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Peptide Functionalized Oxime Hydrogels with Tunable Mechanical Properties and Gelation Behavior

Fei Lin[†], Jiayi Yu[†], Wen Tang[†], Jukuan Zheng[†], Adrian Defante[†], Kai Guo[†], Chrys Wesdemiotis^{†,§}, and Matthew L. Becker^{*,†,||}

[†]Department of Polymer Science, The University of Akron, Akron, Ohio 44325, United States

[§]Department of Chemistry, The University of Akron, Ohio 44325, United States

^{||}Center for Biomaterials in Medicine, Austen Bioinnovation Institute in Akron, Akron, Ohio 44308, United States

Abstract

We demonstrate the formation of polyethylene glycol (PEG) based hydrogels via oxime ligation and the photo-initiated thiol-ene 3D patterning of peptides within the hydrogel matrix post-gelation. The gelation process and final mechanical strength of hydrogels can be tuned using pH and the catalyst concentration. The time scale to reach the gel point and complete gelation can be shortened from hours to seconds using both pH and aniline catalyst, which facilitates the tuning of the storage modulus from 0.3 kPa to over 15 kPa. Azide and alkene functionalized hydrogels were also synthesized and we have shown the post gelation “click” type Huisgen 1,3 cycloaddition and thiolene-based radical reactions for spatially defined peptide incorporation. These materials are the initial demonstration for translationally relevant hydrogel materials that possess tunable mechanical regimes attractive to soft tissue engineering and possess atom neutral chemistries attractive for post gelation patterning in the presence or absence of cells.

Keywords

hydrogel; oxime; PEG; click chemistry; thiolene

Introduction

Hydrogels represent a diverse class of polymeric networks that absorb significant amount of water without dissolution. Hydrogels are utilized widely in tissue engineering, drug and protein delivery, cell culture, and sensors due to their unique and highly tunable chemical, mechanical and transport properties.¹⁻⁵ In biomedical applications, cells and tissues sense and respond to the rigidity of their microenvironments and are able to alter their migration, signaling, differentiation and proliferation responses.⁶⁻⁹ It is essential that hydrogels targeted to tissue engineering scaffolds match the mechanical properties of target tissues.^{10, 11} Therefore, the biomaterials community continually searches for new hydrogel materials where the mechanical properties and network formation times are highly tunable.¹²⁻¹⁶

*Corresponding Author becker@uakron.edu.

Supporting Information

All synthesis and characterization details of all molecules are available free of charge in the supporting information section via the Internet at <http://pubs.acs.org>.

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Polyethylene glycol (PEG) based hydrogels are utilized frequently due to the lack of cytotoxicity, unique hydration properties and ease of end group modification for crosslinking or network formation.¹⁷⁻¹⁹ Although much success can be attributed to the fabrication of PEG hydrogels via conventional photo-initiated free-radical chain polymerization,²⁰⁻²² trends have been moving toward building hydrogels through more robust, selective, efficient, and tunable chemical reactions.^{23, 24} Various types of “click” chemistry strategies have been employed to prepare hydrogels, including Michael-addition²⁵⁻²⁷, thiol-ene radical addition^{28, 29}, tetrazine–norbornene addition^{30, 31}, tetrazine-alkene photo click reaction³², copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC)³³⁻³⁶ or metal-free strain-promoted alkyne-azide cycloadditions (SPAAC)³⁷⁻³⁹, etc. While these strategies are used as crosslinking methodologies, there have been increasing interests to combine several “click” reactions into one system to create more complex and biologically-patterned microenvironments. Anseth and coworkers demonstrated that photo-initiated thiol-ene chemistry can be employed to spatiotemporally incorporate peptides and proteins in PEGs hydrogels via tetrazine-norbornene addition³⁰, CuAAC³⁶ or SPAAC.^{29, 37} Hawker et al also utilized thiol-ene reactions to immobilize bioactive and diagnostic molecules to hydrogel microarray surfaces⁴⁰. Dynamic 3D patterning of biological cues were developed via CuAAC where Cu(I) was produced by photo initiated Cu(II) reduction.³⁴ Shoichet and co-workers further exploited thiol-maleimide Michael-addition for spatial patterning of agarose hydrogels.^{41, 42}

Recently, the condensation between aldehyde or ketone groups and aminooxy functional groups, which is also known as an oxime ligation, has grown in popularity as an emerging “click” reaction (Figure 2(c)). It occurs readily in aqueous solutions without major side reactions,^{43, 44} and is used widely for the conjugation and labeling of biological molecules, cell surface modification, scaffolds preparation, and injectable hydrogels⁴⁵⁻⁴⁸. Though an oxime bond is more stable than a hydrazone⁴³, it has been show to be reversible in some biologically relevant environments⁴⁹. Oxime ligation is particularly interesting due to its pH sensitive reaction kinetics, dependence on catalyst concentration, and varying degrees of reversibility.^{44, 50} For fast reaction speeds, it is necessary to maintain a slightly acidic reaction environment where the proton serves as a catalyst. In neutral or slightly basic conditions, nucleophilic catalysts such as aniline^{44, 51} are necessary to obtain the desired reaction rates and extent of conversion. Currently, significant efforts have been focused on the development of non-toxic catalysts, like 4-aminophenylalanine⁵². These unique features inspire us to create hydrogels with tunable properties such as mechanical strength and gelation time by modulating the reaction conditions.

Our experimental results show using oxime ligation to fabrication hydrogels with tunable mechanical and gelation properties, as well as chemical functionalities for post-gelation biological cues incorporation is an attractive and highly versatile strategy. We have designed and synthesized two precursors: bi-aldehyde functionalized PEG (PEG-bCHO) (Figure 2(a)) and a 4-arm aminooxy crosslinker (Figure 2(b)) molecule. Using identical precursor compositions, we found that the hydrogel properties were influenced greatly by both pH and the presence or absence of an aniline catalyst. The gelation process was completed in minutes at a pH of 4.5 and the resulting storage modulus was 14 kPa, while at pH of 7.4 the gelation process takes hours to form a much softer gel with a modulus of 0.3 kPa. The aniline catalyst helped to improve the gel mechanical properties and shorten the gelation process. In addition, azide functionalized oxime hydrogels were also prepared for post-gelation incorporation of RGD peptides via CuAAC and SPAAC. 3D photo patterning of peptides via thiol-ene chemistry was also explored in alkene derived oxime hydrogels.

Materials and Methods

Materials

All commercial reagents and solvents were purchased from Aldrich or Fisher Scientific and used without further purification unless noted otherwise. All reactions were performed under a blanket of nitrogen unless noted otherwise.

Instrumentation

NMR spectra were obtained by Varian NMRS 300 MHz spectrometer. All chemical shifts are reported in ppm (δ), and referenced to the chemical shifts of residual solvent resonances (^1H NMR CDCl_3 7.27 ppm, D_2O 4.80 ppm, DMSO-d_6 2.50 ppm); ^{13}C CDCl_3 77.00 ppm, DMSO-d_6 39.50 ppm). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, br = broad singlet, m = multiplet. FT-IR spectra were recorded on a SHIMADZU MIRacle 10 ATR-FTIR, and Raman spectra were recorded on a LabRAM HR 800 Spectrophotometer. Fluorescence microscopy images were recorded on OLYMPUS IX 81 Microscope and are unaltered. Size exclusion chromatographic analyses (SEC) were performed using a Waters 150-C Plus instrument equipped with three HR- Styragel columns [100 Å, mixed bed (50/500/103/104 Å), mixed bed (103, 104, 106 Å)], and a differential refractometer (Waters 410) detector. THF was used as eluent with a flow rate of 1.0 mL/min at 30 °C. The molecular mass and mass distribution were calculated from polystyrene standards. Electrospray ionization (ESI) was performed using a HCT Ultra II quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an electrospray ionization source. MALDI-TOF mass spectra were carried out on a Bruker Ultraflex-III TOF/TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA) equipped with a Nd:YAG laser (355 nm). All spectra were measured in positive reflection mode. Trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB, Aldrich, >98%) and sodium trifluoroacetate served as matrix and cationizing salt, respectively.

Synthesis of Bi-aldehyde Functionalized PEG Precursor (PEG-bCHO)

The synthesis route is described as shown in Scheme 1(a). Both PEG (4.6k) and 4-formylbenzoic acid were vacuum dried overnight prior to use. In a 250 mL round flask, PEG (4600 g/mol, 9.2 g, 2 mmol), 4-(N,N-dimethylamino) pyridinium-4-toluenesulfonate (DPTS, 0.5 g, 1.5 mmol) and 4-formylbenzoic acid (1.2 g, 8 mmol) were dissolved in 50 mL of anhydrous DMF. The reaction mixture was placed in an ice bath for 10 min, followed by the addition of 1,3-diisopropyl carbodiimide (DIPC, 1.5 mL, 10 mmol) via syringe. The white precipitation was observed in minutes. The reaction was allowed to warm up to room temperature and stir for 24 h. After centrifugation to remove solids, the top layer solution was diluted with 1 L of methanol. The resulting solution was kept at -20 °C overnight. The resulting white solid was isolated by centrifugation and washed with cold methanol three times followed by ethyl ether twice. After vacuum drying, a white solid product (8.2 g, yield 82%) was stored -20 °C for future use. ^1H NMR (300 MHz, CDCl_3): δ =10.09 (s, 1H, -PhCHO), 8.21 (m, 2H, aromatic H), 7.95 (m, 2H, aromatic H), 4.50 (t, 2H, -COOCH₂CH₂O-), 3.84 (t, 2H, -COOCH₂CH₂O-), 3.63 (s, ~220 H, -CH₂CH₂O-, PEG main chain). FT4IR (cm^{-1}): 2881, 1719, 1701, 1466, 1359, 1341, 1279, 1240, 1202, 1145, 1095, 1059, 961, 841, 760, 681.

Synthesis of 4-arm Aminoxy Crosslinker

The synthesis route is described as shown in Scheme 1(b). Intermediate 1 was first prepared via conventional esterification. Briefly, in a 100 mL round bottom flask, pentaerythritol (1 g, 7.5 mmol), DPTS (0.25 g, 0.75 mmol) and (Boc-aminoxy)acetic acid (5.8 g, 30 mmol) were dissolved in 30 mL of anhydrous DMF. The reaction mixture was placed in an ice bath

for 10 min, followed by the addition of DIPC (5.5 mL, 36 mmol) via syringe. White precipitation was observed within minutes. The reaction formulation was allowed to warm to room temperature and was stirred for 24 h. After removal of the solid by filtration, the collected solution was concentrated for chromatography purification on silica gel. (Ethyl acetate: hexane=3:1(v/v)). The resulting product was a white solid (7.9 g, yield 65%). ^1H NMR (300 MHz, CDCl_3): δ =7.93 (s, 4H, $-\text{NHCO}-$), 4.47 (s, 8H, $-\text{NHOCCH}_2\text{CO}-$), 4.26 (s, 8H, $-\text{COOCH}_2\text{C}-$), 1.49 (s, 36H, $(\text{CH}_3)_3\text{CO}$). ^{13}C NMR (300 MHz, CDCl_3): δ =169.06, 159.27, 82.41, 72.38, 61.91, 42.76, 28.15.

The 4-arm aminooxy crosslinker was achieved through the deprotection of tert-butyloxycarbonyl (BOC) group from intermediate 1. Briefly, in a 100 mL round bottom flask, intermediate 1 (2.00 g, 2.4 mmol) was dissolved in HCl/dioxane solution (20 mL, 4M). The solution was allowed to stir at room temperature for 24 h. The white precipitate was isolated by filtration and washed with dioxane three times followed by ethyl ether twice. After drying in vacuum, product came out as a white solid (1.35 g, quantitative). ^1H NMR (300 MHz, CDCl_3): δ =4.85 (s, 2H, $^+\text{NH}_3\text{OCH}_2\text{CO}-$), 4.45 (s, 2H, $-\text{COOCH}_2\text{C}-$). FT-IR (cm^{-1}): 3710-2159 broad, 1743, 1679, 1513, 1409, 1368, 1205, 1115, 1060, 1015, 887, 873, 717.

Synthesis of Azide-aminooxy Extender (azide- ONH_2)

The chemical structure of azide- ONH_2 is shown in Scheme 1(c). It was synthesized in a similar process as 4-arm aminooxy crosslinker. Detailed synthesis procedure was discussed in supporting information. ^1H NMR (300 MHz, D_2O): δ = 4.67-4.73 (m, 4H, $^+\text{NH}_3\text{OCH}_2\text{COO}-$), 4.15-4.25 (m, 4H, $-\text{CH}_2\text{COOCH}_2\text{C}-$), 3.42-3.52 (m, 4H, $-\text{CCH}_2\text{N}_3$).

Synthesis of Alkene-aminooxy Extender (alkene- ONH_2)

The chemical structure of alkene- ONH_2 is shown in Scheme 1(d). It was synthesized in a similar process as 4-arm aminooxy crosslinker. Detailed synthesis procedure was discussed in supporting information. ^1H NMR (300 MHz, D_2O): 5.75-6.05 (m, 1H, $-\text{CH}=\text{CH}_2$), 5.10-5.50 (m, 3H, $-\text{COOCH}_2\text{CHOOC}-$, $-\text{CH}=\text{CH}_2$), 4.55-4.80 (m, 4H, $^+\text{NH}_3\text{OCH}_2\text{COO}-$), 4.30-4.50 (m, 2H, $-\text{COOCH}_2\text{CHOOC}-$), 3.95-4.15 (m, 2H, $-\text{OCH}_2\text{CH}=\text{CH}_2$), 3.78(d, 2H, $-\text{CHCH}_2\text{OCH}_2\text{CH}=\text{CH}_2$).

Model Reaction of Oxime Ligation (PEG-b-alkene)

The oxime ligation reaction is described in Scheme 1(e). In a 4 mL glass vial, PEG-bCHO (100 mg, 0.02 mmol) and o-(pent-4-en-1-yl) hydroxylamine (intermediate 2, Supporting Information) (8.4 mg, 0.06 mmol) was dissolved in acetic buffer (pH 4.5) and stirred at room temperature for 10 min. The reaction mixture was subjected for MALDI-TOF characterization without purification. Pure sample was obtained by dialyzation in deionized water (molecular weight (MW) cut off 3000 g/mol, cellulose membrane, Pierce), followed by lyophilization. ^1H NMR (300 MHz, CDCl_3): δ =8.05-8.10 (m, 2H, aromatic H), 7.58-7.68 (m, 2H aromatic H), 5.75-5.95 (m, 1H, $\text{CH}_2=\text{CHCH}_2-$), 4.93-5.13 (m, 2H, $\text{CH}_2=\text{CHCH}_2-$), 4.40-4.55(t, 2H, $-\text{COOCH}_2\text{CH}_2\text{O}-$), 4.15-4.25 (t, 2H, $-\text{CH}_2\text{CH}_2\text{ON}=\text{C}-$), 3.80-3.90(m, 2H, $-\text{COOCH}_2\text{CH}_2\text{O}-$), 3.55-3.75 (s, ~220H, $-\text{CH}_2\text{CH}_2\text{O}-$, PEG main chain), 2.10-2.25 (m, 2H, $\text{CH}_2=\text{CHCH}_2\text{CH}_2\text{CH}_2\text{O}-$), 1.75-1.90 (m, 2H, $\text{CH}_2=\text{CHCH}_2\text{CH}_2\text{CH}_2\text{O}-$).

Synthesis of Functionalized Peptides

Two kinds of peptides bearing “clickable” groups were synthesized via standard solid phase Fmoc methodology on a CEM Discovery automated peptide synthesis machine. Peptides were cleaved from resin using standard conditions (45 min, 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), 2.5% water (by volume)) and precipitated in cold diethyl

ether. The crude solid product was isolated by centrifuge, washed twice with diethyl ether and dialyzed in deionized water (molecular weight (MW) cut off 500 g/mol, cellulose membrane, Pierce), followed by lyophilization. As to alkyne-RGD-biotin (sequence Alkyne-GRGDSK(Biotin)-COOH), 5-hexynoic acid was coupled to N-terminus using standard amino acid coupling condition before cleavage. For fluorescein-5(6)-isothiocyanate (FITC)-RGD-thiol (sequence FTIC-GRGDSCS), FITC (Sigma) was coupled to N-terminus in DMF overnight before cleavage.^{53, 54} Alkyne-RGD-Biotin: ESI calculated 939.1, found 939.5. FTIC-RGD-thiol: ESI calculated 1070.1, found 1070.5. Excitation 492 nm, emission 512 nm (PBS buffer, pH 7.4).

Hydrogel Fabrication and Characterization

Citrate-phosphate buffer (10 mM) was used with different pH (2.5, 4.5, 6.0, 6.6, 6.8, 7.0, 7.2, 7.4, and 7.6) for hydrogel formation. Hydrochloric acid-potassium chloride buffer (pH 1.5) was used as the highest acid concentration condition. Concentration of the aniline catalyst was controlled as (0.1 wt %) when used. Hydrogels were prepared with the stoichiometric balanced formulations (1:1 total aminooxy: aldehyde), and total precursor concentration was 10 wt %. The two precursors were pre-dissolved separately in the respective buffer solutions to control the pH. Mixing of the two solutions resulted in the formation of oxime hydrogels. For oscillatory shear measurement, mixture of PEG-bCHO solution and 4-arm aminooxy crosslinker solution was placed in a silicon mold for 12 h to make sure the gelation reactions are complete. Hydrogels were then soaked in phosphate buffered saline (PBS, 10 mM, pH 7.0) overnight to reach the swollen equilibrium. The equilibrium modulus measurements were recorded using a ARES G2 Rheometer (TA Instrument) using 8 mm parallel plate geometry at room temperature, and the gel thickness was around 1 mm. Hydrogels were immersed in PBS during the whole testing process. Frequency ramped from 100 rad s⁻¹ to 0.1 rad s⁻¹ with 10% strain. To evaluate the gel formation kinetics, storage modulus (G') and loss modulus (G'') were observed at the constant frequency 10 rad s⁻¹ as a function of time with a strain 10%. The top of gel formulation was covered with silicon oil to avoid dehydration.

Azide Derived Hydrogel Fabrication and Further Functionalization

The citrate-phosphate buffer (10 mM, pH 4.5) was used as the gelation medium. The ratio between the total aminooxy and aldehyde stoichiometry was controlled precisely at 1:1. The concentration of PEGs was 10 wt%. First the PEGs and bi-aminooxy extender (0.1 eq. to aldehyde) were mixed in buffer solution and stirred for 10 min, followed by the addition of the 4-arm aminooxy crosslinker (0.9 eq. to aldehyde) solution. After 12 h of gelation time, the hydrogels were immersed in PBS (pH 7.0) overnight to reach the swollen equilibrium for further peptide functionalization.

Azide hydrogels were incubated overnight in alkyne-RGD-biotin solution (1 mg/mL) in PBS, together with copper sulfate (0.1 mg/mL) and sodium ascorbate (1 mg/mL). Hydrogels were then placed in fresh media for 2 d to remove copper salt and any unbonded peptides. For FT-IR testing, the solvent exchange was further carried out in deionized water for 1 d before lyophilization. The dried gel sponge was then characterized by FT-IR.

For the protein binding assay, after the copper salt removal, peptide functionalized hydrogels were incubated in tetramethylrhodamine-5(6)-isothiocyanate (TRITC) labeled streptavidin PBS solution (10 ug/mL) for 2 h, followed by incubation in fresh media overnight to remove unbound protein. The resulting hydrogels were imaged by fluorescence microscopy. A control experiment was conducted with plain azide gel without peptide conjugation.

Alkene Derived Hydrogels Fabrication and 3D Biochemical Patterning

Alkene hydrogels were fabricated similarly to azide hydrogels with the exception of using an alkene aminooxy extender to replace the azide one. The thickness of the hydrogel was 1 mm or less. Alkene hydrogels were incubated in PBS containing Irgacure 2959 (0.1 mg/mL) and the patterning agent FTIC-RGD-thiol (0.01 mg/mL) for 30 min. Using conventional photolithographic techniques, gels were exposed to ultraviolet light (365 nm wavelength) through a 300 mesh TEM grid mask for 2 min. After the patterning was complete, hydrogels were washed overnight with fresh media to remove any unbound peptides. The final patterned hydrogels were imaged by fluorescence microscopy.

Results and Discussion

Hydrogel Precursor Synthesis

Bis-aldehyde functionalized PEG (PEG-bCHO) was synthesized via carbodiimide coupling with 4-formylbenzoic acid, in the presence of DPTS and DIPC. The aldehyde group of 4-formylbenzoic acid is highly reactive due to electron deficiency. DPTS/DIPC was employed as catalyst system to obtain high reaction efficiency and yields under mild conditions⁵⁵. The complete conversion of hydroxyl to aldehyde was verified by matrix-assisted desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Figure 1(a)), which shows a single distribution. The monoisotopic mass of each peak matches well with that expected for PEG-bCHO (e.g., for [103mer+Na]⁺, found 4839.7 Da vs calculated 4839.6 Da). The difference between neighboring peaks is equal to the mass of a -CH₂CH₂O- repeating unit (44.0 Da). Analysis by size-exclusion chromatography (SEC) with polystyrene standard indicated a number average molecular weight (M_n) of 5200 Da (polydispersity index (PDI) = 1.08) (Supporting Information, Figure S1, Table S1). It is higher than the M_n of PEG raw materials, which was measured to be 4860 Da.

Prior to hydrogel fabrication, a model reaction was conducted to verify the reaction efficiency and reaction rate of oxime ligation. Intermediate 2 (Supporting Information) was used as a model substrate and coupled to PEG-bCHO in pH 4.5 buffer solution. The raw formulation was submitted to MALDI-TOF-MS without purification after mixing the reagents for 10 min. The single mono-modal distribution of the mass spectra (Figure 1(b)) demonstrates the complete conversion of aldehyde to oxime. The monoisotopic mass peak at 5005.5 Da in Figure 1(b) corresponds to the peak at 4839.7 Da in Figure 1(a) after end capping with o-(pent-4-en-1-yl) hydroxylamine. In SEC analysis, the M_n of the PEG-b-alkene is 5390 Da (PDI=1.12) (Supporting Information, Figure S1, Table S1). The exhibited fast reaction rate and high reaction efficiency of the oxime ligation outlined the promising strategy to fabricate the hydrogels.

Hydrogel fabrication and chemical characterization

Hydrogels were formed using a 10% (mass) precursor solution containing a 1:1 ratio of aldehyde to aminooxy functionalities (Figure 2(d)). FT-IR was used to examine the chemical structure of resulted hydrogels. In Figure 2(g), both the aldehyde C=O stretch at 1701 cm⁻¹ and the C-C stretch at 1202 cm⁻¹ (black line) of PEG-bCHO are much weaker in the spectra of crosslinked hydrogels (red line), indicating that the high conversion of the aldehyde groups was achieved in the network formation process. A new stretch at 1762 cm⁻¹ within the hydrogels is assigned to the ester C=O stretch of the 4-arm aminooxy crosslinker. Raman spectroscopy was also used to further verify this crosslinking process (Figure 2(h)). The characteristic stretch of the oxime C=N bond is found as a sharp peak at 1612 cm⁻¹ (red line). Meanwhile, the small peak at 1612 cm⁻¹ (black line) is assigned to the phenyl stretch of PEG-bCHO, which is shifted to 1565 cm⁻¹ (red line) after the oxime ligation due to the conjugation of π electrons⁵⁶.

pH Control Over Hydrogel Mechanical Properties and Gelation Kinetics

It is very important to control the intrinsic mechanical properties of the hydrogels, which would influence the interaction between the scaffolds and surrounding biological environment, including cell behavior, gene expression, and tissue regeneration.⁵⁷⁻⁶⁰ Recently we have shown that mechanical properties of polyethylene glycol dimethacrylate (PEGDM) hydrogels have a significant impact in modulating human osteoarthritic chondrocyte behaviors and tissue formation.⁹ In addition, recent reports by Ifkovitsa and coworkers found that injectable hyaluronic acid hydrogels with high mechanical strength can improve the post-myocardial infarction remodeling process.⁶¹

To quantitate the influence of pH on hydrogel formation, a series of buffer solutions with different pH values, from 1.5 to 7.6, were used as the gel formation media. The gel precursor composition was kept identical (10%, mass), as well as the 1:1 ratio of aldehyde to aminoxy functional groups. Figure 3(a) shows the modulus-frequency curves of two hydrogels formed in buffer at two different pH values. At a pH of 4.5, the resulting gel has a storage modulus 14.1 ± 1.2 kPa. It is nearly 50 times stronger than the gel formed at pH of 7.4, which resulted in a storage modulus of only 0.3 ± 0.1 kPa. Figure 2(e) and 2(f) demonstrate the strength difference by appearance. The plot in Figure 3(b) shows the pH control over the resulting storage modulus. In the first region from pH 1.5 to 4.5, the storage modulus increases with increasing pH. In the pH range between 4.5 and 7.4, the storage modulus decreases with an increase in pH, especially in slightly basic environments. The storage modulus of gels at pH of 7.0 is 6.2 ± 0.9 kPa, while at pH of 7.4 it drops to 0.3 ± 0.1 kPa. There is no coherent hydrogel formation at pH 7.6 which results in a very viscous fluid.

The mechanical strength of the hydrogels is directly related to the crosslink density, which is determined by functional group conversion. The theoretical gel-point conversion for the step-growth polymerization can be calculated by the Flory-Stockmayer equation^{62, 63}

$$\rho = \frac{1}{\sqrt{r(1 - f_{\text{aldehyde}})(1 - f_{\text{aminoxy}})}} \quad (1)$$

where ρ is the gel-point conversion, r is the stoichiometric ratio, and f_{aldehyde} and f_{aminoxy} are the degree of functionality for PEG-bCHO and 4-arm linker, respectively. In the current system, we have $r=1$, $f_{\text{aldehyde}}=2$, and $f_{\text{aminoxy}}=4$. The critical conversion is approximately 0.58 to achieve gelation. As to oxime ligation, the reaction conversion and equilibrium is largely influenced by pH.^{44, 50} When pH decreases from 4.5 to 1.5, the reverse reactions are favored due to the protonation of imines ($\text{pKb} \sim 10$), which is the rate-limiting step for oxime hydrolysis. The equilibrium of the oxime ligation shifts to the left and the functional group conversion and crosslink density are reduced. As a result, the obtained hydrogels are softer and have a smaller storage modulus. Meanwhile, at pH values of 1.5 and 2.5, the gel was formed immediately after mixing the two precursors (see gelation kinetic data, Figure 4(b)). The fast gelation largely restricts the mobility of both aldehyde and aminoxy, and further crosslink density is reduced. On the other hand, fast gelation results in microscopic inhomogeneity, and then leads to weaker hydrogels.⁶⁴ In the pH range from 4.5 to 7.6, the reaction conversion is reduced due to the increase of pH, and the critical conversion is never reached at pH 7.6. The optimal environment for oxime ligation is mildly acidic. Under these conditions, the attenuated basicity of the aminoxy groups leaves it unprotonated for further attack on the aldehyde, and the imine is also unprotonated due to the α -effect⁶⁵, which suspends the hydrolysis process.

In the time dependent gelation experiment (Figure 4(a)), the crossover of storage modulus (G') and loss modulus (G'') represents the gel point, which means the transformation from a

viscous liquid to a viscoelastic solid. The storage modulus plateau is treated as the complete of gelation. In the entire pH range we tested, from strongly acidic to slightly basic, a decrease in acid concentration lead to the faster gel point and complete gelation time (Figure 4(b)). At pH 1.5 and 2.5, the gel point is not detected due to the fast gelation behavior; only the complete gelation time is obtained (240 s at pH 1.5 and 400 s at pH 2.5). At pH 7.4, the gel point is achieved after 5 h, and 10 h is required for complete gelation. This process is kinetically controlled. The oxime bond formation is catalyzed by acid, which activates aldehyde by protonation and accelerates the dehydration step. When pH decreases, the reaction rate is increased, and then the critical conversion 0.58 and final conversion is achieved faster. It indicates that less time for gelation point and complete gelation is required.

Influence of Aniline Catalyst on Hydrogel Mechanical Properties and Gelation Kinetics

Additional studies quantified the influence of the nucleophilic catalyst, aniline, on hydrogel mechanical properties and the gelation behavior with a pH ranging from 6.6 to 7.6. Aniline is a widely used catalyst for oxime gelation and has been shown to improve the reaction rate and efficiency^{44, 66}. As shown in Figure 5(a), aniline greatly influences the mechanical strength of hydrogels. The catalyst leads to a large increase in the storage modulus, especially at neutral and slightly basic conditions. At pH 7.4, the storage modulus of hydrogels with aniline is 4.7 ± 0.3 kPa, while the storage modulus without aniline is only 0.3 ± 0.1 kPa. Particularly at pH 7.6, there was no gel formation in the absence of catalyst. Following the addition of catalyst, gels with the storage modulus of 2.4 ± 0.2 kPa were obtained. The gelation kinetics are also catalyst dependent. The time scale of both the gel point (Figure 5(b)) and time to complete gelation (Figure 5(c)) is reduced from hours to minutes with the addition of aniline. Adding catalyst results in stronger hydrogels by increasing the functional group conversion and crosslink density, and enables the critical conversion to be reached at pH 7.6. At the same time, aniline also accelerates oxime ligation, and reduces the time necessary to achieve critical conversion.^{44, 51}

Oxime Hydrogels with Chemical Functionalities and its Further Derivation with Peptides

Azide and alkene functionalized hydrogels were prepared for further conjugation of biological molecules via a cascade approach as shown in Figure 6(a). The 2-arm aminooxy precursor with azide (azide-ONH₂, Scheme 1(c)) or alkene (Scheme 1(d)) handles was used as the chain extender. It reacted with PEG-bCHO, resulting in a new precursor formulation in situ. Aldehyde groups are present on the chain end, while the other two arms contain azide or alkene groups. The content of the PEG with “clickable” groups in aldehyde mixtures was controlled via feed ratio between the extender and PEG-bCHO. The 4-arm crosslinker solution was added into aldehyde mixtures to form crosslinked hydrogels.

Azide incorporation into oxime hydrogels was confirmed via FT-IR spectroscopy. The characteristic azide stretch peak is present at 2103 cm^{-1} (Figure 6(b1), black line). Azide groups provide a platform to functionalize hydrogels using azide-alkyne Huisgen cycloaddition, which is widely used in biomedical engineering for bioconjugation and tissue labeling applications⁶⁷⁻⁷⁰. Alkyne-RGD-biotin was used as a model peptide to verify the availability of azide groups for post-gelation reactions. The disappearance of the azide stretch peak after the “click” cyclization reaction indicates the peptide incorporation into hydrogel matrix was successful (Figure 6(b1), red line). An additional protein binding experiment was conducted with tetramethylrhodamine-5(6)-isothiocyanate (TRITC) labeled streptavidin.^{71, 72} The strong red fluorescence of labeled streptavidin protein confirms the existence and bioavailability of biotin in the hydrogels (Figure 6(b2)). The dark region is the glass slide background. The reaction and binding sites are described in Figure 6(b3). The

azide derivatized hydrogels also showed chemical reactivity with 4-dibenzocyclooctynol derived PEG³⁹ via SPAAC (Data not shown).

Oxime hydrogels bearing alkene groups were also utilized for peptide conjugation via photo-initiated thiol-ene reactions. In the Raman spectra of the alkene functionalized gels in Figure 6(c1) (black line), the stretch of the alkene is not observed due to the overlap with the imine peak between 1600 cm⁻¹ and 1700 cm⁻¹. Following the photo-initiated addition of 2-mercaptoethanol with Irgacure 2959 as the radical formation initiator, the C-S stretch peak at 630 cm⁻¹ is observed (Figure 6(c1), red line), which indicates the occurrence of the thiol-ene radical addition. This reaction is further used to create spatial patterns of peptides in oxime hydrogels using a simple photomask. Fluorescein-5-isothiocyanate (FTIC) conjugated RGD with pendant thiol groups (FTIC-RGD-SH) was employed as the model bioactive signal to explore the potential for photochemical patterning. Photocoupling was achieved using a UV light source (365 nm) and the biocompatible initiator Irgacure 2959. A square pattern of fluorescent-labeled peptide was generated with a 300 mesh TEM copper grid functioning as a mask (Figure 6(c2)). The contrast of the exposed square areas and covered lines is obvious, although background fluorescence was observed due to light scattering in the photo pattering process. The tethered sites are described in Figure 6 (c3).

Conclusion

In conclusion, we have demonstrated the fabrication of covalently crosslinked PEG-based hydrogels via oxime ligation. The crosslinking process is both pH and catalyst dependent. The tunable mechanical properties are related to the condensation equilibrium, reaction rate and the precursors mobility⁷³. The results demonstrate that oxime ligation is an efficient method for the fabrication of hydrogels with variable mechanical and gelation kinetics using identical precursors. At 10 wt% concentrations, the storage modulus can be controllably varied from 0.3 kPa to 15 kPa and the gel formation times can be tuned from seconds to hours. Oxime hydrogels with controlled amounts of residual azide and alkene groups were also obtained. The hydrogels were successfully used for pattern formation and peptide incorporation via robust and highly efficient reaction conditions. These materials are the initial demonstration for translationally relevant hydrogel materials that possess tunable mechanical regimes attractive to soft tissue engineering and possess atom neutral chemistries attractive for post gelation patterning in the presence or absence of cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Reference

1. Qiu Y, Park K. Environment-sensitive hydrogels for drug delivery. *Advanced Drug Delivery Reviews*. 2001; 53(3):321–339. [PubMed: 11744175]
2. Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA. Hydrogels in Regenerative Medicine. *Advanced Materials*. 2009; 21(32-33):3307–3329. [PubMed: 20882499]
3. Lee KY, Mooney DJ. Hydrogels for Tissue Engineering. *Chemical Reviews*. 2001; 101(7):1869–1880. [PubMed: 11710233]

4. Dong L, Agarwal AK, Beebe DJ, Jiang H. Adaptive liquid microlenses activated by stimuli-responsive hydrogels. *Nature*. 2006; 442(7102):551–554. [PubMed: 16885981]
5. Hoare TR, Kohane DS. Hydrogels in drug delivery: Progress and challenges. *Polymer*. 2008; 49(8): 1993–2007.
6. Nemir S, West J. Synthetic Materials in the Study of Cell Response to Substrate Rigidity. *Ann Biomed Eng*. 2010; 38(1):2–20. [PubMed: 19816774]
7. Nemir S, Hayenga HN, West JL. PEGDA hydrogels with patterned elasticity: Novel tools for the study of cell response to substrate rigidity. *Biotechnology and Bioengineering*. 2010; 105(3):636–644. [PubMed: 19816965]
8. Carr LR, Krause JE, Ella-Menye J-R, Jiang S. Single nonfouling hydrogels with mechanical and chemical functionality gradients. *Biomaterials*. 2011; 32(33):8456–8461. [PubMed: 21903259]
9. Smith Callahan LA, Ganos AM, Childers EP, Weiner SD, Becker ML. Primary human chondrocyte extracellular matrix formation and phenotype maintenance using RGD-derivatized PEGDM hydrogels possessing a continuous Young's modulus gradient. *Acta Biomaterialia*. 2013; 9(4): 6095–6104. [PubMed: 23291491]
10. Cloyd J, Malhotra N, Weng L, Chen W, Mauck R, Elliott D. Material properties in unconfined compression of human nucleus pulposus, injectable hyaluronic acid-based hydrogels and tissue engineering scaffolds. *Eur Spine J*. 2007; 16(11):1892–1898. [PubMed: 17661094]
11. Seal BL, Otero TC, Panitch A. Polymeric biomaterials for tissue and organ regeneration. *Materials Science and Engineering: R: Reports*. 2001; 34(4-5):147–230.
12. Jeon O, Bouhadir KH, Mansour JM, Alsberg E. Photocrosslinked alginate hydrogels with tunable biodegradation rates and mechanical properties. *Biomaterials*. 2009; 30(14):2724–2734. [PubMed: 19201462]
13. Forget A, Christensen J, Lüdeke S, Kohler E, Tobias S, Matloubi M, Thomann R, Shastri VP. Polysaccharide hydrogels with tunable stiffness and provasculogenic properties via α -helix to β -sheet switch in secondary structure. *Proceedings of the National Academy of Sciences*. 2013; 110(32):12887–12892.
14. Herrick WG, Nguyen TV, Sleiman M, McRae S, Emrick TS, Peyton SR. PEG-Phosphorylcholine Hydrogels As Tunable and Versatile Platforms for Mechanobiology. *Biomacromolecules*. 2013; 14(7):2294–2304. [PubMed: 23738528]
15. Song F, Zhang L-M. Enzyme-Catalyzed Formation and Structure Characteristics of A Protein-Based Hydrogel. *The Journal of Physical Chemistry B*. 2008; 112(44):13749–13755. [PubMed: 18855437]
16. Nguyen MK, Lee DS. Oligo(amidoamine)s hydrogels with tunable gel properties. *Chemical Communications*. 2010; 46(20):3583–3585. [PubMed: 20358122]
17. Mano JF, Sousa RA, Boesel LF, Neves NM, Reis RL. Bioinert, biodegradable and injectable polymeric matrix composites for hard tissue replacement: state of the art and recent developments. *Composites Science and Technology*. 2004; 64(6):789–817.
18. Zhu J. Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials*. 2010; 31(17):4639–4656. [PubMed: 20303169]
19. Browning MB, Russell B, Rivera J, Höök M, Cosgriff-Hernandez EM. Bioactive Hydrogels with Enhanced Initial and Sustained Cell Interactions. *Biomacromolecules*. 2013; 14(7):2225–2233. [PubMed: 23758437]
20. Sawhney AS, Pathak CP, Hubbell JA. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co-poly(α -hydroxy acid) diacrylate macromers. *Macromolecules*. 1993; 26(4):581–587.
21. Elisseeff J, McIntosh W, Anseth K, Riley S, Ragan P, Langer R. Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks. *Journal of Biomedical Materials Research*. 2000; 51(2):164–171. [PubMed: 10825215]
22. Callahan LAS, Ganos AM, McBurney DL, Dilisio MF, Weiner SD, Horton WE, Becker ML. ECM Production of Primary Human and Bovine Chondrocytes in Hybrid PEG Hydrogels Containing Type I Collagen and Hyaluronic Acid. *Biomacromolecules*. 2012; 13(5):1625–1631. [PubMed: 22559049]

23. Nimmo CM, Shoichet MS. Regenerative Biomaterials that “Click”: Simple, Aqueous-Based Protocols for Hydrogel Synthesis, Surface Immobilization, and 3D Patterning. *Bioconjugate Chemistry*. 2011; 22(11):2199–2209. [PubMed: 21995458]
24. Azagarsamy MA, Anseth KS. Bioorthogonal Click Chemistry: An Indispensable Tool to Create Multifaceted Cell Culture Scaffolds. *ACS Macro Letters*. 2012; 2(1):5–9. [PubMed: 23336091]
25. Elbert DL, Pratt AB, Lutolf MP, Halstenberg S, Hubbell JA. Protein delivery from materials formed by self-selective conjugate addition reactions. *Journal of Controlled Release*. 2001; 76(1-2):11–25. [PubMed: 11532309]
26. Pritchard CD, O’Shea TM, Siegwart DJ, Calo E, Anderson DG, Reynolds FM, Thomas JA, Slotkin JR, Woodard EJ, Langer R. An injectable thiol-acrylate poly(ethylene glycol) hydrogel for sustained release of methylprednisolone sodium succinate. *Biomaterials*. 2011; 32(2):587–597. [PubMed: 20880573]
27. Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, Hubbell JA. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics. *Proceedings of the National Academy of Sciences*. 2003; 100(9):5413–5418.
28. Lin C-C, Raza A, Shih H. PEG hydrogels formed by thiol-ene photo-click chemistry and their effect on the formation and recovery of insulin-secreting cell spheroids. *Biomaterials*. 2011; 32(36):9685–9695. [PubMed: 21924490]
29. Fairbanks BD, Schwartz MP, Halevi AE, Nuttelman CR, Bowman CN, Anseth KS. A Versatile Synthetic Extracellular Matrix Mimic via Thiol-Norbornene Photopolymerization. *Advanced Materials*. 2009; 21(48):5005–5010.
30. Alge DL, Azagarsamy MA, Donohue DF, Anseth KS. Synthetically Tractable Click Hydrogels for Three-Dimensional Cell Culture Formed Using Tetrazine-Norbornene Chemistry. *Biomacromolecules*. 2013; 14(4):949–953. [PubMed: 23448682]
31. Zhou H, Woo J, Cok AM, Wang M, Olsen BD, Johnson JA. Counting primary loops in polymer gels. *Proceedings of the National Academy of Sciences*. 2012; 109(47):19119–19124.
32. Fan Y, Deng C, Cheng R, Meng F, Zhong Z. In Situ Forming Hydrogels via Catalyst-Free and Bioorthogonal “Tetrazole-Alkene” Photo-Click Chemistry. *Biomacromolecules*. 2013; 14(8):2814–2821. [PubMed: 23819863]
33. Truong V, Blakey I, Whittaker AK. Hydrophilic and Amphiphilic Polyethylene Glycol-Based Hydrogels with Tunable Degradability Prepared by “Click” Chemistry. *Biomacromolecules*. 2012; 13(12):4012–4021. [PubMed: 23134321]
34. Adzima BJ, Tao Y, Kloxin CJ, DeForest CA, Anseth KS, Bowman CN. Spatial and temporal control of the alkyne-azide cycloaddition by photoinitiated Cu(II) reduction. *Nat Chem*. 2011; 3(3):256–259. [PubMed: 21336334]
35. Hu X, Li D, Zhou F, Gao C. Biological hydrogel synthesized from hyaluronic acid, gelatin and chondroitin sulfate by click chemistry. *Acta Biomaterialia*. 2011; 7(4):1618–1626. [PubMed: 21145437]
36. Polizzotti BD, Fairbanks BD, Anseth KS. Three-Dimensional Biochemical Patterning of Click-Based Composite Hydrogels via Thiolene Photopolymerization. *Biomacromolecules*. 2008; 9(4):1084–1087. [PubMed: 18351741]
37. DeForest CA, Polizzotti BD, Anseth KS. Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments. *Nat Mater*. 2009; 8(8):659–664. [PubMed: 19543279]
38. DeForest CA, Sims EA, Anseth KS. Peptide-Functionalized Click Hydrogels with Independently Tunable Mechanics and Chemical Functionality for 3D Cell Culture. *Chemistry of Materials*. 2010; 22(16):4783–4790. [PubMed: 20842213]
39. Zheng J, Smith Callahan LA, Hao J, Guo K, Wesdemiotis C, Weiss RA, Becker ML. Strain-Promoted Cross-Linking of PEG-Based Hydrogels via Copper-Free Cycloaddition. *ACS Macro Letters*. 2012; 1(8):1071–1073. [PubMed: 23205321]
40. Gupta N, Lin BF, Campos LM, Dimitriou MD, Hikita ST, Treat ND, Tirrell MV, Clegg DO, Kramer EJ, Hawker CJ. A versatile approach to high-throughput microarrays using thiol-ene chemistry. *Nat Chem*. 2012; 4(5):424–424.

41. Wosnick JH, Shoichet MS. Three-dimensional Chemical Patterning of Transparent Hydrogels. *Chemistry of Materials*. 2007; 20(1):55–60.
42. Wylie RG, Shoichet MS. Three-Dimensional Spatial Patterning of Proteins in Hydrogels. *Biomacromolecules*. 2011; 12(10):3789–3796. [PubMed: 21853977]
43. Kalia J, Raines RT. Hydrolytic Stability of Hydrazones and Oximes. *Angewandte Chemie International Edition*. 2008; 47(39):7523–7526.
44. Dirksen A, Hackeng TM, Dawson PE. Nucleophilic Catalysis of Oxime Ligation. *Angewandte Chemie International Edition*. 2006; 45(45):7581–7584.
45. Zeng Y, Ramya TNC, Dirksen A, Dawson PE, Paulson JC. High-efficiency labeling of sialylated glycoproteins on living cells. *Nat Meth*. 2009; 6(3):207–209.
46. Heredia KL, Tolstyka ZP, Maynard HD. Aminooxy End-Functionalized Polymers Synthesized by ATRP for Chemoselective Conjugation to Proteins. *Macromolecules*. 2007; 40(14):4772–4779.
47. Grover GN, Lam J, Nguyen TH, Segura T, Maynard HD. Biocompatible Hydrogels by Oxime Click Chemistry. *Biomacromolecules*. 2012; 13(10):3013–3017. [PubMed: 22970829]
48. Grover GN, Braden RL, Christman KL. Oxime Cross-Linked Injectable Hydrogels for Catheter Delivery. *Advanced Materials*. 2013; 25(21):2937–2942.
49. Orbán E, Mez G, Schlage P, Csík G, Kuli Ž, Ansorge P, Fellinger E, Möller H, Manea M. In vitro degradation and antitumor activity of oxime bond-linked daunorubicin-GnRH-III bioconjugates and DNA-binding properties of daunorubicin-amino acid metabolites. *Amino Acids*. 2011; 41(2):469–483. [PubMed: 20953647]
50. Jencks WP. Studies on the Mechanism of Oxime and Semicarbazone Formation I. *Journal of the American Chemical Society*. 1959; 81(2):475–481.
51. Dirksen A, Dawson PE. Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling. *Bioconjugate Chemistry*. 2008; 19(12):2543–2548. [PubMed: 19053314]
52. Blanden AR, Mukherjee K, Dilek O, Loew M, Bane SL. 4-Aminophenylalanine as a Biocompatible Nucleophilic Catalyst for Hydrazone Ligations at Low Temperature and Neutral pH. *Bioconjugate Chemistry*. 2011; 22(10):1954–1961. [PubMed: 21932849]
53. Bryson DI, Zhang W, Ray WK, Santos WL. Screening of a branched peptide library with HIV-1 TAR RNA. *Molecular BioSystems*. 2009; 5(9):1070–1073. [PubMed: 19668873]
54. Bahta M, Liu F, Kim S-E, Stephen AG, Fisher RJ, Burke TR. Oxime-based linker libraries as a general approach for the rapid generation and screening of multidentate inhibitors. *Nat. Protocols*. 2012; 7(4):686–702.
55. Moore JS, Stupp SI. Room temperature polyesterification. *Macromolecules*. 1990; 23(1):65–70.
56. Lee M, Lee J-P, Rhee H, Choo J, Gyu Chai Y, Kyu Lee E. Applicability of laser-induced Raman microscopy for in situ monitoring of imine formation in a glass microfluidic chip. *Journal of Raman Spectroscopy*. 2003; 34(10):737–742.
57. Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*. 2003; 24(24):4337–4351. [PubMed: 12922147]
58. Gunn JW, Turner SD, Mann BK. Adhesive and mechanical properties of hydrogels influence neurite extension. *Journal of Biomedical Materials Research Part A*. 2005; 72A(1):91–97. [PubMed: 15536643]
59. Marklein RA, Soranno DE, Burdick JA. Magnitude and presentation of mechanical signals influence adult stem cell behavior in 3-dimensional macroporous hydrogels. *Soft Matter*. 2012; 8(31):8113–8120.
60. Banerjee A, Arha M, Choudhary S, Ashton RS, Bhatia SR, Schaffer DV, Kane RS. The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells. *Biomaterials*. 2009; 30(27):4695–4699. [PubMed: 19539367]
61. Ifkovits JL, Tous E, Minakawa M, Morita M, Robb JD, Koomalsingh KJ, Gorman JH, Gorman RC, Burdick JA. Injectable hydrogel properties influence infarct expansion and extent of postinfarction left ventricular remodeling in an ovine model. *Proceedings of the National Academy of Sciences*. 2010; 107(25):11507–11512.
62. Flory PJ. Molecular Size Distribution in Three Dimensional Polymers. II. Trifunctional Branching Units. *Journal of the American Chemical Society*. 1941; 63(11):3091–3096.

63. Stockmayer WH. Theory of Molecular Size Distribution and Gel Formation in Branched-Chain Polymers. *The Journal of chemical physics*. 1943; 11(2):45–55.
64. Tibbitt MW, Kloxin AM, Sawicki LA, Anseth KS. Mechanical Properties and Degradation of Chain and Step-Polymerized Photodegradable Hydrogels. *Macromolecules*. 2013; 46(7):2785–2792.
65. Sander EG, Jencks WP. Equilibria for additions to the carbonyl group. *Journal of the American Chemical Society*. 1968; 90(22):6154–6162.
66. Byeon J-Y, Limpoco FT, Bailey RC. Efficient Bioconjugation of Protein Capture Agents to Biosensor Surfaces Using Aniline-Catalyzed Hydrazone Ligation. *Langmuir*. 2010; 26(19):15430–15435. [PubMed: 20809595]
67. Tornøe CW, Christensen C, Meldal M. Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *The Journal of Organic Chemistry*. 2002; 67(9):3057–3064. [PubMed: 11975567]
68. Gierlich J, Burley GA, Gramlich PME, Hammond DM, Carell T. Click Chemistry as a Reliable Method for the High-Density Postsynthetic Functionalization of Alkyne-Modified DNA. *Organic Letters*. 2006; 8(17):3639–3642. [PubMed: 16898780]
69. Agard NJ, Prescher JA, Bertozzi CR. A Strain-Promoted [3 + 2] Azide-Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. *Journal of the American Chemical Society*. 2004; 126(46):15046–15047. [PubMed: 15547999]
70. Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA, Bertozzi CR. Copper-free click chemistry for dynamic in vivo imaging. *Proceedings of the National Academy of Sciences*. 2007; 104(43):16793–16797.
71. Deng X, Friedmann C, Lahann J. Bio-orthogonal “Double-Click” Chemistry Based on Multifunctional Coatings. *Angewandte Chemie International Edition*. 2011; 50(29):6522–6526.
72. Weber P, Ohlendorf D, Wendoloski J, Salemme F. Structural origins of high-affinity biotin binding to streptavidin. *Science*. 1989; 243(4887):85–88. [PubMed: 2911722]
73. Boekhoven J, Poolman JM, Maity C, Li F, van der Mee L, Minkenberg CB, Mendes E, van EschJan H, Eelkema R. Catalytic control over supramolecular gel formation. *Nat Chem*. 2013; 5(5):433–437. [PubMed: 23609096]

Figure 1.

MALDI-TOF mass spectra of (a) Bi-aldehyde functionalized PEG (PEG-bCHO); (b) Model reaction product between PEG-bCHO and o-(pent-4-en-1-yl) hydroxylamine (Intermediate 2) show monomodal distributions and complete reaction conversion.

Figure 2.

(a) The chemical structure of bi-aldehyde-functionalized PEG (PEG-bCHO); (b) The chemical structure of 4-arm aminooxy crosslinker; (c) The general reaction scheme for the oxime ligation. The reaction is pH and nucleophile catalyst dependent; (d) A schematic of the hydrogel network formation via oxime bond ligation using a 4-arm aminooxy crosslinker; (e) Photos of hydrogels formed at pH 7.4 and hydrogels formed at pH 4.5 (f) show clearly the mechanical differences in the hydrogels with the fast crosslinking kinetics. Gels obtained at pH 4.5 are stronger than those at pH 7.4 at similar time intervals. (g) The FT-IR spectra of PEG-bCHO (black) and hydrogels (red) clearly show the extent of oxime formation in the hydrogels; (h) Raman spectra of phenyl and C=N transitions for PEG-bCHO (black) and hydrogels (red) complement the FT-IR data showing the extent of reaction during the gel formation.

Figure 3.

The extent of crosslinking and mechanical properties are highly dependent on pH. (a) Modulus vs. Frequency behavior of hydrogels formed at pH 4.5 and 7.4 show that the gels formed at low pH are much stronger at comparable time intervals; (b) The storage modulus (G')-pH plot (frequency 10 rad/s, strain 10%) show that the mechanical properties can be tuned via pH. The data are displayed as the mean and the error bars represent the standard deviation of three independent measurements for each condition.

Figure 4.

The gelation kinetics are highly dependent on pH. (a) Modulus-time behavior of hydrogels formed at pH 4.5; (b) The plot of pH vs time captures the gel point and the times to reach complete gelation. At low pH the gelation time is too fast to capture. The asterisks (*) in (b) represent time intervals that are too short to detect. The data represent the mean and the error bars represent the standard deviation of three independent samples for each condition.

Figure 5. Influence of aniline catalyst (0.1 wt%) on hydrogels storage modulus (a), time to reach the gel point (b) and the complete gelation time (c) as a function of pH. The data represent the mean and the error bars represent the standard deviation of three independent samples for each condition.

Figure 6.

(a) Fabrication procedure of oxime hydrogels with “clickable” groups is a two-step process. PEG-bCHO was initially mixed together with Azide-ONH₂ (or alkene-ONH₂) for minutes, followed by the addition of 4-arm aminooxy crosslinker. (b1) FT-IR spectra of azide functionalized hydrogels before (black) and after (red) “click” conjugation with alkyne-RGD-biotin peptides. (b2) Fluorescence microscopy image of azide hydrogels after bonding with TRITC labeled streptavidin. Red areas are from labeled hydrogels and black areas are the glass side background. (b3) Schematic of the “click” cycloaddition reaction and the binding sites for peptide or protein conjugation. (c1) Raman spectra of alkene hydrogels before (black) and after (red) the thiol-ene addition of 2-mercaptoethanol. (c2) Fluorescence microscopy image of alkene functionalized hydrogels after 3D patterning with FTIC-RGD-thiol. (c3) Schematic of thiol-ene addition sites. (Scale bar 100 μm)

Scheme 1.

(a) Synthesis route of bi-aldehyde functionalized PEG Precursor (PEG-bCHO, n=104). (b) Synthesis route of 4-arm aminooxy crosslinker. (c) Chemical structure of azide-aminooxy extender (azide-ONH₂). (d) Chemical structure of alkene-aminooxy extender (alkene-ONH₂). (e) Model reaction of oxime ligation between PEG-bCHO and o-(pent-4-en-1-yl) hydroxylamine (Intermediate 2).