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In Situ Forming Hydrogels via Catalyst-Free and Bioorthogonal "Tetrazole–Alkene" Photo-Click Chemistry

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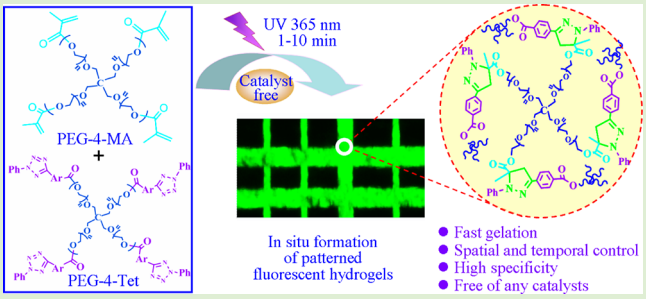
ABSTRACT: *In situ* forming hydrogels were developed from 4-arm poly(ethylene glycol)–methacrylate (PEG-4-MA) and –tetrazole (PEG-4-Tet) derivatives through catalyst-free and bioorthogonal "tetrazole–alkene" photo-click chemistry. PEG-4-MA and PEG-4-Tet (M_n = 10 kg/mol) were soluble at 37 °C in phosphate buffer (PB, pH 7.4, 10 mM) at total polymer concentrations ranging from 20 to 60 wt % but formed fluorescent hydrogels upon 365 nm UV irradiation at an intensity of 20.6, 30.7, or 60 mW/cm^2. The gelation times ranged from ca. 50 s to 5 min, and storage moduli varied from 0.65 to 25.2 kPa depending on polymer concentrations and degrees of Tet substitution in PEG-4-Tet conjugates. The cell experiments via an indirect contact assay demonstrated that these "tetrazole–alkene" photo-click PEG hydrogels were noncytotoxic. The high specificity of photo-click reaction renders thus obtained PEG hydrogels particularly interesting for controlled protein release. Notably, *in vitro* release studies showed that cytochrome *c* (CC), γ-globulins (Ig), and recombinant human interleukin-2 (rhIL-2) all were released from PEG hydrogels in a sustained and quantitative manner over a period of 14–20 days. Importantly, released CC and rhIL-2 exhibited comparable biological activities to native CC and rhIL-2, respectively. These results confirm that "tetrazole–alkene" photo-click reaction is highly compatible with these loaded proteins. This photo-controlled, specific, efficient, and catalyst-free click chemistry provides a new and versatile strategy to *in situ* forming hydrogels that hold tremendous potentials for protein delivery and tissue engineering.



INTRODUCTION

In situ forming hydrogels have attracted widespread interests for biomedical applications including controlled drug release and tissue engineering because they offer several unique advantages such as easy and homogeneous encapsulation of cells and bioactive molecules, minimally invasive implantation, and excellent match to defects of any shapes.[1−5] In the past decade, various physical and chemical cross-linking strategies have been explored to obtain *in situ* forming hydrogels.[6] For example, hydrogels have formed *in situ* based on thermosensitive sol–gel polymers,[7−9] polylactide stereocomplexation,[10,11] and electrostatic interactions.[12] These physical hydrogels could readily encapsulate different proteins like interleukin-2, pigment epithelium-derived factor, human growth hormone, and fibroblast growth factor.[13−16] The release of proteins is, however, generally fast. Moreover, physically cross-linked hydrogels are weak and often exhibit poor tissue dwell time. In contrast, chemically cross-linked hydrogels formed through covalent cross-linking between polymer hydrogel precursors offer better stability, longer durability, and improved mechanical properties. *In situ* forming chemical hydrogels have been developed by different mechanisms such as photoinitiated radical polymerization,[17,18] Michael-type addition chemistry,[19−23] enzymatic cross-linking,[24−26] thiol–ene photopolymerization,[27−29] Schiff-base reaction,[30,31] and oxime reaction.[32] Hennink et al. reported that photopolymerized biodegradable hydrogels gave controlled release of BSA in 2 months.[33] Vermonden et al. reported that hyaluronan-based hydrogels released bradykinin peptide in a diffusion-controlled manner over a period of about 10 days.[34] It should be noted, nevertheless, that these chemical cross-linking strategies suffer drawbacks of using photoinitiator or enzyme that potentially introduces toxicity or immune response and/or low specificity that leads to cross-reactions with proteins and cells.

In recent years, click chemistry with fast reaction and superior specificity has appeared as an emerging approach to prepare *in situ* forming hydrogels.[35,36] For example, *in situ* forming hydrogels have been developed based on azide–alkyne cycloaddition from derivatives of poly(vinyl alcohol),[37] PEG,[38−41] poly(*N*-isopropylacrylamide),[42] and polysaccharide.[43] However, toxic copper catalyst is required for azide–alkyne reaction. Interestingly, Anseth and Becker recently reported that hydrogels were formed from PEG-azides and strained alkyne-flanked peptides/PEG without the need of a copper catalyst.[44−46] The resulting hydrogels demonstrated excellent cytocompatibility. The synthesis of strained alkyne, however, involves multisteps with low overall yields. Shoichet and Marra reported that catalyst-free Diels–Alder click

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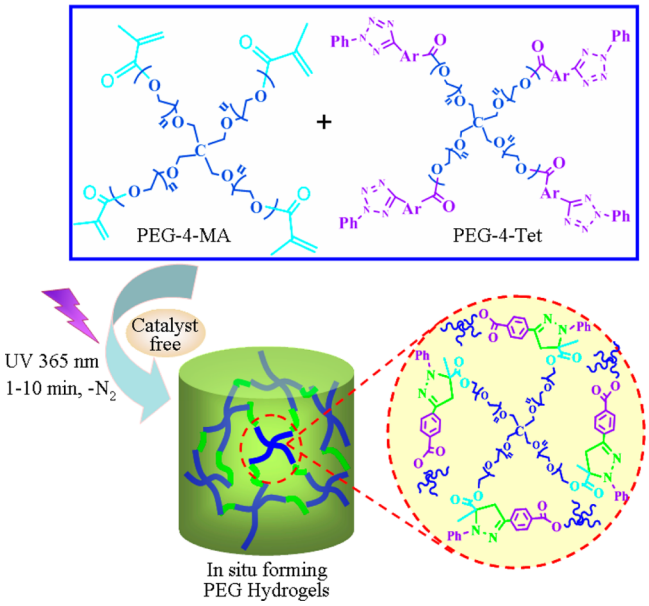
chemistry yielded hyaluronan hydrogels with excellent cytocompatibility and controlled release of proteins like insulin and lysozyme for over 21 days.[47,48] These Diels–Alder click hydrogels, however, had relatively long gelation times (about 21 min) and low storage moduli (18–680 Pa). Very recently, Anseth et al. reported that hydrogels are formed within minutes through tetrazine–norbornene inverse electron demand Diels–Alder reaction.[49]

In this paper, we report on catalyst-free, efficient, and bioorthogonal "tetrazole-alkene" photo-click chemistry as a novel and versatile strategy to fabricate rapidly *in situ* forming PEG hydrogels for facile encapsulation and sustained release of proteins (Scheme 1). Lin et al. discovered that "tetrazole–alkene" photo-click reaction could be used to selectively label alkene-containing proteins in HeLa and *Escherichia coli* cells without obvious toxicity.[50,51] Moreover, "tetrazole–alkene" click reaction yielded fluorescent conjugates.[50] This "tetrazole–alkene" photo-click approach would be ideally suited for preparation of *in situ* forming hydrogels in that (i) it inherits the great features of click chemistry such as high specificity and quantitative conversion but does not require use of toxic catalyst, (ii) it has also unique advantages of photopolymerization including excellent spatial and temporal control over reaction but without use of toxic photoinitiator, and (iii) it provides intrinsically fluorescent hydrogels that could on one hand be used to monitor hydrogel formation and on the other hand facilitate study of hydrogel fate *in vitro* and *in vivo*. Here, *in situ* formation of "tetrazole–alkene" photo-click PEG hydrogels, mechanical behaviors of hydrogels, and encapsulation and controlled release of model and therapeutic proteins were investigated.

Scheme 1. Rapidly *in Situ* Forming Poly(ethylene glycol) (PEG) Hydrogels Prepared from 4-Arm PEG–Methacrylate (PEG-4-MA) and 4-Arm PEG–Tetrazole (PEG-4-Tet) Derivatives via Catalyst-Free and Bioorthogonal "Tetrazole–Alkene" Photo-Click Reaction



EXPERIMENTAL SECTION

Materials. Toluene and dichloromethane (DCM) were dried by refluxing over sodium wire and CaH_2, respectively, and distilled prior to use. Four-arm poly(ethylene glycol) (PEG-4-OH, M_n = 10 kDa, PegBio Co., Ltd.) was dried by azeotropic distillation of toluene. Triethylamine (TEA, Aldrich) was dried over calcium hydride and distilled prior to use. Methacrylic anhydride (MA, Merck), dicyclohexylcarbodiimide (DCC, 99%, Alfa Aesar), 4-(dimethylamino)pyridine (DMAP, 99%, Alfa Aesar), 4-formylbenzoic acid (98%, TCI), benzenesulfonohydrazide (98%, TCI), aniline (Alfa Aesar), 3-mercaptopropionic acid (Alfa Aesar), cytochrome *c* from equine heart (CC, ≥95%, Sigma), γ-globulins from bovine blood (Ig, ≥98%, Sigma), and recombinant human interleukin 2 (rhIL-2, ≥98%, PeproTech) were used as received.

Characterization. ^{1}H NMR spectra were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument using deuterated chloroform ($CDCl_3$), deuterated water (D_2O), or deuterated dimethyl sulfoxide (DMSO-d_6) as solvents. Rheological analysis was performed with RS6000 (Thermo-Fisher, Germany) using parallel plates (20 mm diameter) configuration at 37 °C in oscillatory mode. The rheometer was equipped with a UV light source (LED-300, Dr. Gröbel) with a wavelength of 365 nm and an intensity of 20.6 or 30.7 mW/cm^2. The evolution of storage modulus (G') and loss modulus (G'') was recorded as a function of irradiation time. A gap of 0.5 mm, a frequency of 1 Hz, and a strain of 1% were applied to maintain the linear viscoelastic regime. A solvent trap was used to avoid water evaporation. The gelation time, defined as the time point where $G' = G''$, was determined in triplicate. Circular dichroism (CD) was performed in a quartz cuvette at 37 °C and a protein concentration of 100 μg/mL using an AVIV 410 spectrophotometer (AVIV Biomedical Inc., Lakewood, NJ). CD spectra were recorded over a wavelength range of 190–250 nm.

Synthesis of 4-(2-Phenyl-2*H*-tetrazol-5-yl)benzoic Acid (Tet). Tet was prepared in three steps according to a previous report.[52] First, to a solution of 4-formylbenzoic acid (0.75 g, 5.0 mmol) in 50 mL of ethanol was added benzenesulfonohydrazide (0.86 g, 5.0 mmol). The mixture was stirred for 30 min. The resulting hydrazone adduct was isolated by precipitation in 100 mL of DI water, filtration, and drying for 1 day. Yield = 1.30 g (85.4%). Then, arene diazonium salts were obtained by dropwise adding a solution of $NaNO_2$ (0.173 g, 2.5 mmol) in H_2O (1 mL) into a cooled mixture of aniline (0.23 g, 2.5 mmol) in 4 mL of water–ethanol (1:1) and 0.65 mL of concentrated HCl. Finally, the arene diazonium salt prepared above was added slowly to the hydrazone (0.60 g, 2.0 mmol) in pyridine (15 mL). The mixture was stirred at ice–salt bath for 6 h and then extracted with 30 mL of ethyl acetate for three times. The combined organic solution was precipitated in 120 mL of HCl (3 M). The product was collected by filtration and drying. Yield = 0.21 g (39.4%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.27 (s, 1H, −COO*H*); 8.18 (t, 4H, C_6H_4); 8.30, 7.72, and 7.65 (m, 5H, C_6H_5).

Synthesis of 4-Arm PEG–Tetrazole (PEG-4-Tet) Derivatives. Four-arm PEG–tetrazole (PEG-4-Tet) derivatives were prepared by carbodiimide chemistry using DCC and DMAP as coupling agents. In a typical experiment, DCC (0.248 g, 1.2 mmol) and DMAP (7.33 mg, 0.06 mmol) were added under an argon atmosphere to a solution of PEG-4-OH (0.25 g, 0.025 mmol) and 4-(2-phenyl-2*H*-tetrazol-5-yl)benzoic acid (0.160 g, 0.6 mmol) in DCM (8 mL). The reaction mixture was stirred overnight at room temperature. The product was recovered by precipitation in a mixture of cold diethyl ether/THF (10/1, v/v), filtration, and drying in vacuo. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20–8.25 (m, 4H, C_6H_4 of Tet); 7.53, 7.60, and 8.35 (m, 5H, C_6H_5 of Tet); 4.53 (t, 2H, −COOCH_2−); 3.64 (m, PEG methylene protons). The degree of substitution (DS) of Tet was estimated by ^{1}H NMR to be 71.9%. In a similar way, PEG-4-Tet with DS of 65.6% and 81.6% were prepared at starting Tet/hydroxyl groups of PEG-4-OH molar ratios of 5 and 8, respectively.

Synthesis of 4-Arm PEG-Methacrylate (PEG-4-MA) Derivatives. TEA (60.7 mg, 0.6 mmol) and DMAP (4.89 mg, 0.04 mmol) were added under an argon atmosphere to a solution of PEG-4-OH (0.25 g, 0.025 mmol) and methacrylic anhydride (0.06 g, 0.4 mmol) in toluene (2 mL). The mixture was stirred at 70 °C for 24 h. The product was isolated by precipitation in cold diethyl ether/hexane/methanol (10/1/1, v/v), filtration, and drying in vacuo. Yield: 60.5%.

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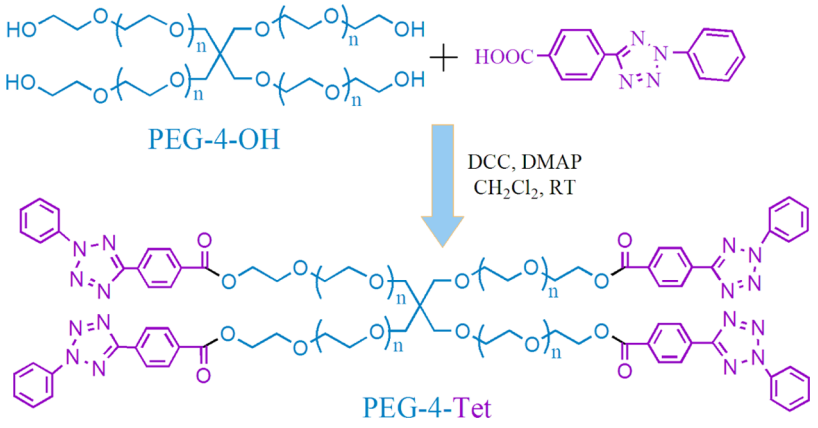
Scheme 2. Synthesis of PEG-4-Tet Derivative



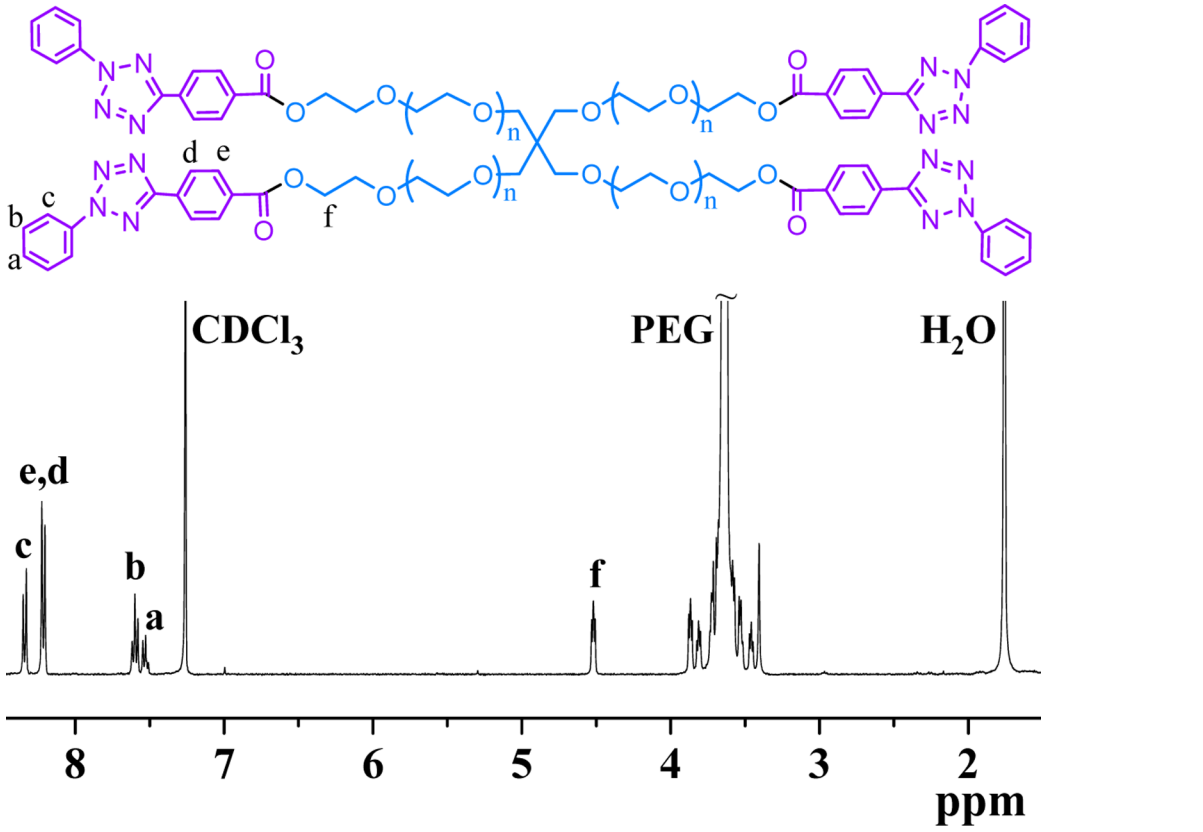


Figure 1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of PEG-4-Tet (DS 81.6%) (Table 1, entry 3).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.54 and 6.10 (s, 2H, $-COC(CH_3)$-CH_2), 4.28 (t, 2H, $-COOCH_2-$), 3.61 (m, PEG main chain protons), 1.91 (s, 3H, $-COC(CH_3)CH_2$). DS of MA was estimated by ^{1}H NMR to be 80.0%.

***In Situ* Forming PEG Hydrogels.** PEG-4-Tet and PEG-4-MA were separately dissolved in phosphate buffer (PB, pH 7.4, 10 mM) at concentrations ranging from 20 to 60 wt %. Equal volume (0.5 mL) of PEG-4-Tet and PEG-4-MA solutions was well mixed and irradiated with UV (365 nm, 60.0 mW/cm^2) for 10 min. The resulting cylindrical hydrogels were 2 mm in height and 10 mm in diameter. Rheological analyses were carried out with RS6000 rheometer equipped with a UV light source with a wavelength of 365 nm and an intensity of 20.6 or 30.7 mW/cm^2.

Gel Content and Swelling Test. To determine the gel content, 0.5 g of PEG hydrogels was lyophilized and weighed (W_d). The dry hydrogels were extensively extracted with dichloromethane to a constant weight. The remaining hydrogels were lyophilized and weighed (W_s). The gel content was expressed as $W_s/W_d \times 100\%$. The experiments were performed in triplicate.

To determine the swelling ratio, PEG hydrogels (about 0.5 g) were prepared and accurately weighed (W_i). The hydrogels were incubated with 3 mL of PB at 37 °C. At regular time intervals, the medium was removed carefully and the hydrogels were gently blotted dry and weighed (W_t). The swelling ratio was determined from the equation $W_t/W_i \times 100\%$. The medium was refreshed once a day. The experiments were performed in triplicate.

Cytocompatibility of Hydrogel. The cytocompatibility of preformed "tetrazole−alkene" photo-click PEG hydrogels was studied by an indirect contact method, as reported by Hennink et al.[53] L929 fibroblast cells were seeded in 24-well plates at a cell density of 1.0×10^4 cells/well and maintained in a medium supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. After 4 h cell culture, the medium was refreshed and sterilized hydrogels were placed on top of the cells. The cells were cultured for another 1, 2, or 4

days. Then, the medium was replaced by 100 μL of fresh medium followed by adding 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL). The cells were incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 100 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The amount of cells was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. The experiments were conducted in triplicate, and the results presented were the average data with standard deviation.

***In Vitro* Protein Release.** The release profiles of CC and Ig from PEG hydrogels were studied using a dialysis tube at 37 °C in PB. Hydrogels were prepared as described above, and protein release studies were performed at a protein loading content of 1.0 mg/mL. At desired time intervals, 2.0 mL of release media was taken out and replenished with an equal volume of fresh media. The amounts of released proteins were determined by bicinchoninic acid (BCA) protein assay kit (R-Protagen Co., Ltd.) using a multifunctional microplate reader (Multiskan Flash, Thermo, excitation at 560 nm). The release behavior of rhIL-2 from PEG hydrogels was studied at 37 °C using a similar method except that a lower protein loading content of 0.4 mg/mL was used and release media contained 0.01 wt % of SDS to prevent precipitation of rhIL-2. The amount of rhIL-2 released was determined by using ELISA measurement (excitation at 450 nm). The release experiments were conducted in triplicate. The results presented are the average data with standard deviations.

Activity of CC and rhIL-2 Released from PEG Hydrogels. The concentrations of released CC and rhIL-2 were determined by the BCA and ELISA assays, respectively. The electron transfer activity of CC was measured by examining the catalytic conversion of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The released CC following dilution with PB buffer to a final concentration of 4.0 μg/mL was placed in a quartz cuvette. 10 μL of hydrogen peroxide solution (0.045 M) and 100 μL of ABTS solution (1 mg/mL) in PB were added. The absorbance at 418 nm of the oxidized product was monitored every 15 s for 3.0 min. Native CC at a concentration of 4.0 μg/mL was used as a control.

The antitumor activity of released rhIL-2 was studied in melanoma B16 cells by MTT assays. The cells were seeded in a 96-well plate at a cell density of 1.0×10^4 cells/well and maintained in a medium supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. After 4 h cell culture, 10 μL of released rhIL-2 (10 μg/mL) was added to each well. The cells were incubated at 37 °C in an atmosphere containing 5% CO_2 for 24 h. The medium was aspirated and replaced by 100 μL of fresh medium, and 10 μL of MTT solution (5 mg/mL) was added. The cells were incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The relative cell viability was evaluated by comparing the absorbance at 490 nm with control wells containing only cell culture medium. The experiments were conducted in triplicate, and the results presented were the average data with standard deviation.

RESULTS AND DISCUSSION

Synthesis of PEG-4-Tet and PEG-4-MA Derivatives. PEG-4-Tet derivatives were prepared by conjugating 4-(2-phenyl-2*H*-tetrazol-5-yl)benzoic acid to PEG-4-OH (M_n = 10 kg/mol) using DMAP and DCC as coupling agents (Scheme 2). ^{1}H NMR showed besides resonance characteristic of PEG (δ 3.64) also signals at δ 7.53−8.35 and 4.53 attributable to the aromatic protons of Tet moieties and methylene protons next to the ester group, respectively (Figure 1). The degree of substitution (DS, defined as the percentage of Tet substituents relative to initial hydroxyl groups in 4-arm PEG) could be estimated by comparing the integrals of signals at δ 4.53 and 3.64. The results showed that PEG-4-Tet derivatives with DS of 65.6%, 71.9%, and 81.6% were obtained at Tet to hydroxyl groups in PEG-4-OH molar feed ratios of 5/1, 6/1, and 8/1, respectively (Table 1). PEG-4-MA derivative was synthesized by reacting PEG-4-OH (M_n = 10 kg/mol) with methacrylic anhydride in the presence of TEA and DMAP. ^{1}H NMR displayed besides peak at δ 3.61 due to PEG also signals at δ 6.10, 5.54, and 1.91 attributable to the methacrylate protons (data not shown). The degree of methacrylate substitution was determined to be 80.0% by comparing the integrals of signals at δ1.91 (methyl protons of MA) and δ 3.61.

Table 1. Synthesis of PEG-4-Tet Derivatives

entry	Tet/hydroxyl groups of PEG-4-OH (mol/mol)	DS (^{1}H NMR)[a] (%)	yield (%)
1	5/1	65.6	90.0
2	6/1	71.9	91.4
3	8/1	81.6	90.0

[a]Degree of substitutions (DS), defined as the percentage of Tet substituents relative to initial hydroxyl groups in 4-arm PEG, were determined from ^{1}H NMR by comparing the integrals of signals at δ 4.53 and 3.64.

***In Situ* Forming Hydrogels and Gelation Time.** Both PEG-4-MA and PEG-4-Tet were soluble at 37 °C in PB at concentrations ranging from 20 to 60 wt %. The rheology measurements showed that a mixture of PEG-4-MA and PEG-4-Tet (DS 81.6%) at an MA/Tet molar ratio of 1/1 and a PEG-4-MA concentration of 30 wt % formed hydrogels upon 365 nm UV irradiation at an intensity of 30.7 mW/cm^2 (Figure 2A). The "tetrazole−alkene" cycloaddition reaction between Tet group in PEG-4-Tet and MA group in PEG-4-MA upon UV irradiation was assumed to be the cross-linking mechanism (Scheme 3). We observed bubbles during formation of hydrogels, confirming release of nitrogen as a byproduct of the reaction. The storage modulus (G') increased rapidly and leveled off in ca. 10 min, indicating fast and complete "tetrazole−alkene" photo-click reaction. Gelation time, defined as the time point where $G' = G''$ (loss modulus), was approximately 70 s. It is interesting to note that this "tetrazole−alkene" photo-click reaction forms hydrogels much faster as compared to reported catalyst-free click hydrogels, e.g., by azide-strained alkyne (gelation time: 5−17 min)[44,46] and Diels−Alder click chemistry (gelation time: 21 min).[48] Lin et al. reported that rate constant of "tetrazole−alkene" photo-click cycloaddition reaction was around 10 000 times higher than that of strain-promoted click cycloaddition reaction.[54] It should further be noted that this photo-click strategy not only affords fast gelation at an MA/Tet molar ratio of 1/1 and a PEG-4-MA concentration of 30 wt % upon 365 nm UV irradiation but also allows excellent spatial and temporal control over hydrogel formation. Figure 2B shows that by selective exposure to UV light hydrogels with designed pattern and strong fluorescence at around 460 nm were formed. The fluorescence of hydrogels comes from pyrazoline cycloadducts,[52] which can be used to monitor hydrogel formation as well as to facilitate study of hydrogel fate *in vitro* and *in vivo*. The progress of "tetrazole−alkene" photo-click reaction over hydrogel formation was also monitored by FTIR. The results showed clearly that the absorbance at 1637 cm^{-1} due to $\nu_{C=C}$ decreased rapidly upon UV irradiation (365 nm, 60.0 mW/cm^2) and completely disappeared in 180 s (Figure 2C), further confirming fast and

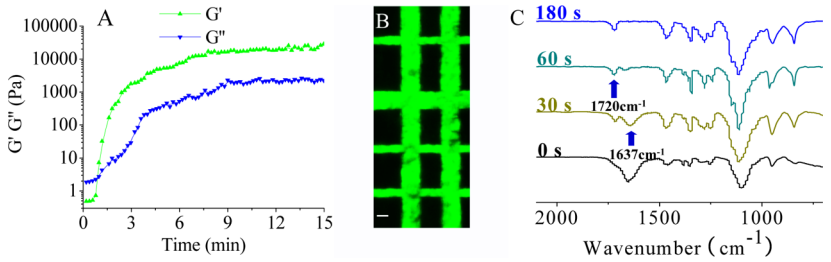
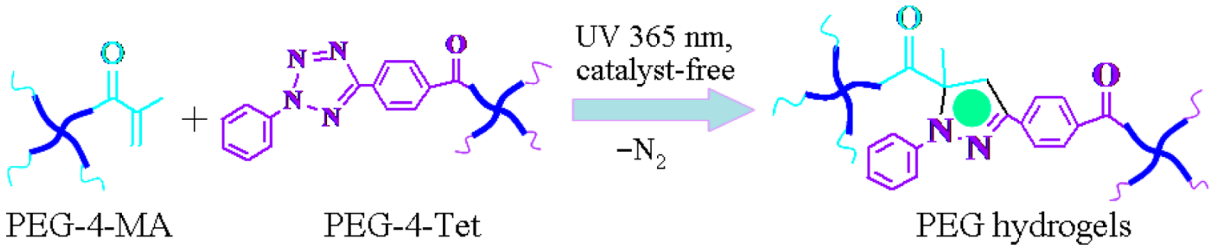


Figure 2. PEG hydrogels prepared from PEG-4-Tet (DS 81.6%) and PEG-4-MA solution in PB at 37 °C with an MA/Tet molar ratio of 1/1 and a PEG-4-MA concentration of 30 wt %: (A) evolution of storage modulus (G') and loss modulus (G'') upon UV irradiation (365 nm, 30.7 mW/cm^2); (B) fluorescence image of patterned PEG hydrogels formed under UV irradiation (365 nm, 60.0 mW/cm^2) using photomasks (scale bar = 100 μm); and (C) FTIR spectra of PEG-4-MA and PEG-4-Tet (DS 81.6%) solution following different UV irradiation times (365 nm, 60.0 mW/cm^2). The absorbance at 1720 and 1637 cm^{-1} was attributed to $\nu_{C=O}$ and $\nu_{C=C}$, respectively.

Scheme 3. "Tetrazole–Alkene" Photo-Click Cycloaddition Reaction



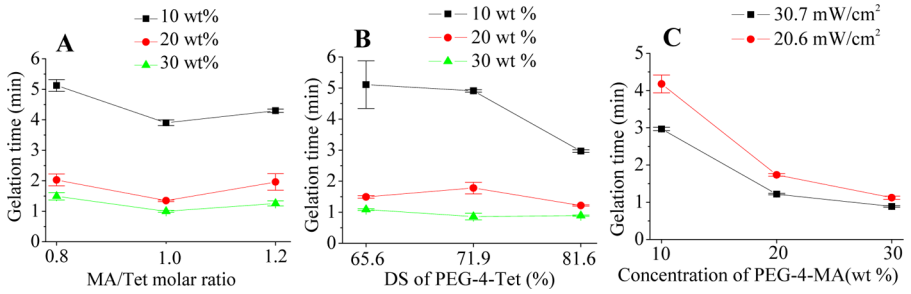


Figure 3. Gelation times of PEG-4-Tet and PEG-4-MA mixture in PB at 37 °C under 365 nm UV irradiation: (A) as a function of MA/Tet molar ratios using PEG-4-Tet (DS 81.6%) and 30.7 mW/cm^2 UV irradiation; (B) as a function of DS of PEG-4-Tet at an MA/Tet molar ratio of 1/1 and 30.7 mW/cm^2 UV irradiation; and (C) as a function of PEG-4-MA concentrations and UV intensities (20.6 or 30.7 mW/cm^2) using PEG-4-Tet (DS 81.6%) at an MA/Tet molar ratio of 1/1. The data are presented as mean ± SD (n = 3).

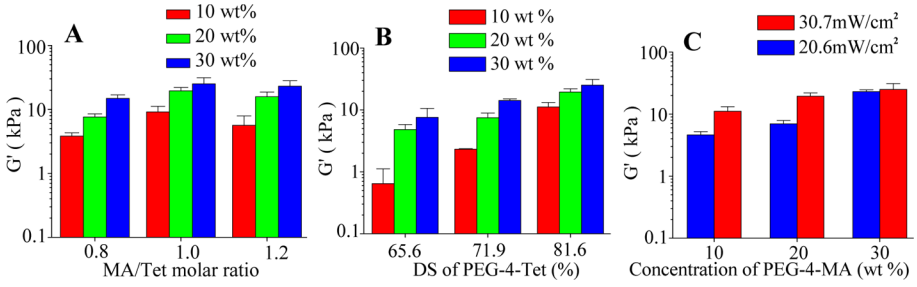


Figure 4. Storage moduli (G') of photo-click PEG hydrogels formed in PB at 37 °C under 365 nm UV irradiation: (A) as a function of MA/Tet molar ratios using PEG-4-Tet (DS 81.6%) at 30.7 mW/cm^2 UV irradiation; (B) as a function of DS of PEG-4-Tet at an MA/Tet molar ratio of 1/1 and 30.7 mW/cm^2 UV irradiation; and (C) as a function of PEG-4-MA concentrations and UV intensities (20.6 or 30.7 mW/cm^2) using PEG-4-Tet (DS 81.6%) at an MA/Tet molar ratio of 1/1. The data are presented as mean ± SD (n = 3).

quantitative "tetrazole–alkene" photo-click reaction under UV irradiation.

Gelation time is a critical parameter for *in situ* forming hydrogels in that slow gelation might result in diffusion of hydrogel precursors into surrounding areas, low encapsulation of bioactive molecules, and/or even failure of gel formation *in vivo*.[24] Here, influences of polymer concentration, MA/Tet molar ratio, DS of PEG-4-Tet derivatives, and UV intensity on gelation time were systematically investigated. Figure 3A shows that at a constant polymer concentration and UV intensity (365 nm, 30.7 mW/cm^2) gelation time increased when MA/Tet molar ratio deviated from 1, signifying importance of equivalent

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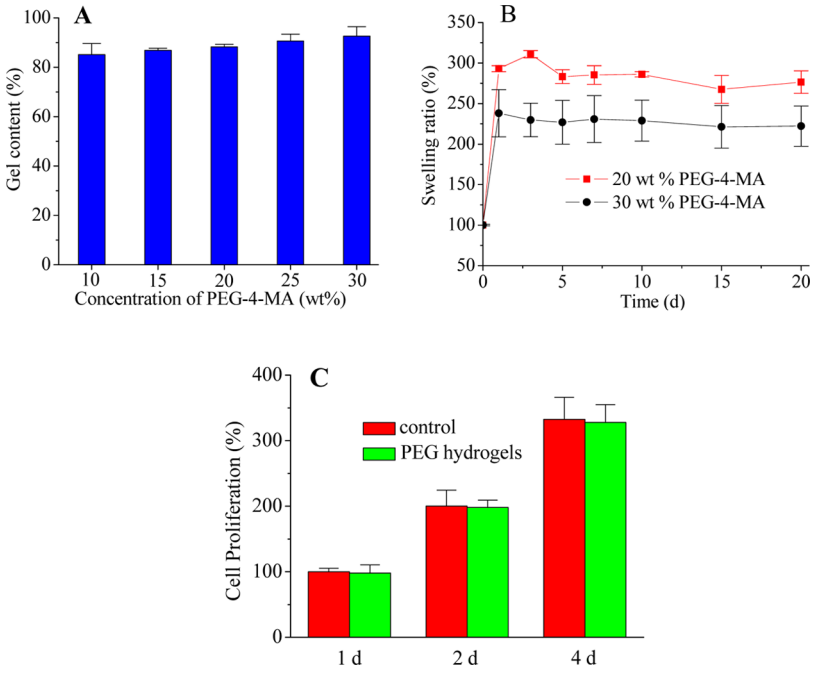

Figure 5. Properties of photo-click PEG hydrogels formed from PEG-4-Tet (DS 81.6%) and PEG-4-MA solution in PB at 37 °C under UV irradiation (365 nm, 60.0 mW/cm^2) for 10 min with an MA/Tet molar ratio of 1/1: (A) gel contents as a function of PEG-4-MA concentrations; (B) swelling ratios of hydrogels as a function of PEG-4-MA concentrations; and (C) proliferation of L929 fibroblast cells in the presence of photo-click PEG hydrogels for 1, 2, and 4 days. The cells cultured on tissue culture plastic without presence of PEG hydrogels were used as a control. The amount of cells was determined by MTT assays. The data are presented as mean ± SD (n = 3).

MA and Tet groups for fast gelation. At a constant MA/Tet molar ratio, gelation time decreased considerably with increasing PEG-4-MA concentrations from 10 to 30 wt %, likely due to increase in the number of functional groups per volume of gel precursor solutions. Figure 3B displays that at a concentration of 10 wt % PEG-4-MA, gelation time decreased with increasing DS of PEG-4-Tet derivatives from 65.6% to 81.6%, due to increase of cross-linking density. At high polymer concentration of 20 and 30 wt % PEG-4-MA, gelation time was not much influenced by DS of PEG-4-Tet. Figure 3C shows that gelation time decreased with increasing light intensities from 20.6 to 30.7 mW/cm^2. In brief, by changing polymer concentration, MA/Tet molar ratio, DS of PEG-4-Tet derivatives, and UV intensity, hydrogels with gelation times ranging from 50 s to 5 min could readily be obtained via "tetrazole−alkene" photo-click cross-linking.

Properties of Photo-Click PEG Hydrogels. The mechanical properties of photo-click PEG hydrogels were examined as a function of polymer concentration, MA/Tet molar ratio, and UV intensity at 37 °C. The results showed that at a constant MA/Tet molar ratio of 1/1 and UV intensity of 30.7 mW/cm^2, storage modulus (G') of photo-click PEG hydrogels increased from 9.2 to 25.2 kPa with increasing PEG-4-MA concentrations from 10 to 30 wt % (Figure 4A). Moreover, at a constant PEG-4-MA concentration, storage moduli of hydrogels decreased when MA/Tet molar ratio deviated from 1 (e.g., 0.8 or 1.2), in accordance with equivalent coupling reaction between MA and Tet. The storage moduli of PEG hydrogels increased with increasing DS of PEG-4-Tet derivatives (Figure 4B), likely due to increased cross-linking density. Figure 4C shows that at a constant polymer concentration of 10 or 20 wt % PEG-4-MA and an MA/Tet molar ratio of 1/1, storage moduli of hydrogels increased with increasing UV intensity from 20.6 to 30.7 mW/cm^2, indicating that network evolution is not complete at the lower UV dosage. At a high polymer concentration of 30 wt % PEG-4-MA, however, UV intensity had little influence on storage moduli of hydrogels due to a high concentration of functional groups per volume of gel precursors. According to the Flory−Stockmayer theory of gelation, the critical gel point conversion (ρ_c) was determined to be 0.45, indicating that hydrogels form readily from PEG-4-MA and PEG-4-Tet at an MA/Tet molar ratio of 1/1. These results demonstrate that PEG hydrogels obtained by "tetrazole−alkene" photo-click chemistry offer broad storage moduli ranging from 0.65 to 25.2 kPa.

Gel content studies showed that photo-click hydrogels based on PEG-4-Tet (DS 81.6%) had high gel contents of 85.2−92.7% at PEG-4-MA concentrations ranging from 10 to 30 wt % (Figure 5A), indicating efficient cross-linking via "tetrazole−alkene" photo-click reaction. The swelling measurements in phosphate buffered saline (PBS, pH 7.4, 150 mM NaCl) at 37 °C demonstrated that hydrogels formed from 20 and 30 wt % PEG-4-MA reached equilibrium swelling in 1 day and remained unchanged in 20 days (Figure 5B), indicating that these hydrogels had excellent stability and little hydrolytic degradation under physiological conditions. Hydrogels formed at a higher concentration of 30 wt % PEG-4-Tet exhibited a relative lower swelling ratio of 230%, most probably associated with their higher cross-linking density. Notably, MTT assays using L929 fibroblast cells revealed that these preformed photo-click PEG hydrogels had no adverse effect on cell growth in 4 days (Figure 5C), indicating that no cytotoxic compounds are leaching out of the "tetrazole−alkene" photo-click hydrogels.

Loading and *in Vitro* Release of Proteins. The high specificity of photo-click reaction renders thus-formed PEG hydrogels particularly interesting for protein release. The loading and release of proteins were studied at 37 °C and pH 7.4 using three different proteins, i.e., CC, Ig, and rhIL-2.

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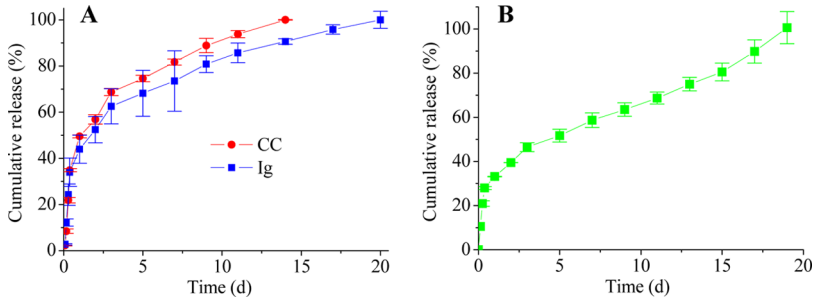

Figure 6. *In vitro* protein release profiles from photo-click PEG hydrogels of PEG-4-Tet (DS 81.6%) and PEG-4-MA in PB at an MA/Tet molar ratio of 1/1 and 20 wt % PEG-4-MA formed by 10 min UV irradiation (365 nm, 60.0 mW/cm^2). (A) Release of CC and Ig and (B) release of rhIL-2. The data are presented as mean ± SD (n = 3).

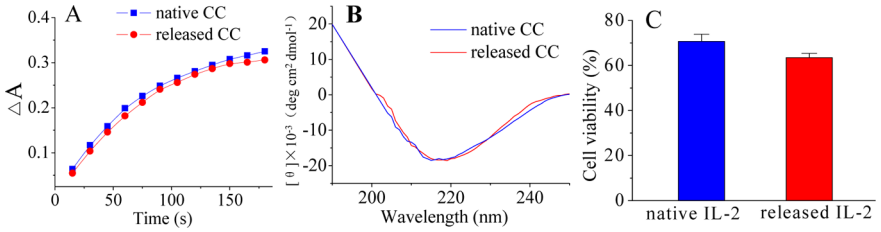

Figure 7. Activity and secondary structure of proteins released from photo-click PEG hydrogels at 20.0 wt % PEG-4-MA in PB and an MA/Tet molar ratio of 1/1 under UV irradiation (365 nm, 60.0 mW/cm^2) for 10 min. (A) Oxidation of ABTS catalyzed by native CC and released CC at a concentration of 4.0 μg/mL, (B) CD spectra of native CC and released CC at a concentration of 100 μg/mL, and (C) antitumor activity of native rhIL-2 and released rhIL-2 (dosage: 10 μg/mL) in B16 cells by MTT assays. The data are presented as mean ± SD (n = 3).

The proteins were loaded easily and homogeneously into hydrogels by mixing protein solution and polymer precursor solutions before UV irradiation. Interestingly, *in vitro* release studies showed that CC, Ig, and rhIL-2 all were released from hydrogels in a sustained and quantitative manner over a period of 14−20 days (Figure 6), suggesting that neither irreversible aggregation nor covalent bonding of proteins to the polymer chains had occurred. The release of Ig was somewhat slower as compared to that of CC (20 vs 14 days), most likely due to a larger size of Ig. It is known that large-sized proteins have a lower diffusion rate.[55]

The enzymatic activity of CC released from photo-click PEG hydrogels was examined using ABTS assay.[56] The results showed that there was no significant difference in UV adsorption of oxidized ABTS catalyzed either by CC released from hydrogels or by the native CC (Figure 7A), indicating that released proteins maintain their bioactivity. Moreover, far-UV CD analysis showed that CC released from photo-click PEG hydrogels possessed a similar CD profile to native CC in the region between 190 and 250 nm, indicating that secondary structure of CC protein has not been altered (Figure 7B). Notably, MTT assays using B16 cells showed that rhIL-2 (100 ng/well) released from photo-click PEG hydrogels had similar *in vitro* antitumor activity to native rhIL-2 under otherwise the same conditions (Figure 7C). It is evident, therefore, that catalyst-free and bioorthogonal "tetrazole−alkene" photo-click strategy provides cytocompatible hydrogels that are particularly interesting as matrices for sustained protein release. Notably, the PEG hydrogels can be easily developed into biodegradable hydrogels by the incorporation of enzyme-responsive peptide or hydrolytic polyester segments to achieve on-demand protein delivery.[33,57,58]

CONCLUSIONS

We have demonstrated that catalyst-free and bioorthogonal "tetrazole−alkene" photo-click chemistry is a simple, versatile, and efficient strategy to prepare rapidly *in situ* forming and yet robust hydrogels. Notably, these photo-click hydrogels have several unique features: (i) They exhibit tunable gelation times from tens of seconds to several minutes, which renders them interesting to be applied as injectable hydrogels. (ii) They allow excellent spatial and temporal control over hydrogel formation, while no toxic photoinitiator is needed. (iii) Like traditional click chemistry, "tetrazole−alkene" photo-click reaction has high conversion and specificity, which avoids possible protein denaturation and deactivation. However, copper catalyst is not required. (iv) They release proteins quantitatively and in a sustained manner and furthermore released proteins maintain their bioactivity. (v) They show strong fluorescence that can be used to monitor hydrogel formation as well as hydrogel fate *in vitro* and *in vivo*. This "tetrazole−alkene" photo-click strategy has opened a new and versatile avenue to rapidly *in situ* forming hydrogels that hold a tremendous potential for protein delivery and tissue engineering.

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Notes

The authors declare no competing financial interest.

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