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Enzymatic Cross-Linking of Dynamic Thiol-Norbornene Click Hydrogels

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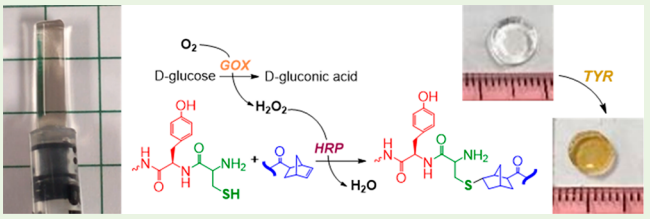
ABSTRACT: Enzyme-mediated in situ forming hydrogels are attractive for many biomedical applications because gelation afforded by enzymatic reactions can be readily controlled not only by tuning macromer compositions, but also by adjusting enzyme kinetics. For example, horseradish peroxidase (HRP) has been used extensively for in situ cross-linking of macromers containing hydroxyl-phenol groups. The use of HRP to initiate 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 the 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 cross-linking. 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) was used as a cross-linker for forming enzymatically and orthogonally polymerized hydrogel. For HRP-initiated PEG-peptide hydrogel cross-linking, gelation efficiency was significantly improved via adding tyrosine residues on the peptide cross-linkers. Interestingly, these additional tyrosine residues did not form permanent dityrosine cross-links following HRP-induced gelation. As a result, they remained available for tyrosinase-mediated secondary cross-linking, which dynamically increased hydrogel stiffness. In addition to material characterizations, we also found that both PEG- and gelatin-based hydrogels exhibited excellent cytocompatibility for dynamic 3D cell culture. The enzymatic thiol-norbornene gelation scheme presented here offers a new cross-linking mechanism for preparing modularly and dynamically cross-linked hydrogels.



KEYWORDS: *dynamic hydrogels, horseradish peroxidase, glucose oxidase, thiol-norbornene click chemistry*

1. INTRODUCTION

Hydrogels prepared from orthogonal cross-linking methods have tremendous potential in drug delivery and tissue engineering applications.[1−3] In particular, thiol-norbornene click reaction is advantageous in hydrogel cross-linking 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 cross-linking, 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 at any given sizes and shapes without sacrificing modularity of the cross-linking.

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 cross-linking). To this end, horseradish peroxidase (HRP) has emerged as a highly useful enzyme for in situ cross-linking of hydroxyl-phenol (e.g., hydroxyphenyl-acetic acid (HPA), tyramine, or tyrosine) or vinyl-modified polymers into hydrogels.[23−25] HRP initiates hydrogel cross-linking 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)

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and glucose).[26] For example, Kim et al. used HRP/GOX initiated cross-linking 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 cross-linking 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 cross-linking 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 cross-linking.[30] As demonstrated by Zavada et al., PEG diallyl ether (PEGDAE) and ethoxylated trimethylolpropane tri(3-mercaptopropionate) (ETTMP) can be successfully cross-linked 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 min 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 cross-linking of modular and dynamic thiol-norbornene hydrogels under ambient and aqueous conditions.

In this contribution, we present the use of HRP to initiate cross-linking of modular thiol-norbornene hydrogels. Differing from the cross-linking of hydroxyl-phenyl (e.g., tyramine) containing macromers into hydrogels, HPR-initiated thiol-norbornene hydrogelation exhibits characteristic modularity in hydrogel cross-linking. Specifically, we used 8-arm PEG-norbornene (PEG8NB) or gelatin-norbornene (GelNB) as the norbornene-modified macromer for cross-linking with multifunctional thiol (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 cross-linking efficiency and postgelation dynamic stiffening of PEG-peptide hydrogels. Similar to other HRP-based hydrogel cross-linking, 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 (0.1 vol %) of TFA as the starting solvent mobile phase. A linear gradient of acetonitrile was used to separate the products through a semiprep 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 cross-linked 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 s. The precursor solution was immediately pipetted in between two glass slides separated by 1 mm-thick spacers. Gelation occurred within 5 min at room temperature. The hydrogels cross-linked 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 s before pipetted in a Teflon mold with 8 mm diameter cavities. Hydrogel discs were obtained after 5 min of gelation.

To stiffen hydrogels using mushroom tyrosinase (MT), we cross-linked PEG8NB hydrogels by tyrosine-containing peptide (thiol to norbornene ratio was fixed at 1). Prior to MT-mediated stiffening, hydrogels were swollen in PBS for 24 h to wash off un-cross-linked species. To induce dynamic stiffening, hydrogels were submerged in 1 kU/mL MT for 6 h. Afterward, MT was removed via swelling hydrogels in PBS for 24 h, 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 cross-linked 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 cross-linkers, HRP, H_2O_2 (or GOX and glucose) were mixed and vortexed for 5 s. 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

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immediately after mixing and at intervals of every 2 min 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, and H_2O_2 at different thiol to norbornene ratios (keeping PEGNB concentration constant at 3.5 wt %) were mixed in deuterium oxide for 10 min. 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 ($W_{first\ dried}$). The dried gels were incubated in ddH_2O at 37 °C overnight to remove uncross-linked species. Afterward, swollen weights were obtained; swollen gels were dried and weighed again to obtain the second dried weight ($W_{second\ dried}$). Gel fraction (eq 1) was determined by the ratio of the second dried weight over the first dried weight:

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

Hydrogel mass swelling ratios (q, eq 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 cross-linked 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 min. 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 Cross-Linked PEG-Peptide Hydrogels. MT was used to induce dynamic stiffening of PEG8NB-peptide hydrogels. Twenty-four hrs after cell encapsulation, the gels were incubated in 1 kU/ml MT for 6 h to induce stiffening. Afterward the enzyme was removed via swelling in culture media for 24 h. To observe the effect of matrix stiffening on cell morphology and cytoskeletal organization, we fixed and stained cell-laden hydrogels 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 10× objective, with Z-stacked of 10 slices and 10 μm per slice. Immunofluorescence images were captured at 20× 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. Although HRP has been previously used to initiate cross-linking 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 (Figure 1A). To test this hypothesis, we first mixed different combinations of PEG8NB (20 kDa), DTT, HRP, and H_2O_2 in test tubes and evaluated gelation speed using a simple tilt-test. As shown in Figure 1B,

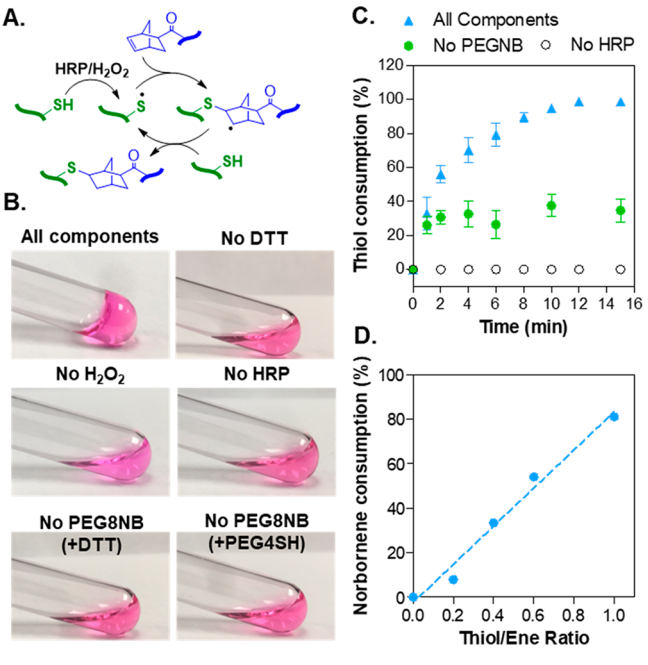

Figure 1. HRP-mediated cross-linking of thiol-norbornene click reactions. (A) Schematic of HRP/H_2O_2-induced thiyl radical generation and subsequent thiol-norbornene cross-linking. (B) Gelation tilt-test. All components: 200 U/mL HRP, 0.5 mM H_2O_2, 3.5 wt % PEG8NB, and 14 mM DTT. (C) Thiol consumption 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).

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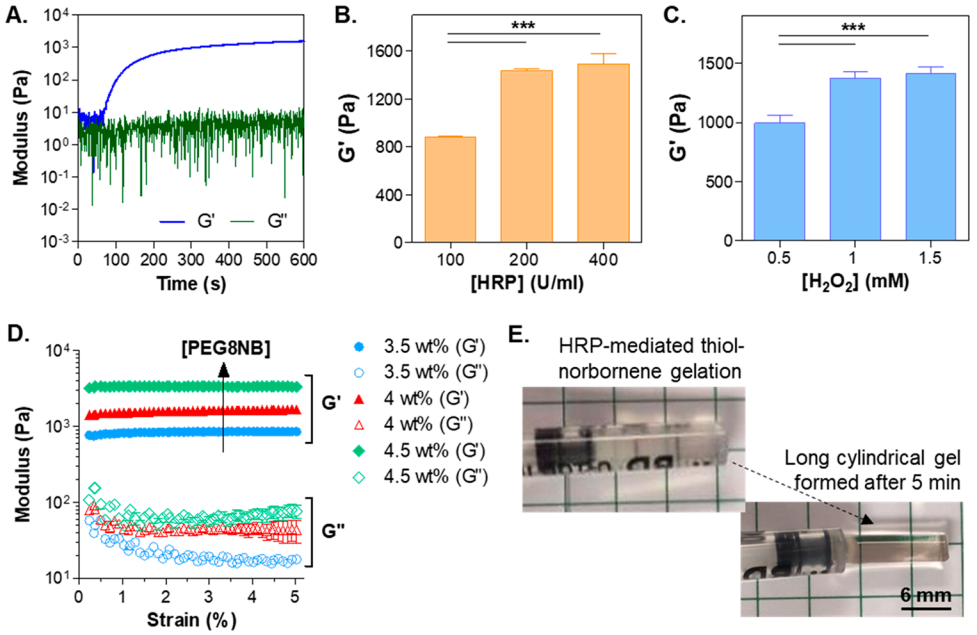

Figure 2. Characterization of HRP-mediated thiol-norbornene hydrogelation. (A) In situ rheometry of HRP-initiated thiol-norbornene gelation (all components: 3.5 wt % PEG8NB, 14 mM DTT, 200 U/ml HRP, 0.5 mM H_2O_2). Effect of (B) HRP concentrations and (C) H_2O_2 concentration on shear moduli of PEG8NB-DTT 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-cross-linked 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).

gelation occurred within a few minutes only when all four components (PEG8NB, DTT, HRP, H_2O_2) were included. The cross-linking was clearly triggered by enzymatic reaction because gelation did not occur without HPR or H_2O_2. Furthermore, gelation was not due to norbornene homopolymerization (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 complete gelation while permitting solution-based assay. Note that not all formulations formed hydrogels, especially at early reaction time points (in thiol consumption test) and low thiol/ene ratios (in norbornene consumption test). Figure 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 (Figure 1C. No HRP group), suggesting that uncatalyzed disulfide bond formation was not a concern within the 15 min reaction time.

Using proton NMR, we analyzed chemical shifts of norbornene group (Figure 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 toward thiol groups (Figure 1C, no PEGNB group). Because 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, Figure 2A).[16] Enzymatic cross-linking of DTT and PEG8NB into hydrogels required relatively low concentration of HRP (∼100 U/mL, Figure 2B) and H_2O_2 (∼0.5 mM, Figure 2C). Through adjusting PEG8NB macromer contents (i.e., 3.5, 4, and 4.5 wt %), gel cross-linking density and modulus ($G' \approx 1$ to 3 kPa, Figure 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 (Figure 2E). Thiol-norbornene hydrogels cross-linked by the HRP/H_2O_2 system also appeared to maintain good fidelity of the syringe mold. In principle, this enzymatic cross-linking 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 Cross-Linking of PEG-Peptide Hydrogels. After demonstrating the feasibility of HRP-initiated thiol-norbornene hydrogel cross-linking using DTT as a cross-linker, we asked if bis-cysteine peptide linkers can be used to form PEG-peptide hydrogels. Peptide cross-linkers 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

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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). Although 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 min (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 cross-linking 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 dithiol cross-links formation.[46] Figure 3A illustrates the potential mechanism of

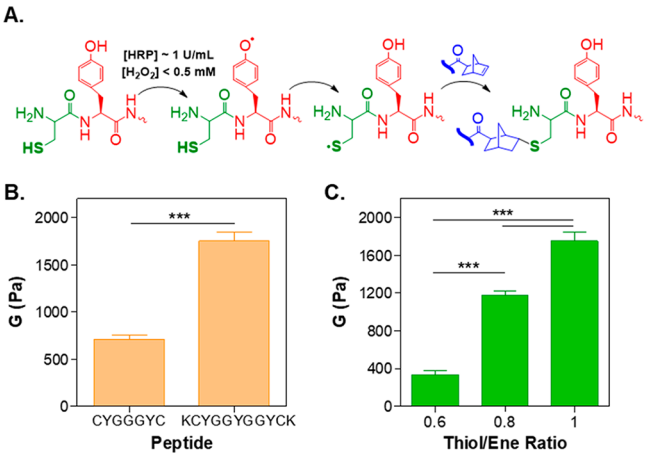

Figure 3. Effect of tyrosine residue on HRP-mediated cross-linking of thiol-norbornene PEG-peptide hydrogels. (A) Proposed schematic of thiyl radical generation via tyrosine residues. Effect of (B) tyrosine concentration and (C) thiol to norbornene ratio on the shear moduli of hydrogels (n = 3, mean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

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 dityrosine cross-linking or norbornene-tyrosine reaction, as control experiments using a cysteine-free peptide (i.e., KGYGGYGGYGK) did not yield hydrogel cross-linking (data not shown). In order to obtain gelation in a more manageable time frame, 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 cross-linked within 10 min and shear moduli of these hydrogels (as characterized by strain-sweep rheometry) were higher when using peptide linker containing more tyrosine residues (Figure 3B). Additional gelation tests using off-stoichiometric ratios of thiol/norbornene led to gels with tunable moduli (Figure 3C), a typical characteristic of modularly cross-linked 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 (Figure 4A). Gelation of PEG8NB and CYGGGYC peptide using this scheme was successful and the degree of hydrogel cross-linking was dose-dependently and almost linearly tuned in the presence of 1 to 10 mM glucose (Figure 4B). However, when glucose concentration was raised to above 10 mM, hydrogels were formed with lower moduli, suggesting a reduced cross-linking 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 cross-linking efficiency of enzymatic thiol-norbornene hydrogels to that of UV cross-linked gels with the same macromer compositions. In terms of gel fraction (Figure 4C), hydrogels cross-linked by the HRP/GOX/glucose system (87.4 ± 2.1) were comparable to that of UV cross-linked gels (87.8 ± 2.4), suggesting high cross-linking efficiency of the HRP-mediated thiol-norbornene reactions. However, when gels were cross-linked 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 cross-linking efficiency in gels formed by the HRP/H_2O_2 system when comparing with gels formed by HRP/GOX tandem enzymatic reactions (i.e., higher q and lower G' in HRP/H_2O_2 system Figure 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 postgelation (0−24 h). Hydrogels were placed in PBS immediately after gelation. As shown in Figure 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 1 h postgelation. After 5 h, O_2 content in hydrogel increased to ~6%. Oxygen level in the hydrogel returned to almost normoxia after 24 h, 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 cross-linking. 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-cross-linked 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 Cross-Linked Gelatin-Based Thiol-Norbornene Hydrogels. To demonstrate the versatility of

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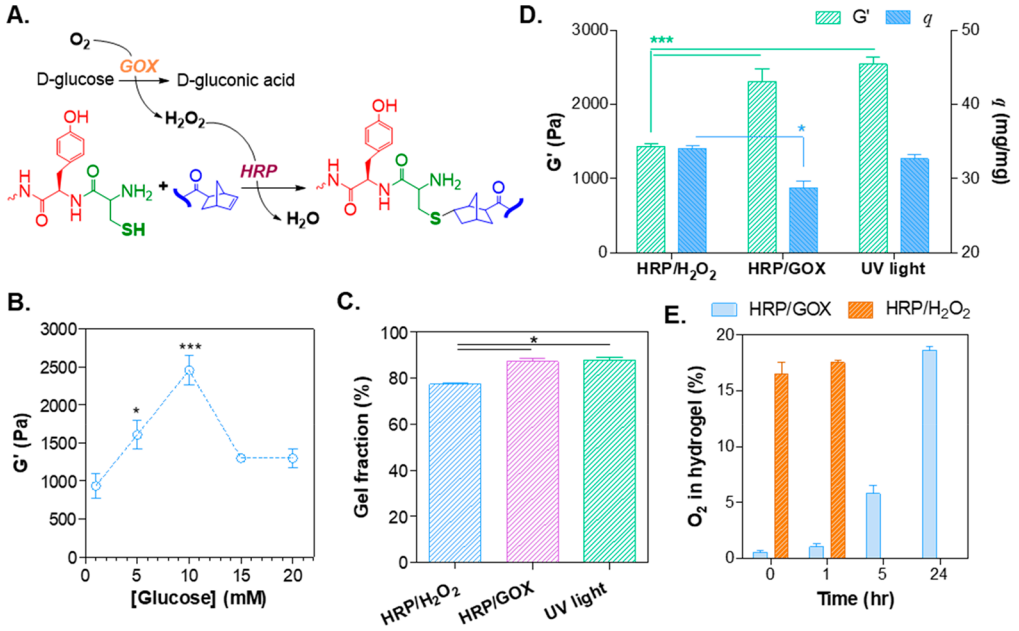

Figure 4. Thiol-norbornene gelation initiated by tandem enzymatic reactions. (A) Schematic of thiol-norbornene hydrogel formation via GOX and HRP-mediated cross-linking. (B) Effect of glucose concentration on shear moduli of dual enzyme-cross-linked thiol-norbornene hydrogels (1 U/mL HRP, 10 U/ml GOX, 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-cross-linked gels were prepared with 3.5 wt % PEG8NB and 14 mM 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 min. (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 h because O_2 levels were close to normoxia in both measurements (All experiments: $n = 3$, mean ± SEM, $*p < 0.05$ $***p < 0.001$).

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 cross-linked into hydrogels with highly tunable stiffness. Gel moduli were controlled by adjusting either gelatin content (Figure 5A) or thiol to norbornene ratio (Figure 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 Figure 4C demonstrated an extremely low oxygen level within the first hour of gelation (<1%), which might not be ideal for cell 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 cross-link 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

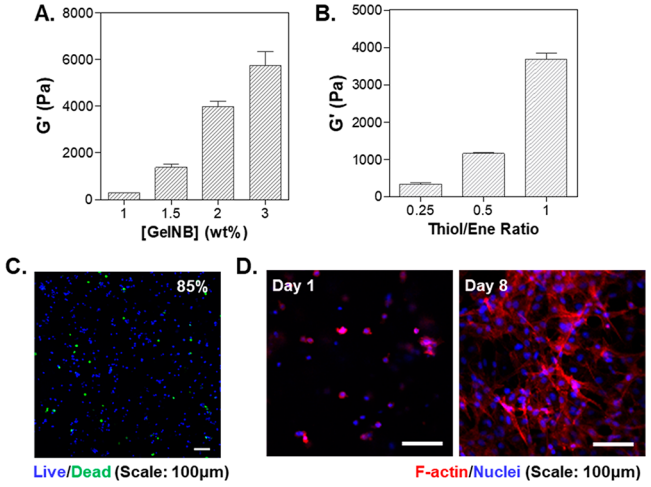

Figure 5. Cytocompatibility of HRP-mediated GelNB-PEG4SH hydrogels. Effect of (A) gelatin content and (B) thiol-to-norbornene ratio on shear moduli of GelNB-PEG4SH hydrogels (100 U/mL HRP, 10 U/mL GOX, 10 mM glucose. $n \geq 3$, mean ± SEM). (C) Live/Dead staining images of NIH/3T3 fibroblasts cultured in GelNB-PEG4SH hydrogels 24 h 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.

HRP.[58] By quantifying the numbers of live and dead cells using live/dead staining and confocal imaging, we found that enzymatically cross-linked GelNB-PEG4SH hydrogels displayed good cytocompatibility with above 85% of the encapsulated cells remained alive after 24 h of encapsulation

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(Figure 5C). Moreover, encapsulated cells proliferated significantly after 8 days of culture (Figure 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 cross-linked 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 Cross-Linked PEG-Peptide Hydrogels. Figure 3 has clearly shown that tyrosine residues on bis-cysteine peptide linker facilitate thiyl radical generation and thiol-norbornene hydrogel cross-linking. 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 (Figure 2A). We then asked if hydroxyl side group on tyrosine residues can be exploited for mushroom tyrosinase (MT)-mediated postgelation dynamic stiffening (Figure 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 cross-linked thiol-norbornene PEG-peptide hydrogels could indeed be dynamically stiffened using exogenously added MT (incubation for 6 h. Figure 6B, C), 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 exhibited characteristic yellow/brown color as shown in Figure 6B.[33,39,40] These additional DOPA dimers resulted in higher gel cross-linking density and shear modulus (Figure 6C). Enzymatic stiffening occurred in hydrogels cross-linked by peptides with two or three tyrosine residues, as well as gels cross-linked 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]

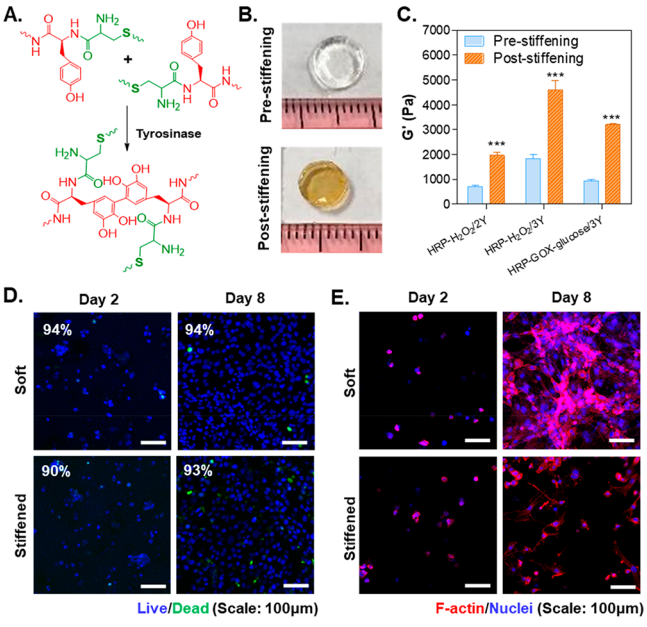

Figure 6. Orthogonal enzymatic reactions for cross-linking and dynamic stiffening of PEG-peptide hydrogels. (A) Schematic of MT-induced postgelation dynamic cross-linking. (B) Photographs of enzymatically cross-linked PEG-peptide (2.5 wt % PEG8NB and KCYGGYGGYCK (3Y) thiol-norbornene hydrogels pre- and poststiffening. Gel cross-linking was initiated by 1 U/mL HRP, 10 U/mL GOX, and 10 mM glucose. Stiffening was induced by incubating the swollen gels in PBS containing 1 kU/mL MT. (C) Shear moduli of hydrogels pre- and poststiffening. HRP = 1 u/mL, HRP/H_2O_2 hydrogels were made with 3 wt % PEG8NB, whereas HRP/GOX-glucose was made with 2.5 wt % PEG8NB (n = 3, mean ± SEM, ***p < 0.001). (D) Live/dead staining images 48 h after encapsulation. Stiffened groups were treated with 1 kU/mL MT for 6 h on day 1. Hydrogels were made with 3 wt % PEG8NB-KCYGGYGGYCK (1 U/mL HRP, 0.5 mM H_2O_2, $G' \approx 1500$ Pa). (E) F-actin and nuclei staining of NIH/3T3 fibroblasts encapsulated in soft or MT-stiffened gels.

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 cross-linkers susceptible to 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 h). Live/dead staining results showed that both groups displayed good cytocompatibility, with 90% or more cell survived the enzymatic encapsulation process. (Figure 6D). On day 1, the cells exhibited rounded morphology in both groups (Figure 6E). However, after 8 days of culturing, F-actin staining results showed distinct differences in cell morphology between the soft and stiffened hydrogels. While cells cultured in the nontreated (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 (Figure 5D). On the other hand, although 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 (Figure 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 non-degradable "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 dityrosine cross-links 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 increase in cell density were seen in both groups after 8 days. Cell density increase indicated that

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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 Figure 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 (Figure 6) have confirmed that the tyrosine residues not only facilitated HRP-mediated thiol-norbornene gel cross-linking, but also remained 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 (Figure 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 cross-linked enzymatically via HRP. Uniquely, the PEG8NB-peptide hydrogel system exhibited additional dynamic and enzymatic stiffening feature that has not been reported in other HRP-cross-linked 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 cross-linked thiol-norbornene hydrogels address the limitation of light attenuation issue in photopolymerization while retaining the modularity of the thiol-norbornene cross-linking. The enzyme-mediated cross-linking 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 cross-linked 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 cross-linking 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 cross-link 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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Notes

The authors declare no competing financial interest.

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