Enzymatic crosslinking of dynamic thiol-norbornene click hydrogels

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Abstract

Enzyme-mediated *in situ* forming hydrogels are attractive for many biomedical applications because gelation afforded by the enzymatic reactions can be readily controlled not only by tuning macromer compositions, but also by adjusting enzyme kinetics. For example, horseradish peroxidase (HRP) has been used extensively for *in situ* crosslinking of macromers containing hydroxyl-phenol groups. The use of HRP on initiating thiol-allylether polymerization has also been reported, yet no prior study has demonstrated enzymatic initiation of thiol-norbornene gelation. In this study, we discovered that HRP can generate thiyl radicals needed for initiating thiol-norbornene hydrogelation, which has only been demonstrated previously using photopolymerization. Enzymatic thiol-norbornene gelation not only overcomes light attenuation issue commonly observed in photopolymerized hydrogels, but also preserves modularity of the crosslinking. In particular, we prepared modular hydrogels from two sets of norbornene-modified macromers, 8-arm poly(ethylene glycol)-norbornene (PEG8NB) and gelatin-norbornene (GelNB). Bis-cysteine-containing peptides or PEG-tetra-thiol (PEG4SH) were used as crosslinkers for forming enzymatically and orthogonally polymerized hydrogels. For HRP-initiated PEG-peptide hydrogel crosslinking, gelation efficiency was significantly improved via adding tyrosine residues on the peptide crosslinkers. Interestingly, these additional tyrosine residues did not form permanent dityrosine crosslinks following HRP-induced gelation. As a result, they remained available for tyrosinase-mediated secondary crosslinking, which dynamically increases hydrogel stiffness. In addition to material characterizations, we also found that both PEG- and gelatin-based hydrogels provide excellent cytocompatibility for dynamic 3D cell culture. The enzymatic thiol-norbornene gelation scheme presented here offers a new crosslinking mechanism for preparing modularly and dynamically crosslinked hydrogels.

***Keywords*:** Dynamic hydrogels, Horseradish peroxidase, Glucose oxidase, Thiol-norbornene click chemistry.

1. Introduction

Hydrogels prepared from orthogonal crosslinking methods have tremendous potential in drug delivery and tissue engineering applications.[1-3] In particular, thiol-norbornene click reaction is advantageous in hydrogel crosslinking owing to the rapid and quantitative reactivity between thiol- and norbornene-functionalized macromers.[4-9] The modular and orthogonal reactivity of thiol-norbornene click reaction has been used to fabricate a diverse array of biomaterials, including bulk hydrogel,[10-12] colloidal gel,[13, 14] as well as cell surface coating.[15] Current modular thiol-norbornene hydrogels are exclusively prepared from photopolymerizations initiated by ultraviolet (UV) light, visible light, or two photon irradiation.[4, 5, 9, 16-20] While photopolymerization affords spatial-temporal control in crosslinking, hydrogels formed by photochemistry are typically limited in thickness/depth due to light attenuation in thick/dark samples. For clinical applications, UV light absorption by the skin also reduces the utility of photopolymerized hydrogels.[21] It will be ideal if the synthetically simple thiol-norbornene hydrogels could be prepared with high injectability and any given sizes and shapes without sacrificing modularity of the crosslinking.

The light attenuation issue of photopolymerization can be overcome by exploiting enzymatic reaction capable of generating thiyl radicals needed for the initiation of thiol-norbornene reaction.[22] The use of enzyme to catalyze thiol-norbornene click reaction also has the advantage of independent and modular controls over gelation kinetics and final gel properties. This is particularly important as gelation speed and final gel properties are often coupled together in conventional click hydrogels (i.e., higher macromer contents/functionalities are required for faster gelation, which leads to higher degree of gel crosslinking). To this end, horseradish peroxidase (HRP) has emerged as a highly useful enzyme for *in situ* crosslinking of hydroxyl-phenol (e.g., hydroxyphenylacetic acid (HPA), tyramine, or tyrosine) or vinyl-modified polymers into hydrogels.[23-25] HRP initiates hydrogel crosslinking by generating radical species in the

presence of hydrogen peroxide (H_2O_2), which is provided either through exogenous addition or generated *in situ* through tandem enzymatic reactions (i.e., HRP with Glucose oxidase (GOX) and glucose).[26] For example, Kim *et al.* used HRP/GOX initiated crosslinking to form gelatin-based hydrogel with tunable mechanical property and gelation time.[27] These hydrogels also exhibited high cytocompatibility for encapsulation of human dermal fibroblasts. More recently, Gantumur and colleagues reported a crosslinking mechanism in which HRP was used as both the catalyst and the supplier of H_2O_2.[28] It was hypothesized that HRP oxidizes thiol moieties on itself to generate H_2O_2. This self-oxidization process was accelerated with high concentration of glucose and HRP. In addition to catalyzing crosslinking of hydroxyl-phenol-modified polymers into hydrogels, HRP was recently used to catalyze Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization,[29] as well as thiol-allyether[22] and tetrazine-norbornene hydrogel crosslinking.[30] As demonstrated by Zavada *et al*., PEG diallyl ether (PEGDAE) and ethoxylated trimethylolpropane tri(3-mercaptopropionate) (ETTMP) can be successfully crosslinked to form hydrogels with HRP and H_2O_2.[22] However, the gel points for HPR-initiated thiol-allylether gelation were on the order of 10 minutes using moderately high HRP concentrations (~100-300 U/mL).[22] Nevertheless, HRP provides diverse routes for preparing hydrogels suitable for various biomedical applications. To the best of our knowledge, however, no prior study has utilized HRP or other enzyme to initiate the crosslinking of modular and dynamic thiol-norbornene hydrogels under ambient and aqueous conditions.

In this contribution, we present the use of HRP to initiate crosslinking of modular thiol-norbornene hydrogels. Differing from the crosslinking of hydroxyl-phenyl (e.g., tyramine) containing macromers into hydrogels, HPR-initiated thiol-norbornene hydrogelation exhibits characteristic modularity in hydrogel crosslinking. Specifically, we used 8-arm PEG-norbornene (PEG8NB) or gelatin-norbornene (GelNB) as the norbornene-modified macromers for crosslinking with multi-functional thiols (e.g., dithiothreitol (DTT), 4-arm PEG-thiol (PEG4SH), or

bis-cysteine-bearing peptide) into step-growth hydrogels. H_2O_2 needed for activating HRP was supplied either exogenously or generated *in situ* via GOX and glucose. In addition to studying the parameters critical for initiating enzymatic reaction, we examined the effect of tyrosine residue on crosslinking efficiency and post-gelation dynamic stiffening of PEG-peptide hydrogels. Similar to other HRP-based hydrogel crosslinking, the system exhibits high cytocompatibility for *in situ* cell encapsulation under proper reaction conditions. Finally, we explored the additional tyrosine residues on the peptide linker for enzyme-mediated dynamic gel stiffening.

2. Material and Methods:

2.1 Materials

8-arm poly(ethylene glycol) (PEG-OH) (20 kDa) was purchased from JenKem Technology; HRP (220 U/mg) and mushroom tyrosinase (MT, 845 U/mg) were purchased from Worthington. GOX (111 U/mg) was acquired from Amresco. All other chemicals were purchased from Fischer Scientific and used without further purification unless otherwise stated. 8-arm PEG-ester-norbornene (PEG8NB, ~95% substitution) and photoinitiator lithium aryl phosphinate (LAP) were synthesized as described previously.[5, 31, 32]

2.2 Peptide synthesis and purification

All peptides were synthesized using standard solid-phase peptide synthesis in an automated microwave-assisted peptide synthesizer (CEM Liberty 1) using Fmoc-protected amino acids. Peptide cleavage was performed using a cleavage cocktail containing 7.6 mL trifluoroacetic acid (TFA), 0.2 mL triisopropylsilane (TIS), 400 mg phenol, and 0.2 mL double distilled water. The peptides were cleaved from the resin for ~3 h at room temperature and precipitated in cold ethyl ether. The cleaved peptides were dried in vacuo and purified by reverse phase HPLC (PerkinElmer Flexar system) using 95%/5% (v/v) water/acetonitrile with trace (0.1vol.%) of TFA as the starting solvent mobile phase. A linear gradient of acetonitrile was used to separate the products through a semi-prep scale peptide C18 column at 5 mL/min flow rate. The separation

processes were monitored with a UV/vis detector at 280 nm (for peptides with tyrosine residue) or 220 nm (for peptides without tyrosine residue). Purified peptides were characterized with liquid chromatography coupled with mass spectrometry (Agilent Technologies, 1200 series LC/MS system).

2.3 Hydrogel fabrication

To fabricate HRP/H_2O_2 mediated thiol-norbornene hydrogel, macromer PEG8NB was crosslinked with either DTT or bis-cysteine-bearing peptides (i.e. CGGGC, CYGGGYC, CGGYGGC, KCYGGYGGYCK). Specifically, to make a 1:1 thiol-to-norbornene ratio ($R_{thiol/ene}$ =1) of PEG8NB-KCYGGYGGYCCK hydrogel, 2.5 wt% of PEG8NB and 10 mM of KCYGGYGGYCK (final concentrations) were dissolved in phosphate buffer solution (PBS) at pH 7.4. HRP (1 U/ml) and H_2O_2 (0.5 mM) were added to the solution, followed by vortexing for ~5 seconds. The precursor solution was immediately pipetted in between two glass slides separated by 1-mm-thick spacers. Gelation occurred within 5 minutes at room temperature. The hydrogels crosslinked from PEG8NB and tyrosine-free linker (CGGGC or DTT) or with GelNB and PEG4SH were also prepared following the same procedure but with a more concentrated HRP (100 to 200 U/ml).

For dual enzyme (HRP/GOX)-mediated gelation, PEG8NB-DTT and PEG8NB-peptide hydrogels were prepared following the similar procedures described above. Briefly, 3 wt% PEG8NB and 12 mM (final concentrations) DTT or peptides were dissolved in PBS with 1 U/ml (for tyrosine-containing peptides), or 200 U/ml HRP (for CGGGC or DTT), 1 U/ml GOX, and 10 mM glucose. The solution was vortexed for ~5 seconds before pipetted in a Teflon mold with 8-mm diameter cavities. Hydrogel discs were obtained after 5 minutes of gelation.

To stiffen hydrogels using mushroom tyrosinase (MT), PEG8NB hydrogels were crosslinked by tyrosine-containing peptide (thiol to norbornene ratio was fixed at 1). Prior to MT-mediated stiffening, hydrogels were swollen in PBS for 24 hrs to wash off un-crosslinked species. To induce dynamic stiffening, hydrogels were submerged in 1 kU/ml MT for 6 hrs. Afterwards, MT

was removed via swelling hydrogels in PBS for 24 hrs, followed by rheological measurements of hydrogel shear modulus.

2.4 Rheometry

Rheological measurements were conducted with circular hydrogel discs fabricated between two glass slides. Gel discs were punched out with an 8 mm biopsy punch. The hydrogels were carefully transferred to the rheometer platform prior to initiating the measurements. Storage and loss moduli (G' and G") of the hydrogels were determined using a Bohlin CVO 100 digital rheometer fitted with an 8-mm diameter parallel geometry. Frequency sweep was first performed to determine the frequency at which the viscoelastic properties are independent of the imposed stress or strain (i.e., linear viscoelastic (LVE) region). For most covalently crosslinked hydrogels, a frequency of 1 Hz typically falls within the LVE region. The rheological measurements were performed in strain-sweep mode with the strain ranging from 0.1% to 5%, and the oscillation frequency was kept constant at 1 Hz.

For *in situ* gelation experiments, precursor PEG8NB solution containing thiol crosslinkers, HRP, H_2O_2 (or GOX and glucose) were mixed and vortexed for 5 seconds. Immediately after vortexing, 7 μL of the mixture was placed on the lower plate and the geometry was lowered to 90 μm. A layer of mineral oil was applied on the edge of the plate geometry head to prevent dehydration.

2.5 Norbornene and Thiol Consumption

The thiol conversion study was conducted with precursor solutions containing linear PEGNB, DTT, HRP and H_2O_2. Briefly, 3.5 wt% PEGNB, 14 mM DTT, 200 U/ml HRP, and 1.5 mM of H_2O_2 were mixed together and portions of the solution (25 μL) were collected immediately after mixing and at intervals of every 2 minutes afterward. Remaining thiol contents were determined using Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid. ThermoFisher Scientific) following the

manufacturer's protocol. The thiol concentration left at each specific time point was used to calculate the amount of thiol that had been consumed.

As for norbornene consumption, mixtures of linear PEGNB, DTT, HRP, H_2O_2 at different thiol to norbornene ratios (keeping PEGNB concentration constant at 3.5 wt%) were mixed in deuterium oxide for 10 minutes. Polymer samples for ^{1}H NMR analysis were prepared at a concentration of 20 mg/ml. The reaction mixtures were then subjected to analysis using Bruker Avance III 500 Hz NMR. The amount of norbornene left after the reaction for each $R_{thiol/ene}$ ratio was calculated using the ratio of the integration of the norbornene peaks at 6.00 to 6.36 ppm over the integration of the PEG backbone region from 4.21 – 4.37 ppm.

2.6 Characterization of gel fraction

Hydrogels were formed with PEG8NB, DTT, HRP and H_2O_2 (or GOX/glucose); each gel was prepared from 45 µL of precursor solution. Immediately after gelation, hydrogels were dried in vacuo and weighed to obtain first dried weight ($W_{1st\ dried}$). The dried gels were incubated in ddH_2O at 37 °C overnight to remove un-crosslinked species. Afterwards, swollen weights were obtained; swollen gels were dried and weighed again to obtain the second dried weight ($W_{2nd\ dried}$). Gel fraction (**Equation 1**) was determined by the ratio of the 2nd dried weight over the 1st dried weight:

$$\text{Gel fraction} = \frac{W_{2nd\ dried}}{W_{1st\ dried}} \quad (1)$$

Hydrogel mass swelling ratios (q, **Equation 2**) were calculated using the following equation:

$$q = \frac{W_{swollen}}{W_{2nd\ dried}} \quad (2)$$

2.7 In-gel oxygen measurements

A needle-type oxygen probe connected to Microx4 oxygen sensor (PreSens Precision Sensing GmbH) was used to obtain the oxygen concentrations within the gels. The needle of the oxygen probe was inserted into the gel at specified time points. After needle penetration, the

optical fiber of the probe was extended to the tip of the needle so that it was exposed to the gel but remained housed within the needle.

2.8 NIH/3T3 fibroblast encapsulation

Cytocompatibility of the enzymatically crosslinked thiol-norbornene hydrogel was evaluated using murine NIH/3T3 fibroblasts acquired from American Type Culture Collection (ATCC). Cells were maintained in high glucose Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin before performing cell encapsulation. All macromer components used for cell encapsulation were sterilized by passing through sterile 0.22 μm syringe filter. For cell encapsulation, a solution of 3 wt% PEG8NB, 13 mM KCYGGYGGYCK peptide, 1 mM CRGDS peptide, 1 U/ml HRP, and 0.5 mM H_2O_2 were mixed together, followed by gently suspending NIH/3T3 cells into the precursor solution (final cell density: 2×10^6 cells/ml). The mixture was then added to 1 mL syringes (with the top cut open) and allowed to gel for 5 minutes. After that, cell-laden gels were transferred into a 24-well plate. GelNB-PEG4SH cell-laden hydrogels were prepared following similar steps but with a higher HRP concentration (100 U/ml) and without the addition of CRGDS. To evaluate cell viability after encapsulation and throughout culturing period, the encapsulated cells were stained with NucBlue®, which labels nuclei of all cells, and NucGreen®, which stains cells with compromised plasma membranes (i.e., dead cells). The numbers of live (all cells minus dead cells) and dead cells were imaged with a confocal microscope and counted using ImageJ software.

2.9 Dynamic stiffening of enzymatically crosslinked PEG-peptide hydrogels

MT were used to induce dynamic stiffening of PEG8NB-peptide hydrogels. 24 hrs after cell encapsulation, the gels were incubated in 1 kU/ml MT for 6 hours to induce stiffening. Afterwards the enzyme was removed via swelling in culture media for 24 hrs. To observe the effect of matrix stiffening on cell morphology and cytoskeletal organization, cell-laden hydrogels were fixed and stained for cell nuclei and F-actin. Specifically, at predetermined time points after

encapsulation, cell-laden hydrogels were fixed with 4% paraformaldehyde and permeabalized with saponin solution following a published protocol.[33, 34] Next, rhodamine phalloidin and DAPI were used to stain for F-actin and nuclei, respectively. Live/Dead and immunofluorescence stained samples were imaged with Olympus Fluoview FV100 laser scanning microscopy. Live/Dead images were captured at 10x objective, with Z-stacked of 10 slices and 10 μm per slice. Immunofluorescence images were captured at 20x objective, with Z-stacked of 10 slices and 2 μm per slice.

2.10 Statistics

All experiments were performed independently for three times and with a minimum of three samples per conditions. Statistical significance was evaluated using a two-tail t-test in Prism 5 software. Single, double, and triple asterisks represent $p<0.05$, 0.01, and 0.001 respectively.

3. Results and Discussion

3.1 Characterization of HRP-mediated thiol-norbornene gelation

While HRP has been previously used to initiated crosslinking of thiol-allylether hydrogels,[22] its utility on initiating thiol-norbornene gelation has not been reported. We reasoned that thiyl radicals generated by HRP can propagate to the strained norbornene bond, creating a carbon-center radical to abstract hydrogen from another thiol group. A stable thioether bond is subsequently formed, thus completing the step-growth cycle (**Fig. 1A**). To test this hypothesis, we first mixed PEG8NB (20 kDa), DTT, HRP, and H_2O_2 in test tubes and evaluated gelation speed using a simple tilt-test. As shown in **Fig. 1B**, gelation occurred within a few minutes only when all four components (PEG8NB, DTT, HRP, H_2O_2) were included. The crosslinking was clearly triggered by enzymatic reaction because gelation did not occur without HPR or H_2O_2. Furthermore, gelation was not due to norbornene homo-polymerization (i.e., no DTT) or entirely by HRP-mediated disulfide bond formation (i.e., no PEG8NB groups). To ensure that gelation was a result of HRP-mediated thiol-norbornene reaction, we performed thiol and norbornene

consumption tests using linear PEGNB, DTT, HRP, and H_2O_2. Linear PEGNB was used to prevent crosslinking while permitting solution-based assay since not all formulations formed hydrogels, especially at early reaction time points (in thiol consumption test) and low thiol/ene ratios (in norbornene consumption test). **Fig. 1C** shows that a time-dependent depletion of thiols only occurred in the presence of all necessary components (i.e., PEGNB, DTT, and HRP/H_2O_2). Limited thiol consumption (~30%) was detected in the presence of DTT and HRP, which could be attributed to HRP-catalyzed disulfide bond formation. It should be noted that, in the absence of HRP, no thiol consumption was detected (**Fig. 1C**. No HRP group), suggesting that un-catalyzed disulfide bond formation was not a concern within the 15 minutes reaction time.

Using proton NMR, we analyzed chemical shifts of norbornene group (**Fig. 1D**) and established a linear and quantitative relationship of norbornene consumption as a function of thiol-to-norbornene ratio ($R_{thiol/ene}$). It is worth noting that there was an incomplete norbornene consumption even when $R_{thiol/ene}$ reached unity. The lower than expected and incomplete norbornene consumption could be a result of the HRP reactivity towards thiol groups (**Fig 1C**. No PEGNB group). Since the $R_{thiol/ene}$ values were calculated based on the amounts of thiol and norbornene groups added in the solutions, partial consumption of thiol by HRP would reduce the actual thiol-to-norbornene ratio, which could explain why a lower than expected norbornene consumption was obtained.

Using *in situ* rheometry, we demonstrated a rapid gelation kinetics, which was on par with the visible light initiated thiol-norbornene gelation system (gel point ~80 s, **Fig. 2A**).[16] Enzymatic crosslinking of DTT and PEG8NB into hydrogels required relatively low concentration of HRP (~100 U/mL, **Fig. 2B**) and H_2O_2 (~0.5 mM, **Fig. 2C**). Through adjusting PEG8NB macromer contents (i.e., 3.5, 4, and 4.5 wt%), gel crosslinking density and modulus (G' ~ 1 to 3 kPa, **Fig. 2D**) could be readily tuned in a range relevant to many normal and diseased tissues, including stem cell differentiation,[35-37] tumor progression,[34, 38-41] and fibrosis.[42, 43] More importantly, unlike light-mediated photochemistry that has light attenuation issue, especially in

dark samples, we showed that HRP-catalyzed thiol-norbornene hydrogels can be used to form hydrogels with higher depth/thickness since enzymatic reactions occurs simultaneously throughout the dimension of the vessel (**Fig. 2E**). Thiol-norbornene hydrogels crosslinked by the HRP/H_2O_2 system also appeared to maintain good fidelity of the syringe mold. In principle, this enzymatic crosslinking scheme can be adapted for injectable delivery of thiol-norbornene hydrogels, which have an ideal network structure and can conform the size and shape of the delivery site.

3.2 Tyrosine-assisted enzymatic crosslinking of PEG-peptide hydrogels

After demonstrating the feasibility of HRP-initiated thiol-norbornene hydrogel crosslinking using DTT as a crosslinker, we asked if bis-cysteine peptide linkers can be used to form PEG-peptide hydrogels. Peptide crosslinkers are advantageous in promoting cell fate processes, such as protease-mediated matrix cleavage. As a proof-of-concept, we designed a model peptide linker containing only terminal cysteines and internal glycine residues (i.e., CGGGC) and tested gelation under 1 mM H_2O_2 and a range of HRP concentrations (i.e., 1 to 200 U/mL). While gelation occurred at high HRP concentrations (100-200 U/mL) as expected, no sol-gel transition was observed when HRP concentration was lower to 5 U/mL even after 30 minutes (data not shown). We then examined whether adding soluble tyrosine could promote HRP-mediated thiol-norbornene gelation as this approach was reported to improve HRP-induced crosslinking of thiolated polymers,[44] as well as the gelation efficiency of photopolymerized thiol-norbornene hydrogels.[45] Unfortunately, soluble tyrosine also did not assist thiol-norbornene PEG-peptide gelation using 5 U/mL HRP (data not shown).

We next tested whether placing tyrosine residue on the cysteine-containing peptide linkers would enhance HRP-mediated thiyl radical generation. This approach was inspired by another recent work where tyrosine/cysteine dually labeled protein was used to facilitate HRP-mediated di-thiol crosslinks formation.[46] **Fig. 3A** illustrates the potential mechanism of tyrosine-assisted

thiyl radical generation. Experimentally, we used a tyrosine containing model peptide CYGGGYC for HRP-mediated thiol-norbornene gelation tests. Surprisingly, immediate gelation was obtained at 5 U/mL HRP and 1 mM H_2O_2, suggesting that adding tyrosine residues on the peptide sequence improved thiyl radical generation and hence thiol-norbornene gelation. It is worth noting that gelation was not due to HRP-mediated di-tyrosine crosslinking or norbornene-tyrosine reaction, as control experiments using a cysteine-free peptide (i.e., KGYGGYGGYGK) did not yield hydrogel crosslinking (data not shown). In order to obtain gelation in a more manageable timeframe, we intentionally reduced the concentration of HRP and H_2O_2 to 1 U/mL and 0.5 mM, respectively. Under these conditions, PEG-peptide thiol-norbornene hydrogels could be crosslinked within 10 minutes and shear moduli of these hydrogels (as characterized by strain-sweep rheometry) were higher when using peptide linker containing more tyrosine residues (**Fig. 3B**). Additional gelation tests using off-stoichiometric ratios of thiol/norbornene led to gels with tunable moduli (**Fig. 3C**), a typical characteristic of modularly crosslinked PEG-peptide thiol-norbornene hydrogels. It should also be noted that the above results were obtained without altering the concentrations of PEG8NB macromer (i.e., 3 wt%), HRP (i.e., 1 U/mL), or H_2O_2 (i.e., 0.5 mM), further providing flexibility in preparing hydrogels with highly tunable properties.

3.3 HRP/GOX dual enzymatic thiol-norbornene gelation

Next, we explored whether thiol-norbornene gelation could be achieved using enzyme-catalyzed tandem reactions. Specifically, H_2O_2 needed for HRP-catalyzed thiol-norbornene gelation was generated in tandem by GOX, glucose, and dissolved oxygen (**Fig. 4A**). Gelation of PEG8NB and CYGGGYC peptide using this scheme was successful and the degree of hydrogel crosslinking was dose-dependently and almost linearly tuned in the presence of 1 to 10 mM glucose (**Fig. 4B**). However, when glucose concentration was raised to above 10 mM, hydrogels were formed with lower moduli, suggesting a reduced crosslinking efficiency. This was likely due to an inhibition effect of higher H_2O_2 to HRP and/or GOX (i.e., more H_2O_2 would be generated at

higher glucose concentrations).[47, 48] We further compared crosslinking efficiency of enzymatic thiol-norbornene hydrogels to that of UV crosslinked gels with the same macromer compositions. In terms of gel fraction (**Fig. 4C**), hydrogels crosslinked by the HRP/GOX/glucose system (87.4 ± 2.1) were comparable to that of UV crosslinked gels (87.8 ± 2.4), suggesting high crosslinking efficiency of the HRP-mediated thiol-norbornene reactions. However, when gels were crosslinked by the HRP/H_2O_2 system, a slightly lower gel fraction (77.5 ± 1.0) was obtained, which could be attributed to HRP inactivation caused by bolus addition of H_2O_2.[49-51] Further characterizations of mass swelling ratio (q) and shear modulus (G') of the hydrogels confirmed a lower crosslinking efficiency in gels formed by the HRP/H_2O_2 system when comparing to gels formed by HRP/GOX tandem enzymatic reactions (i.e., higher q and lower G' **Fig. 4D**).

To gain insights into the effect of tandem HRP/GOX enzymatic thiol-norbornene reactions on the oxygen contents during gelation, we used a needle-type oxygen probe to detect concentrations of dissolved oxygen inside the two groups of hydrogels at various time points post-gelation (0-24 hr). Hydrogels were placed in PBS immediately after gelation. As shown in **Fig. 4E**, oxygen contents inside the hydrogels formed by HRP/H_2O_2-initiated gelation remained close to normoxia after gelation. This is not surprising, as no dissolved oxygen was needed in HRP/H_2O_2-mediated reaction. However, in the HRP/GOX/glucose gelation system, severe hypoxia (~ 1%) was detected within one hour post-gelation. After 5 hours, O_2 content in hydrogel increased to ~6%. Oxygen level in the hydrogel returned to almost normoxia after 24 hours, presumably due to oxygen diffusion into the gel over time. The increased 'in-gel oxygen' results suggested that no GOX was permanently trapped in the hydrogel after crosslinking. The highly efficient enzyme-initiated PEG-peptide thiol-norbornene hydrogel system is advantageous as injectable cell-responsive matrices for tissue engineering applications. Furthermore, the transient hypoxia occurred within the dual enzyme-crosslinked thiol-norbornene hydrogels may be exploited to

improve 3D vascularization and cytokine secretion from mesenchymal stem cells in the future.[52, 53]

3.4 Enzymatically crosslinked gelatin-based thiol-norbornene hydrogels

To demonstrate the versatility of the HRP mediated gelation, hydrogels were formed with norbornene-modified gelatin (GelNB),[54] an attractive macromer used extensively in many biomedical applications due to its intrinsic biocompatibility and degradability.[39, 55-57] Because GelNB and PEG4SH were both multifunctional macromers, HRP concentration was lowered to ~100 U/ml (instead of 200 U/ml for PEG8NB) to achieve a more manageable gelation time. GelNB and PEG4SH readily crosslinked into hydrogels with highly tunable stiffness. Gel moduli were controlled by adjusting either gelatin content (**Fig. 5A**) or thiol to norbornene ratio (**Fig. 5B**). Hypothetically, GOX/glucose system is more ideal for cell encapsulation because GOX-generated H_2O_2 would be consumed by HRP soon after its production. On the other hand, exogenously added H_2O_2 would present a much higher initial H_2O_2 concentration for the encapsulated cells. However, the in-gel oxygen measurements results shown in **Fig. 4C** demonstrated an extremely low oxygen level within the first hour of gelation (<1%), which might not be ideal for cells survival. Other potential challenges with the HRP/GOX/glucose system as a mean to supply H_2O_2 lie in the fact that the remaining GOX within the hydrogel can continuously consume glucose within the culture media to generate gluconic acid. In addition, the remaining HRP, GOX and glucose could also crosslink the pH indicator phenol red in the media, which may hinder its buffering effect on pH changes. Therefore, we chose HRP/H_2O_2 system for cell encapsulation studies. To minimize potential cytotoxicity, H_2O_2 concentration was lowered to 0.5 mM. A recent study on HRP/H_2O_2 enzymatic reaction reported by Park *et al.* has concluded that any initial H_2O_2 concentration below 0.063 wt% (~18 mM) is a safe level for cell culture and almost all residual H_2O_2 would be converted to water and oxygen by HRP.[58] By quantifying the numbers of live and dead cells using live/dead staining and confocal imaging, we found that enzymatically crosslinked GelNB-PEG4SH

hydrogels displayed good cytocompatibility with above 85% of the encapsulated cells remained alive after 24 hours of encapsulation (**Fig. 5C**). Moreover, encapsulated cells proliferated significantly after 8 days of culture (**Fig. 5D**). Since gelatin is susceptible to protease-mediated degradation, the encapsulated cells were able to form extensive and interconnected network following local matrix degradation. All in all, the enzymatically crosslinked GelNB-PEG4SH hydrogels are capable of supporting long term cell survival as well as providing favorable platform for cell expanding and proliferation.

3.5 Dynamic stiffening of enzymatically crosslinked PEG-peptide hydrogels

Fig. 3 has clearly shown that tyrosine residues on bis-cysteine peptide linker facilitate thiyl radical generation and thiol-norbornene hydrogel crosslinking. One potential mechanism responsible for this gelation is that the hydroxyl and thiol groups are in close proximity on the peptide linker. It is likely that the hydroxyl group on tyrosine residue regains its hydrogen atom following thiyl radical generation (**Fig. 2A**). We then asked if hydroxyl side group on tyrosine residues can be exploited for mushroom tyrosinase (MT)-mediated post-gelation dynamic stiffening (**Fig. 6A**). We have previously developed similar strategies to dynamically stiffen PEG-peptide hydrogels for controlling cell fate processes.[39, 40] We found that the enzymatically crosslinked thiol-norbornene PEG-peptide hydrogels could indeed be dynamically stiffened using exogenously added MT (incubation for 6 hours. **Fig. 6B**, **6C**), suggesting that the hydroxyl-phenol groups on tyrosine remained protonated following HRP-mediated gelation. Upon the addition of MT, these tyrosine residues were catalyzed to DOPA dimers that exhibit characteristic yellow/brown color as shown in **Fig. 6B**.[33, 39, 40] These additional DOPA dimers resulted in increased gel crosslinking density and shear modulus (**Fig. 6C**). Enzymatic stiffening occurred in hydrogels crosslinked by peptides with two or three tyrosine residues, as well as gels crosslinked with HRP/H_2O_2 or HRP/GOX/glucose systems. Most importantly, the degrees of stiffening (from 2 to 5 kPa) were relevant to the mechanics of many normal and diseased tissues.[59-62]

3.6 Cell encapsulation and dynamic stiffening of cell-laden hydrogels

Fibroblast has been known to play a critical role during normal wound healing, where the stiffness of the ECM increases significantly.[63] Our enzyme-mediated matrix stiffening strategy can be used to mimic this process and examine how matrix stiffening would regulate fibroblasts behavior. To investigate how dynamic stiffening affects cell fate, we encapsulated NIH/3T3 fibroblasts in PEG8NB-peptide hydrogels, with the peptide crosslinkers susceptible to mushroom tyrosinase (MT)-mediated on-demand stiffening. The PEG8NB-peptide hydrogels were divided into two groups: the control group, which received no MT treatment and the stiffened group, which underwent dynamic stiffening (i.e., treated with 1 kU/ml MT for 6 hours). Live/dead staining results show that both groups displayed good cytocompatibility, with 90% or more cell survived the enzymatic encapsulation process. (**Fig. 6D**). On day 1, the cells exhibited rounded morphology in both groups (**Fig. 6E**). However, after 8 days of culturing, immunofluorescence staining results show distinct differences in cell morphology between the soft and stiffened hydrogels. While cells cultured in the non-treated (or soft) gels exhibited extensive and significant cell spreading with many cells connected to each other, those in the stiffened hydrogels remained mostly as single cells. The extensive spreading in the soft group was similar to that observed in cells encapsulated within soft GelNB-PEG4SH gels (**Fig. 5D**). On the other hand, while the PEG8NB-peptdie hydrogels used here did not contain protease-sensitive linkers, some cells in the stiffened gels still exhibited spreading and/or irregular protrusions after 8 days of culture (**Fig. 6E**). We reasoned that these cell protrusions were permitted by network defects and/or gradual hydrolysis of ester bonds located between the norbornene moiety and the PEG backbone. We have previously shown that hydrolysis of ester bonds in gels composed of 'PEG-ester-NB' macromer encouraged a higher degree of cell spreading when compared with gels formed by 'PEG-amide-NB'.[64] Nonetheless, these results suggested that soft gels exhibited appropriate mechanical strength to allow for more cell spreading. Due to the additional di-tyrosine crosslinks within the stiffened gels network, the mesh sizes of these hydrogels were smaller and could impose physical strain to

restrict gel degradation and cell spreading. This behavior is likely not exclusive to NIH/3T3 cells, our result agreed with previous studies where soft gels were also shown to allow more spreading than stiffened ones.[39] It is also important to note that, the minimal cell spreading behavior within the stiffened group was mainly due to matrix stiffening and not due to cell death, because pronounced increased in cell density were seen in both groups after 8 days. Cell density increase indicated that NIH/3T3 fibroblasts were viable and able to proliferate even in stiffened hydrogels. While we did not perform rheological measurements for cell-laden hydrogels, all hydrogels underwent MT-mediated stiffening changed their color to dark brown (data not shown. Similar to **Fig. 6B**), indicating that the gels were indeed stiffened. Future studies may be conducted to correlate the degree of enzymatic matrix stiffening and mechanotransduciton in the encapsulated cells. Nonetheless, our stiffening experiments (**Fig. 6**) have also that the tyrosine residues not only facilitated HRP-mediated thiol-norbornene gel crosslinking, but were also available for MT-mediated stiffening to dynamically affect cell fate processes.

Another important issue to note is that cell viability was slightly lower in the GelNB-PEG4SH gels (**Fig. 5C**) than in the PEG-peptide gels. We reasoned that this was due to the higher concentration of HRP used (i.e., 100 U/mL for GelNB-PEG4SH gels and 1 U/mL for PEG-peptide gels) during encapsulation process, which could have adverse effect on fibroblasts. Although less likely, the differences in radical generation mechanism of the HRP-mediated thiol-norbornene system with and without the incorporation of tyrosine residues could also be a reason for the viability discrepancy. Regardless of the macromers used, we have shown that cell-laden thiol-norbornene hydrogels could be readily crosslinked enzymatically via HRP. Uniquely, the PEG8NB-peptide hydrogel system exhibited additional dynamic and enzymatic stiffening feature that has not been reported in other HRP-crosslinked gels. If desired, these tyrosine residues can be explored for labeling/patterning of receptor binding ligands, a strategy reported recently by our group.[33] Overall, the enzymatically crosslinked thiol-norbornene hydrogels address the limitation of light attenuation issue in photopolymerization while retaining the modularity of the

thiol-norbornene crosslinking. The enzyme-mediated crosslinking mechanism can be utilized in a wide range of applications ranging from injectable cell-laden hydrogels to *in vitro* dynamic cell culture platforms.

4. Conclusion

In summary, we have developed the first orthogonal enzymatic thiol-norbornene click reaction suitable for forming modularly crosslinked hydrogels under ambient conditions. Furthermore, we discovered that HRP can be used to initiate gelation of macromers other than those containing hydroxyl-phenyl groups. Most importantly, the hydrogels can be dynamically stiffened by means of tyrosinase-mediated crosslinking owing to the preservation of tyrosine residues following the initial thiol-norbornene click gel reaction. The modular and dynamic hydrogels described in this contribution offer researchers an attractive alternative to form modularly crosslink and dynamic hydrogels without the concerns of light attenuation in thick samples or potential cell damage caused by UV light irradiation.

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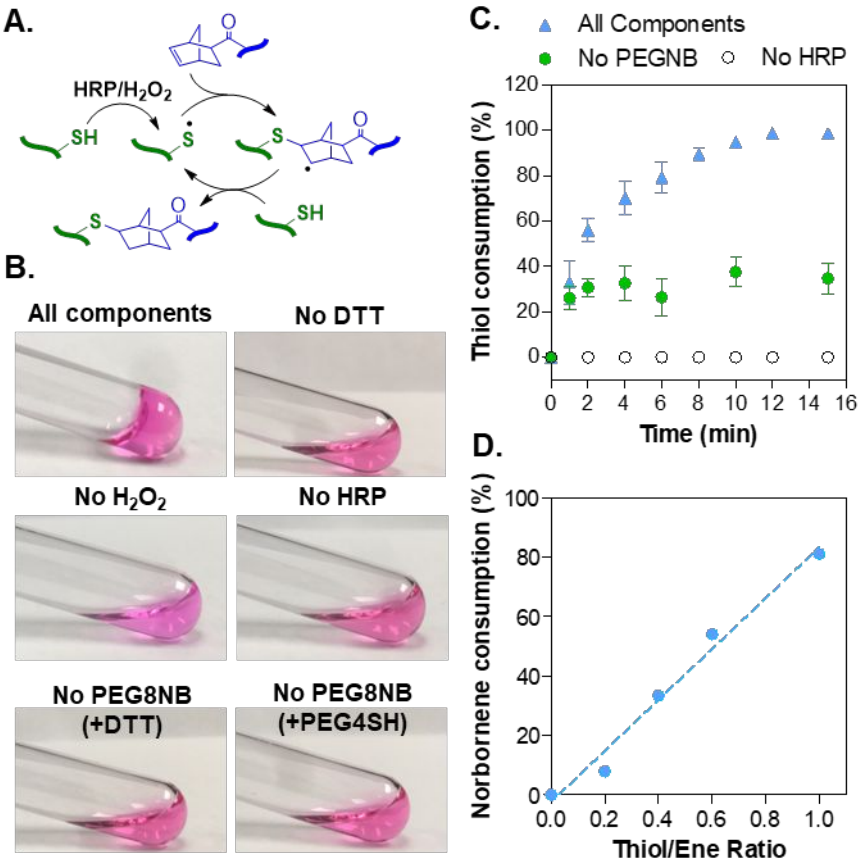

Figure 1. HRP-mediated crosslinking of thiol-norbornene click reactions. (A) Schematic of HRP/H_2O_2-induced thiyl radical generation and subsequent thiol-norbornene crosslinking. (B) Gelation tilt-test. All components: 200 U/mL HRP, 0.5 mM H_2O_2, 3.5 wt% PEG8NB, and 14 mM DTT. (C) Thiol consumption as a function of reaction time. (D) Norbornene consumption as a function of thiol-norbornene ratio (i.e., Thiol/Ene Ratio, calculated using the actual molarity of thiol and norbornene groups added to the reactions).

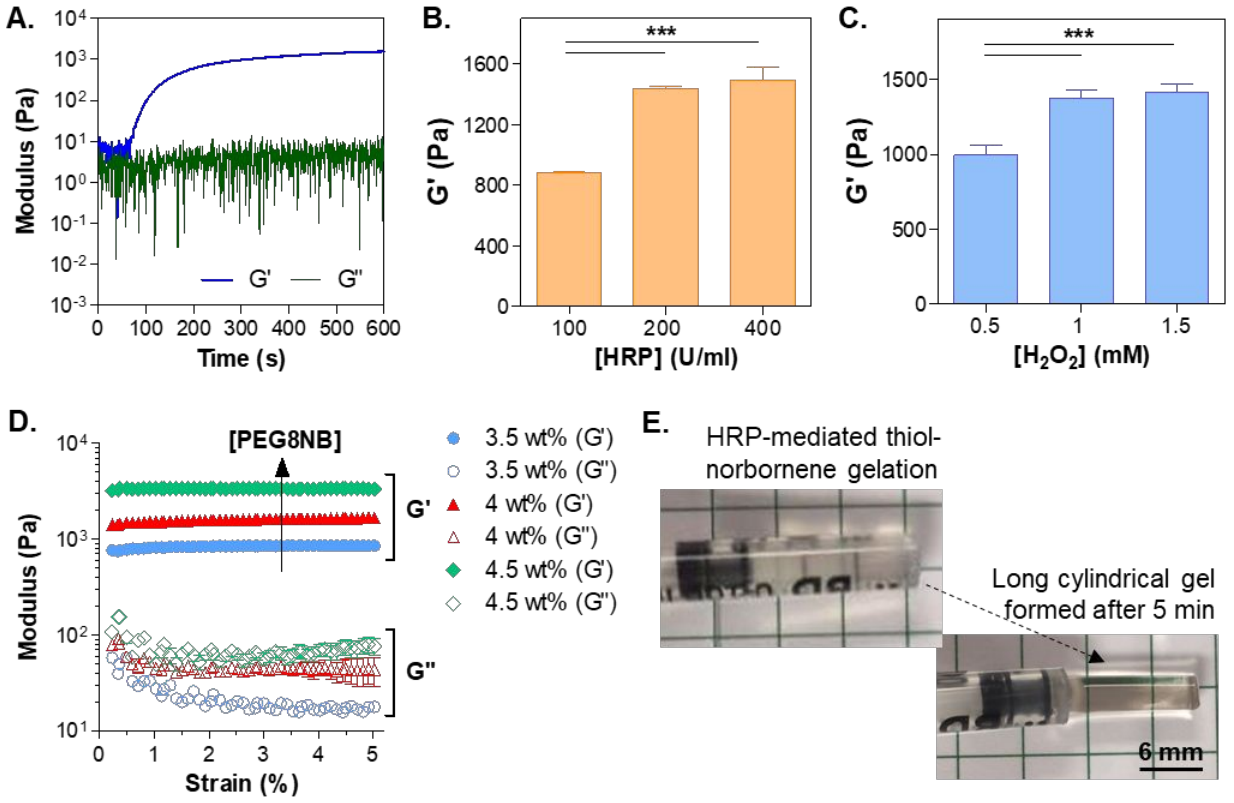

Figure 2. Characterization of HRP-mediated thiol-norbornene hydrogelation. (A) In situ rheometry of HRP-initiated thiol-norbornene gelation (all components: 3.5 wt% PEG8NB, 14 mM DTT, 200 U/ml HRP, 0.5 mM H_2O_2). Effect of (B) HRP concentrations and (C) H_2O_2 concentration on shear moduli of PEG8NB-DTT hydrogels. Gelation was formed with 3.5 wt% PEG8NB, and 14 mM DTT, $R_{thiol/ene}$=1. N = 3, mean ± SEM. ***p<0.001). (D) Strain-sweep rheometry of thiol-norbornene hydrogels formed with different macromer contents (Circles, triangles, and diamonds represent 3.5, 4, and 4.5 wt% PEG8NB, respectively. $R_{thiol/ene}$ = 1). (E) HRP-crosslinked thiol-norbornene hydrogel with a diameter of ~4 mm and a length of ~15 mm (200 U/mL HRP, 0.5 mM H_2O_2, 3.5 wt% PEG8NB, and 14 mM DTT).

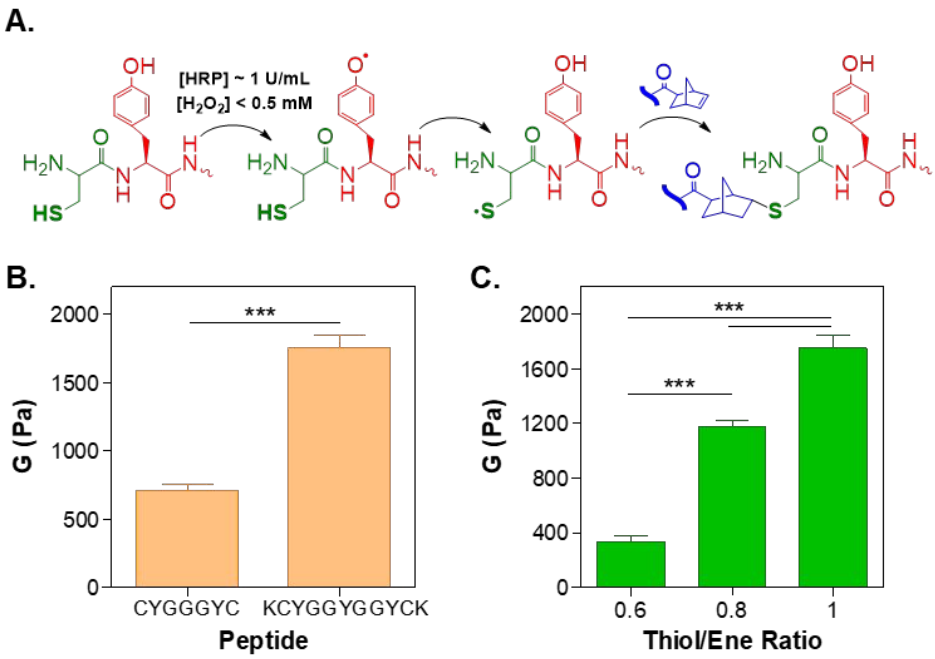

Figure 3. Effect of tyrosine residue on HRP-mediated crosslinking of thiol-norbornene PEG-peptide hydrogels. (A) Proposed schematic of thiyl radical generation via tyrosine residues. Effect of (B) tyrosine concentration and (C) thiol to norbornene ratio on the shear moduli of hydrogels (n=3, Mean ± SEM, ***p<0.001).

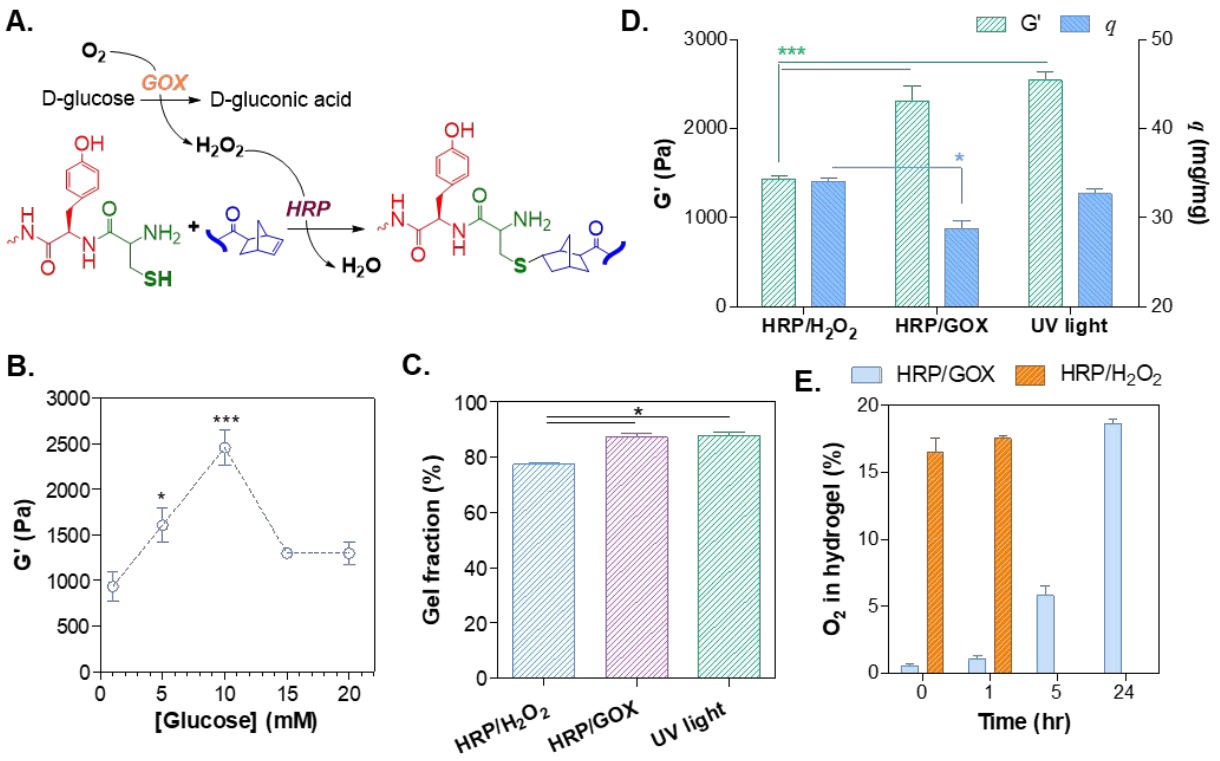

Figure 4. Thiol-norbornene gelation initiated by tandem enzymatic reactions. (A) Schematic of thiol-norbornene hydrogel formation via GOX and HRP-mediated crosslinking. (B) Effect of glucose concentration on shear moduli of dual enzyme-crosslinked thiol-norbornene hydrogels (1 U/mL HRP, 10 U/ml GOX, 3 wt% PEG8NB, and 12 mM CYGGGYC). (C) Gel fraction of hydrogels formed by HRP/H_2O_2, HRP/GOX, and UV light-mediated thiol-norbornene polymerization. Enzyme-crosslinked gels were prepared with 3.5 wt.% PEG8NB and 14mM DTT using HRP (200 U/mL), H_2O_2 (1 mM), or with HRP (200 U/mL), GOX (10 U/ml), and glucose (10 mM). UV (365 nm) light-polymerized gels were formed with 1 mM LAP with light irradiation for 2 minutes. (D) Swelling ratio (q) and shear modulus (G') of the hydrogels as described in (C). (E) Oxygen contents within hydrogels formed by HRP/GOX (1 U/ml HRP, 1 U/mL GOX, 10 mM glucose) and HRP/H_2O_2 (1 U/mL HRP, 0.5 mM H_2O_2). In the HRP/H_2O_2 group, data were recorded only at 0 and 1-hr since O_2 levels were close to normoxia in both measurements (All experiments: n=3, Mean ± SEM, *p<0.05 ***p<0.001).

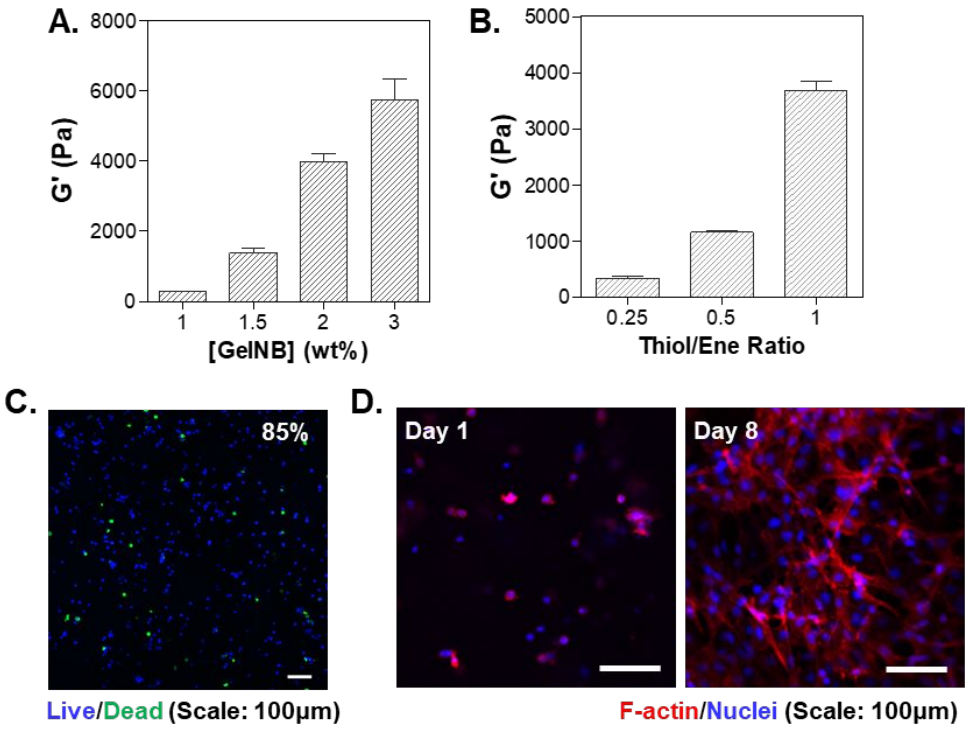

Figure 5. **Cytocompatibility of HRP-mediated GelNB-PEG4SH hydrogels**. Effect of gelatin content (A) and thiol-to-norbornene ratio (B) on shear moduli of GelNB-PEG4SH hydrogels (100 U/ml HRP, 10 U/ml GOX, 10 mM glucose. n>=3, Mean ± SEM). (C) Live/Dead staining images of NIH/3T3 fibroblasts cultured in GelNB-PEG4SH hydrogels 24 hrs after encapsulation. (D) Fluorescence staining images of F-actin and nuclei in the encapsulated NIH/3T3 fibroblasts. Cell-laden gels were formed with 1.5 wt% GelNB-PEG4SH, 100 U/ml, and 0.5 mM H_2O_2.

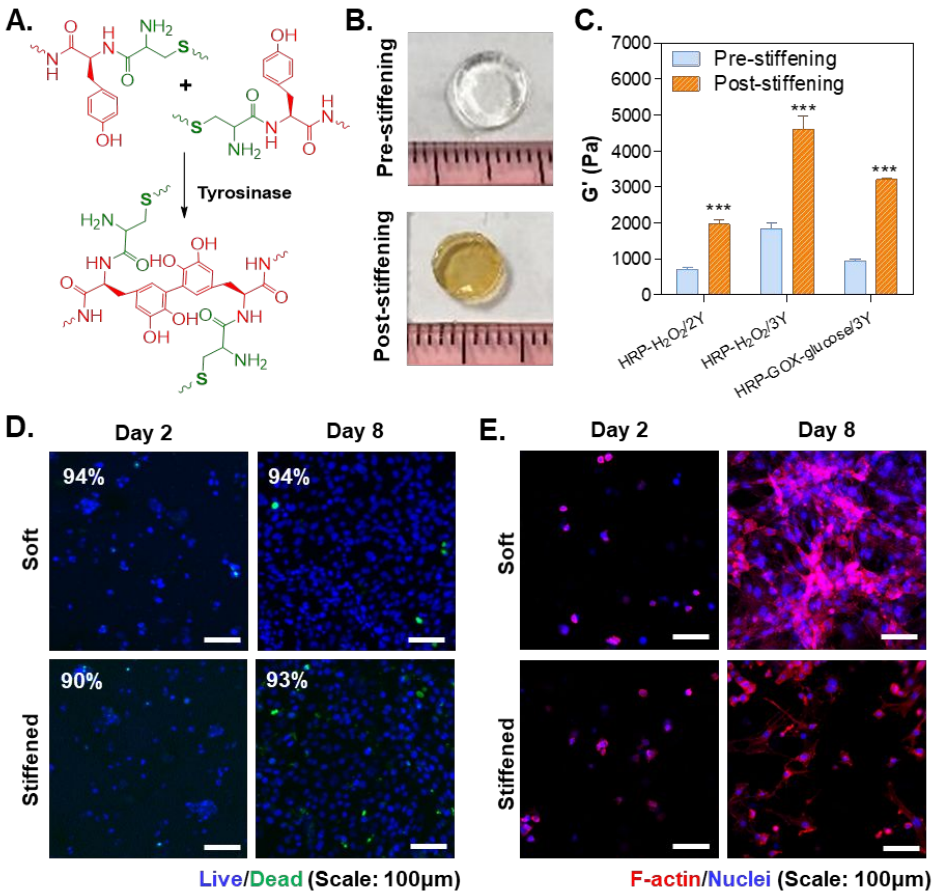

Figure 6. Orthogonal enzymatic reactions for crosslinking and dynamic stiffening of PEG-peptide hydrogels. (A) Schematic of MT-induced post-gelation dynamic crosslinking. (B) Photographs of enzymatically crosslinked PEG-peptide (2.5 wt% PEG8NB and KCYGGYGGYCK (3Y) thiol-norbornene hydrogels pre- and post-stiffening. Gel crosslinking was initiated by 1 U/mL HRP, 10 U/mL GOX, and 10mM glucose. Stiffening was induced by incubating the swollen gels in PBS containing 1 kU/mL MT. (C) Shear moduli of hydrogels pre- and post-stiffening. HRP = 1 u/ml, HRP/H_2O_2 hydrogels were made with 3 wt% PEG8NB, while HRP/GOX-glucose were made with 2.5 wt% PEG8NB (n=3, Mean ± SEM, ***p<0.001). (D) Live/dead staining images 48 hrs after encapsulation. Stiffened group were treated with 1 kU/ml MT for 6 hours on day 1. Hydrogels were made with 3 wt% PEG8NB-KCYGGYGGYCK (1 U/ml HRP, 0.5 mM H_2O_2, G' ~ 1,500 Pa). (E) F-actin and nuclei staining of NIH/3T3 fibroblasts encapsulated in soft or MT-stiffened gels.

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