

**Dually degradable click hydrogels for controlled degradation
and protein release**

Journal:	<i>Journal of Materials Chemistry B</i>
Manuscript ID:	TB-ART-03-2014-000496.R1
Article Type:	Paper
Date Submitted by the Author:	21-May-2014
Complete List of Authors:	Kharkar, Prathamesh; University of Delaware, Materials Science and Engineering Kloxin, April; University of Delaware, Chemical & Biomolecular Engineering and Materials Science & Engineering Kiick, Kristi; University of Delaware, Materials Science and Engineering

1 **Dually degradable click hydrogels for controlled**
2 **degradation and protein release**

3
4
5
6

7 **Prathamesh M. Kharkar,^a April M. Kloxin,^{*ab} and Kristi L. Kiick^{*acd}**

8

9 ^aDepartment of Materials Science and Engineering, University of Delaware,
10 Newark, DE 19716, USA. E-mail: akloxin@udel.edu; kiick@udel.edu

11

12 ^bDepartment of Chemical and Biomolecular Engineering, University of Delaware,
13 Newark, DE 19716, USA

14

15 ^cBiomedical Engineering, University of Delaware, Newark, DE 19716, USA

16

17 ^dDelaware Biotechnology Institute, University of Delaware, Newark, DE 19716,
18 USA

19

1 **1. Introduction**

2

3 Click reactions have garnered significant interest in the broader areas of materials
4 science and bioconjugation owing to their fast reaction kinetics, high
5 regioselectivity, and efficient reaction yields, all under mild conditions.¹⁻⁴ Many
6 click chemistries have been applied to the production of materials, including the
7 traditional azide-alkyne, Diels-Alder, Michael addition, thiol-ene, and oxime
8 reactions.^{3,5} In particular, click reactions that do not require a catalyst or initiator
9 and are free of byproducts, such as the reaction of maleimides and thiols, are
10 useful for biological applications owing to their cytocompatibility in the presence
11 of proteins, cells, or tissues.^{6,7} Utilizing these reactions, injectable hydrogels can
12 be easily created as delivery vehicles for therapeutics, particles, or cells.⁸⁻¹⁰ In this
13 application, temporal changes in material properties caused by degradation allow
14 the controlled release of therapeutics, the elaboration of secreted matrix by
15 encapsulated or infiltrating cells, or the spreading, migration, and release of
16 encapsulated cells.¹¹⁻¹³

17

18 Cleavage of the click linkages provides an attractive and relatively cost-effective
19 approach to incorporate degradability without the use of more complex
20 components, such as degradable peptides or proteins. Recent studies have
21 demonstrated the degradability of click crosslinks under mechanical^{14, 15} and
22 thermal^{16, 17} stresses; however, such reaction conditions can limit the translation
23 of these approaches into clinical applications owing to the limited
24 cytocompatibility of their associated stimuli. Overcoming this limitation, Baldwin

1 and Kiick have recently introduced thiol-maleimide click reactions in solution and
2 within PEG-heparin hydrogels that are sensitive to reducing microenvironments
3 found *in vivo*.^{10, 18} Opportunities to exploit these strategies for controlled delivery
4 of encapsulated cargo molecules, however, have not yet been demonstrated.

5
6 Despite recent technological advances, the delivery of therapeutic proteins (e.g.,
7 Trastuzumab, Bevacizumab, Rituximab) and small molecule drugs (e.g.,
8 Fluorouracil, Paclitaxel) remains a major challenge in the treatment of many
9 diseases, including cancer.¹⁹ In approaches for cancer treatment, delivery to the
10 site of a tumor is critical for therapeutic success and minimization of side
11 effects.²⁰ Injectable hydrogel-based drug carriers offer advantages for these
12 applications, enabling the efficient encapsulation of cargo molecules while
13 maintaining bioactivity for localized delivery at a preprogrammed rate or
14 responsive manner.²¹⁻²⁴ Depending on the cargo molecule of interest, the rate of
15 release can be controlled by diffusion, degradation, affinity, or a combination of
16 these mechanisms through hydrogel design. Degradation-mediated release is a
17 versatile approach for the temporally controlled delivery of numerous payloads,
18 from hydrophilic proteins to small molecules caged within nanoparticles, without
19 chemical modification of the therapeutic, which can affect drug efficacy and
20 clinical translation.²⁵⁻²⁷ Several strategies have been employed to incorporate
21 degradability within the hydrogel by inclusion of labile crosslinks, including
22 esters,^{28, 29} photolabile groups,³⁰⁻³² and enzyme-sensitive linkers.^{25, 33} As will be
23 elaborated below, linkers that are sensitive to reductants are attractive and simple

1 for controlled release in cancerous tissues, which have elevated levels of sulfur-
2 containing compounds.³⁴
3
4 Accordingly, reduction-sensitive disulfide linkages have been widely used for
5 intracellular delivery of DNA, siRNA, proteins, and therapeutic drugs.³⁵⁻³⁹ These
6 strategies rely on rapid destabilization of the drug carrier due to reduction of
7 disulfide bonds in the presence of glutathione (GSH) tripeptides, one of the major
8 sulfur-containing compounds found at elevated levels within cancerous tissues
9 and cells.^{40, 41} Since the intracellular concentration of GSH (ca. 0.5 mM to 10
10 mM) is 100 to 1000 times higher than the extracellular concentration (ca. 0.001
11 mM to 0.02 mM), efficient intracellular delivery of cargo molecules has been
12 achieved using disulfide chemistry.^{42, 43} However, the rapid rate of degradation of
13 disulfide linkages provides limited control over material degradation and cargo
14 release, and GSH-sensitive linkers that permit controlled extracellular delivery
15 over days to weeks thus have been less explored. In addition, since the
16 concentration of GSH is higher in carcinoma tissues than in healthy tissues due to
17 abnormal proliferative activities of cancer cells,^{40, 41, 44} reducing sensitive
18 chemistries incorporated within drug delivery carriers offer great potential for
19 localized cancer treatment. To address this need and opportunity, we present
20 reducing microenvironment-sensitive hydrogels that undergo tunable degradation
21 on the order of days to weeks for controlled protein delivery, demonstrating the
22 broad utility of the click bond cleavage and thiol exchange reaction as a general

1 strategy not only to control degradation but also to control the release of cargo
2 molecules locally from a bioinert delivery vehicle.

3

4 Specifically, we describe the development of multimode, degradable
5 poly(ethylene glycol) (PEG) hydrogels using Michael-type addition and exchange
6 reactions by incorporation of select thioether succinimide crosslinks. These
7 hydrogels are composed of multifunctional PEG crosslinked using thiol-
8 maleimide click chemistry and can undergo degradation by two mechanisms: i)
9 cleavage of click linkages and thiol exchange reactions in the presence of GSH
10 and ii) ester hydrolysis. To achieve this, multiarm PEG macromers were
11 functionalized with different mercaptoacids and reacted with maleimide-
12 functionalized PEG, creating hydrogels that degrade by either hydrolytic *or*
13 hydrolytic and thiol-exchange mechanisms. Hydrogel degradation was monitored
14 in physiologically-relevant GSH microenvironments via oscillatory rheometry
15 and volumetric swelling measurements to assess the degradation kinetics. The
16 ability to incorporate and selectively release a cargo molecule was investigated by
17 monitoring, via fluorescence spectroscopy, the release of bovine serum albumin
18 (BSA) as a model protein. The ability to precisely control hydrogel degradation
19 and thus the release profile of cargo molecules using cleavage of click linkages
20 offers exciting avenues for designing biomaterials for drug delivery and tissue
21 engineering applications.

22

23 **2. Methods and Materials**

24

1 **2.1 Materials**

2
3 4-arm hydroxyl-functionalized poly(ethylene glycol) (PEG-4-OH, 10000 g mol⁻¹),
4 4-arm thiol-functionalized PEG (PEG-4-SH, 10000 g mol⁻¹), and linear
5 maleimide-functionalized PEG (PEG-2-MI, 5000 g mol⁻¹) were purchased from
6 JenKem Technology USA Inc. (Allen, TX). 3-Mercaptopropionic acid (MP), 4-
7 mercaptophenylacetic acid (MPA), p-toluenesulfonic acid monohydrate (PTSA),
8 triethylamine (TEA), dithiothreitol (DTT), and glutathione (GSH) were purchased
9 from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) and all solvents
10 were obtained from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin
11 labeled with Alexa Fluor 488 (BSA-488) was purchased from Life Technologies
12 (Grand Island, NY). All commercially available reagents were used as received
13 without further purification unless otherwise noted.

15 **2.2 Synthesis of mercaptoacid-based PEG-thiols**

16
17 PEG was modified with MP or MPA functional groups based on modified
18 versions of previously published protocols.^{8, 10} Briefly, PEG-4-OH (0.1 mmol),
19 mercaptoacid (4 mmol), PTSA (0.04 mmol), and toluene (20 mL) were added to
20 an oven-dried round-bottom flask equipped with a reflux condenser. The reaction
21 setup was purged with nitrogen under room temperature. The reaction (**Scheme 1**)
22 was heated to reflux (110 °C) and stirred for 48 hours, and generated water was
23 collected by using a Dean-Stark trap. Upon completion, the reaction was cooled to
24 room temperature, and the functionalized PEG precipitated three times in ethyl
25 ether. The product was recovered by vacuum filtration and rinsed with 2-propanol

1 followed by hexane. The dried polymer product (1 equiv) was reduced in toluene
2 using DTT (1 equiv) and TEA (1 equiv) for 5 hours, under inert atmosphere. The
3 finished reaction was acidified with TFA (1.1 equiv), and the polymer was again
4 precipitated in ethyl ether and recovered by filtration. Subsequently, the polymer
5 was dissolved in methanol, and the mixture was filtered through a 0.22 μm filter
6 followed by precipitation in 2-propanol and vacuum filtration. The solid product
7 was rinsed with copious amounts of 2-propanol and hexane. The final dried
8 polymer was obtained by removal of residual solvents under reduced pressure.
9 The degree of thiol functionalization of the polymer was characterized via ^1H
10 NMR spectroscopy, using a Bruker AV 400 NMR spectrometer (Bruker
11 Daltonics, Billerica, MA) with CDCl_3 as the solvent and TMS as the reference.

12

13 *PEG-4-MP*

14 The general procedure for synthesis of PEG-thiol was followed using MP as the
15 mercaptoacid to yield PEG-4-MP. The final polymer was obtained as a white
16 solid (0.6 g, 74% yield). The functionality was estimated to be 92% based on
17 integration of the proton neighboring the ester linkage relative to the PEG
18 backbone protons. (**Fig. S1 B**).

19 ^1H NMR (400 MHz, CDCl_3) δ : 4.28 (8H, t), 3.90-3.35 (900H, bs), 2.82-2.62
20 (16H, m), 1.68 (4H, t).

21

22 *PEG-4-MPA*

1 The general procedure for synthesis of PEG-thiol was followed using MPA as the
2 mercaptoacid to yield PEG-4-MPA. The final polymer was obtained as a white
3 solid (0.54 g, 66% yield). The functionality was estimated to be 90% based on
4 integration of the proton neighboring the ester linkage relative to the PEG
5 backbone protons (**Fig. S1 C**).

6 ^1H NMR (400 MHz, CDCl_3) δ : 7.24-7.08(16H, m), 4.24 (8H, t), 3.90-3.35 (900H,
7 bs), 3.42-3.39 (4H, s).

8

9 **2.3 Gelation time and rheology characterization**

10

11 Hydrogel precursor solutions were prepared by dissolution of thiol- and
12 maleimide-functionalized PEG (5% w/w) in citric acid buffer (pH 5) and
13 phosphate-buffered saline (pH 7.4), respectively. Slightly acidic conditions
14 allowed tuning of the gelation time (i.e., increased gelation time) due to the
15 reduced nucleophilicity of thiolate species under acidic conditions;^{10, 45} these
16 polymerization conditions previously have been shown to be effective for use in
17 cell/protein studies in vitro.⁴⁶ Gelation time was studied qualitatively using the
18 tube inversion method. Briefly, the hydrogel precursor solutions were mixed (100
19 μL) and immediately pipetted into a glass vial. In five-second intervals, vials were
20 inverted to observe if the solution flowed. The timepoint at which the solution did
21 not flow was recorded as the gelation time.

22

23 For rheological studies, the hydrogels were formed directly on the rheometer
24 (AR-G2, TA instruments, USA) by mixing the precursor solutions (1:1

1 maleimide:thiol molar ratio resulting in 5 % w/w hydrogels), immediately
2 pipetting onto a Peltier plate at 25 °C, and commencing measurements (120 µm
3 gap). Gelation at room temperature ensured that the gelation time was sufficiently
4 slow to allow good mixing of precursor solutions on the Peltier plate prior to
5 gelation. This also allowed the gels to form homogeneously so that all gels had
6 similar moduli prior to protein release experiments. The gelation time and final
7 shear modulus of the hydrogel were determined using rheometry experiments.
8 Frequency sweeps were performed to determine the linear viscoelastic regime
9 (0.01 to 10 % strain at 6 rad/s). Using a 20-mm diameter parallel plate geometry,
10 time-sweep measurements were obtained within the linear viscoelastic regime (1
11 % constant strain mode at a frequency of 6 rad/s) at 25 °C.

12

13 **2.4 Hydrogel degradation characterization**

14

15 For hydrogel degradation studies, polymer precursor solutions (5% w/w) were
16 mixed in a 1:1 maleimide:thiol molar ratio and pipetted into a cylindrical mold
17 (diameter = 4.6 mm, thickness = 1.8 mm). The solutions were allowed to gel for
18 two hours at room temperature to ensure maximum possible crosslink density was
19 achieved for all samples. The rheological data showed that once the gels have
20 been formed (i.e., stable storage moduli is achieved at 30 min), the moduli remain
21 consistent through 2 hours. The resulting hydrogels were washed with PBS and
22 incubated, at room temperature, in 5 mL of PBS buffer (pH 7.4) containing GSH
23 (0 mM, 0.01 mM or 10 mM) over the experimental time period. The pH of the
24 buffer after GSH addition was adjusted to a pH of 7 by addition of 0.1 M sodium

1 hydroxide. Degradation was monitored by measuring volumetric swelling and
2 shear modulus. For the shear modulus measurements, time sweeps were
3 performed within the linear viscoelastic regime for each sample (2 rad/s, 2%
4 strain, and 0.25 N normal force in order to prevent hydrogel slip).

5

6 **2.5 Volumetric swelling and mesh size calculations**

7

8 Hydrogel discs (diameter = 4.6 mm, thickness = 1.8 mm) were placed in PBS
9 buffer with 0 mM, 0.01 mM, or 10 mM GSH at room temperature and gently
10 rocked. Samples were removed at respective time points, and the diameters of
11 hydrogels were measured using a Vernier caliper, whereas the height was
12 determined using the rheometer gap values. Volume of the hydrogel at each time
13 points was determined based on measured diameter and height and assuming
14 cylindrical geometry. The % volumetric swelling at each time point was
15 calculated by normalizing to the volume of the gel immediately after formation
16 (day 0 before equilibrating with PBS).

17

18 **2.6 Protein release**

19

20 For protein release experiments, polymer precursor solutions (5% w/w) were
21 mixed in a 1:1 maleimide:thiol molar ratio along with BSA-488 (loading
22 concentration 1.2 mg/ml) and added to a cylindrical mold (diameter = 4.6 mm,
23 thickness = 1.8mm). The solutions were allowed to gel for two hours at room
24 temperature. Hydrogel discs were immediately washed with PBS thrice to remove
25 any non-encapsulated BSA-488 and then gently rocked at room temperature in 5

1 mL of PBS buffer with GSH (10 mM). The amount of BSA-488 present in the
2 hydrogel was calculated by subtracting the amount of BSA-488 released during
3 wash steps from the amount of BSA-488 that was initially loaded into the gel. At
4 each time point, a 100- μ L aliquot of the sink solution was removed for protein
5 release measurements and replaced by 100 μ L of fresh GSH in PBS. The released
6 BSA-488 was quantified by fluorescence measurements using a microplate reader
7 (Synergy H4, BioTek Inc., Winooski, VT) taking into account the cumulative
8 sample dilution due to removal and addition of fresh GSH in PBS at each time
9 point measurement (see Supporting Information). To estimate the concentration of
10 released BSA-488, a calibration curve for the fluorescence of BSA-488 as a
11 function of its concentration was acquired. The release of the BSA was monitored
12 via SDS-PAGE analysis of the solutions removed at each timepoint; 7 μ L of sink
13 solution containing released protein was loaded onto a standard sodium dodecyl
14 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for analysis. The
15 concentration of protein in each band was quantified with densitometry analysis
16 using the gel analysis function in ImageJ (version 1.46).

17

18 **2.7 Statistical analysis**

19

20 Results are expressed as mean \pm standard error of mean (SEM) unless otherwise
21 specified. Monomer synthesis reactions were performed in duplicate. Hydrogel
22 formation experiments were performed in triplicate. Degradation and protein
23 release studies were performed in duplicate with 3 hydrogels per condition at each

1 experimental time point. Statistical comparisons were based on analysis of
2 variance (ANOVA) and $p < 0.05$ was considered statistically significant.

3 4 **3. Results and Discussion**

5 6 **3.1 Hydrogel compositions for control of degradation**

7
8 Many natural and synthetic polymers have been used for hydrogel formation, with
9 polymer selection partly dictated by the application of interest.⁵ PEG-based
10 hydrogels are well suited for drug delivery applications owing to their
11 biocompatibility, lack of protein binding sites, and the ease of engineering their
12 properties.⁴⁷ The facile functionalization of the hydroxyl end groups of PEG
13 allows the incorporation of different chemical functionalities for hydrogel
14 formation in the presence of proteins and cells *and* for controlled degradation.
15 Exploiting these advantages, PEG-OH was functionalized with alkyl (MP) and
16 aryl (MPA) based mercaptoacids utilizing established protocols.^{8, 10} These thiol
17 end groups act as nucleophiles and react rapidly with maleimide functional groups
18 to form crosslinks by a Michael-type addition reaction. Michael-type addition
19 reactions are highly efficient and versatile reactions that occur under
20 physiological conditions without byproducts and have been used to crosslink
21 cytocompatible hydrogels.⁴⁸⁻⁵⁰ Here, the composition of the hydrogel was varied
22 to enable microenvironment-controlled degradation and protein release (**Fig. 1**).
23 PEG-4SH-based hydrogels (**Control**), which contain water stable ether bonds,
24 served as a non-degradable control owing to lack of any degradable functional
25 groups. Owing to the presence of ester linkages, MP-based hydrogels (one

1 degradable group, **D1E**) undergo ester hydrolysis, whereas the MPA-based
2 hydrogels (two degradable groups, **D2ER**) undergo ester hydrolysis and click
3 bond cleavage and thiol exchange reactions.

4

5 **3.2 Consistent hydrogel formation**

6

7 Dynamic time sweep experiments were conducted to study hydrogel gelation
8 kinetics and final hydrogel moduli. Data were acquired within the linear
9 viscoelastic regime. After vortexing the precursor solutions, the storage and loss
10 moduli were recorded as a function of time. Representative results for the **D2ER**
11 hydrogel formation are shown in **Fig. 2A**. The crossover point (i.e., $G'=G''$),
12 which is an indirect measurement of the gel point, was not observed due to the
13 rapid onset of gelation before the first data point was acquired; the gelation time
14 thus was semi-qualitatively determined by the tube inversion method.⁵¹ Faster
15 gelation was observed for **D2ER** (~20 sec) compared to **D1E** (~35 sec) and
16 **Control** (~40 sec) hydrogels; this rapid gelation is consistent with the reported
17 kinetics of thiol-maleimide reactions.⁵² The time difference for gelation between
18 **D2ER**, **D1E**, and **Control** can be attributed to the thiol reactivity (**D1E** and
19 **Control**, alkylthiols $pK_a = 10.2$; **D2ER**, arylthiols $pK_a = 6.6$).^{53, 54} The difference
20 in thiol reactivity of **Control**, **D1**, and **D2ER** essentially arises from the
21 mesomeric effect provided by the aromatic ring in the case of **D2ER**, making it
22 more nucleophilic than **Control** and **D1E**.

23

1 Presence of aromatic ring in **D2ER** results in higher nucleophilicity due to
2 mesomeric effect, which dictates the thiol reactivity. With increasing time, the
3 storage modulus (G') increases rapidly without a significant increase in loss
4 modulus (G''). These data highlight the elastic nature of the network. Time to
5 achieve final storage moduli varied depending upon the identity of thiol groups,
6 which again can be attributed to the thiol reactivity (**D2ER**: ~15 minutes; **D1E**:
7 ~30 min; and **Control**: ~34 min). Although the experiments were performed at
8 the room temperature (25 °C), the gelation time and time to achieve the final
9 storage moduli can be further decreased by forming hydrogels at elevated
10 temperatures.

11

12 Material modulus is directly correlated with the crosslink density as per the theory
13 of rubber elasticity.⁵⁵ The final storage moduli, which is defined as the value of G'
14 after reaching plateau, for **Control**, **D1E**, and **D2ER** hydrogels were examined to
15 compare the consistency in crosslink density between the different compositions
16 (**Fig. 2B**). The final post-gelation, equilibrium-swollen G' were recorded after
17 complete gelation for **Control**, **D1E**, and **D2ER**. As indicated in the figure, the
18 post gelation equilibrium G' was ~2.3 kPa for all three compositions. There were
19 no statistically significant differences between the final plateau moduli of the
20 various gels (one-way ANOVA, $p = 0.88$), indicating that the use of different
21 thiols did not affect the final crosslink density substantially. Side reactions such as
22 disulfide formation and maleimide ring hydrolysis alter the reactivity of the thiol
23 and maleimide groups and thus could affect the number of functional groups

1 available for hydrogel formation (**Scheme S1**), potentially decreasing the final
2 moduli for a particular composition. The lack of a statistically significant
3 difference between the final equilibrium G' values thus also suggests that there
4 were no significant differences in the extent of these side reactions for the various
5 hydrogel compositions. These results suggest that differences in gelation for
6 **Control**, **D1E**, and **D2ER** did not significantly contribute to network defects due
7 to strict 1:1 stoichiometry and relative rate of Michael-type addition as compared
8 to other defect forming side reactions. Further, the molecular weight of the PEG
9 chains for the gel compositions investigated here was selected to minimize any
10 looping, unreacted functionalities, and other related network defects based on
11 studies of related PEG hydrogels in the literature.⁴⁵ The similarity of the initial
12 crosslink densities between the **Control**, **D1E**, and **D2ER** hydrogels allow the
13 study of the degradation kinetics by direct monitoring of changes in G' as a
14 function of time.

15

16 **3.3. Degradation in a reducing microenvironment**

17

18 In order to evaluate the most rapid hydrogel degradation that might be observed in
19 physiologically relevant reducing microenvironments, as well as to evaluate the
20 associated degradation mechanism, the higher GSH of 10 mM first was examined.
21 Potential degradation mechanisms for each hydrogel composition are described in
22 **Fig. 3**. Thioether succinimide linkages formed using arylthiols (**D2ER** hydrogels)
23 can undergo thiol exchange in the presence of exogenous thiols (a GSH-rich
24 microenvironment) in contrast to alkylthiols (**Control** and **D1E**), which are stable

1 within the experimental time frame (stable at $t < 6$ days).¹⁸ The presence of ester
2 linkages in the **D1E** and **D2ER** hydrogels allows degradation by ester hydrolysis
3 over longer time scales (stable at $t > 1$ month to 2 years depending upon
4 neighboring groups).⁵⁶ A potential hindrance to degradation by the thiol exchange
5 mechanism is possible hydrolysis of the maleimide ring, which leads to ring
6 opening and the formation of an irreversible crosslink. However, the rate of
7 maleimide ring hydrolysis is significantly slower (by one order of magnitude)
8 than the competing click cleavage and thiol exchange reaction ($k = 3.7 \times 10^{-2} \text{ h}^{-1}$
9 for click cleavage and thiol exchange, $k = 3.3 \times 10^{-3} \text{ h}^{-1}$ for maleimide ring
10 hydrolysis).¹⁸ Consequently, we assume that changes in G' for **D2ER** hydrogels
11 are dominated by mainly thiol-exchange reactions in reducing microenvironments
12 and by ester hydrolysis in non-reducing microenvironments.

13

14 Oscillatory rheometry and volumetric swelling measurements were used to study
15 the degradation of the hydrogels (defined here as the scission of network
16 crosslinks) under thiol-rich reducing conditions. Degradation kinetics were
17 assessed by measuring the storage moduli of hydrogel discs that were suspended
18 in solutions containing 10 mM GSH (**Fig. 4A**). The storage moduli at each time
19 point were normalized to the initial modulus for that gel composition directly after
20 formation (day 0 before equilibrating with PBS), where the initial gel has a
21 normalized modulus of 100%. As illustrated in the figure, the **Control** and **D1E**
22 samples exhibited an initial decrease in G' to approximately 80% of the
23 normalized value within 24 hours, but did not exhibit any further rapid decrease in

1 moduli after this point. The initial decrease can be attributed to the equilibrium
2 swelling that occurs after hydrogel formation. No significant change was
3 observed in G' post-equilibrium swelling for **Control** hydrogels, which was
4 expected since no degradable functional groups are present within these
5 hydrogels. A slight decrease in modulus over time was observed for **D1E**
6 hydrogels, which can be attributed to ester hydrolysis (calculated first-order rate
7 constant, $k = 3.33 \times 10^{-5} \text{ min}^{-1}$). This rate constant compares well with the typical
8 ester linkage hydrolysis rate constant in hydrophilic polymer networks (1.33×10^{-5}
9 to $7.33 \times 10^{-6} \text{ min}^{-1}$) corresponding to half lives of 6 to 32 days.⁵⁷ The
10 degradation rate constant for **D1E** was found to be statistically different from the
11 **Control** (two-tailed P value = 0.005), highlighting the role of ester linkages in the
12 degradation of **D1E** (**Fig. S3 and S4**). In contrast, a rapid decrease in G' was
13 observed for **D2ER** hydrogels, and the reverse gel point, defined as complete
14 hydrogel dissolution, was observed after approximately 4 days (at 5700 minutes).
15 The rapid decrease in G' indicates a substantial decrease in crosslink density and
16 can be attributed to the reversibility of the thiol-maleimide reaction and the
17 consequent thiol exchange reactions that occur in the presence of GSH. As the
18 rates of ester hydrolysis and maleimide ring hydrolysis are slow, the rapid rate of
19 degradation of **D2ER** highlights the role of click bond cleavage and thiol
20 exchange reaction as leading cause of hydrogel degradation.

21

22 Temporal changes in the volumetric swelling also were examined for the **Control**,
23 **D1E**, and **D2ER** hydrogels that were suspended in 10 mM GSH (**Fig. 4B**). All

1 three hydrogel compositions showed an initial increase in the swelling as the
2 hydrogels achieved equilibrium swelling. The **Control** and **D1E** hydrogels
3 remained stable after this initial swelling event ($t > \sim 24$ hours), whereas the
4 volumetric swelling for **D2ER** hydrogels continued increasing until complete
5 degradation (gel dissolution) occurred at 5700 minutes. The continuous increase
6 in the swelling before complete degradation for **D2ER** is consistent with a bulk
7 degradation mechanism, as well as with the rheometric measurements where the
8 increases in swelling are commensurate with observed decreases in modulus.
9 Overall, these results indicate that well-defined hydrogels can be designed to
10 degrade in a reducing microenvironment with selection of arylthiol-based
11 thioether succinimide linkages. Such a system could prove useful in the design of
12 hydrogels for controlled and local delivery of anti-cancer drugs.

13

14 **3.4 Influence of GSH concentration on hydrogel degradation**

15

16 To reject the possibility that the degradation of **D2ER** hydrogels under high
17 [GSH] conditions was substantially affected by ester hydrolysis, the mechanical
18 properties of **D2ER** hydrogels were monitored in solutions lacking GSH (0 mM
19 GSH). In addition, since thiol exchange reactions are dependent on GSH
20 concentration, we investigated an additional condition (0.01 mM GSH, **D2ER**
21 hydrogel), which mimics the extracellular GSH concentration. The storage moduli
22 (G') of hydrogels were measured at predetermined time points. The **Control**
23 hydrogel exhibited an initial decrease in G' over the first 24 hours, after which G'
24 did not change, irrespective of GSH concentration (0 and 10 mM, **Fig. S3**). As

1 discussed in Section 3.3, the initial changes in G' can be attributed to equilibrium
2 swelling. The constant moduli observed for timepoints after 24 hours indicate that
3 the polymeric crosslinks are stable within the experimental timeframe and do not
4 undergo any significant degradation. For **D1E** hydrogels, the storage moduli
5 initially decreased, which again can be attributed to equilibrium swelling (**Fig.**
6 **S4**). However, for the **D1E** hydrogel, the decrease in storage moduli continued
7 past 24 hours, which would be consistent with degradation via ester hydrolysis as
8 discussed above.

9
10 As shown in **Fig. 5**, the storage moduli varied as a function of GSH concentration
11 for **D2ER** hydrogels. For the 0 mM GSH condition, G' initially decreased during
12 the first 24 hours, owing to equilibrium swelling, followed by a slow decrease in
13 G' to 81% of its initial normalized value. The decrease after 24 h can be attributed
14 to ester hydrolysis ($k = 1.35 \times 10^{-5} \text{ min}^{-1}$, $t_{1/2} = 35 \text{ days}$). For 0.01 mM GSH, G'
15 decreases rapidly and complete gel degradation was observed at approximately 8
16 days ($t \sim 200 \text{ h}$), indicating that the degradation mechanism in the presence of
17 glutathione is dominated by the reversibility of the thiol-maleimide reaction and
18 the resulting thiol exchange that is possible in the presence of GSH. Further, in
19 comparison with solutions containing 10 mM GSH, these data highlight the
20 dependence of the rate of **D2ER** hydrogel degradation on GSH concentration. At
21 lower GSH concentration, the free thiol groups ($\sim 14 \mu\text{M}$) generated due to the
22 click bond cleavage compete with the free GSH thiols ($\sim 10 \mu\text{M}$), since the
23 concentration is comparable. In this case, the GSH concentration is a limiting

1 factor, and the rate of degradation is significantly slower for 0.01 mM compared
2 to the 10 mM GSH condition, in which GSH is present in a large excess. Overall,
3 these results indicate that the **D2ER** hydrogels can undergo ester hydrolysis, but
4 the rate of ester hydrolysis is very slow (under 0 mM GSH $k \sim 10^{-5} \text{ min}^{-1}$). As
5 rapid degradation of D2ER hydrogels is observed under reducing conditions (10
6 mM GSH $k \sim 10^{-3} \text{ min}^{-1}$), the data clearly indicate that the click bond cleavage
7 and thiol exchange reaction is the primary mechanism for gel degradation.
8 Further, to verify that **D2ER** hydrogels can undergo complete degradation via
9 ester hydrolysis, **D2ER** hydrogels were subjected to basic conditions to accelerate
10 ester bond hydrolysis (0.1 M sodium carbonate buffer, pH 11.5) and exhibited
11 complete degradation within 24 hours in the absence of a reducing
12 microenvironment, confirming the dual degradability of the hydrogels.

13

14 To further investigate the mode of degradation, temporal changes in the
15 volumetric swelling were monitored for **D2ER** hydrogels suspended in various
16 reducing microenvironments (**Fig. 5B**). During the first 24 hours, the volumetric
17 swelling increases for all three conditions, which can be attributed to initial
18 hydrogel equilibrium swelling. After 24 hours, the volumetric swelling
19 continuously increases for the 0.01 mM and 10 mM condition over the course of
20 degradation, which is consistent with rheometric measurements. A bulk
21 degradation mechanism is indicated by this continuous increase in the swelling as
22 a function of time.^{25, 58}

23

1 3.5 Degradation Kinetics

2
3 Regression analysis was conducted to obtain further insight into the degradation
4 mechanism of **D2ER** hydrogels and the kinetics of associated degradation
5 reactions (**Fig. 6**). When exposed to 0 mM GSH, the decrease in storage moduli
6 can be attributed to ester hydrolysis. Owing to the highly swollen nature of the
7 hydrogels, and since the buffer is present in large excess, the water concentration
8 during the degradation time period can be assumed to be relatively constant.
9 Hence, the reaction kinetics was observed to be pseudo-first order with a rate
10 constant $1.87 \times 10^{-5} \pm 5.83 \times 10^{-6} \text{ min}^{-1}$. The differences in the rate of ester
11 hydrolysis calculated for the **D2ER** (here) and **D1E** hydrogels (above) can be
12 attributed to local hydrophobic domains associated with aryl thiols in the **D2ER**
13 gels, consistent with a previously reported study by Schoenmakers *et al.* in which
14 the rate of ester hydrolysis varied with local hydrophobicity.⁵⁹ When **D2ER** gels
15 were exposed to 0.01 mM GSH, a rapid decrease in G' was observed, consistent
16 with the occurrence of both thiol exchange reactions and ester hydrolysis.
17 Because the theoretical concentration of thiol groups from PEG is comparable to
18 that of the thiol groups from GSH (see above), the rate of hydrogel degradation is
19 dependent both on the concentration of degradable functional groups (which
20 correlate with the crosslink density with 2 degradable groups per crosslink) and
21 the concentration of GSH. Consistent with this, the hydrogel degradation kinetics
22 were observed to be second order, with a rate constant $5.03 \times 10^{-6} \pm 0.16 \times 10^{-6}$
23 $\text{mM}^{-1} \text{ min}^{-1}$. With a higher concentration of GSH, the **D2ER** hydrogel rapidly
24 degrades. At 10 mM GSH, the GSH is present in large excess (~ 3 orders of

1 magnitude as compared to thiols present in the hydrogel), and thus the
2 concentration of GSH can be assumed to be constant during the experimental time
3 frame. Thus, the rate of degradation is dependent on only the crosslink density,
4 and first order degradation kinetics regression analysis yields a rate constant of
5 $1.75 \times 10^{-3} \pm 0.26 \times 10^{-3} \text{ min}^{-1}$. The degradation rate constants for the **Control**,
6 **D1E**, and **D2ER** hydrogels are been summarized in **Table S1**.

7

8 **3.6 Controlled release of a model protein**

9

10 The ability to tune the rate of degradation by varying crosslink chemistry offers
11 opportunities to utilize these hydrogels for the controlled release of therapeutics in
12 response to the reducing microenvironment or at a preprogrammed rate by ester
13 hydrolysis. To study the applicability of these hydrogels for controlled release
14 applications, a fluorescently-tagged model protein, bovine serum albumin (BSA-
15 488), was encapsulated during hydrogel formation. BSA-488 was chosen as a
16 model protein for release studies since the hydrodynamic diameter ($\sim 7.2 \text{ nm}$)⁶⁰ is
17 comparable to the estimated hydrogel mesh size ($\sim 9 \text{ nm}$). The size of the BSA
18 and mesh size calculated for the hydrogels suggest that these materials would be
19 useful for tailored release by hydrogel degradation, upon which the mesh size
20 becomes large enough to facilitate protein release. Similarly, bioactive proteins
21 (e.g., growth factors), therapeutic-laden nanoparticles, or even cells could be
22 released by this mechanism.

23

1 The release of BSA-488 was monitored by measuring fluorescence as a function
2 of time. The percent cumulative release was plotted as a function of time for all
3 three compositions (**Fig. 7A**). Approximately 40 % of BSA-488 was initially
4 released from all hydrogel compositions (**Control**, **D1E**, and **D2ER** hydrogels).
5 This release may be attributed to the increase in mesh size associated with initial
6 equilibrium swelling. The effective diffusion coefficient (D_e) was calculated using
7 a modified form of Fick's law^{61, 62} and the value was found to be $\sim 1.56 \times 10^{-8}$
8 $\text{cm}^2 \text{sec}^{-1}$ (see Supporting Information). This value of D_e is in agreement with
9 previously reported D_e values for BSA release from PEG hydrogels.⁶³ **D2ER**
10 hydrogels, which undergo rapid degradation in reducing microenvironments
11 owing to thiol exchange reactions, exhibited degradation-dependent release, with
12 ~ 95 % of the cargo released after approximately 4 days, commensurate with when
13 complete hydrogel degradation was observed. This result suggests that the
14 degradation reaction broadly modulates the release of the cargo molecule. Here,
15 the D_e was found to be $5.70 \times 10^{-8} \text{cm}^2 \text{sec}^{-1}$. The difference between the effective
16 diffusion coefficients for the hydrogel compositions correlates with the
17 degradation profile of these hydrogels.

18

19 SDS PAGE was employed to assess the molecular mass of the released BSA as an
20 indirect measure of its stability during encapsulation and release from the various
21 hydrogel compositions (**Fig. 7B**). Lanes 2 and 3 in the figure, which served as
22 controls, were loaded with BSA-488 in PBS buffer containing 10 mM GSH
23 prepared at two different time points (i.e., just before electrophoresis and before

1 starting the release experiment for BSA-488, respectively). Lane 4, 5, and 6 were
2 loaded with sink solution containing released BSA-488 from the **Control**, **D1E**,
3 and **D2ER** hydrogels, respectively. No major differences were observed between
4 the band locations. These results suggest that there were no substantial changes in
5 the overall hydrodynamic volume or molecular weight of the protein during
6 encapsulation and release. Densitometry analysis was carried out using NIH
7 Image J software. The band intensity from lane 3 was normalized to 100%, and
8 compared with the band intensity of released BSA from the **Control** (~33%),
9 **D1E** (~36%), and **D2ER** (~90%) hydrogels. The results correlated well with the
10 protein release data obtained using fluorescence measurements. Taken together,
11 these results suggest the utility of GSH-responsive hydrogels as a drug carrier for
12 controlled cargo release applications. However, for applications where rapid
13 release (~1 to 3 hours) of cargo is desired in response to reducing
14 microenvironment, disulfide linkages still may be more appropriate.

15

16 Few studies have reported the use of dually degradable hydrogels for tissue
17 engineering and cell encapsulation applications,^{28, 64} and the use of dually
18 degradable hydrogels for controlled release applications has been limited.
19 Recently, Wang and co-workers investigated use of dually degradable hydrogels
20 for protein release studies by incorporating an enzymatically degradable
21 hyaluronic acid based backbone and chemically cleavable disulfide linkages.⁶⁵
22 Depending on concentration of hyaluronidase and GSH, the hydrogel exhibited
23 significant degradation within the first ~1.5 to 5 hours, and complete release of a

1 cargo molecule (stromal cell-derived factor 1 α , 100 ng) was achieved within
2 approximately 8 hours. The click cleavage and subsequent thiol exchange system
3 presented here undergoes degradation on a significantly longer timescale (~ 4
4 days) offering advantages for controlled drug delivery, where wider control over
5 degradation can help transition to clinical applications. In addition, incorporation
6 of ester linkages affords long term clearance of these hydrogels from *in vivo*
7 microenvironments due to ester hydrolysis and subsequent degradation.

8

9 **4. Conclusions**

10

11 In this work, we report dually degradable PEG hydrogels in which degradation
12 can be tailored, without affecting hydrogel formation, by the Michael-type
13 addition of select functional groups that yield crosslinks with tunable, and
14 previously unexplored, degradation mechanisms. This facile approach enables
15 hydrogel formation by broadly useful thiol-maleimide click chemistry employing
16 arylthiols, while eliminating the need for the additional incorporation of more
17 complex and potentially costly labile chemistries within the crosslinker to
18 facilitate degradation, such as enzyme-labile peptides. The rate of hydrogel
19 degradation was found to be dependent upon the chemistry of linker, the number
20 of degradable crosslinks, and the concentration of the reducing
21 microenvironment. The release of a model protein from these hydrogels
22 demonstrates the potential of these matrices and approaches for controlled release
23 applications in thiol-rich reducing microenvironments. Control of degradation
24 rates permitted a 2.5-fold difference in protein release for the dually degradable

1 (D2ER) as compared to the non-degradable (Control) or single-mode degradable
2 (D1E) hydrogels. In principle, this strategy could easily be employed for
3 controlled release over different time frames using combinations of these thiol
4 functional groups within a single hydrogel or utilized in conjunction with more
5 elaborate degradable chemistries when desired for more complex degradation and
6 release profiles. The degradation of hydrogels by cleavage of click linkages
7 presents considerable opportunities in the design of materials for controlled drug
8 delivery and soft tissue engineering applications.

9

10 **5. Acknowledgements**

11

12 Research reported in this publication was supported by Institutional Development
13 Award (IDeA) from the National Institute of General Medical Sciences of the
14 National Institutes of Health under grant number P20GM103541 and the
15 University of Delaware Research Foundation. The authors thank Dr. Wilfred
16 Chen for use of the plate reader. The authors thank Rachel Kennel for technical
17 help with precursor solution and hydrogel preparation. Additionally, the authors
18 would like to thank Matthew Rehmann, Megan Smithmyer, Lisa Sawicki, and
19 Kelsi Skeens for feedback on earlier versions of this manuscript.

20

21

22

1 **6. References**

2

- 3 1. J. E. Moses and A. D. Moorhouse, *Chem Soc Rev*, 2007, 36, 1249-1262.
- 4 2. R. Manetsch, A. Krasinski, Z. Radic, J. Raushel, P. Taylor, K. B.
5 Sharpless and H. C. Kolb, *J Am Chem Soc*, 2004, 126, 12809-12818.
- 6 3. C. M. Nimmo and M. S. Shoichet, *Bioconjug Chem*, 2011, 22, 2199-2209.
- 7 4. H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew Chem Int Ed Engl*,
8 2001, 40, 2004-2021.
- 9 5. P. M. Kharkar, K. L. Kiick and A. M. Kloxin, *Chem Soc Rev*, 2013, 42,
10 7335-7372.
- 11 6. R. J. Pounder, M. J. Stanford, P. Brooks, S. P. Richards and A. P. Dove,
12 *Chem Commun*, 2008, 5158-5160.
- 13 7. M. A. Azagarsamy and K. S. Anseth, *ACS Macro Lett*, 2013, 2, 5-9.
- 14 8. T. Nie, A. Baldwin, N. Yamaguchi and K. L. Kiick, *J Control Release*,
15 2007, 122, 287-296.
- 16 9. H. Wang, A. Han, Y. Cai, Y. Xie, H. Zhou, J. Long and Z. Yang, *Chem*
17 *Commun*, 2013, 49, 7448-7450.
- 18 10. A. D. Baldwin and K. L. Kiick, *Polym Chem*, 2013, 4, 133-143.
- 19 11. M. W. Tibbitt, B. W. Han, A. M. Kloxin and K. S. Anseth, *J Biomed*
20 *Mater Res A*, 2012, 100, 1647-1654.
- 21 12. C. Adelow, T. Segura, J. A. Hubbell and P. Frey, *Biomaterials*, 2008, 29,
22 314-326.
- 23 13. F. P. Brandl, A. K. Seitz, J. K. Tessmar, T. Blunk and A. M. Gopferich,
24 *Biomaterials*, 2010, 31, 3957-3966.
- 25 14. J. N. Brantley, K. M. Wiggins and C. W. Bielawski, *Science*, 2011, 333,
26 1606-1609.
- 27 15. K. M. Wiggins, J. N. Brantley and C. W. Bielawski, *Acs Macro Letters*,
28 2012, 1, 623-626.
- 29 16. T. Dispinar, R. Sanyal and A. Sanyal, *J Polym Sci A Polym Chem*, 2007,
30 45, 4545-4551.

- 1 17. K. C. Koehler, K. S. Anseth and C. N. Bowman, *Biomacromolecules*,
2 2013, 14, 538-547.
- 3 18. A. D. Baldwin and K. L. Kiick, *Bioconjug Chem*, 2011, 22, 1946-1953.
- 4 19. D. S. Dimitrov, *Methods Mol Biol*, 2012, 899, 1-26.
- 5 20. K. K. Jain, in *Applications of Biotechnology in Oncology*, Springer, 2014,
6 pp. 617-669.
- 7 21. B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini and N.
8 A. Peppas, *Adv Mater*, 2009, 21, 3307-3329.
- 9 22. T. R. Hoare and D. S. Kohane, *Polymer*, 2008, 49, 1993-2007.
- 10 23. Y. Li, J. Rodrigues and H. Tomas, *Chem Soc Rev*, 2012, 41, 2193-2221.
- 11 24. T. Vermonden, R. Censi and W. E. Hennink, *Chem Rev*, 2012, 112, 2853-
12 2888.
- 13 25. A. A. Aimetti, A. J. Machen and K. S. Anseth, *Biomaterials*, 2009, 30,
14 6048-6054.
- 15 26. Y. Fu and W. J. Kao, *Expert Opin Drug Deliv*, 2010, 7, 429-444.
- 16 27. T. Diab, E. M. Pritchard, B. A. Uhrig, J. D. Boerckel, D. L. Kaplan and R.
17 E. Guldborg, *J Mech Behav Biomed Mater*, 2012, 11, 123-131.
- 18 28. S. Sahoo, C. Chung, S. Khetan and J. A. Burdick, *Biomacromolecules*,
19 2008, 9, 1088-1092.
- 20 29. M. S. Rehmman, A. C. Garibian and A. M. Kloxin, *Macromol Symp*, 2013,
21 329, 58-65.
- 22 30. A. M. Kloxin, A. M. Kasko, C. N. Salinas and K. S. Anseth, *Science*,
23 2009, 324, 59-63.
- 24 31. A. M. Kloxin, M. W. Tibbitt, A. M. Kasko, J. A. Fairbairn and K. S.
25 Anseth, *Adv Mater*, 2010, 22, 61-+.
- 26 32. D. R. Griffin, J. L. Schlosser, S. F. Lam, T. H. Nguyen, H. D. Maynard
27 and A. M. Kasko, *Biomacromolecules*, 2013, 14, 1199-1207.
- 28 33. J. Patterson and J. A. Hubbell, *Biomaterials*, 2010, 31, 7836-7845.
- 29 34. G. K. Balendiran, R. Dabur and D. Fraser, *Cell Biochem Funct*, 2004, 22,
30 343-352.

- 1 35. F. Meng, W. E. Hennink and Z. Zhong, *Biomaterials*, 2009, 30, 2180-
2 2198.
- 3 36. R. Cheng, F. Feng, F. Meng, C. Deng, J. Feijen and Z. Zhong, *J Control*
4 *Release*, 2011, 152, 2-12.
- 5 37. X. J. Cai, H. Q. Dong, W. J. Xia, H. Y. Wen, X. Q. Li, J. H. Yu, Y. Y. Li
6 and D. L. Shi, *J Mater Chem*, 2011, 21, 14639-14645.
- 7 38. J. Liu, Y. Pang, W. Huang, X. Huang, L. Meng, X. Zhu, Y. Zhou and D.
8 Yan, *Biomacromolecules*, 2011, 12, 1567-1577.
- 9 39. H. Y. Wen, H. Q. Dong, W. J. Xie, Y. Y. Li, K. Wang, G. M. Pauletti and
10 D. L. Shi, *Chem Commun*, 2011, 47, 3550-3552.
- 11 40. E. Ferruzzi, R. Franceschini, G. Cazzolato, C. Geroni, C. Fowst, U.
12 Pastorino, N. Tradati, J. Tursi, R. Dittadi and M. Gion, *Eur J Cancer*,
13 2003, 39, 1019-1029.
- 14 41. A. I. Fiaschi, A. Cozzolino, G. Ruggiero and G. Giorgi, *Eur Rev Med*
15 *Pharmacol Sci*, 2005, 9, 361-367.
- 16 42. J. M. Estrela, A. Ortega and E. Obrador, *Crit Rev Clin Lab Sci*, 2006, 43,
17 143-181.
- 18 43. G. Wu, Y. Z. Fang, S. Yang, J. R. Lupton and N. D. Turner, *J Nutr*, 2004,
19 134, 489-492.
- 20 44. J. Gajewska, M. Szczypka, K. Pych, A. Borowka and T. Laskowska-Klita,
21 *Neoplasma*, 1994, 42, 167-172.
- 22 45. K. M. Schultz, A. D. Baldwin, K. L. Kiick and E. M. Furst,
23 *Macromolecules*, 2009, 42, 5310-5316.
- 24 46. K. G. Robinson, T. Nie, A. D. Baldwin, E. C. Yang, K. L. Kiick and R. E.
25 Akins, Jr., *J Biomed Mater Res A*, 2012, 100, 1356-1367.
- 26 47. C. C. Lin and K. S. Anseth, *Pharm Res*, 2009, 26, 631-643.
- 27 48. E. A. Phelps, N. O. Enemchukwu, V. F. Fiore, J. C. Sy, N. Murthy, T. A.
28 Sulchek, T. H. Barker and A. J. Garcia, *Adv Mater*, 2012, 24, 64-70, 62.
- 29 49. Y. Lei and T. Segura, *Biomaterials*, 2009, 30, 254-265.
- 30 50. C. L. McGann, E. A. Levenson and K. L. Kiick, *Macromol Chem Phys*,
31 2013, 214, 203-213.

- 1 51. D. Gupta, C. H. Tator and M. S. Shoichet, *Biomaterials*, 2006, 27, 2370-
2 2379.
- 3 52. L.-T. T. Nguyen, M. T. Gokmen and F. E. Du Prez, *Polym Chem*, 2013, 4,
4 5527-5536.
- 5 53. J. P. Danehy and Paramesw.Kn, *J Chem Eng Data*, 1968, 13, 386-&.
- 6 54. T. V. DeCollo and W. J. Lees, *J Org Chem*, 2001, 66, 4244-4249.
- 7 55. L. R. G. Treloar, *The physics of rubber elasticity*, Oxford University Press,
8 1975.
- 9 56. P. M. Gschwend and D. M. Imboden, *Environmental organic chemistry*,
10 John Wiley & Sons, 2005.
- 11 57. Y. S. Jo, J. Gantz, J. A. Hubbell and M. P. Lutolf, *Soft Matter*, 2009, 5,
12 440-446.
- 13 58. P. J. Martens, S. J. Bryant and K. S. Anseth, *Biomacromolecules*, 2003, 4,
14 283-292.
- 15 59. R. G. Schoenmakers, P. van de Wetering, D. L. Elbert and J. A. Hubbell, *J*
16 *Control Release*, 2004, 95, 291-300.
- 17 60. M. C. Branco, D. J. Pochan, N. J. Wagner and J. P. Schneider,
18 *Biomaterials*, 2010, 31, 9527-9534.
- 19 61. J. Siepmann and N. A. Peppas, *Adv Drug Deliver Rev*, 2012, 64, 163-174.
- 20 62. J. Siepmann and F. Siepmann, *Int J Pharm*, 2008, 364, 328-343.
- 21 63. S. P. Zustiak and J. B. Leach, *Biotechnol Bioeng*, 2011, 108, 197-206.
- 22 64. A. Raza and C. C. Lin, *Macromol Biosci*, 2013, 13, 1048-1058.
- 23 65. S. Y. Choh, D. Cross and C. Wang, *Biomacromolecules*, 2011, 12, 1126-
24 1136.

25

26

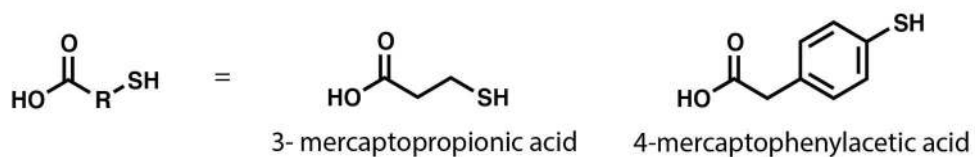
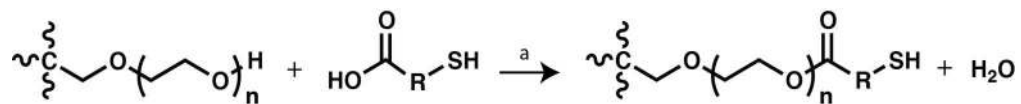
27

28

1 **FIGURES**

2

3



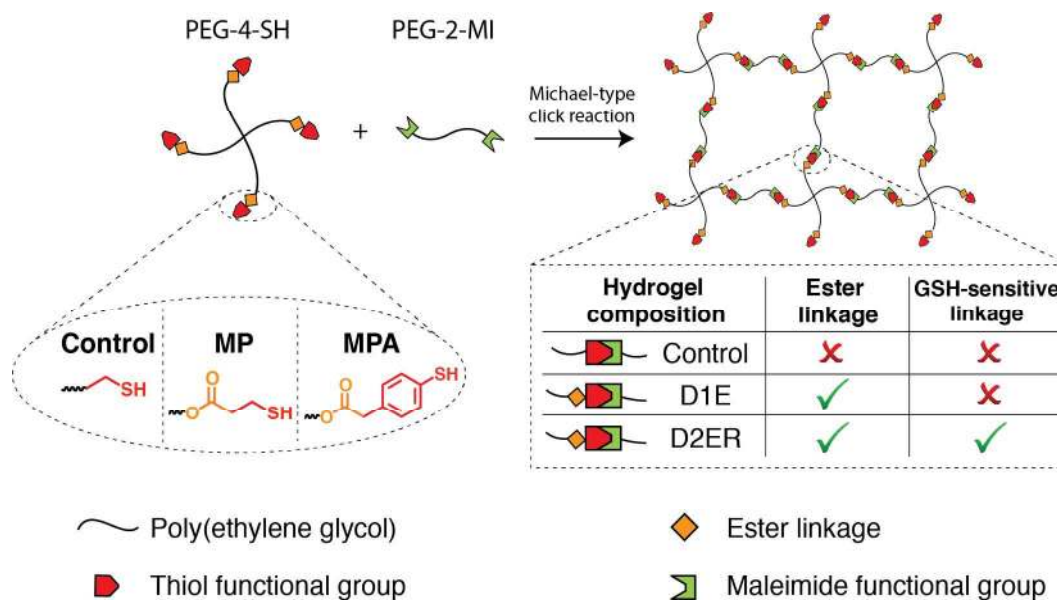
4

5

6

7 **Scheme 1. Functionalization of 4-arm PEG.** Schematic of the synthesis of thiol-8 functionalized PEG. (a: Toluene, PTSA, $\sim 110^\circ\text{C}$)

1



2

3

4

5

Fig. 1. Hydrogel formation via click reaction. Degradable PEG hydrogels were

6

synthesized by Michael type addition reaction between thiol functionalized 4-arm

7

PEG (PEG-4-SH) and maleimide functionalized linear PEG (PEG-2-MI). The

8

thiol-functionalized macromers were synthesized by esterification of PEG using

9

two different mercaptoacids (Scheme 1). The identity of the thiol was varied to

10

tune the degradability of the hydrogels (**Control**: no degradable groups; **D1E**: one

11

degradable group per crosslink (i.e., ester linkage); and **D2ER**: two degradable

12

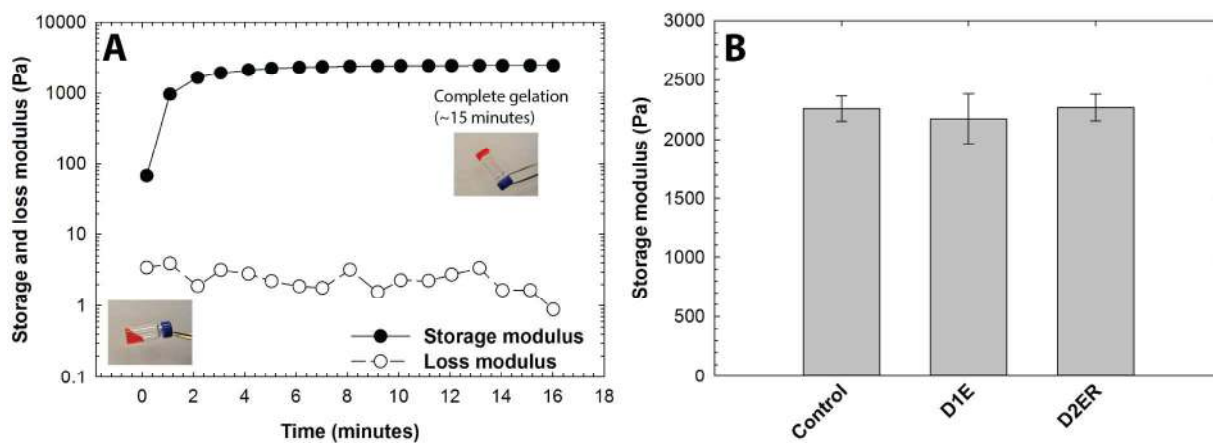
groups per crosslink (i.e., ester and reducing environment susceptible click =

13

linkages).

14

1



2

3

4 **Fig. 2. Modulus evolution during hydrogel formation. A)** Time-sweep

5 measurements on an oscillatory rheometer were utilized to monitor hydrogel

6 formation (**D2ER** hydrogel shown). Although formation of a gel is clearly

7 observed, samples polymerize too quickly for measurement of the gel point with

8 rheometry. To estimate the time to initial gelation, the tube-tilt method was

9 utilized (inset images), where faster gelation was observed for **D2ER** (~20 s) as10 compared to **Control** (~40 s) and **D1E** (~35 s) hydrogels. For better visual

11 assessment, Allura Red AC dye was added to the precursor solution (0.5 mg/ml)

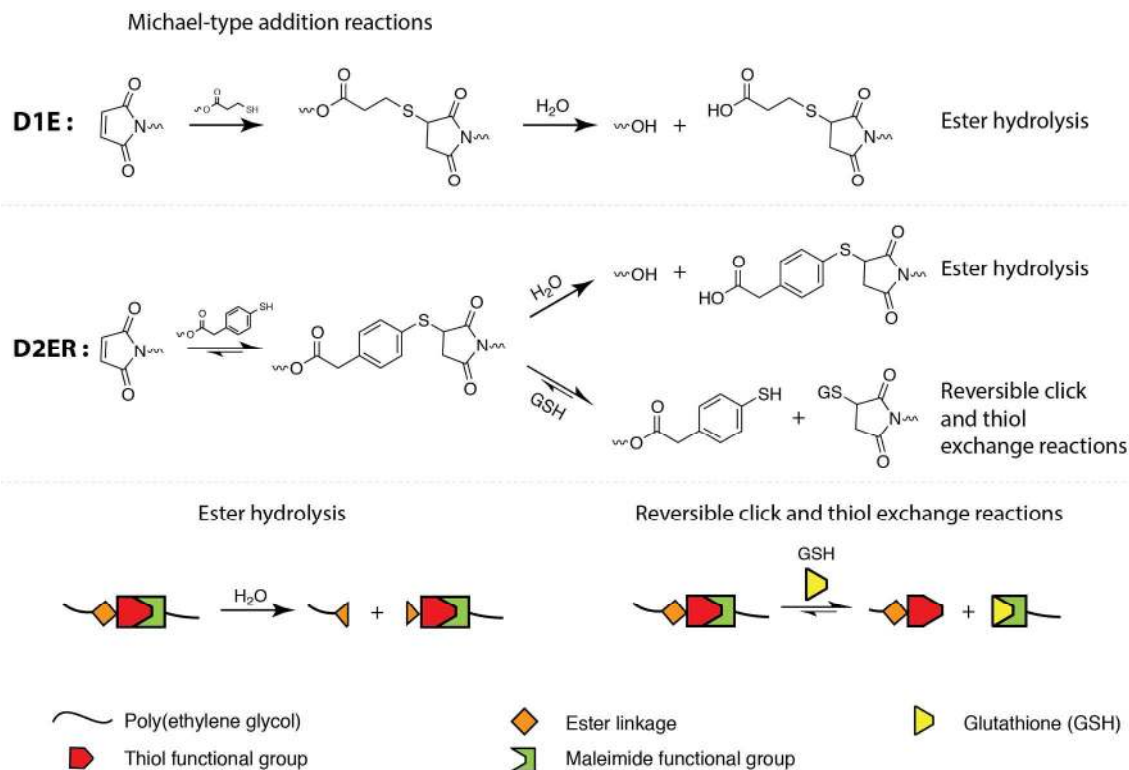
12 for tube-tilt measurements. **B)** Irrespective of the identity of the thiol used for the

13 hydrogel formation, the storage moduli for all three hydrogels post-gelation were

14 statistically similar, indicating similar structural and mechanical properties. The

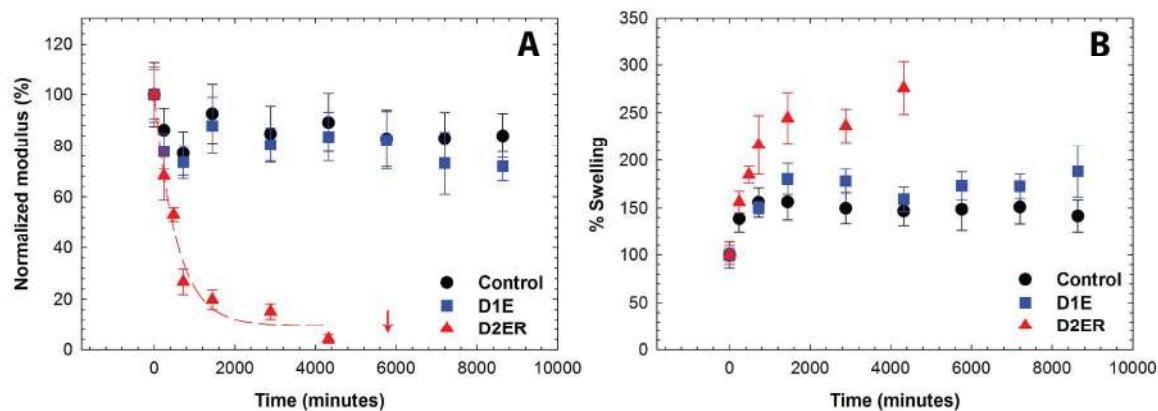
15 data shown illustrate the mean ($n = 3$), with error bars showing the standard error.

16



1
2
3
4
5
6
7
8
9
10
11
12

Fig. 3. Multimode hydrogel degradation. Schematic of the click bond cleavage and thiol exchange reaction of thioether succinimide linkages under a glutathione (GSH) reducing microenvironment and by ester hydrolysis. The **D1E** hydrogels can only undergo degradation by ester hydrolysis. **D2ER** hydrogels can undergo degradation by ester hydrolysis and by thiol exchange reactions, owing to the presence of arylthiol-based thioether succinimide linkages. Owing to the lack of degradable functional groups, control hydrogels do not degrade in aqueous reducing microenvironments. The rate and extent of the click bond cleavage depends on the Michael donor reactivity and thiol pK_a .



1

2

3 **Fig. 4. Hydrogel degradation in reducing microenvironment by cleavage of**4 **click bonds.** Degradation of the hydrogel in a thiol-rich reducing5 microenvironment (10 mM GSH) was studied by monitoring **A)** the storage6 modulus and **B)** % volumetric swelling at discrete time points. All compositions

7 exhibit an initial change in properties over 24 h as equilibrium swelling occurs.

8 Due to the presence of the arylthiol-based thioether succinimide crosslinks, **D2ER**

9 hydrogels exhibited rapid bulk degradation by click cleavage and thiol exchange

10 reactions. The arrow indicates the time point when reverse gelation was observed.

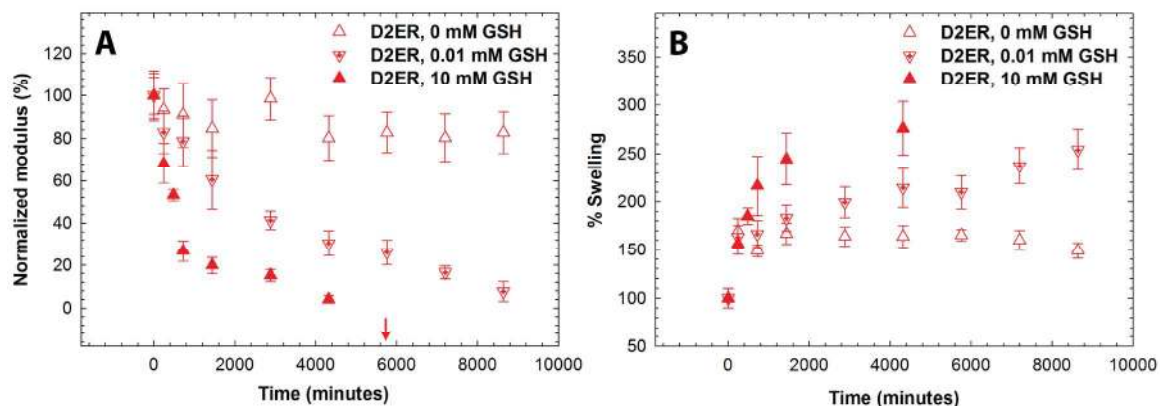
11 **D1E** and **Control** hydrogels were relatively stable during the experimental time

12 frame due to the absence of GSH-sensitive crosslinks. The data shown illustrate

13 the mean ($n = 6$), with error bars showing the standard error.

14

1



2

3

4 **Fig. 5. Influence of GSH concentration on hydrogel degradation.** The effect of5 GSH concentration (0, 0.01, and 10 mM) on **D2ER** hydrogel degradation was6 studied by analyzing **A)** the decrease in the storage modulus, and **B)** the %

7 volumetric swelling. The dependence of the decrease in moduli on GSH

8 concentration indicates that the click cleavage and thiol exchange reaction is the

9 dominant degradation mechanism for the **D2ER** hydrogels. The increase in

10 volumetric swelling as a function of time before the reverse gel point confirms

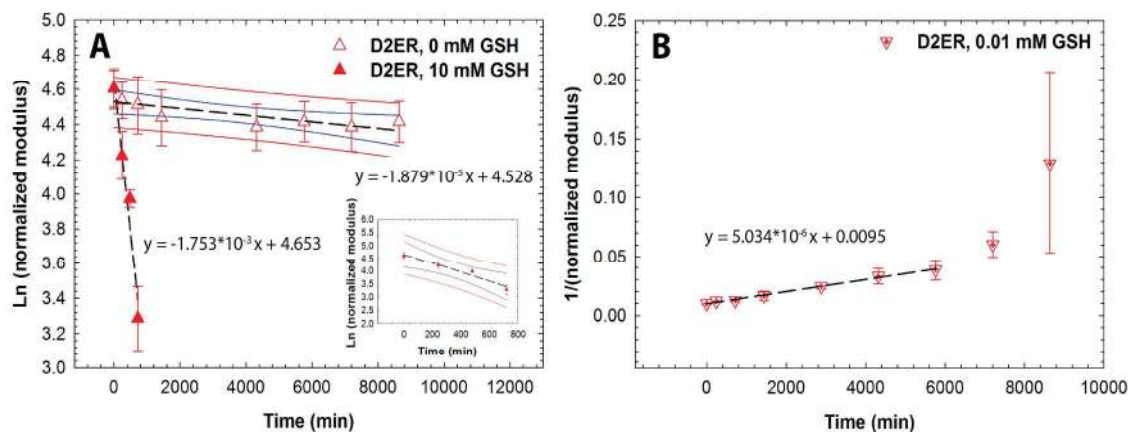
11 bulk degradation of hydrogels. The arrow indicates the time point when reverse

12 gelation was observed for the 10 mM GSH condition. The data shown illustrate

13 the mean (n = 6), with error bars showing the standard error.

14

1



2

3

4 **Fig. 6. Reducing environment-dependent degradation kinetics. A) D2ER**

5 hydrogels exhibited first order degradation kinetics in a strong reducing

6 microenvironment (10 mM GSH), whereas limited degradation is observed in a

7 thiol-lacking microenvironment (0 mM GSH), owing to the slow rate of ester

8 hydrolysis. Data point for 0 mM GSH at 2880 minutes was identified as a
9 significant outlier (Grubb's test, $p < 0.05$) and hence omitted during regression10 analysis. **B) D2ER** hydrogels followed second order reaction kinetics in a weak

11 reducing microenvironment (0.01 mM GSH). Later time points were omitted

12 during the regression analysis, due to large standard error, which can be attributed

13 to experimental limitations when handling soft, more liquid-like degraded gels.

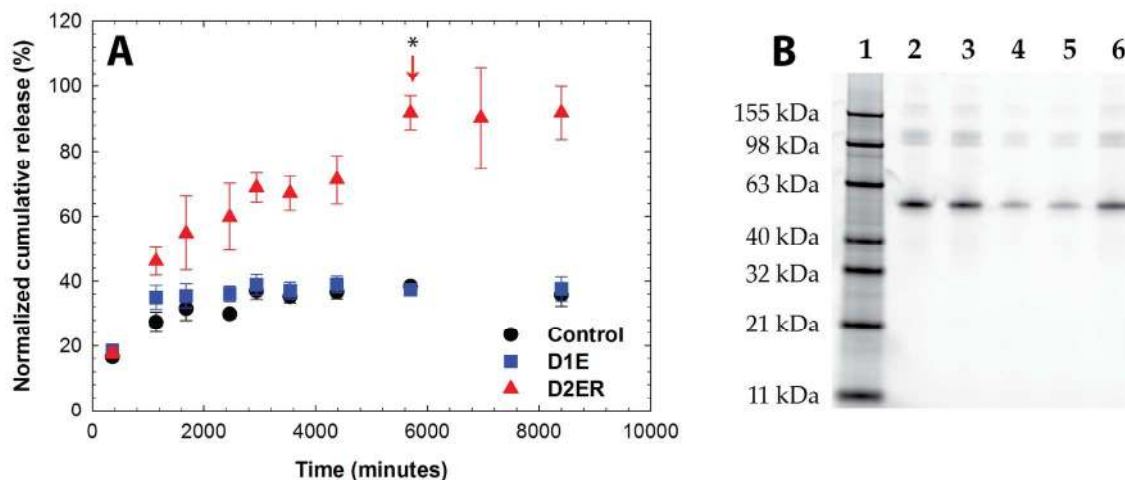
14 As a whole, this study highlights the dependence of hydrogel degradation on GSH

15 concentration. The data shown illustrate the mean ($n = 6$), with error bars showing

16 the standard error. Black line indicates the linear fit using regression analysis.

17 Blue and red lines indicate 95% confidence and prediction bands.

18



1

2 **Fig. 7. Protein release in a reducing microenvironment.** A) Release of a
3 fluorescently-labeled model cargo protein, bovine serum albumin (BSA-488), was
4 monitored using fluorometry. The arrow indicates the time point when reverse
5 gelation (complete gel dissolution) was observed for the **D2ER** hydrogel. While
6 some protein is initially released from all compositions upon gel equilibrium
7 swelling, release from the **Control** and **D1E** hydrogels after this is minimal,
8 owing to no or slow hydrolytic degradation, respectively, over the time course of
9 the experiment. Substantial, statistical differences ($p < 0.05$ for time points after
10 complete hydrogel degradation) in protein release are observed as the **D2ER**
11 hydrogel rapidly degrades by the click cleavage and thiol-mediated exchange
12 mechanism in addition to hydrolytic degradation. Differences in the release
13 profile of BSA-488 from **D2ER**, **D1E**, and **Control** hydrogels highlight that the
14 delivery of cargo molecules is controlled by hydrogel degradation. The data
15 shown illustrate the mean ($n = 6$), with error bars showing the standard error. **B**)
16 SDS-PAGE analysis of released protein. Lane 1: protein ladder; Lane 2: free
17 BSA-488; Lane 3: free BSA-488 suspended in reducing microenvironment (10

1 mM GSH/PBS) with hydrogel precursor solution; Lane 4, 5, 6: supernatant after
2 protein release from **Control**, **D1E**, and **D2ER** hydrogel, respectively. No major
3 differences in the locations of the free BSA and released BSA band are observed,
4 confirming that the protein remained intact during encapsulation and release.
5 Further, analysis of the band intensity by densitometry further supports the
6 relative amounts of protein released from each gel composition as determined by
7 fluorescence (**Control**: ~33%; **D1E**: ~36%; and **D2ER**: ~90%).

8