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Red Light Activation of Tetrazine–Norbornene Conjugation for Bioorthogonal Polymer Cross-Linking across Tissue

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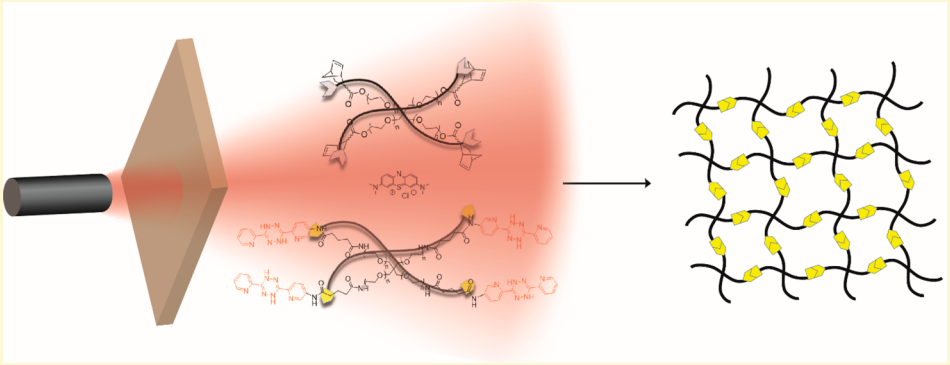
Supporting Information



ABSTRACT: Light-triggered chemistry has been used extensively in polymer cross-linking for the preparation of hydrogels. However, most photoclick reactions rely on the use of UV light that is easily absorbed by and damaging to cells and tissue. We report the utilization of red light-catalyzed oxidation of dihydrogen tetrazine for activation of tetrazine–norbornene inverse electron-demand Diels–Alder conjugation. The activation takes place rapidly under physiological conditions and was applied in polymer conjugation and cross-linking to form hydrogels. Gelation was demonstrated to occur behind a dermal tissue model with a thickness of 1 cm, and the cross-linking process is nontoxic to mesenchymal stem cells, allowing encapsulation of hMSC within the gel matrix.

INTRODUCTION

Phototriggered chemistry has long been utilized in materials science because of the ability of light to provide precise spatial and temporal control over chemical processes.[1,2] Many of the photochemical reactions fulfill the requirements of click chemistry, which are fast and efficient and proceed under mild conditions, and have found their uses in polymer science, including polymer conjugation and cross-linking to form polymeric biomaterials.[3,4] Most photoclick reactions, however, utilize ultraviolet (UV) or short wavelength visible (vis) light, each of which is strongly absorbed by skin and tissue,[1−6] limiting their use in clinical applications. In addition, high-energy UV light can be damaging to biological species, including bioactive peptides, enzymes, and DNA. Development of chemistry responding to photons in the 600–900 nm wavelength, the so-called optical therapeutic window in which there is minimal absorption of light by water and tissue,[7] is the key for the transition of light-induced click reactions into biomedical fields.

Several strategies have been developed to convert low-energy long wavelength visible light into energy suitable for the initiation of certain coupling reactions. For example, two-photon near-infrared (near-IR) light generated by a 700 nm femtosecond pulsed laser has been employed to trigger tetrazole–ene cycloaddition for efficient live cell imaging.[8] However, this method not only requires high-intensity pulsed lasers but also provides very small focal volumes, making it impractical for many applications. The Lawrence group reported the preparation of a vitamin B_{12} derivative photoinitiator that can be activated by red light (660 nm) to trigger free radical-induced cross-linking of poly(ethylene glycol) bisacrylate.[9] In a recent work, Barner-Kowollik and co-workers utilized upconversion nanoparticles (UCNPs) to convert single-photon NIR light into internal UV light to activate tetrazole–ene cycloaddition and demonstrated efficient polymer end group modification and coupling by light activation underneath a tissue spacer.[10] While the reaction was reported not to affect the bioreactivity of a biotin derivative, the cytotoxicity effects of the UCNP and the in situ-generated UV light on living cells were not examined.

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Within the bioorthogonal chemistry toolbox, the inverse electron-demand Diels–Alder (IEDDA) addition of tetrazines to alkene or alkyne dienophiles has gained attention because of the extremely high reactivity of the conjugation process.[11] The tetrazine–norbornene (Tz–Nb) additions have been employed in polymer conjugation and coupling,[12] cellular imaging,[13] DNA ligation,[14] and preparation of bioorthogonally cross-linked hydrogels.[15−17] While a range of norbornene compounds with conjugation handles such as amine or carboxylic are commercially available, tetrazine compounds with such reactive groups are generally synthesized on demand with overall yields in the range of 20–30%.[18] Tetrazine compounds with carboxylate substitution have high reactivity toward dienophiles but are not stable in aqueous media.[18] Other reactive tetrazines with enhanced stability, via selected substitution on the aromatic groups, have been successfully applied for molecular ligation under physiological conditions.[19,20] Trout et al. reported that dipyridyl tetrazine retained 98 and 83% fidelity in a PBS solution at 25 °C after 2 and 24 h, respectively.[19]

Tz compounds are generally prepared by oxidation of dihydrotetrazine (dHTz) precursors, and recent developments in tetrazine ligation have introduced very mild oxidation conditions such as photocatalytic or enzymatic activation for the preparation of small Tz molecules.[19] We postulated that the dHTz group can be incorporated into polymers for subsequent oxidation by long wavelength visible light to facilitate the Tz–Nb addition in situ. Here, we report the application of one-photon red light for catalytic activation of IEDDA under physiological conditions for facile polymer conjugation. The chemistry was applied in polymer cross-linking to prepare hydrogels triggered by light behind a layer of biomimetic phantom tissue, and the bio-orthogonality of the reaction was assessed.

EXPERIMENTAL SECTION

General Considerations. Four-arm PEG (molar mass of 10000 g mol^{-1}) was purchased from Jenkem Technology. All other chemicals were purchased from Sigma-Aldrich and used as received. 5-Oxo-5-({6-[6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl]pyridin-3-yl}-amino)pentanoic acid [compound **S2** (Supporting Information)] was prepared following a previously published procedure with some modification,[12] and MeO-PEG-Nb, MeO-PEG-SH, and four-arm PEG-Nb were prepared by esterification of the corresponding polymers with norbornene carboxylic acid (*endo*/*exo* mixture).[21] Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance III 400 instrument with a 5 mm broadband autotunable probe with Z-gradients at 293 K. Chemical shifts are reported as δ in parts per million and referenced to the chemical shift of the residual solvent resonances ($CDCl_3$, ^1H, δ = 7.26 ppm; $(CD_3)_2SO$, ^1H, δ = 2.50 ppm).

Synthesis of Four-Arm PEG-dHTz. Compound **S2** (0.44 g, 1.2 mmol) was dissolved in DMF (10 mL) followed by addition of EDC (0.36 g, 1.8 mmol) and NHS (0.2 g, 1.8 mmol). The solution was stirred at ambient temperature for 30 min, and four-arm PEG-NH_2 (2 g, 0.2 mmol) was added. The solution was stirred for an additional 20 h and concentrated in vacuo. The residue was taken up in CH_2Cl_2 (20 mL), washed with water (10 mL) and brine (10 mL), dried, concentrated, and precipitated in Et_2O twice to yield the product as an orange solid (1.7 g, ∼84% yield): ^1H NMR ($CDCl_3$, 400 MHz) δ 9.42 (s), 8.72 (d, $^2J_{HH}$ = 2.5 Hz), 8.59 (dt, $^2J_{HH}$ = 4.7 Hz, $^3J_{HH}$ = 5.3, 1.4 Hz), 8.48 (s), 8.25 (dd, $^2J_{HH}$ = 8.8, 2.6 Hz, 1H), 8.05, (d, $^2J_{HH}$ = 6.6 Hz). 7.92 (d, $^2J_{HH}$ = 7.8 Hz), 7.38 (t, $^3J_{HH}$ = 7.4 Hz), 6.71 (t, $^3J_{HH}$ = 5.3 Hz), 3.6–3.7 (m), 3.38 (s), 2.49 (t, $^3J_{HH}$ = 7.4 Hz), 2.21 (t, $^3J_{HH}$ = 7.3 Hz, overlapped with water), 2.05 (m).

Preparation of Four-Arm PEG-Tz by Catalytic Red Light Activation. Four-arm PEG-dHTz (50 mg, 4.9 μmol) was dissolved in a PBS solution (2 mL) containing methylene blue (1 mg mL^{-1}), and the solution was irradiated with red light (wavelength of 625 nm) while being stirred for 10 min. The solution was dialyzed against excess water for 20 h using dialysis tubing with a molecular weight cutoff (MWCO) of 3.5 kDa and freeze-dried to give the product as a pink solid (48 mg, 96% yield): ^1H NMR ($CDCl_3$, 400 MHz) δ 9.71 (s), 9.03 (d, $^2J_{HH}$ = 2.56 Hz), 9.0 (m), 8.76 (m), 8.62 (dd, $^2J_{HH}$ = 2.29, 5.92 Hz), 8.02 (dt, $^3J_{HH}$ = 6.09 Hz, $^2J_{HH}$ = 1.85 Hz), 7.8 (m), 6.8 (bs), 3.6–3.7 (m), 3.38 (s), 2.49 (t, $^3J_{HH}$ = 7.4 Hz), 2.21 (t, $^3J_{HH}$ = 7.3 Hz), 2.05 (m, overlapped with water).

Size Exclusion Chromatography (SEC). SEC analyses of polymer samples were performed using a Shimadzu modular system comprising a DGU-20A3R degasser unit, a SIL-20A HT autosampler, a 10.0 μm bead size guard column (50 mm × 7.8 mm) followed by three KF-805L columns (300 mm × 8 mm, bead size of 10 μm, pore size maximum of 5000 Å), and an RID-10A differential refractive index detector. The temperature of the columns was maintained at 40 °C using a CTO-20A oven. The eluent was dimethylacetamide (CHROMASOLV Plus for high-performance liquid chromatography), and the flow rate was kept at 1.0 mL min^{-1} using an LC-20AD pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to 2 × 10^6 g mol^{-1}. Polymer solutions at approximately 2 mg mL^{-1} were prepared and filtered through 0.45 μm PTFE filters before injection.

UV–Vis Spectroscopy. UV–vis measurements were taken using a Cary spectrometer. A quartz cuvette with a transparent window above 220 nm was used, and the recorded absorbance values were corrected for background and solvent absorbance. In a typical kinetic study, PEG-dHTz was dissolved in a PBS solution (pH 7.4) containing methylene blue (5 μM) at a concentration of 10 mM and the solution was subjected to irradiation with red light (625 nm wavelength, 10 mW cm^{-1} intensity) generated by a light-emitting diode light source (WheeLED, Mightex) equipped with a liquid light guide. At certain time intervals, a small aliquot was withdrawn and diluted to a polymer concentration of ∼0.1 mM for UV–vis analysis.

Kinetic Study of the Photocatalytic Oxidation by ^1H NMR. Four-arm PEG-dHTz was dissolved in a PBS solution (pH 7.4) containing methylene blue (5 μM) at a concentration similar to the concentration used in the hydrogel preparation (5 wt %, 5 mM). The solution was placed in a glass vial with a diameter of 12 mm and irradiated with red light (10 mW cm^{-1}) from top to bottom without being stirred to simulate the static condition of hydrogel synthesis. After predetermined time periods, the solution was freeze-dried and redissolved in $CDCl_3$ for NMR analysis. The conversion of the dHTz group was assessed by integration of the chemical shift at 8.51 ppm, which corresponds to the protons of the dHTz ring, compared to the chemical shift at 6.74 ppm, which corresponds to the amide proton linked to the PEG chain that does not change during the photocatalytic oxidation process. Two other sets of kinetic studies were undertaken with (i) the same solution under ambient light in the laboratory and (ii) the polymer solution without methylene blue but with red light irradiation.

The photocatalytic oxidation of dHTz follows first-order reaction kinetics; thus, the rate constant (k_{rxn}) can be determined using the following equation:

$$\ln \frac{C}{C_o} = -k_{rxn}t \tag{1}$$

The quantum yield of the photocatalytic oxidation is the ratio between the reaction rate and the rate of light absorption by methylene blue and can be calculated as

$$\Phi = \frac{N_A h c \varepsilon k_{rxn}}{\varepsilon \lambda I} \tag{2}$$

where N_A is Avogadro's number (6.022 × 10^{-23} mol^{-1}), h is Planck's constant (6.626 × 10^{-34} J s), c is the speed of light (3 × 10^8 m s^{-1}), ε

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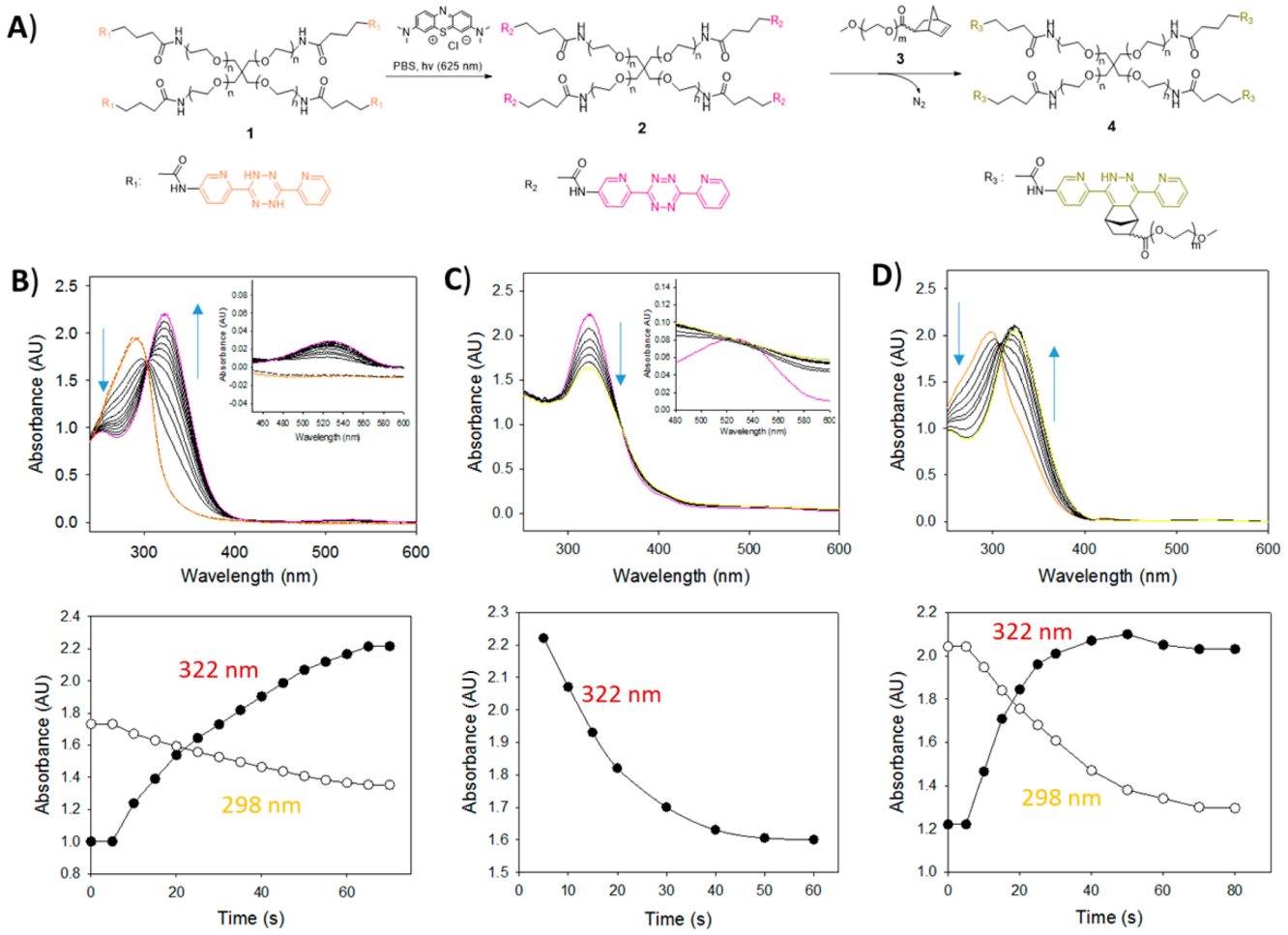

Figure 1. (A) Chemical structure of four-arm PEG-dHTz and its oxidation by red light and methylene blue to form PEG-Tz, which reacts with MeO-PEG-Nb to form the conjugated polymer product via a dihydropyridazine link. (B) Polymer **1** has a maximal absorbance at 298 nm in a PBS solution (pH 7.4) that decreases with red light treatment (625 nm, 10 mW cm^{-1}) in the presence of methylene blue (5 μM). At the same time, the formation of a peak with maximal absorbance at 322 nm and a very low intensity peak with maximal absorbance at 525 nm was observed; this change was not seen in the same solution without light irradiation (dashed line). (C) Upon addition of MeO-PEG-Nb, the consumption of the absorbance at 322 nm was observed. The maximal absorbance of the dihydropyridazine product is similar to that of the tetrazine group. The consumption of the peak at 525 nm could not be followed because the MeO-PEG-Nb spectrum also has a peak with maximal absorbance at 533 nm (see Figure S12). (D) Time course of the one-pot reaction of **1** and **3** in a solution containing methylene blue (5 μM) under red light irradiation (625 nm, 10 mW cm^{-1}) monitored by UV−vis spectroscopy.

is the molar absorptivity of methylene blue at 625 nm (40117 cm^{-1} M^{-1}), λ is the representative wavelength of the light source (625 nm), and I is the average intensity of light across the sample.

Because methylene blue was not consumed during the reaction and both Tz and dHTz groups do not absorb light at 625 nm, the average intensity can be calculated using the Beer−Lambert law assuming attenuation is minimal and constant:

$$\log\left(\frac{I_0}{I}\right) = \varepsilon C_{MB} l \tag{3}$$

where C_{MB} is the concentration of methylene blue and l is the thickness of the polymer solution.

Rheology Analysis. Rheology studies were performed using an Anton Paar Physica rheometer with a plate−plate configuration with the lower plate made of quartz and the upper plate made of stainless steel with a diameter of 12 mm. The liquid light guild was connected below the lower plate. In a typical experiment, a solution (50 μL) containing a mixture of the polymer precursor was placed on the lower plate, the upper plate was lowered to a measurement gap of 0.3 mm, and the test was started by applying a 1% strain with a frequency of 1 Hz on the sample. The light was turned on approximately 100−200 s after the start of the rheology measurement.

Rheology Measurement of Gelation behind Phantom Tissues. The dermal tissue model was first prepared by making solutions containing predetermined intralipid and melanin. Agarose was then added to form a 1 wt % solution, and the mixture was heated at 60 °C until a homogeneous solution was obtained. The solution was then allowed to cool in a mold that is made from a 5 mL plastic syringe capped with Parafilm at one end. The resultant gels have a diameter of 3 cm and a thickness of 1 cm. In the experiment involving phantom skin tissue, the skin mimic was taped at the edge of the gels underneath the quartz plate.

Three-Dimensional (3D) Stem Cell Encapsulation. Cell culture tests were performed with commercial hMSCs (Lonza). Cells were cultured on tissue culture flasks by following the manufacturers' instructions and then trypsinized with TrypLE Express to detach the cells from the culture surfaces. The cells were centrifuged for 3 min at 0.3 g, and the supernatant was discarded. Precursor solutions of PEG-Nb and PEG-dHTz were weighed out in stoichiometric proportions and made up in complete culture medium to give a final concentration of 10% (w/v) polymer. The cell suspension was then added (cell seeding concentration of ~50000 cells per 100 μL of polymer solution) followed by an aliquot of methylene blue solution to reach a final concentration of 5 μM). The solution was mixed using a pipet to

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ensure homogeneity. The solution was then irradiated with red light (625 nm) for 10 min. The cell-laden gel was then rinsed twice with hMSC medium and incubated with serum-containing medium overnight. Twenty-four hours after seeding, gel samples were stained with the live/dead solution consisting of 4 μM ethidium homodimer-1 and 2 μM calcein AM (Life Technologies), which were made up in D-PBS. The medium was removed from the wells, and the gels were incubated in the live/dead solution (100 μL per well) for 30 min at 37 °C and 5% CO_2. The cells were rinsed once with PBS and imaged under either a Nikon Eclipse Ti–Li with Spot Xplorer camera or a Nikon Eclipse LV 100ND with Nikon DS-Ri2 camera.

RESULTS AND DISCUSSION

We first explored light catalytic activation of a four-arm poly(ethylene glycol) (PEG) containing a dHTz end group (Figure 1A). PEG is commercially available in a wide range of molecular masses and architectures and is commonly used in protein conjugation,[22] micelle preparation for drug delivery,[23] and hydrogels for tissue engineering applications.[2,15,16,24,25] Thus, bioorthogonal conjugation of the PEG polymer is of significant utility in materials science. The dHTz-COOH was first synthesized and conjugated to a four-arm PEG amine via carbodiimide coupling to obtain dHTz-functionalized polymer **1** (see the Supporting Information). Notably, the carbodiimide coupling of dHTz-COOH is highly efficient with quantitative conversion (99%) of the amine end group, as seen by integration of the ^{1}H NMR spectrum (Figure S6). In comparison, conjugation of the Tz-COOH to four-arm PEG was previously reported to achieve up to 75% conversion even with excess Tz-COOH in the reaction mixture.[16] Several tetrazine compounds were also reported to decompose in the presence of common coupling reagents such as 1-hydroxybenzotriazole and *N*,*N*′-diisopropylcarbodiimide,[26] which generally leads to a decrease in coupling efficiency.

Oxidation of the dHTz group to the Tz group at the polymer chain end by red light (625 nm, 10 mW cm^{-1}) in a PBS solution (pH 7.4) containing methylene blue (5 μM) was followed by the change in the UV–vis absorbance spectrum of the solution. As seen in Figure 1B, the rapid disappearance of the peak at 298 nm characteristic of the dHTz end group was observed, followed by the appearance of an absorbance at 322 nm corresponding to the Tz group. Upon addition of methoxy-PEG-Nb (polymer **3**), the Tz absorbance value from the UV–vis spectrum rapidly decreased (Figure 1C), indicating the consumption of the Tz group. A mixture of **1** with **3** and methylene blue in PBS (pH 7.4) also displayed the formation of the dihydropyridazine product under red light irradiation (Figure 1D) in a one-pot reaction. A similar reaction could also be observed in deionized water; however, our interest lies in the utilization of the conjugation under a biological environment (e.g., cell culture media), and therefore, PBS (pH 7.4) was consistently used in our experiments. It is noteworthy that photocatalytic oxidation does not occur in polar organic solvents such as dimethyl sulfoxide, ethanol, and acetonitrile; however, it can proceed in an aqueous/organic solvent mixture such as a water/acetonitrile mixture, providing access to conjugation of the hydrophobic component with low solubility in pure water. Methylene blue is known to generate singlet oxygen ($[^1O_2]$); however, addition of sodium azide, which is known as a $[^1O_2]$ scavenger, did not impede the reaction, which suggests that the mechanism of oxidation does not depend on the $[^1O_2]$ species.[19]

We then sought to isolate the four-arm PEG-Tz for use in further coupling via the reactive Tz group. Thus, the polymer solution after red light treatment was dialyzed to remove methylene blue and salts, followed by lyophilization to give the pure polymer product. ^{1}H NMR spectra (Figure 2A) of the

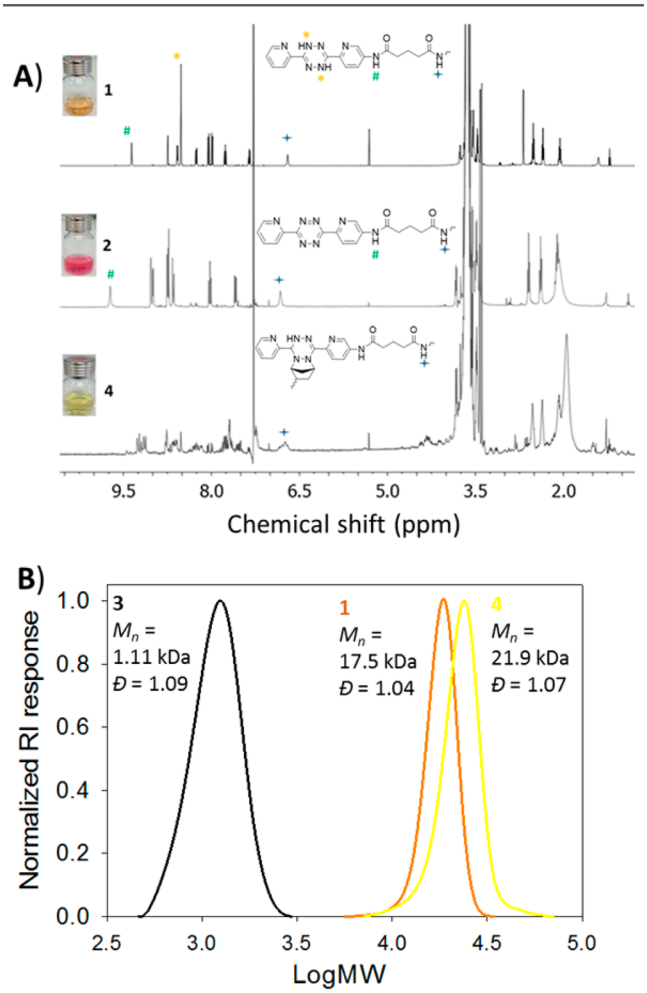

Figure 2. (A) ^{1}H NMR spectra of polymers **1**, **2**, and **4** ($CDCl_3$, 400 MHz). Polymers **2** and **4** were purified by dialysis and lyophilization. (B) SEC traces of the polymer precursors before photocatalytic oxidation and the polymer product. The calculated M_n value is higher than the M_n of the actual value (~10 kDa) of the polymer because of the polystyrene used as a standard in the molecular weight calibration curve.

polymer compounds indicate the near complete loss (98%) of the chemical shifts characteristic of the dHTz group and the appearance of the chemical shifts corresponding to the Tz group under photocatalytic oxidation conditions (see Figures S6, S7, and S11 for full assignments of the chemical shifts). We noticed a small percentage [0.5% based on ^{1}H NMR (Figure S6)] of polymer **1** being oxidized to form **2** during the amide coupling step to four-arm PEG-amine; however, polymer **1** is highly stable under refrigerator storage (at 2 °C) with no change in the NMR spectrum after 5 months.

^{1}H NMR analysis of the reaction kinetics (Figures S13 and S14) reveals a rate constant of 0.078 ± 0.003 s^{-1} and a corresponding quantum yield of 0.041 ± 0.002 under the investigated condition (methylene blue concentration of 5 μM and red light irradiation at 625 nm with an intensity of 10 mW cm^{-1}). In addition, the control experiments without either methylene blue or red light irradiation show no change in the chemical shifts of the dHTz, indicating that both components

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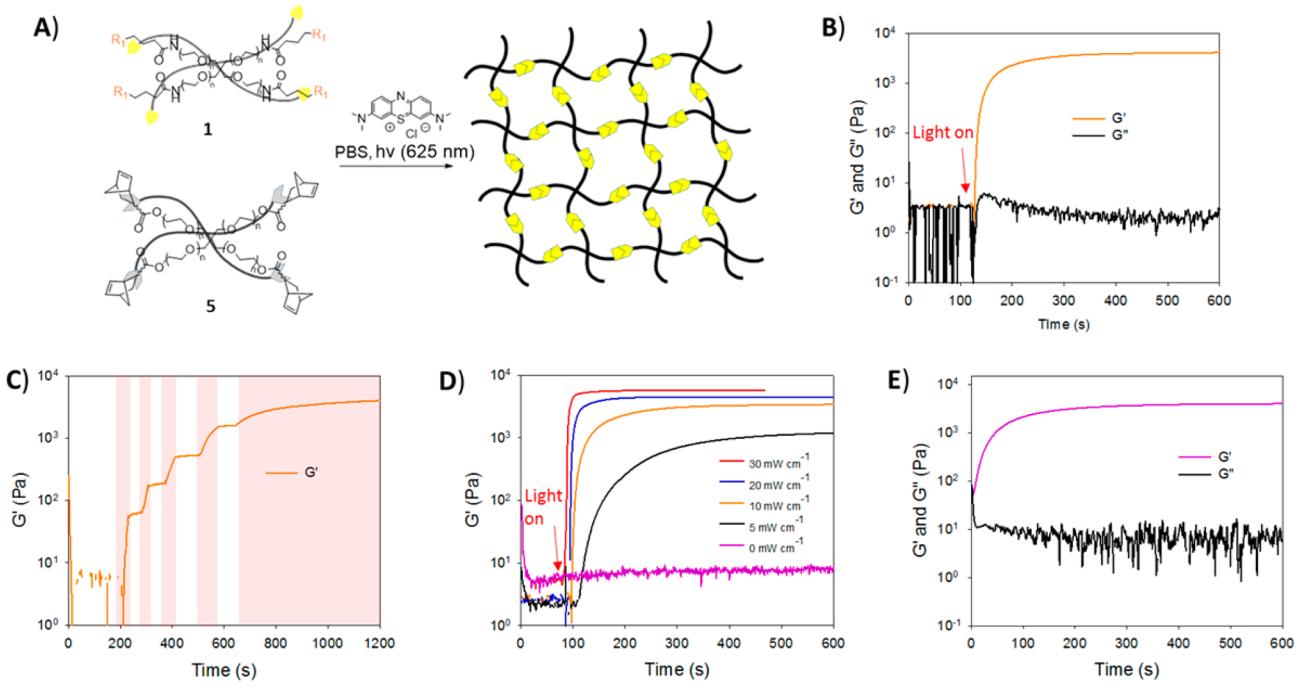

Figure 3. (A) Cross-linking scheme for the preparation of a [4+4] hydrogel network cross-linked by photocatalytic activation of IEDDA. (B) Rheological data for the cross-linking process showing the evolution of storage (G') and loss (G'') as a function of time for the polymer solution (polymers **1** and **5**) in PBS (pH 7.4) containing methylene blue (5 μM). (C) Temporal changes in G' and G'' under the effect of red light. Polymerization was seen to proceed under a period of light irradiation (highlighted area). (D) Polymerization of the polymer solution using different light intensities. (E) Evolution of G' and G'' as a function of time for the polymer system via Tz−Nb cross-linking (polymers **2** and **5**) in a PBS solution (pH 7.4). Because of the preparation time for sample loading before the rheology measurement had commenced, cross-linking may have occurred when **2** and **5** were mixed at the loading step; thus, G' was higher than G'' at time zero.

are required for the oxidation of the dHTz group. Under fume cupboard light and at ambient temperature, we observed ~34% of polymer **1** (in a PBS solution with methylene blue) being oxidized to polymer **2** after 1 h (Figure S16); this is likely due to methylene blue absorbing (with a lower molar absorptivity) in the blue light region. Purification of the polymer solutions via dialysis and lyophilization resulted in the polymers displaying colors characteristic of the end groups such as the pink color from the Tz group and the yellow color of the Tz−Nb ligation product. SEC also displays a shift to a higher molecular weight of the polymer product after the conjugation compared to those of the starting polymers, confirming the success of polymer ligation (Figure 2B).

Tetrazines in biological media have been reported to display cross reactivity with nucleophiles such as a free thiol group;[27] thus, the stability of end groups R1 and R2 were tested against a PBS solution containing cysteine (1 mM). After incubation at 37 °C for 1 h, UV−vis spectra of the compounds indicate complete reversal of the peak with a maximal absorbance at 322 nm back to the peak with a maximal absorbance at 298 nm (Figure S17), while dHTz group fidelity was retained under the same condition. This indicates that the Tz group was reduced back to the dHTz group in the presence of excess thiol. Analysis of ^{1}H NMR spectra of polymer **3** with MeO-PEG-SH before and after incubation in a PBS solution at 37 °C for 1 h further confirms the reduction of the loss of the Tz group and thiol group as well as the formation of the dHTz group (Figures S18 and S19). In a solution containing both MeO-PEG-SH and MeO-PEG-Nb, the in situ forming Tz group was observed to react exclusively with the Nb group (Figure S20). These results suggest that the dHTz group can be used as a latent handle for temporal conjugation under physiological conditions, thus broadening the facility of Tz−Nb coupling in biological applications.

The isolated four-arm PEG-Tz can also be used for reaction with MeO-PEG-Nb in organic solvents such as chloroform, and this reaction has a much slower rate, with complete conversion after 12 h followed by UV−vis spectroscopy (Figure S21), compared to the reaction in aqueous solution. The accelerated reaction rate of IEDDA in water has been reported previously[12,28,29] and is explained by the stabilization effect of aqueous solvent on the tetrazine−ene complex before the elimination of nitrogen.[28]

Another aspect of the study is the utilization of light-activated Tz−Nb coupling in polymer cross-linking for hydrogel preparation (Figure 3A). Thus, four-arm PEG-Nb (polymer **5**) was prepared to react with **1** under conditions similar to those mentioned above, and the kinetics of polymerization was followed by rheological analysis. The cross-linking was observed to commence within 10 s of red light irradiation, and complete gelation occurred in 300 s, producing a hydrogel with a modulus of ~4.1 kPa (Figure 3B). The temporal control of the gelation process by light was also demonstrated by turning the light off at various time periods (after exposure to light for 15, 20, 40, and 80 s) during the cross-linking process, which effectively halted the polymerization (Figure 3C). The increase in storage modulus resumed when the light was turned on. The modulus of the gel system after periods of on−off light exposure is lower than the modulus of the gel system under continuous light exposure; e.g., after exposure to light for 35 s, the G' value for the on−off system is 176 Pa and the G' value for the continuous system is 192 Pa. A G' value of 3.7 kPa was

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obtained for the gel in the on−off experiment after a total exposure time of 600 s. The lower G' value of the gel in the on−off experiment compared to continuous light exposure has also been observed in other light-triggered gelation processes;[30] however, the mechanism for this is unclear.

In addition, the rate of cross-linking can also be tuned by changing the intensity of light irradiation from 5 to 30 mW cm^{-1}. Specifically, the induction period after light irradiation is longer at lower light intensities (Figure 3D). At a higher intensity, the gelation rate was found to increase and the formed gel had a modulus that was higher than that of the gel formed by light at a lower intensity within the same period of light treatment. Gelation was not observed in the absence of light irradiation. The increase in the reaction rate was also observed in other photoregulated step-growth polymerization systems and is due to the higher concentration of the reactive species being generated.[30,31] These results therefore present the possibility of fine-tuning the mechanical strength of hydrogels in situ via modulation of light dosage.

Besides methylene blue, rose bengal (λ_{max} = 550 nm) was also found to catalyze the photo-oxidation of dHTz for the activation of Tz−Nb cross-linking (Figure S13). The oxidation is triggered by green light (530 nm wavelength) that corresponds to the absorbance of the photosensitizer. In a separate experiment, **5** was cross-linked with **2** in a PBS solution (pH 7.4) via direct Tz−Nb conjugation and the process was monitored by rheology. Polymerization was found to proceed efficiently, resulting in a hydrogel with a modulus similar to that of the gel prepared by one-pot long wavelength photo-oxidation of the dHTz end group (Figure 3E). ^{1}H NMR analysis of the hydrogel shows some norbornene after cross-linking (Figure S22); however, we were not able to quantify the amount of unreacted Nb. The gel fraction, which is the ratio between the weight of the dried gel and weight of polymer precursors, was found to be 0.96 ± 0.01 and 0.94 ± 0.02 for photocatalytic activation cross-linking and spontaneous Tz−Nb ligation, respectively, indicating a high cross-linking efficiency.

We next examined if red light can activate Tz−Nb cross-linking through dermal tissue models that consist of agarose gels (thickness of 1 cm) containing Intralipid [3% (v/v)] and various concentrations of melanin (Figure 4A). Intralipid is an emulsion (20%) of phospholipid-stabilized soil bean oil that is commonly used to mimic light scattering properties of tissue, while melanin is the main pigment in the skin responsible for UV light absorption and skin color.[9,32] When a pure agarose gel was placed between the incident light and polymer solution, rapid cross-linking similar to gelation without the spacer occurred (Figure 4B, solid line), indicating that light scattering and absorption through the agarose gel are minimal. The cross-linking was impeded when Intralipid was introduced, shown by the longer induction time (~30 s) for gelation and the slower rate of polymerization. Further retardation of the cross-linking was observed by the addition of melanin. In particular, the gel spacer increased the induction time to ~40 s for Fitzpatrick type I−II (light skin, melanin concentration of 9 μg mL^{-1}) and ~60 s for Fitzpatrick type V−VI (dark skin, melanin concentration of 130 μg mL^{-1}) before the onset of gelation. Nevertheless, fast evolution of the storage modulus could be recorded, demonstrating that transdermal light-regulated cross-linking could be achieved for the photocatalytic activation of Tz−Nb cross-linking at a wavelength of 625 nm. In comparison, the cross-linking rate of rose bengal/green light activation was highly hindered when the Intralipid-containing

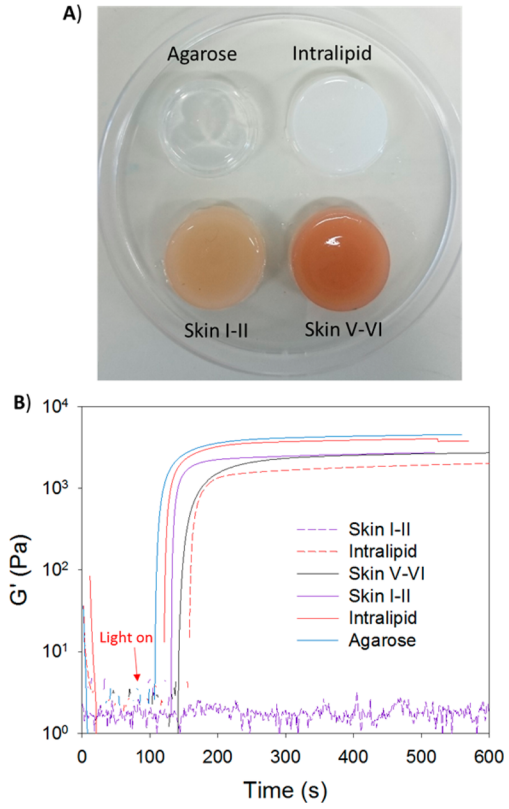

Figure 4. (A) Picture of agarose gels used as a dermal model. (B) Changes in the storage modulus of the polymer solutions behind different types of phantom tissue upon light exposure. Solid lines represent data for the solution containing methylene blue and irradiated with red light (625 nm, 10 mW cm^{-1}), and dashed lines represent data for the solution containing rose bengal and irradiated with green light (530 nm). No polymerization was observed for the rose bengal/green light system when is was blocked by Fitzpatrick type I−II skin.

agarose gel (Figure 4B, dashed line) was placed before the light source. Upon addition of melanin, green light (530 nm) was completely blocked, preventing polymerization.

In situ forming hydrogels are valuable materials to be used as cell-laden scaffolds in regenerative medicine. Thus, we assessed the cytocompatibility of the systems by 3D encapsulation of human mesenchymal stem cells (hMSCs) under conditions investigated as described above (10 wt % polymer concentration and modulus of ~4.1 kPa). The gels were subsequently rinsed to remove methylene blue and subjected to standard culture conditions, and live/dead staining was used to evaluate cell viability. We observed a very high cell viability, indicated by green-stained cells (Figure 5), 24 h postencapsulation, demonstrating the bioorthogonality of the red light-induced cross-linking process.

CONCLUSIONS

In conclusion, we have demonstrated the application of long wavelength photocatalytic activation of Tz−Nb click reaction in polymer conjugation and cross-linking under physiological conditions. We suggest that the dHTz group, because of its stability in a biological solution compared to the Tz group, can be used as a covert handle for temporal bioorthogonal conjugation, allowing for a more flexible and economical synthetic strategy in biological study. In addition, a hydrogel

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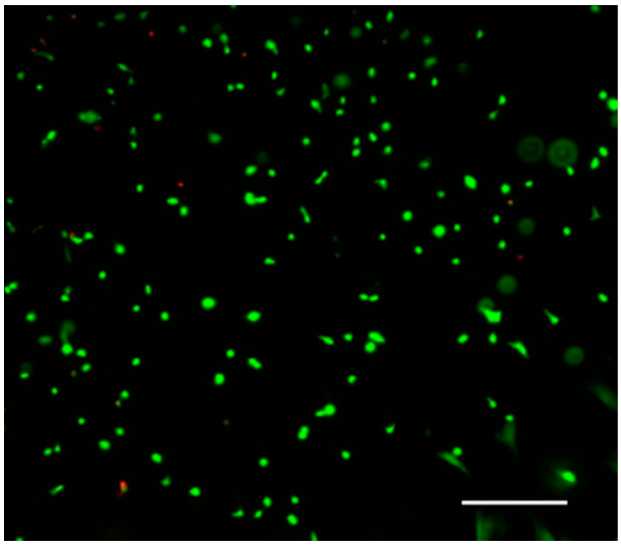

Figure 5. Live/dead staining of hMSC encapsulated in PEG hydrogels prepared by red light-activated Tz−Nb cross-linking after 24 h (green cells are live, and red cells are dead; the scale bar is 50 μm).

was demonstrated to form in situ behind dermal tissue models, which suggests that the system can be applied as injectable hydrogels across the skin and tissue with better control over gelation compared to a spontaneously formed gel from two-component mixing systems.[5,15,16,33]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.7b00561.

Synthesis procedures, NMR spectra of the compounds, experimental details and additional data on rheology measurements, and an experimental description of the cell encapsulation study (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

UCNP, upconversion nanoparticle; IEDDA, inverse electron-demand Diels−Alder; Tz, tetrazine; Nb, norbornene; dHTz, dihydrogen tetrazine; PEG, poly(ethylene glycol)

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