomask. The patterned gels were washed and imaged by confocal microscopy at 10× objective. Images were analyzed via Image J.

Cells in 3D Encapsulation and Characterization: COLO-357 cells, a PCC line, were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin-streptomycin (Gibco, 50 U mL−1 for both antibiotics). Cells were maintained in a standard cell culture incubator (37 °C, 5% CO2). Prior to encapsulation in hydrogels, cells were trypsinized and suspended (to a final cell density of 2 × 104 cells mL−1) in buffer solution consisting of LAP and macromer precursors (i.e., PEG8NB and peptide crosslinker) at desired concentrations. Cell precursor solution (25 µL) was loaded to a 1 mL disposable syringe with cut-open tip and exposed to 365 nm light (5 mW cm−2) for 2 min. These cell-laden gels were cultured in a 24-well plate for two weeks. To evaluate cell viability, cell-laden gels were stained with live/dead staining kit (Biotium; Calcein-AM stained live cells green, ethidium homodimer-1 stained dead cells red) on day 2, 7, 14, and imaged by confocal microscopy (Olympus Fluoview FV1000 laser scanning microscope). 2-stack images (10 µm per slice, 100 µm total) were obtained from minimum of three random areas within the cell-laden gels. In addition, cell metabolic activity was evaluated by AlamarBlue assay (ABD Serotec; 10% in culture media, 2.5 h incubation time).

RNA Isolation, Reverse-Transcription Polymerase Chain Reaction (PCR), and Real-Time PCR: Cell-laden gels were harvested on day 14 in RNase-free microtubes, followed by flash-freezing in liquid nitrogen and storage at −80 °C. Total RNA was isolated from the encapsulated cells with NucleoSpin RNA II kit (Clontech). The concentration and purity of RNA were obtained by NanoDrop 2000 Spectrophotometer (Thermo Scientific). Next, purified RNA samples were converted into complementary DNA (cDNA) with PrimeScript RT Reagent Kit (Clontech, Takara). mRNA expression of selected genes were evaluated by quantitative PCR (qPCR) using SYBR Premix Ex TaqII kit (Clontech) with primers listed in Table S1 (Supporting Information). GAPDH was used as the housekeeping gene. The expression level of genes in the control hydrogel group (i.e., gels prepared by PEG8NB/CGGCC) was used as negative controls, from which to calculate the relative fold change of each mRNA expression in the experimental hydrogel groups (2−ΔΔCt method).

Effect of Spatially Patterned Gel Mechanics on CAFs: Initial PEG-peptide gelation (i.e., 2.5 wt% PEG8NB, 5 × 10−3 M peptide crosslinker (KCYGGYGGYCK), 1 × 10−3 M CRGDS, and 1 × 10−3 μL LAP) was achieved using 2 min of visible-light exposure (5 mW cm−2). Precursor solutions were loaded in a rectangular silicon mold (5 L × 2 W × 1 H mm). After swelling the gels in DPBS overnight, 106 cells mL−1 of pancreatic CAFs, which were derived from KRAS/mutp53-induced pancreatic cancer mouse (i.e., KPC/CAFs, a gift from Prof. Murray Korc of IU School of Medicine), were seeded on top of the rectangular gel and cultured for 2 d. Next, the gels were incubated with soluble FMN (1 × 10−3 M) for 30 min, washed with DPBS to remove excess FMN, and exposed to visible light (3 mW cm−2) for 2 min through a photomask covering half of the gel (2.5 × 2 mm). After the visible light/FMN-induced stiffening process, gels were returned to culture media for another 5 d. On day 8, gels were imaged by bright-field microscopy and confocal microscopy for evaluating cell morphology and YAP localization.

Immunostaining of CAFs on Top of Soft and Stiffened Gel: After CAFs were cultured on top of partially stiffened gel for 8 d, cells were fixed with 4% paraformaldehyde, permeabilized with saponin (1/1000), and blocked with 1% bovine serum albumin (BSA) and 10% FBS at 4 °C for overnight. Sample was stained with the primary antibody, Rabbit anti-YAP (1/200) at 4 °C for overnight, and washed with PBS thrice for 10 min each, and treated with donkey anti-rabbit 488 (1/200) at 4 °C for overnight. Sample were washed again with PBS and stained with F-actin using rhodamine phalloidin (1/200) at 4 °C for overnight and washed. Finally, cell nuclei were counterstained with DAPI (1/1000) for 1 h.
washed stored in ibidi mounting medium to prevent photobleaching. Finally, images were obtained from confocal microscopy at 10x and 20x objectives.

**Statistical Analysis:** All experiments were repeated three times independently with minimum of four samples per condition. Numerical data were analyzed by two-way ANOVA, and reported as mean ± SEM. Single, double, and triple asterisks represent p < 0.05, 0.001, and 0.0001, respectively. p < 0.05 was considered statistically significant. For gene expression analysis, pairs of groups were compared by one-way ANOVA with GraphPad Prism 7 software.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

dynamic hydrogels, flavin mononucleotide, matrix stiffening, step-growth photopolymerization, visible light