

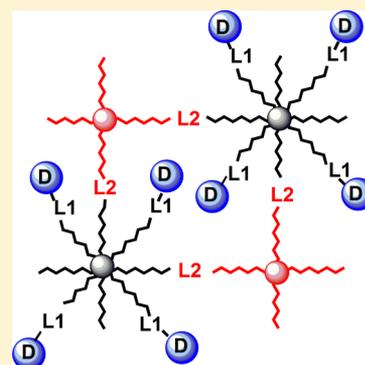
# Biodegradable Tetra-PEG Hydrogels as Carriers for a Releasable Drug Delivery System

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**S** Supporting Information

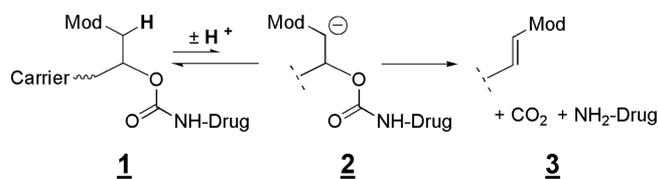
**ABSTRACT:** We have developed an approach to prepare drug-releasing Tetra-PEG hydrogels with exactly four cross-links per monomer. The gels contain two cleavable  $\beta$ -eliminative linkers: one for drug attachment that releases the drug at a predictable rate, and one with a longer half-life placed in each cross-link to control biodegradation. Thus, the system can be optimized to release the drug before significant gel degradation occurs. The synthetic approach involves placing a heterobifunctional connector at each end of a four-arm PEG prepolymer; four unique end-groups of the resultant eight-arm prepolymer are used to tether a linker-drug, and the other four are used for polymerization with a second four-arm PEG. Three different orthogonal reactions that form stable triazoles, diazines, or oximes have been used for tethering the drug to the PEG and for cross-linking the polymer. Three formats for preparing hydrogel–drug conjugates are described that either polymerize preformed PEG–drug conjugates or attach the drug postpolymerization. Degradation of drug-containing hydrogels proceeds as expected for homogeneous Tetra-PEG gels with minimal degradation occurring in early phases and sharp, predictable reverse gelation times. The minimal early degradation allows design of gels that show almost complete drug release before significant gel-drug fragments are released.



## INTRODUCTION

We have recently developed a drug-delivery system (DDS) that uses sets of linkers that self-cleave by a  $\beta$ -elimination reaction to release the native drug. Drug release occurs in a highly predictable manner, and with half-lives of cleavage spanning from hours to months.<sup>1,2</sup> In this approach (Scheme 1), a

Scheme 1



macromolecular carrier is connected to a linker that is attached to a drug or pro-drug via a carbamate group (1); the  $\beta$ -carbon has an acidic carbon–hydrogen bond (C–H) and also contains an electron-withdrawing “modulator” (Mod) that controls the  $pK_a$  of that C–H. Upon proton loss (2), a rapid  $\beta$ -elimination occurs to cleave the linker–carbamate bond and release the free drug (3). The rate of linker cleavage is proportional to the acidity of the proton, which is controlled by the electron withdrawing effect of the modulator.

Initially, we used 40 kDa polyethylene glycol (PEG) as a circulating carrier for drug half-life extension.<sup>1</sup> However, with slowly cleaving linkers, the  $t_{1/2}$  of the released drug is limited by the renal elimination  $t_{1/2}$  of the PEG conjugate, which is  $\leq 1$  week in humans. Since we are interested in half-life extension to

weeks or months, we developed a DDS using our  $\beta$ -eliminative linkers to tether drugs to noncirculating hydrogel implants. Although the implants are primarily intended for subcutaneous placement, they could as well be used for intravitreal, intra-articular, and other compartmental injections. Since there is no renal elimination of the hydrogel–drug conjugate over the period of drug release, the in vivo elimination  $t_{1/2}$  of a released drug reflects the  $t_{1/2}$  of the linker used.<sup>2</sup>

Most alternative drug delivery implants encapsulate drugs noncovalently within a polymer cross-linked with ester bonds such that pore size prevents drug diffusion and release; drug release occurs by diffusion as the ester cross-links hydrolyze and pore sizes enlarge.<sup>3–5</sup> However, the concomitant encapsulation–polymerization process often requires solvents that are detrimental to peptidic drugs. Also, polymer degradation via ester hydrolysis generates carboxylic acids that acidify the polymer interior and denature or modify sensitive proteins.

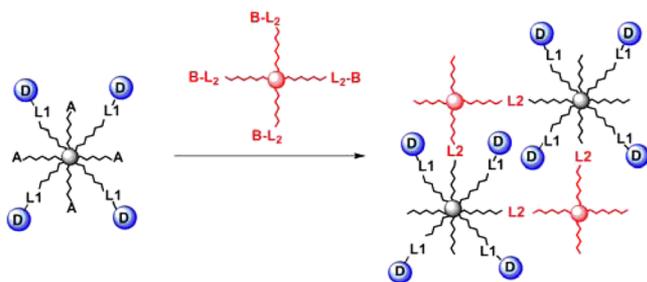
As a matrix for our DDS, we chose Tetra-PEG hydrogels, a relatively new class of polymer network that is fabricated by combining two four-arm PEG polymers carrying mutually reactive end-groups (for a recent review, see ref 6). Tetra-PEG gels have been intensively studied and shown to possess near-ideal polymer properties with minimal network defects, and resemble the diamond lattice.

**Received:** November 24, 2014

**Revised:** December 16, 2014

**Published:** January 13, 2015

Our hydrogels are prepared from two PEG prepolymers that each contain mutually reactive end-groups—designated as A and B (Figure 1)—that react to form polymers that contain a



**Figure 1.** Formation of a hydrogel from an 8-arm PEG having four drugs **D** tethered via **L1** linkers and four connecting groups, **A**, by reaction with four **B** connecting groups of a 4-arm PEG containing cleavable cross-linkers **L2**.

$\beta$ -eliminative cleavage site **L2** in the cross-links. We tether a drug via another faster-cleaving  $\beta$ -eliminative releasable linker, **L1**, to one of these prepolymers.

In our first-generation hydrogel DDS,<sup>2</sup> an average of four of eight cyclooctyne (CO) end groups of PEG<sub>40 kDa</sub>-(CO)<sub>8</sub> were randomly tethered to a drug via linkers **L1**, Figure 2; the remaining CO end groups were reacted with 4-arm PEG<sub>20 kDa</sub>-(NH-L2(Mod)-N<sub>3</sub>)<sub>4</sub> (see Nomenclature section) containing linkers **L2**. Assuming normal distribution of the random drug attachment, the 8-arm PEG–drug conjugates contained on average only ~27% 4-arm CO modifications; 3- and 5-arm modifications occur at 22% each and 2- and 6-arm modifications at 11% each. Although this heterogeneity does not affect the drug release rate, polymerization does not produce homogeneous Tetra-PEG hydrogels having four cross-links per monomer. As expected consequences of the network imperfections—for, example <4 cross-links per monomer in the polymer—early release of gel-fragments—including those attached to drug—are amplified, and the time to reverse gelation ( $t_{RG}$ ) is unpredictable. In addition, the heterogeneous 8-arm prepolymer cannot be purified, and may present challenges in reproducible manufacturing of the DDS.

In the present work, we solve the above network imperfection problems by developing second-generation hydrogels in which *exactly* 4 equiv of drug is attached to four arms of an 8-arm PEG, and *exactly* 4 arms are used for cross-linking. The key synthesis strategy, depicted in Figure 3, was to create a path that would allow attachment of two different orthogonal functional groups, **A** and **X**, on each of the arms of a 4-arm PEG

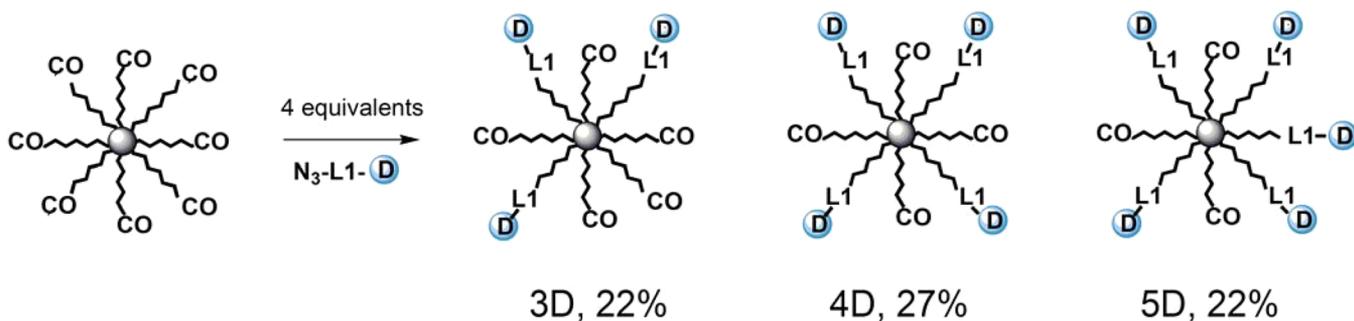
to give the product designated as prepolymer A. Prepolymer A is prepared by coupling PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>4</sub> with a <sup>15</sup>N,<sup>13</sup>C-disubstituted-Lys-HSE (A-Lys(X)-HSE) containing click chemistry participants that can undergo sequential orthogonal ligations; substituent **X** specifically ligates to **Y** of **Y-L1-drug** to attach the drug and substituent **A** specifically ligates to **B** end-groups on the second prepolymer **B**, PEG<sub>20 kDa</sub>-(NH-L2(Mod)-B)<sub>4</sub>, to form the gel.

## RESULTS

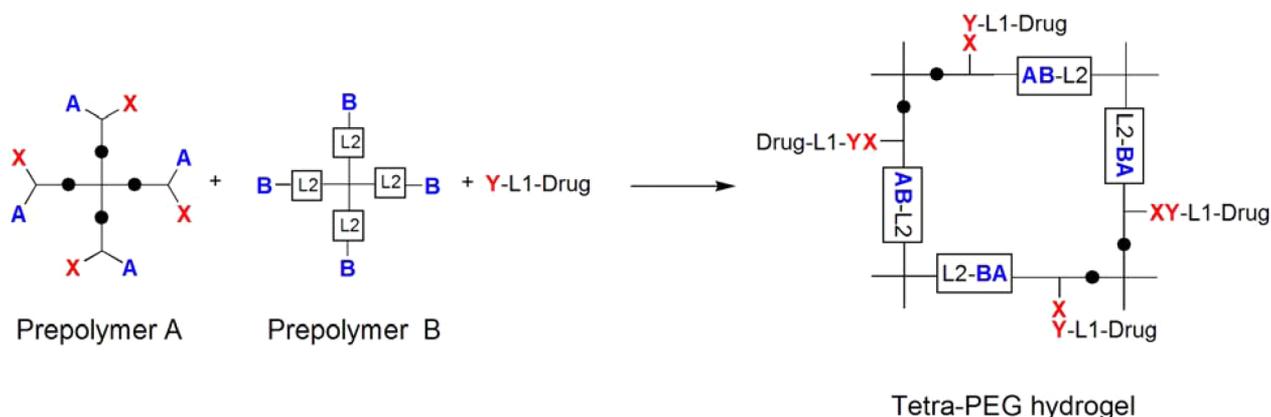
**Orthogonal Coupling Reactions.** We used three different bioorthogonal reactions to achieve the desired connections: **X** + **Y** to tether 4 equiv of **Y-L1-drug** to a [A-Lys(X)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> to give prepolymer A, and A + B to cross-link prepolymer A with a 4-armed prepolymer B, PEG<sub>20 kDa</sub>-(NH-L2(Mod)-B)<sub>4</sub>. The reactions used were (a) strain-promoted azide–alkyne cycloaddition (SPAAC) to convert cycloalkynes and azides to 1,2,3-triazoles, (b) inverse electron demand Diels–Alder (IEDDA) to convert an electron-poor 1,2,4,5-tetrazine and electron-rich alkene dienophile to a diazine, and (c) condensation of an alkoxyamine and a ketone to form an oxime. Structures of components are given in Figure 4 and reaction rates and gelation times for 5% w/v gels are given in Table 1.

For SPAAC, the reaction rate is rather insensitive to azide structure but can be greatly varied by modification of the alkyne. We used the cyclooctynes dibenzocyclooctyne (DBCO),<sup>7</sup> monofluorocyclooctyne (MFCO),<sup>8</sup> and aryl-less cyclooctyne (ALO),<sup>9</sup> which vary over a ~1000-fold range in reaction rates with aliphatic azides.<sup>10</sup> For IEDDA reactions we used norbornene (Nb) as the dienophile and 4-(6-methyl-1,2,4,5-tetrazine)phenylacetic acid (MeTz-PAA) as its reaction partner;<sup>11</sup> these have a good balance of stability in neutral aqueous media, reasonable reaction rate, and cost. The rates of IEDDA reactions can be greatly varied by modifying either the tetrazine or the dienophile.<sup>11</sup> The rates of oxime formation can vary greatly with pH and the concentration of aniline catalyst used.<sup>12,13</sup>

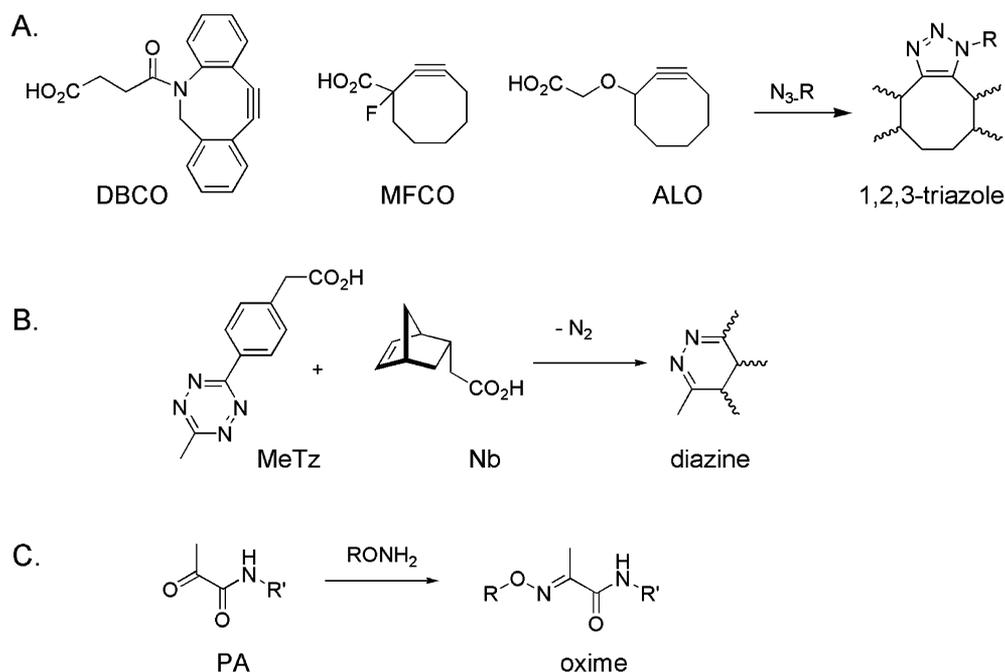
**Heterobifunctional Prepolymer Synthesis.** While this work was in progress, we found that commercially available PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>4</sub> could contain up to ~15% impurities having 1- to 3 amine groups per PEG (SI Text 1). These impurities were usually carried forward through chemical modifications and were likely responsible for minor HPLC peaks present in gel prepolymers. In addition, the prepolymers derived from PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>1–3</sub> are anticipated to interfere with the polymer network of ideal Tetra-PEG gels; such impurities



**Figure 2.** First generation random loading of a Tetra-PEG hydrogel precursor; only 27% of the product PEG prepolymers are expected to contain exactly 4 free CO groups.



**Figure 3.** Second-generation hydrogel–drug conjugates. Prepolymer A contains four X end-groups that couple with Y for attachment of a drug having a cleavable linker, L1, and four A end-groups that react with B groups of prepolymer B to form a Tetra-PEG gel.



**Figure 4.** A. Cyclooctynes used in SPAAC reactions. B. Methyltetrazine (MeTz) and Norbornene (Nb) used in IEDDA. C. Alkoxyamine and pyruvamide (PA) used in oxime formation.

**Table 1.** Reaction Rates and Gelation Times Using Orthogonal Connecting Groups

reagent	reagent	product	reaction rate $k$ , $M^{-1} s^{-1}$	gelation time, min
DBCO <sup>a</sup>	R-N <sub>3</sub>	triazole	2.0 <sup>b</sup>	1.4
MFCO <sup>a</sup>	R-N <sub>3</sub>	triazole	0.25	18
ALO <sup>a</sup>	R-N <sub>3</sub>	triazole	0.010	560
Nb	MeTz	diazine	0.091 <sup>c</sup>	60
RONH <sub>2</sub>	pyruvamide	oxime	<sup>d</sup>	
RONH <sub>2</sub>	-NHCOPhCH=O	oxime	<sup>d</sup>	7 <sup>e</sup>

<sup>a</sup>For aliphatic azides in water; reported rates in MeCN are about 10-fold slower. <sup>b</sup>Excellent agreement with reported rate of  $2.1 M^{-1} s^{-1}$  in water;<sup>14</sup> rates with DBCO are consistently ~5-fold faster ( $\sim 10 M^{-1} s^{-1}$ ) when the azide contained DEAC. <sup>c</sup>The rate can be modified by using alternative tetrazines;<sup>11</sup> however, faster reacting tetrazines are inconveniently less stable. <sup>d</sup>Dependent on pH and catalyst. <sup>e</sup>Using 100 mM aniline catalyst, pH 4.5; no gel formed in 21 h at pH 4.5–7.4 without aniline.

result in unformed cross-links in the gel and could alter the time of reverse gelation ( $t_{RG}$ ). For the present purpose, we accepted such imperfections but are seeking a solution for future studies.

**Prepolymer A.** A key common intermediate for prepolymer A was [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub>, **7** (Scheme 2). PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>4</sub> was treated with Fmoc-Lys(Boc)-OSu in MeCN/DIPEA to give the acylated PEG, **4**, followed by removal of Boc with TFA/DCM to give [Fmoc-Lys(H)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub>, **5**. The latter was acylated with 1.2 equiv of an activated ester, exemplified by DBCO-OSu, in MeCN/DIPEA, to give [Fmoc-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub>, **6**, which was cleanly unblocked in 0.5 to 1 h by treatment with 5% 4-methylpiperidine/DMF to give the key intermediate [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub>, **7**. The intermediate [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> was then acylated with an activated ester (HSE or Pfp) of a second cyclooctyne such as MFCO or ACO, a dienophile such as Nb-acetic acid, or a keto-acid such as pyruvate to give the corresponding heterobifunctional

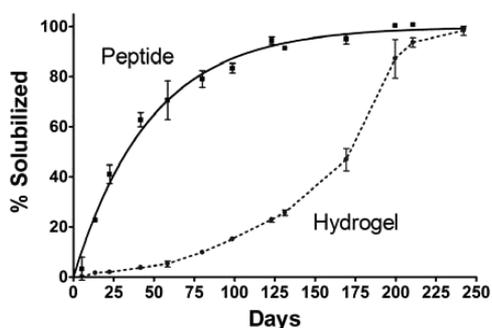


hydrogels (Figure 5) using the above coupling groups. Following are descriptions of the formats along with a specific example of drug attachment to each.

**Same Connecting Group Chemistry, Different Reaction Rates.** Kele et al.<sup>15</sup> reported the use of sequential copper-free and copper mediated click chemistry to differentially label one molecule with two different azides. Here, we accomplished a similar objective by using SPAAC. One Lys-amine of [H-Lys(H)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> contained a DBCO group and the other had another CO (MFCO or ALO) that is less reactive toward azides. The four DBCO groups were coupled with a stoichiometric amount of N<sub>3</sub>-L1-drug, and the remaining CO groups were used for polymerization with prepolymer B, PEG<sub>20 kDa</sub>-(NH-L2(Mod)-N<sub>3</sub>)<sub>4</sub>. Here, azide preferentially reacts with the DBCO groups rather than the less reactive MFCO or ALO groups. This results in selective modification of the DBCO groups, and hence a higher proportion of polymers with exactly four modified and four free CO end groups needed for a Tetra-PEG hydrogel.

Using numerical analysis to solve equations for second-order parallel reactions, and the relative rates of alkyl azide reaction with COs (Table 1), we could estimate the expected percent modification of the more reactive DBCO groups after reaction with 1 equiv of azide. We calculated that 81% of the DBCO groups in [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> would be consumed, and 98.5% would react using [ALO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub>. Experimentally, selectivity for DBCO over MFCO or ALO was shown by titrating the loss of DBCO absorbance in polymers with O-(2-azidoethyl)heptaethylene glycol (PEG7-N<sub>3</sub>) (SI Text II). Here, reaction of the azide with DBCO results in loss of UV absorbance, whereas reaction with nonabsorbing COs are UV-transparent. When a control PEG<sub>20 kDa</sub>-(NH-DBCO)<sub>4</sub> or [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> was titrated with 4 equiv of azide, exactly 1 mol of DBCO was consumed per mole of azide. Likewise, for [ALO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> ≥99 mol percent of DBCO (98.5% calculated) was consumed per mole of azide, since ALO is much less reactive toward alkyl azides than DBCO. With [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub>, titration indicated reaction of 81% of the DBCO moieties which is in exact accord with what is calculated from the rates of azide reaction of the two COs.

An example of the utility of this approach (Figure 6) is the attachment of the 39-amino-acid peptide, exenatide, via an <sup>n</sup>N-azido-L1<sup>1</sup> to the DBCO end-groups of [MFCO-Lys(DBCO)-



**Figure 6.** Release of exenatide from a hydrogel formed from [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> prepolymer A and PEG<sub>20 kDa</sub>-(NH-L2(CN)-N<sub>3</sub>)<sub>4</sub> prepolymer B. Peptide release and degelation were recorded at pH 9.4 and extrapolated to pH 7.4 using  $t_{R,pH7.4} = t_{R,pH9.4}(10^{pH9.4-pH7.4})$  (1,2); peptide (—■—) and hydrogel solubilization (—●—).

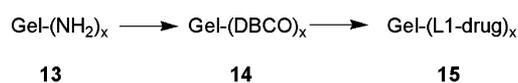
NH]<sub>4</sub>-PEG<sub>20 kDa</sub>. After polymerization with a 4-arm azido-PEG<sub>20 kDa</sub> prepolymer B, the hydrogel had 4.0 μmol (17 mg) of tethered peptide/mL gel and released the drug with the expected cleavage rate.

**Bioorthogonal Reaction Pairs.** With three orthogonal reactions, three pairs can be used for drug attachment to and polymerization of gels. For example, [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> contains four Lys groups in which one amine connects a DBCO for SPAAC while the other contains the orthogonal Nb group for IEDDA.<sup>11</sup> Karver et al.<sup>14</sup> have shown that this cycloaddition pair shows excellent mutual orthogonality and has a good balance of reactivity and stability. Here we show that the rates of Tz coupling with several commercially available Nb analogs suitable for bioconjugation were quite similar (SI Table S1). The Tz-Nb pair has previously been used to cross-link hydrogels,<sup>16</sup> and we have also examined that approach in this study. The IEDDA reaction produces N<sub>2</sub> which we have observed to cause trapped bubbles in the gel when used for polymerization. The gel bubbles can be circumvented by polymerizing while centrifuging or in vacuo, but such restrictions may limit the practical usefulness of such gels. For this reason, we generally use IEDDA to attach a MeTz-linker-drug to the Nb moiety of prepolymer A, and use SPAAC for gel polymerization.

Using this format, we attached a 46 kDa Fab via coupling of a MeTz-L1-Fab to the Nb-groups of [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> followed by polymerization with a PEG<sub>20 kDa</sub>-(NH-L2(Mod)-N<sub>3</sub>)<sub>4</sub> prepolymer B. The final hydrogel released 9 mg of tethered Fab/mL gel at the expected rate.

**Preformed Activated Gels.** Here (Scheme 4), the [DBCO-Lys(H)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> prepolymer was polymerized with

**Scheme 4**



PEG<sub>20 kDa</sub>-(NH-L2(Mod)-N<sub>3</sub>)<sub>4</sub> to give a gel functionalized with four free amine groups per monomeric 4-arm PEG unit. The amino-Tetra-PEG gel, **13**, cast as a small cone, was acylated with DBCO-HSE in about 90% yield to give **14**, which could then be coupled to a N<sub>3</sub>-L1-drug to give drug-gel conjugate **15**. Here, one connecting group is used for polymerization before the other is introduced so, if desirable, the same connecting group can be used for both polymerization and drug attachment.

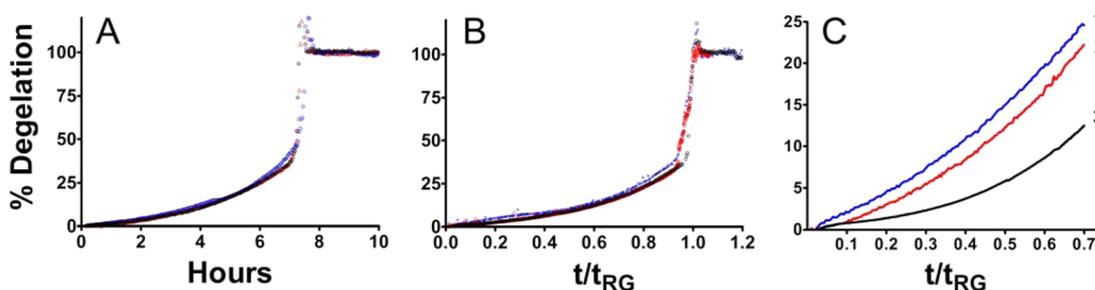
Using this format, we attached 22 kDa N<sub>3</sub>-L1-human growth hormone to a DBCO-derivatized hydrogel to give a gel loaded with 32 mg protein/mL hydrogel. We also prepared a Nb-derivatized gel by polymerizing [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> and PEG<sub>20 kDa</sub>-(NH-L2(Mod)-N<sub>3</sub>)<sub>4</sub> and coupled this with a MeTz-L1-Darpin of ~14 kDa to give a gel with 20 mg protein/mL gel.

**Stability of Connecting Group Linkages.** The azide-CO couplings provide 1,2,3-triazole products, Tz-Nb couplings give diazines and aldehyde/ketone-aminoalkyl couplings provide oximes. Clearly, these products must be stable for the duration of in vivo studies (37 °C, pH 7.4) and under conditions anticipated for storage (pH ~5 to 7). While short-term stability of such linkages is evident from various reports, we are not aware of stability studies over periods of one month and longer. Solubilization of gels cross-linked with these functional groups could not be detected after several months at pH 7.4, 37 °C.<sup>2</sup>

Table 2. Degradation of Hydrogels Prepared with  $\beta$ -Eliminative Linkers Containing the PhSO<sub>2</sub>-pK<sub>a</sub> Modulator in Cross-Links

entry	prepolymer A	cap/erosion probe <sup>a</sup>	prepolymer B	cross-link	$t_{RG}^d$ (h)	AUC 0.7 $t_{RG}$
1	[Nb-Lys(DBCO)-NH] <sub>4</sub> -PEG <sub>20</sub> kDa	none	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	DBCO/N <sub>3</sub>	96 ± 6.7	4.6 ± 0.3
2	[MFCO-Lys(DBCO)-NH] <sub>4</sub> -PEG <sub>20</sub> kDa	none	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	MFCO/N <sub>3</sub>	100 ± 2.8	5.2 ± 0.3
3	PEG <sub>20</sub> kDa-(NH-DBCO) <sub>4</sub>	none	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	DBCO/N <sub>3</sub>	76.0 ± 3.0	4.9 ± 0.6
4	PEG <sub>40</sub> kDa-(NH-DBCO) <sub>8</sub>	PEG7-N <sub>3</sub>	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	DBCO/N <sub>3</sub>	85.5 ± 3.5	6.5 ± 0.9
5	PEG <sub>40</sub> kDa-(NH-DBCO) <sub>8</sub>	PEG7-N <sub>3</sub> <sup>b</sup>	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	DBCO/N <sub>3</sub>	70 ± 4.0	7.6 ± 0.5
6	[MFCO-Lys(DBCO)-NH] <sub>4</sub> -PEG <sub>20</sub> kDa	PEG7-N <sub>3</sub> /DEAC-N <sub>3</sub> <sup>c</sup>	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	MFCO/N <sub>3</sub>	163 ± 5.3	4.1 ± 0.1
7	[Nb-Lys(DBCO)-NH] <sub>4</sub> -PEG <sub>20</sub> kDa	PEG7-N <sub>3</sub> /DEAC-N <sub>3</sub> <sup>c</sup>	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-MeTz) <sub>4</sub>	Nb/Tz	174 ± 7.2	4.0 ± 0.3
8	[Nb-Lys(DBCO)-NH] <sub>4</sub> -PEG <sub>20</sub> kDa	MeTz-COOH/DEAC-MeTz <sup>c</sup>	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	DBCO/N <sub>3</sub>	216 ± 26	3.3 ± 0.5
9	PEG <sub>40</sub> kDa-(NH-DBCO) <sub>8</sub>	PEG7-N <sub>3</sub> /DEAC-N <sub>3</sub> <sup>c</sup>	PEG <sub>20</sub> kDa-(NH-L2(PhSO <sub>2</sub> )-N <sub>3</sub> ) <sub>4</sub>	DBCO/N <sub>3</sub>	118 ± 8	5.1 ± 0.5

<sup>a</sup>4 equiv of cap/erosion probe were used per equivalent of prepolymer A. <sup>b</sup>Capping reagent was added after cross-linking. <sup>c</sup>DEAC represents 37% of the total capping mixture. <sup>d</sup>Values were determined at pH 8.4, extrapolated to pH 7.4 using  $t_{RG,pH7.4} = t_{RG,pH8.4} (10^{pH8.4-pH7.4})^2$ .<sup>2</sup> The error is the SD of  $\geq 3$  replicates.



**Figure 7.** Degelation curves of hydrogels with the PhSO<sub>2</sub>-modulator in cleavable cross-links at pH 8.4, 37 °C. A. Reproducibility of the degelation assay using a gel made from PEG<sub>20</sub> kDa-(NH-DBCO)<sub>4</sub> and PEG<sub>20</sub> kDa-(NH-L2(PhSO<sub>2</sub>)-N<sub>3</sub>)<sub>4</sub> assayed on three different days (Table 2, entry 3). B. Time-normalized degelation curves showing three gels with different  $t_{RG}$ s (Table 2, entries 1, 2, and 6). C. Time-normalized AUC<sub>0.7</sub> curves for a structured gel, C3, (Table 2, entry 8) and randomly formed gels prepared from PEG<sub>40</sub> kDa-(NH-DBCO)<sub>8</sub> and PEG<sub>20</sub> kDa-(NH-L2(PhSO<sub>2</sub>)-N<sub>3</sub>)<sub>4</sub> capped before, C2, (Table 2, entry 4) or after cross-linking, C1 (Table 2, entry 5).

However, assuming Tetra-PEG gels are ideal networks, and that we would have detected 5% or more solubilization of gel components, we calculated that more than 40% of the cross-links could have cleaved without detection of fragments in the supernatant (unpublished results). Thus, gel solubilization is an insensitive assay for bond cleavage of Tetra-PEG gels, and we therefore studied stabilities of simple model systems (SI Table S2).

**Triazole Stability.** DBCO acid was reacted with PEG7-N<sub>3</sub>, and stability of the triazole product was monitored by HPLC at A<sub>254</sub>. At either pH 5.4 (4 °C) or pH 7.4 (4 and 37 °C),  $\leq 5\%$  decomposition was observed after 3 months, indicating a  $t_{1/2}$  of greater than 30 months.

**Diazine Stability.** For diazine stability studies, we prepared a Me-diazine by reaction of a fluorescein-labeled Nb (Nb-AAF) and DNP-labeled MeTz (DNP-Lys(MTzAA)OH) so separation of fluorescein and DNP chromophores by HPLC would allow direct assessment of bond cleavage. After 37 days at either pH 5.4 or pH 7.4 at 4 °C,  $\leq 5\%$  cleavage occurred indicating that  $t_{1/2}$  was  $\leq 1$  yr. At pH 7.4, 37 °C, 8% cleavage was observed at 37 days, indicating a  $t_{1/2}$  of  $\sim 8$  months.

**Oxime Stability.** For oxime stability studies, we prepared soluble oximes between either a glycolamide, pyruvamide, alkyl aldehyde, or *p*-formylbenzamide and a fluorescein-labeled alkoxyamine. Because of the high equilibrium constant of oxime bonds, in a closed system dissociated bonds will reform and cleavage is not apparent.<sup>12</sup> However, if an aldehyde or ketone is included as a trap for the liberated alkoxyamine, oxime hydrolysis is in effect irreversible.<sup>17</sup> The abundance of

aldehydes/ketones in vivo (e.g., glucose) should likewise irreversibly trap released alkoxyamines.

In the present work, oximes formed with an aliphatic fluorescent alkoxyamine were incubated at various pH values in the presence of 0.5 M acetone and hydrolysis monitored as the appearance of the fluorescent acetone oxime. Oximes of pyruvamide and *p*-formylbenzamide were extraordinarily stable to hydrolysis at pH 5.4 to 7.4, with estimated  $t_{1/2}$  values exceeding three years. In contrast, oximes formed from a glycolamide or aliphatic aldehyde had estimated  $t_{1/2}$  values of only 2 and 6 months, respectively, at pH 7.4, 37 °C, and were considered unsuitable for long-term in vivo exposure.

**Hydrogel Degradation.** Previously, to monitor gel solubilization we attached a trace amount of an absorbing "erosion probe" in place of gel cross-links, and monitored solubilization of the probe.<sup>2</sup> We subsequently recognized that since a monomer with an erosion probe has no more than three cross-links, it will be released faster than a monomer with four bonds; hence, the kinetics of probe solubilization were somewhat faster than bulk gel solubilization. In the present work we constructed gels such that the erosion probe did not interfere with gel cross-links. Two formats for erosion probe placement were used. In one, a UV or vis absorbing molecule was stably attached to arms extending from the intact gel network. In the second, the probe was contained within intact cross-links. For example, the dibenzo-triazole product of DBCO-azide coupling ( $\epsilon_{261}$  6600 M<sup>-1</sup> cm<sup>-1</sup>) can be directly monitored as a surrogate for solubilization of PEG fragments.

Because of the time-consuming process of manual determination of gel-degradation profiles, we devised an apparatus that

allows continuous, simultaneous analyses of up to four samples. Cuvettes containing ~2.5 mL buffer and a magnetic stirrer were fitted with an immersed mesh separator above the light path to isolate the gel in the top compartment. Four such cuvettes were placed in temperature-controlled multisample compartment of a spectrophotometer fitted with a magnetic stirrer; a fifth cuvette contained a thermocouple in buffer to ensure temperature constancy. By computer-control of spectrophotometer readings and data collection, the change in absorbance and temperature could be recorded simultaneously. In practice, we recommend that each run contain three replicates and a control standard gel of known  $t_{RG}$ . With this method, degelation curves of degradable Tetra-PEG gels were remarkably reproducible. For example, with the same minimal gel prepared from PEG<sub>20 kDa</sub>-(NH-L2(PhSO<sub>2</sub>)-N<sub>3</sub>)<sub>4</sub> and PEG<sub>20 kDa</sub>-(NH-DBCO)<sub>4</sub> (Table 2, entry 3), the intrarun variation of the reverse gelation time ( $t_{RG}$ ) was  $\pm 6\%$  SD ( $n = 4$ ), and interday variation (Figure 7A) was  $\pm 2\%$  SD ( $n = 3$ ).

Using the automated method for gel degradation analysis, we monitored degradation of different gels containing the same PhSO<sub>2</sub>-modulator in the cleavable cross-linker,<sup>2</sup> and hence expectedly similar degelation kinetics and  $t_{RG}$  values.

To compare the levels of gel fragment solubilization before  $t_{RG}$ , we normalized the degelation curves by plotting percent sol vs  $t/t_{RG}$  (Figure 7B). Then, we determined the area under the curve up to  $0.7 t_{RG}$  (AUC<sub>0.7</sub>). After that point, release of large fragments compromised reproducibility. As shown in Table 2, the AUC<sub>0.7</sub> for the gels developed in the present study are quite similar at  $\sim 3.3\%$  to  $5.2\% \times t$  (entries 1, 2, and 6–8). We also prepared randomly formed gels by first capping 8-arm PEG<sub>40 kDa</sub>-(NH-DBCO)<sub>8</sub> with 4 equiv PEG7-N<sub>3</sub> followed by polymerization with the PEG<sub>20 kDa</sub>-(NH-L2(PhSO<sub>2</sub>)-N<sub>3</sub>)<sub>4</sub> (Table 2, entry 4), or by polymerizing 8-arm PEG<sub>40 kDa</sub>-(DBCO)<sub>8</sub> with 1.0 equiv of PEG<sub>20 kDa</sub>-(NH-L2(PhSO<sub>2</sub>)-N<sub>3</sub>)<sub>4</sub> followed by capping with PEG7-N<sub>3</sub> (Table 2, entry 5). The AUC<sub>0.7</sub> for these random gels was up to 230% higher than the structured gel (Figure 7C); also, whereas up to 25% of the randomly formed gel was solubilized by  $0.7t_{RG}$ , the structured gel showed only  $\sim 12\%$  solubilization. Hence, randomly formed gels release significantly more gel fragments early in degelation than the gels developed here.

In general, gels having the heterobifunctional Lys adapter had longer  $t_{RG}$  values than the minimal gel prepared from two 4-armed PEGs (Table 2, entries 1, 2, 6–8 vs 3). The  $t_{RG}$  of such gels seemed to vary depending on the functional group tethered to the gel. Thus, a pendant MFCO or Nb moiety showed an increase in  $t_{RG}$  of  $\sim 30\%$  (Table 2, entries 1, 2 vs 3), and with two other modulators ( $-\text{CN}$ ,  $\text{O}(\text{CH}_2\text{CH}_2)_2\text{NSO}_2-$ ) in the cross-linker, a tethered exenatide increased the  $t_{RG}$  by  $\sim 35\%$  (unpublished). The largest effect was observed with a pendant hydrophobic coumarin (DEAC) that prolonged  $t_{RG}$  by up to 250% (Table 2, entries 6–8 vs 3). Hence, it seems that tethered chemical moieties may increase the  $t_{RG}$ , possibly by solvation effects that perturb the pK<sub>a</sub> of the acidic C–H bond required for  $\beta$ -eliminative cleavage.

We also compared gels polymerized by MeTz-Nb vs CO-azide coupling. Gels were cast using a MeTz-Nb cross-linker in the manner described here and under centrifugation, so gels either contained or did not contain N<sub>2</sub> bubbles. The  $t_{RG}$  of the gel with bubbles was  $\sim 8\%$  longer than those without, and AUC<sub>0.7</sub> was decreased 15%. Compared to SPACC cross-linked gels, the MeTz-Nb cross-linked gels showed both a slightly reduced  $t_{RG}$  and AUC<sub>0.7</sub>.

**Concomitant Drug Release and Hydrogel Degradation.** The cleavage rates of linkers attaching the drug to the hydrogel, L1, and in the gel cross-links, L2, should be balanced such that most of the drug is released before the gel undergoes significant solubilization, otherwise excessive fragment–drug conjugates may be released. The fraction of free drug ( $D_{\text{free}}/D_{\text{ttl}}$ ) released from the intact hydrogel or gel fragments is given by eq 1

$$D_{\text{free}}/D_{\text{ttl}} = 1 - e^{-k_{L1} * t_{RG}} \quad (1)$$

where  $k_{L1}$  is the rate of drug release and  $t_{RG}$  the reverse gelation time. Substituting  $k_{L1} = \ln 2/t_{1/2,L1}$  into eq 1 gives eq 2.

$$D_{\text{free}}/D_{\text{ttl}} = 1 - e^{-\ln 2 * t_{RG}/t_{1/2,L1}} = 1 - 0.5^{t_{RG}/t_{1/2,L1}} \quad (2)$$

This shows that  $D_{\text{free}}/D_{\text{ttl}}$  can be calculated knowing the  $t_{RG}$  of a gel and the rate of cleavage of an appended releasable drug,  $t_{1/2,L1}$ . Thus, for example, one can calculate the anticipated  $D_{\text{free}}/D_{\text{ttl}}$  in Tetra-PEG gel 8 (Table 2), and the two randomly formed gels 5 and 9 shown in Figure 7C. If a linker–drug with cleavage  $t_{1/2,L1} = 50$  h tethered to each gel, the homogeneous Tetra-PEG gel 8 ( $t_{RG}$  216 h at pH 7.4) would release 95% of the drug before its  $t_{RG}$ , whereas the random gels 5 and 9 ( $t_{RG}$  70 and 118 h at pH 7.4, respectively) would release only 62% and 85%, respectively. For example, in Figure 6 a peptide drug is released with  $t_{1/2,L1}$  of 33 days, and the  $t_{RG}$  is 205 days. Substituting these values into eq 2 indicates that 98% of the drug is released before the gel dissipates. Thus, with a given rate of drug release the  $D_{\text{free}}/D_{\text{ttl}}$  can be empirically adjusted by modifying the  $t_{RG}$ .

## DISCUSSION

We recently described the use of cleavable  $\beta$ -eliminative linkers both to attach drugs to hydrogels for predictable drug release, and to cross-link the polymers for tunable control of biodegradation rates.<sup>2</sup> Our drug-releasing gels were initially prepared by randomly attaching 4 equiv of linker–drug to an 8-arm PEG<sub>40 kDa</sub>, and using the remaining arms to cross-link the gel to a 4-arm PEG<sub>20 kDa</sub>. However, the random distribution of the number of unreacted arms on the 8-arm prepolymer led to a heterogeneous-cross-linked hydrogel that is far different than the homogeneous network of Tetra-PEG gels.

In the present work, we developed an approach to prepare drug-containing Tetra-PEG hydrogels with four cross-links per monomer. This was accomplished by attaching a heterobifunctional connector to each of the four arms of PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>4</sub> so the resultant 8-arm prepolymer contained exactly four connecting end-groups for linker–drug attachment and four for cross-linking. The other prepolymer was also prepared from PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>4</sub> and each of the four arms contained a connecting end-group reactive toward that used in the 8-arm prepolymer, as well as a cleavable linker proximal to the connecting group. Upon mixing the two mature prepolymers, covalent bonds were formed between the four connecting groups of each to form a Tetra-PEG gel containing cleavable cross-links; the drug was attached by a cleavable linker either pre- or postpolymerization. One of the advantages of the present approach over the previous one is the ability to prepare homogeneous prepolymers. This permits analysis and, if necessary, purification of the prepolymers which should be a major benefit in manufacturing reproducibility and in complying with FDA GMP regulations.

For both linker–drug attachment and hydrogel polymerization, we used three well-studied orthogonal reactions: SPAAC to convert alkyl azides and cyclooctynes to triazoles, IEDDA to convert tetrazines and dienophiles to diazines, and condensation of aminoxy-alkyl groups with ketones to form ketooximes. Although not extensively explored here, depending on need the reaction rates of SPAAC or IEDDA could be varied by use of more or less reactive analogs of one of the reactants (e.g., cyclooctyne for SPAAC, tetrazine or alkene for IEDDA, respectively), or in the case of oxime formation the pH or concentration of the aniline catalyst. In the present work, it was convenient to use DBCO as one of the components of the SPAAC reaction because of the ability to monitor its reaction spectrophotometrically and because of its rapid rate of reaction.

The hydrogels were intended for long-term drug delivery over periods of weeks to months. Hence, we studied the stability of the products of the coupling reactions used over appropriately long durations at pH 7.4, 37 °C, as well as lower pH and temperatures intended for storage. Noteworthy was the finding that the commonly used oximes of glyoxamide—conventionally formed by periodate oxidation of N-terminal Ser of proteins<sup>18</sup>—were not stable for prolonged periods ( $t_{1/2}$  ~2 months at pH 7.5, 37 °C). With this exception, products of all reactions studied—triazole-, diazine-, and ketooxime-formation—were shown to be stable under physiological and intended storage conditions for periods exceeding many months.

Drug-containing Tetra-PEG gels could be made by several formats. In one, each of two analogous groups with different reaction rates (e.g., DBCO and MFCO or ALO) is attached to the prepolymer. The fastest reacting groups are first connected to the linker–drug, and the remaining connecting groups are used for polymerization. We show by calculation and experiment that the relative reactivity of the two connecting groups should be ~10-fold to specifically modify >80% of the faster reacting group. A second format involves the use of two orthogonal connecting reactions that do not cross-react (e.g., SPAAC and IEDAA). Here, either group of the prepolymer can first be reacted with a linker–drug and the gel then formed by polymerization using the other connecting groups. Finally, a hydrogel can first be prepared and subsequently coupled to a linker–drug. Here, a preferred method is to prepare an amino-derivatized hydrogel, acylate the amines with a connecting group, and then couple it to a linker–drug; unlike other methods described here, this format enjoys the convenience that the same connecting group can be used for both polymerization and attachment of linker–drug. In all formats, the polymer network structure can assume the same homogeneous network as the well-studied Tetra-PEG gels.

The hydrogels described here were all formed from two 4-arm PEG<sub>20 kDa</sub> prepolymers resulting in Tetra-PEG gels with 10 kDa PEG cross-links that are calculated to have a pore size of ~8 nm. Such gels have previously been shown to present little barrier to diffusion for proteins up to at least ~66 kDa.<sup>2</sup> Gels cast at 5% PEG have a capacity of 5  $\mu$ mol drug/mL and we have loaded them with up to ~20 mg protein/mL. In an attempt to increase capacity, preliminary studies have been performed using analogous prepolymers to form gels with with 2.6 kDa PEG cross-links. These have a calculated pore size of ~5 nm that also allowed good diffusion of 66 kDa BSA ( $t_{1/2}$  = 40 min). Five percent gels formed from these prepolymers have a potential capacity of ~13  $\mu$ mol/mL—over 2.5 times greater

than the current gels—and have been loaded with up to ~30 mg of a 4 kDa peptide per mL of gel.

A major benefit of these Tetra-PEG hydrogels as a drug delivery system is that their degradation profiles are highly predictable and reproducible. Optimally, most drug should be released before the gel undergoes significant solubilization; otherwise, excessive fragment–drug conjugates will be released which may have undesirable properties. Although solubilized fragment–drug conjugates are continuously releasing free drug throughout de-gelation, it seems both prudent and advantageous to minimize their accumulation as much as possible. With randomly formed gels, there was significant premature release of fragments due to monomer components that were attached to others in the gel by two or three cross-links, and the  $t_{RG}$  values were also irregular. In contrast, the Tetra-PEG hydrogels described here show minimal premature fragment release and  $t_{RG}$ 's that are predictable, sharp, and highly reproducible. For example, in Figure 7C, at about 60% of the time of reverse gelation, only about 10% of the Tetra-PEG gels are solubilized compared to 15% to 20% of randomly formed gels. One can design biodegradable hydrogels that discharge the drug before significant levels of fragment–drug conjugates accumulate in one of two ways. First, once a suitable rate is obtained for drug release, the fraction of free drug that released before  $t_{RG}$  can be adjusted by empirically modifying the  $t_{RG}$  according to eq 2 ( $D_{free}/D_{tot} = 1 - 0.5^{t_{RG}/t_{1/2,LI}}$ ). Here, for example, if the  $t_{RG}$  is adjusted to be 3-fold longer than the  $t_{1/2}$  of the drug release rate, almost 90% of the drug is free before the gel dissipates. Thus, we usually use linkers in which the  $t_{1/2}$  of cross-link-cleavage is  $\geq 3$ -fold slower than drug release.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Experimental Procedures and the source of reagents and specialized materials are provided along with their use. Detailed procedures for the synthesis of hydrogel prepolymers, related characterization methods, and hydrogel formation are described in SI Text I. Formats for the preparation of hydrogel–drug conjugates are described in SI Text II. Synthesis and characterization of model compounds for stability studies is presented in SI Text III. Kinetic assays for hydrogel degradation are presented in SI Text IV. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was in part supported by NSF grants 1248239 and 1429972. We thank Louise Robinson for preparing some of the intermediates used in this work, and Ralph Reid for assistance in deriving the equations that describe drug release and degelation kinetics.

## ■ NOMENCLATURE

Polymer nomenclature used: If a sequence, such as an amino acid or peptide is present as a substituent on PEG, then standard IUPAC nomenclature is used and prefaces the PEG used. For example, [Fmoc-Lys(Boc)-NH]<sub>4</sub>-PEG<sub>20 kDa</sub> repre-

sents a four-armed tetra-amino-PEG with each of its arms connected to the C-terminus of Lys containing an  $\alpha$ -Fmoc and  $\epsilon$ -tBoc substituent. If no amino acid or peptide is present, the abbreviated nomenclature designates the chemical substituents and stoichiometry to the right of PEG. Thus, PEG<sub>20 kDa</sub>-[NH-L2(Mod)-N<sub>3</sub>]<sub>4</sub> is a 20 kDa 4-arm amino PEG containing an azido-alkyl linker, L2, activated by a pK<sub>a</sub> modulator, Mod.

## ■ ABBREVIATIONS

HSE, hydroxysuccinimide ester; DEAC, 7-diethylaminocoumarin-3-carboxylic acid; Pfp, pentafluorophenyl ester

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## ■ NOTE ADDED IN PROOF

We recently received samples of PEG<sub>20 kDa</sub>-(NH<sub>2</sub>)<sub>4</sub> (NOF product # PTE-200PA) containing 13% tri- and 87% tetra-amino PEG, and an ion-exchange purified sample containing 6% tri- and 94% tetra-amino (NOF product # PTE-200PA-IP). A portion of each sample was converted to the PEG<sub>20 kDa</sub>-(NH-DBCO)<sub>4</sub> and PEG<sub>20 kDa</sub>-(NH-L2(MePhSO<sub>2</sub>)-N<sub>3</sub>)<sub>4</sub>. After precipitation with MTBE HPLC analysis (308 nm) showed 11% tri-substituted in the unpurified sample, and 3.3% in the purified PEG<sub>20 kDa</sub>-(NH-DBCO)<sub>4</sub>. Hydrogels were prepared from the purified and unpurified prepolymers. Degradation analysis at pH 9.3, 37 °C, showed similar AUC<sub>0.7</sub> values, but a 9% increase in t<sub>RG</sub> (143 vs 157 min at pH 9.4).

## SUPPLEMENTARY INFORMATION

### Biodegradable Tetra-PEG hydrogels as carriers for a releasable drug delivery system

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#### I. Prepolymers and hydrogel formation

##### A. Synthesis of prepolymers A

1. Common intermediate: [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>
2. Acylation of [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>
  - a. [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>
  - b. [ALO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>
  - c. [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>
  - d. [Pyruvamide-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>

##### B. Synthesis of prepolymers B

1. Common intermediate: PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub>
2. Acylation of PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub>  
PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-MeTz]<sub>4</sub>

##### C. Hydrogel formation and gelation time

#### II. Formats for drug-bound Tetra-PEG hydrogel formation

##### A. Same attachment chemistry, different reaction rates.

1. Selectivity of azides for DBCO over MFCO, Nb, and ALO
2. Rate of DBCO-azide gel crosslinking
3. Exenatide hydrogel

##### B. Biorthogonal reaction pairs.

1. Reactions of tetrazines with norbornene and 1-Me-cyclopropene dienophiles
2. Fab hydrogel

##### C. Preformed activated gels.

1. (Amino)<sub>x</sub>-hydrogel
2. (DBCO)<sub>x</sub>-hydrogel
3. Human growth hormone (hGH) hydrogel

#### III. Stability studies

##### A. Synthesis of model compounds for stability studies

1. Triazole
2. Diazine
3. Oximes
  - a. pyruvamide oxime
  - b. glyoxamide oxime
  - c. hexanal and benzaldehyde oximes

##### B. Stability assays of model compounds

#### IV. Hydrogel degelation

##### A. Manual assay

## B. Automated assay

## V. References

### General Materials and Methods

PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>4</sub> was purchased from JenKem (Item A7026, 4ARM-NH2-20K), and DBCO-OSu was purchased from Click Chemistry Tools (Item A133). DMF was stored over an AldraAmine packet (Sigma, Item Z511706) to reduce dimethylamine and water contamination. All other commercially available reagents were of reagent grade purity and were used without further purification. Dialyses were performed using SpectraPor 2 membranes (12-14 kDa molecular weight cutoff). Solutions of DBCO-containing compounds were quantified spectrophotometrically using  $\epsilon_{308} = 13,448 \text{ M}^{-1}\text{cm}^{-1}$ . Solutions of Fmoc-containing substrates were quantified spectrophotometrically using  $\epsilon_{266} = 17847 \text{ M}^{-1}\text{cm}^{-1}$ ,  $\epsilon_{288} = 5137 \text{ M}^{-1}\text{cm}^{-1}$ , and  $\epsilon_{300} = 5380 \text{ M}^{-1}\text{cm}^{-1}$ . Trinitrobenzene sulfonate (TNBS) assays to quantify amines were as described, except a 60 min reaction time was used before readings (1). Under conditions of the TNBS assay (pH 9.3, 1 h)  $\leq 2\%$  of PEG<sub>20kDa</sub>-(NH-Fmoc)<sub>4</sub> was cleaved; therefore, accurate estimates of amine content could be made in the presence of this blocking group. Samples were analyzed on an Agilent Series 1100 HPLC system with a Jupiter 5  $\mu\text{m}$  C<sub>18</sub> column (150 x 4.6 mm) using a 6-min linear gradient of 20-100% acetonitrile (MeCN) with 0.1% TFA at 1 mL/min. The instrument was equipped with an auto-sampler (Agilent G1329A) and a photodiode-array detector (Agilent G1315A). Size exclusion HPLC was performed with a Phenomenex BioSep-SEC-s2000 column (300 x 7.8 mm) using a 20 min isocratic method of 50% MeCN with 0.1% TFA at 1 mL/min. Absorption analyses were performed using a Hewlett-Packard 8453 UV-Vis spectrophotometer. <sup>1</sup>H NMR spectra were obtained at the UCSF Magnetic Resonance Laboratory using a Varian INOVA-400 MHz spectrometer.

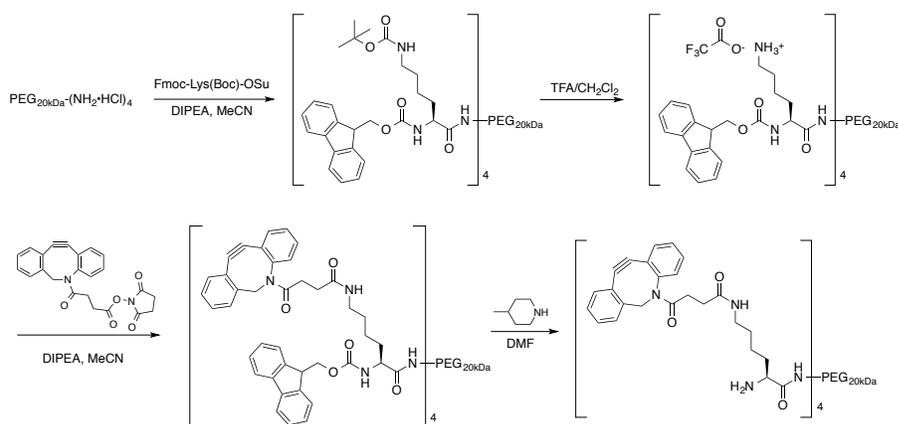
**Capping of amine groups on PEG.** Solutions of 0.5  $\mu\text{mol}$  PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>4</sub> (2  $\mu\text{mol}$  amine by TNBS) in 1.0 mL of MeCN (or 1:1 H<sub>2</sub>O:MeCN) were treated with 20  $\mu\text{mol}$  of either Ac<sub>2</sub>O (1.9  $\mu\text{L}$ ) or AcOSu (3.1 mg). After 30 min at ambient temperature a TNBS assay for amines indicated  $\leq 2.5\%$  of the starting amines remained. We conclude that under pseudo first order conditions treatment of amines with 20 mM acylating agent for 30 min corresponds to  $\sim 6$  half-lives and will result in  $\geq 97.5\%$  capping.

**Purity of PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>4</sub>.** Commercially obtained PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>4</sub> (10.0 mg, 2.0  $\mu\text{mol}$  amine) was dissolved in 1.0 mL of MeCN and treated with a 100 mM solution of Fmoc-OSu in MeCN (50  $\mu\text{L}$ , 5.0  $\mu\text{mol}$ ) and a 100 mM solution of DIPEA in MeCN (50  $\mu\text{L}$ , 5.0  $\mu\text{mol}$ ). Aliquots of 5  $\mu\text{L}$  were periodically injected onto a C<sub>18</sub> HPLC column, which was eluted with H<sub>2</sub>O/0.1% TFA for 2 min followed by a gradient from 0-100% MeCN/H<sub>2</sub>O/0.1% TFA over 10 min. The distribution of PEG<sub>20kDa</sub>-(NH-Fmoc)<sub>1-4</sub> was determined by normalizing the peak area (266 nm detection) to the extinction coefficient for Fmoc and the number of Fmoc groups on the corresponding PEG species. The various species eluted with the following retention volumes: PEG<sub>20kDa</sub>-(NH-Fmoc)<sub>1</sub> - 9.63 mL (2.7 %); PEG<sub>20kDa</sub>-(NH-Fmoc)<sub>2</sub> - 9.81 mL (2.1 %); PEG<sub>20kDa</sub>-(NH-Fmoc)<sub>3</sub> - 9.96 mL (10.0 %); PEG<sub>20kDa</sub>-(NH-Fmoc)<sub>4</sub> - 10.21 mL (85.1 %).

## I. Prepolymers and hydrogel formation

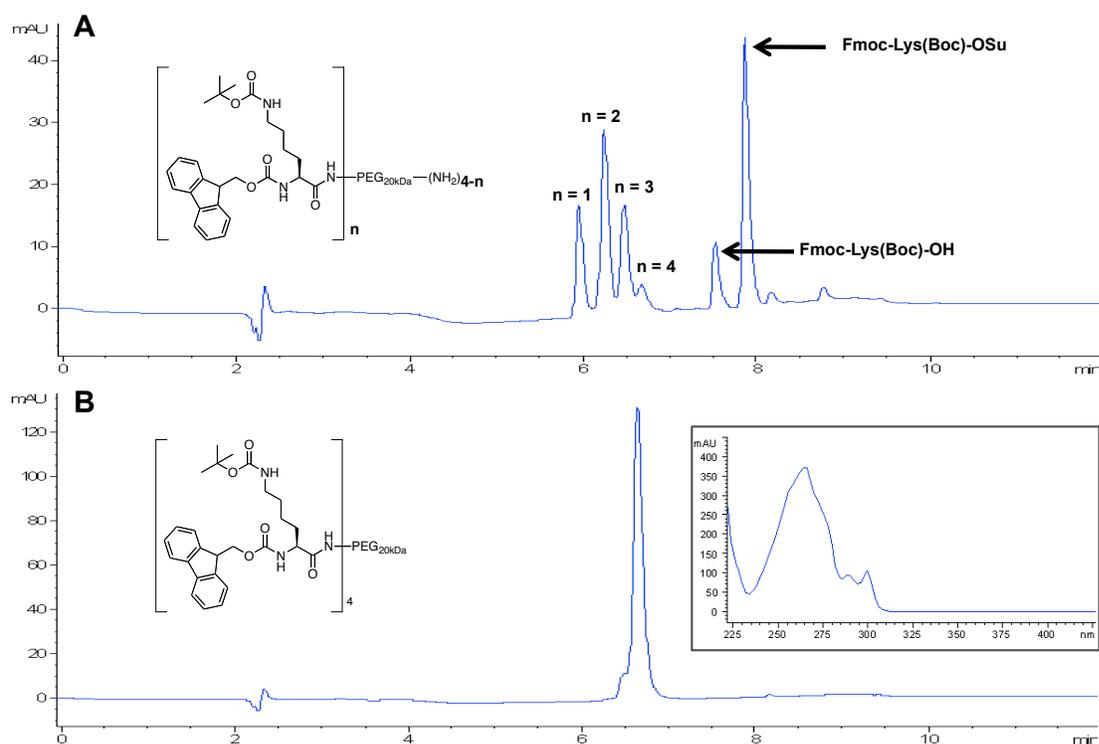
### A. Syntheses of pre-polymers A

1. Common intermediate: [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>



**[Fmoc-Lys(Boc)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>.** Fmoc-Lys(Boc)-OSu (249 mg, 520  $\mu\text{mol}$ ) and DIPEA (139  $\mu\text{L}$ , 800  $\mu\text{mol}$ ) were successively added to a solution of PEG<sub>20kDa</sub>-(NH<sub>2</sub>·HCl)<sub>4</sub> (2.00 g, 100  $\mu\text{mol}$ ) in 20 mL of MeCN. The reaction mixture was stirred at ambient temperature and monitored by C<sub>18</sub> HPLC. The starting material converted to a single product peak via three slower eluting Fmoc-containing intermediates (Fig. SI1), and the reaction was complete within 1.5 h. TNBS assay of the reaction mixture indicated the presence of  $\leq 0.8$  mol% of the starting amine (LOQ = 0.8 mol%). Ac<sub>2</sub>O (38  $\mu\text{L}$ , 400  $\mu\text{mol}$ ) was added to cap unreacted amine, and stirring was continued for 30 min. After concentrating to  $\sim 10$  mL, the reaction mixture was added to 400 mL of MTBE, and the resulting suspension was vigorously stirred for 1 h. The supernatant was decanted, and the residual mixture was filtered to recover the precipitate. The solid material was successively washed with MTBE (3 x 50 mL), Et<sub>2</sub>O (3 x 50 mL), and MTBE (1 x 50 mL). After removal of residual solvent under vacuum, the title compound (2.06 g, 94.5  $\mu\text{mol}$  PEG, 95% yield by weight) was recovered as a white powder.

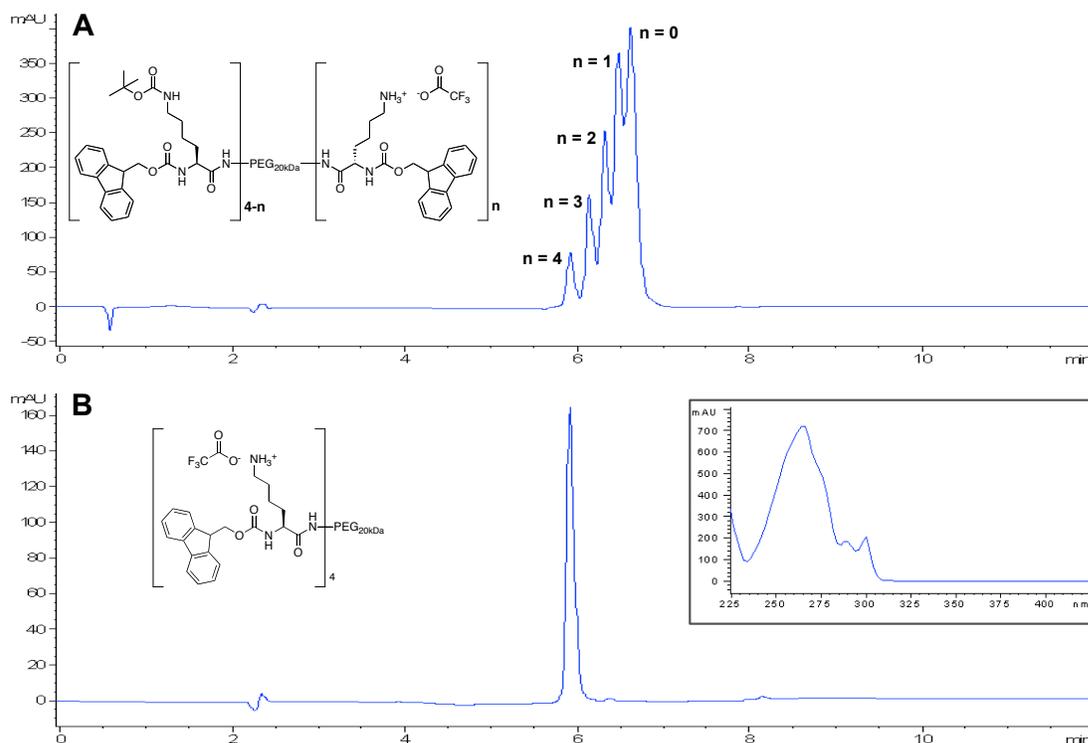
C<sub>18</sub> HPLC, purity was determined at 300 nm: 94% (RV = 6.6 mL) and 6% shoulder (RV = 6.5 mL) attributed to PEG-(NH<sub>2</sub>·HCl)<sub>(n < 4)</sub> contaminant in the starting PEG. Fmoc content was determined using  $\epsilon_{266} = 17847 \text{ M}^{-1}\text{cm}^{-1}$  (H<sub>2</sub>O or MeCN/H<sub>2</sub>O): 4.00  $\mu\text{mol/mL}$  by weight, found 4.06  $\mu\text{mol/mL}$  by Fmoc A<sub>266</sub>.



**Figure S11.** C<sub>18</sub> HPLC analysis ( $\lambda = 300$  nm) of PEG<sub>20kDa</sub>-(NH<sub>2</sub>·HCl)<sub>4</sub> acylation with Fmoc-Lys(Boc)-OSu. A. Reaction mixture at 15 sec; B. Isolated [Fmoc-Lys(Boc)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>; B inset. UV spectrum of HPLC peak at 6.6 min.

**[Fmoc-Lys(NH<sub>2</sub>·TFA)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>.** Trifluoroacetic acid (1.8 mL) was added to a solution of [Fmoc-Lys(Boc)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (802 mg, 36.8  $\mu$ mol) in 1.8 mL of CH<sub>2</sub>Cl<sub>2</sub>. The reaction mixture was stirred at ambient temperature and monitored by C<sub>18</sub> HPLC. The starting material converted to a single product peak via three faster eluting Fmoc-containing amino-PEG intermediates (Fig. S12), and the reaction was complete within 15 min. After concentrating to ~3 mL, the reaction mixture was added dropwise to 80 mL of Et<sub>2</sub>O and was vigorously stirred for 30 min. The supernatant was decanted, and the precipitate recovered by filtration and washed with Et<sub>2</sub>O (5 x 30 mL). After removal of residual solvent under vacuum, the title compound (731 mg, 33.4  $\mu$ mol PEG, 91% yield by weight) was recovered as a white powder.

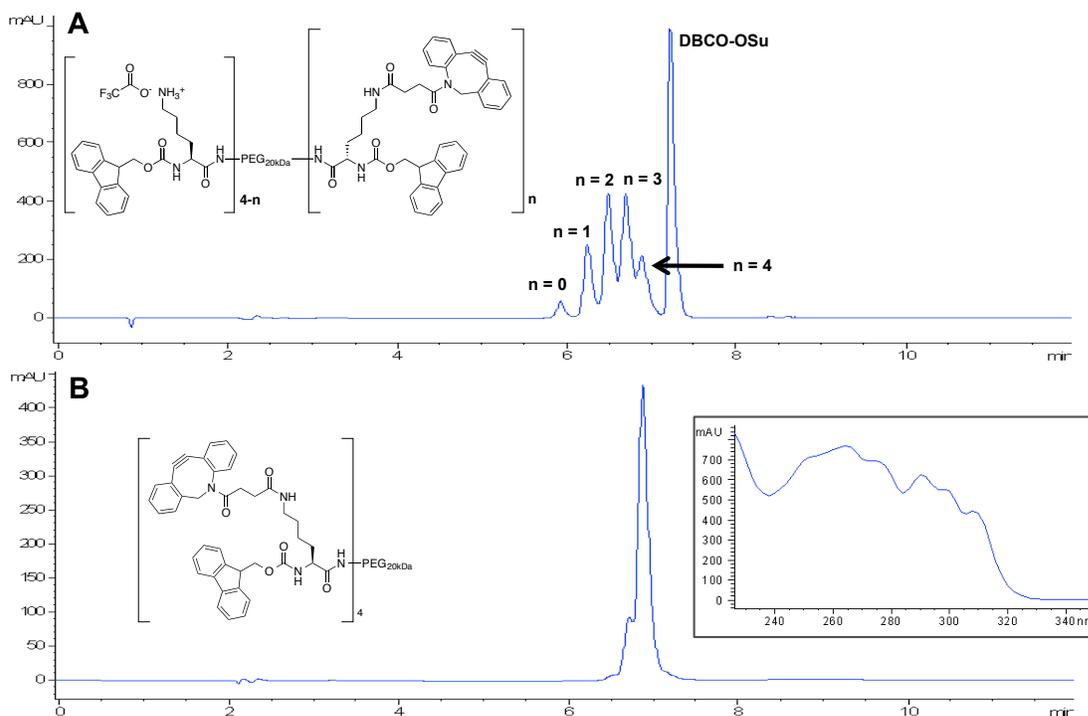
C<sub>18</sub> HPLC, purity was determined at 300 nm: 98% (RV = 5.9 mL). Fmoc content of the title compound was determined using  $\epsilon_{266} = 17,847$  M<sup>-1</sup>cm<sup>-1</sup>: 3.97  $\mu$ mol/mL by weight, 4.01  $\mu$ mol/mL by Fmoc A<sub>266</sub>.



**Figure S12.** C<sub>18</sub> HPLC analysis ( $\lambda = 300$  nm) of TFA deprotection of [Fmoc-Lys(Boc)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>; A. Reaction mixture at  $t = 15$  sec; B. Isolated [Fmoc-Lys(NH<sub>2</sub>•TFA)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>; B inset. UV spectrum of HPLC peak at 5.9 min.

**[Fmoc-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>.** DIPEA (52  $\mu$ L, 300  $\mu$ mol) and DBCO-OSu (69 mg, 170  $\mu$ mol) were successively added to a solution of [Fmoc-Lys(NH<sub>2</sub>•TFA)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (716 mg, 32.8  $\mu$ mol) in 6.5 mL of MeCN. The reaction mixture was stirred at ambient temperature and monitored by C<sub>18</sub> HPLC. The starting material converted to a single product peak via three slower eluting intermediates with an increasing ratio of DBCO:Fmoc that, by 2-component analysis (1), were shown to contain 0, 0.25, 0.5, 0.75 and 1.0 DBCO:Fmoc (Fig. S13). The reaction was complete within 90 min. TNBS assay of the final reaction mixture indicated the presence of  $\leq 1.5$  mol% of the starting amine (LOQ = 1.0 mol%). Ac<sub>2</sub>O (12  $\mu$ L, 130  $\mu$ mol) was added, and stirring was continued for 30 min. After dialysis against MeOH, the title compound was recovered as methanol solution (121  $\mu$ mol DBCO in 25 mL, 92% yield of DBCO A<sub>308</sub>).

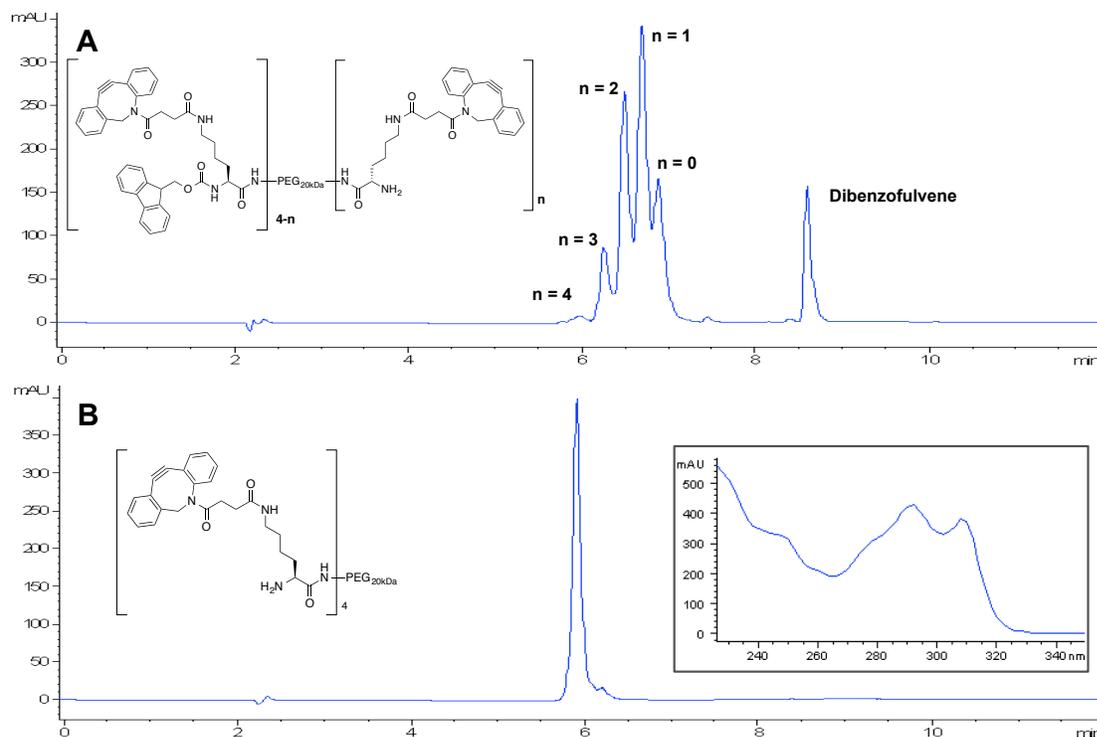
C<sub>18</sub> HPLC, purity was determined at 300 nm: 85% (RV = 6.9 mL) and 15% shoulder (RV = 6.7 mL) attributed to reaction of PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>(n < 4)</sub> contaminant in the starting PEG. The DBCO:Fmoc ratio of the title compound was determined to be 1.06 by 2-component analysis (1) using Fmoc extinction coefficients  $\epsilon_{266} = 17847$  M<sup>-1</sup>cm<sup>-1</sup> and  $\epsilon_{308} = 228$  M<sup>-1</sup>cm<sup>-1</sup> and DBCO  $\epsilon_{266} = 6666$  M<sup>-1</sup>cm<sup>-1</sup> and  $\epsilon_{308} = 13448$  M<sup>-1</sup>cm<sup>-1</sup>.



**Figure S13.** C<sub>18</sub> HPLC analysis ( $\lambda = 300$  nm) of [Fmoc-Lys(NH<sub>2</sub>·TFA)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> acylation with DBCO-OSu: A. Reaction mixture at t = 10 sec; B. Isolated [Fmoc-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>; Inset of B. UV spectrum of HPLC peak at 6.9 min.

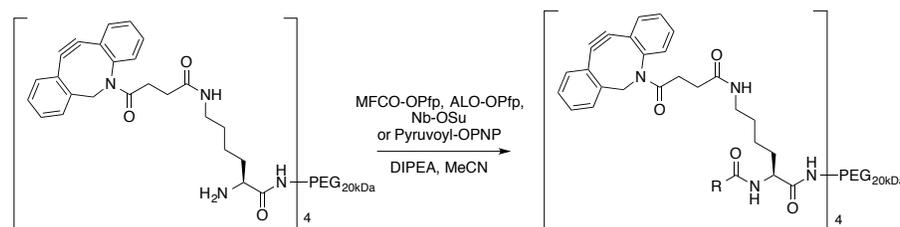
**[H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>.** 4-Methylpiperidine (0.27 mL, 2.3 mmol) was added to a solution of [Fmoc-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (636 mg, 28.2  $\mu$ mol) in 5.1 mL of DMF. The reaction mixture was stirred at ambient temperature and monitored by C<sub>18</sub> HPLC. The starting material converted to a single product peak via three faster eluting DBCO-containing intermediates (Fig. S14), and the reaction was complete within 15 min. The product was precipitated by slow addition to 150 mL of MTBE. The supernatant was decanted, and the solid material was recovered by filtration and washed with MTBE (5 x 15 mL). After removal of residual solvent under vacuum, the solid residue of [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (531 mg) was dissolved in MeCN and gave the UV spectrum of DBCO, with no Fmoc contribution (100  $\mu$ mol DBCO in 25 mL, 88% yield of DBCO A<sub>308</sub>).

C<sub>18</sub> HPLC, purity was determined at 308 nm: 95% (RV = 5.9 mL) and 4% shoulder (RV = 6.2 mL) attributed to reaction of PEG<sub>20kDa</sub>-(NH<sub>2</sub>·HCl)<sub>(n < 4)</sub> contaminant in the starting PEG. DBCO content of the title compound was determined using  $\epsilon_{308} = 13,448$  M<sup>-1</sup>cm<sup>-1</sup>: 3.92  $\mu$ mol/mL by weight, 4.00  $\mu$ mol/mL by DBCO A<sub>308</sub>.



**Figure S14.** C<sub>18</sub> HPLC analysis ( $\lambda = 308$  nm) of Fmoc deprotection of [Fmoc-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>: A. Reaction mixture at 30 sec; B. Isolated [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>; B inset. UV spectrum of HPLC peak at 5.9 min.

## 2. Acylation of [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>



**a. [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>.** DIPEA (15.8  $\mu$ L, 91.1  $\mu$ mol) and MFCO-OPfp (52  $\mu$ mol in 139  $\mu$ L MeCN) were successively added to a solution of [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (40  $\mu$ mol DBCO in 2.0 mL MeCN). The reaction mixture was stirred at ambient temperature and monitored by C<sub>18</sub> HPLC. The starting material converted to a single product peak via three slower eluting DBCO-containing intermediates, and the reaction was complete within 20 hr. TNBS assay of the reaction mixture indicated 1.2 mol% of the starting amine (LOQ = 0.6 mol%). Ac<sub>2</sub>O (3.8  $\mu$ L, 40  $\mu$ mol) was added to cap any unreacted amine, and stirring was continued for 30 min. The reaction mixture was purified by dialysis against MeOH to provide the title compound as a solution in MeOH (32  $\mu$ mol DBCO in 10.0 mL, 81% yield of DBCO A<sub>308</sub>).

C<sub>18</sub> HPLC, purity was determined at 308 nm: 84% (RV = 6.5 mL) and 16% shoulder (RV = 6.4 mL) attributed to reaction of PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>(n < 4)</sub> contaminant in the starting PEG.

**b. [ALO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>.** As above, [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (18.0  $\mu$ mol DBCO in 1 mL MeCN) was acylated with ALO-OPfp (23  $\mu$ mol in 38  $\mu$ L in MeCN) and DIPEA (41  $\mu$ mol, 7.2  $\mu$ L). After stirring overnight, TNBS assay indicated the presence of 0.7

mol% of the starting amine (LOQ = 0.5 mol%). After capping with Ac<sub>2</sub>O and dialyzing against MeOH, the title compound was recovered as a solution in MeOH (15.0 μmol DBCO in 5.0 mL, 83% yield of DBCO A<sub>308</sub>).

C<sub>18</sub> HPLC, purity was determined at 308 nm: single peak (RV = 6.5 mL).

c. **[Nb-Lys(DBCO)-NH]<sub>4</sub>PEG<sub>20kDa</sub>**. As above, [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (18.0 μmol DBCO in 1.00 mL MeCN) was acylated with Nb-OSu (5.8 mg, 23 μmol) and DIPEA (7.2 μL, 41 μmol). After capping with Ac<sub>2</sub>O and dialyzing against MeOH, the title compound was recovered as a solution in MeOH (13.3 μmol DBCO in 5.0 mL, 74% yield of DBCO A<sub>308</sub>).

C<sub>18</sub> HPLC, purity was determined at 308 nm: 83% (RV = 6.4 mL) and 17% shoulder (RV = 6.3 mL) attributed to reaction of PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>(n < 4)</sub> contaminant in the starting PEG.

d. **[Pyruvoyl-Lys(DBCO)-NH]<sub>4</sub>PEG<sub>20kDa</sub>**. As above, [H-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (18.0 μmol DBCO in 1.00 mL MeCN) was acylated with 4-nitrophenyl pyruvate (4.9 mg, 23 μmol) and DIPEA (7.2 μL, 41 μmol). After capping with Ac<sub>2</sub>O and dialyzing against MeOH, the title compound was recovered as a solution in MeOH (13.3 μmol DBCO in 5.00 mL, 75% yield of DBCO A<sub>308</sub>).

C<sub>18</sub> HPLC, purity was determined at 308 nm: 91% (RV = 6.1 mL) and 9% shoulder (RV = 6.3 mL) attributed to reaction of PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>(n < 4)</sub> contaminant in the starting PEG.

## B. Synthesis of pre-polymers B

**(7-Azido-1-(Mod)-2-heptyloxycarbonyl)<sub>4</sub>-PEG<sub>20kDa</sub>** (PEG<sub>20kDa</sub>-[NH-L2(Mod)-N<sub>3</sub>]<sub>4</sub>) prepolymers were prepared as previously described (2).

### 1. Common intermediate: **PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub>**

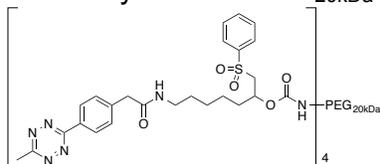
**7-(tert-Butoxycarbonylamino)-1-phenylsulfonylmethyl-2-heptanol**. A 1.0 M solution of trimethylphosphine in THF (2.2 mL, 2.2 mmol) was added dropwise to a stirred solution of 7-azido-1-phenylsulfonyl-2-heptanol (2) (600 mg, 2.0 mmol) in 1.8 mL of THF, resulting in vigorous gas evolution. After 10 min, gas evolution subsided and water (0.2 mL) was added, followed by di-*tert*-butyl dicarbonate (500 mg, 2.3 mmol). After 15 min, the mixture was diluted with EtOAc and washed with sat. aq. NaHCO<sub>3</sub>. The organic phase was washed sequentially with 0.1 N HCl, water, sat. aq. NaHCO<sub>3</sub>, and brine, then dried over MgSO<sub>4</sub>, filtered, and evaporated to give a colorless oil. Chromatography on SiO<sub>2</sub> using a step gradient of hexane and 60:40 hexane/EtOAc provided the product (592 mg, 1.6 mmol, 80%) as a colorless oil. <sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO): δ 7.89 (2H, m), 7.72 (1H, m), 7.63 (2H, m), 6.75 (1H, t, J = 5.2 Hz), 4.82 (1H, d, J = 6 Hz), 3.83 (1H, m), 3.34 (2H, m), 2.85 (2H, q, J = 6.8 Hz), 1.36 (9H, s), 1.6 (4H, m), 1.15 (4H, m).

**7-(tert-Butoxycarbonylamino)-1-phenylsulfonylmethyl-2-heptyl succinimidyl carbonate**. Pyridine (0.16 mL, 2.0 mmol) was added dropwise to a stirred solution of 7-(*tert*-butoxycarbonylamino)-1-phenylsulfonyl-2-heptanol (372 mg, 1.0 mmol) and triphosgene (500 mg, 1.7 mmol) in 15 mL of anhydrous THF. A white precipitate formed. After 10 minutes, the mixture was concentrated to dryness. The residue was dissolved in 15 mL of anhydrous THF and treated with N-hydroxysuccinimide (350 mg, 3.0 mmol) and pyridine (0.25 mL, 3.0 mmol) for 15 minutes. The mixture was evaporated to dryness, and the residue was dissolved in EtOAc, filtered, and washed sequentially with water, 5% KHSO<sub>4</sub>, and brine, then dried over MgSO<sub>4</sub>, filtered, and evaporated to dryness. Chromatography on SiO<sub>2</sub> using a step gradient of 0, 20, 40, and 60% EtOAc in hexane provided the product (300 mg, 0.59 mmol, 59%) as a foam which crystallized from 1:1 EtOAc/hexane. <sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO): δ 7.89 (2H, m), 7.75 (1H,

m), 7.65 (2H, m), 6.74 (1H, t, J = 5.2 Hz), 5.11 (1H, m), 3.95 (1H, dd, J = 8.8, 15.2 Hz), 3.83 (1H, dd, J = 2.8, 15.2 Hz), 2.85 (2H, q, J = 6.8 Hz), 2.81 (4H, s), 1.65 (2H, m), 1.36 (9H, s), 1.30 (2H, m), 1.15 (4H, m).

**PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub>.** A solution of PEG<sub>20kDa</sub>-(NH<sub>2</sub>•HCl)<sub>4</sub> (650 mg, 33 μmol, 132 μmol amine groups) in water (3 mL) was added to 1 M NaHCO<sub>3</sub> (0.26 mL, 0.26 mmol) followed by a solution of 7-(*tert*-butoxycarbonylamino)-1-phenylsulfonyl-2-heptyl succinimidyl carbonate (80 mg, 0.16 mmol). The resulting mixture was allowed to stand for 1 h then assessed for amine content by TNBS assay (7.0% amines detected). The reaction mixture was adjusted to pH 3.5 with acetic acid then dialyzed twice against 50% aqueous MeOH and once against MeOH. The dialysis mixture was concentrated to dryness to give PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub> (235 mg, 11 μmol, 33% after inadvertent material loss) as a white solid. This material was treated with 1:1 TFA:CH<sub>2</sub>Cl<sub>2</sub> (6 mL) for 1 h then concentrated to dryness under reduced pressure. The residue was dissolved in MeOH (10 mL) and again concentrated to dryness. This process was repeated 3 times. The residue was dissolved in 60% MeCN in MeOH (1.6 mL) and treated with 15 mL Et<sub>2</sub>O. The resulting precipitate was collected by centrifugation and washed with Et<sub>2</sub>O (15 mL) to give PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub> (157 mg, 7.3 μmol, yield 67% based on BOC intermediate) as a white solid.

## 2. Acylation of PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub>.



**PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-MeTz]<sub>4</sub>.** A solution of PEG<sub>20kDa</sub>-[NH-L2(PhSO<sub>2</sub>)-NH<sub>2</sub>]<sub>4</sub> (157 mg, 7.3 μmol, 29 μmol amine) in 1 mL of water was treated with a solution of succinidyl 4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenylacetate (11.4 mg, 0.035 mmol) in 1 mL of MeCN and 1 M NaHCO<sub>3</sub> (0.058 mL, 0.058 mmol), measured pH = 6.75, another 0.030 mL of 1 M NaHCO<sub>3</sub> was added to adjust the pH to 7.45. The resulting mixture was kept for 1 h then assessed for amine content by TNBS assay (7.9% amines detected). The reaction mixture was adjusted to pH 4.0 with AcOH then dialyzed against MeOH. The dialysis mixture was concentrated to dryness to give the title compound (133 mg, 0.60 μmol 83%) as a bright pink solid. SEC HPLC, purity was determined at 520 nm: 92% at RV = 6.9 mL.

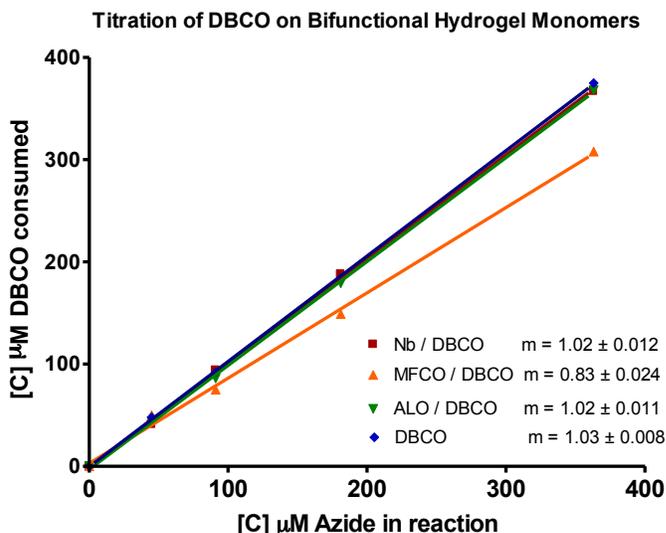
## C. Hydrogel formation and gelation time

Unless otherwise specified, 5% Tetra-PEG hydrogels were prepared as previously described (2). Briefly, equal volumes of solutions of prepolymer A containing 10 mM connector X and prepolymer B containing 10 mM connector Y were mixed. The resulting mixture was immediately cast into a rubber cylindrical disk mold (9 mm diameter x 1 mm high, 64 μL; Electron Microscopy sciences 70326-52) mounted on a silanized glass microscope slide. Sixty μL gels containing Nb-MeTz crosslinks were prepared by casting the mixed prepolymer solution into a 0.6 mL Eppendorf tube and centrifuging at ~300 g for 2 h or in a 9 mm x 1 mm gel disk mold as described above. Gels were allowed to cure for 18 h then transferred to 5 mL of acetate buffer (10 mM, pH 4.5) for storage at 4 °C prior to use in degelation assays. To measure gelation time prepolymer solutions (~50 μL each) were mixed as described above in an Eppendorf tube. Gelation time was defined as the point where the solution transitioned from a liquid to a gel and could no longer be withdrawn by pipette.

## II. Formats for drug-bound Tetra-PEG hydrogel formation

### A. Same orthogonal chemistry, different reaction rates

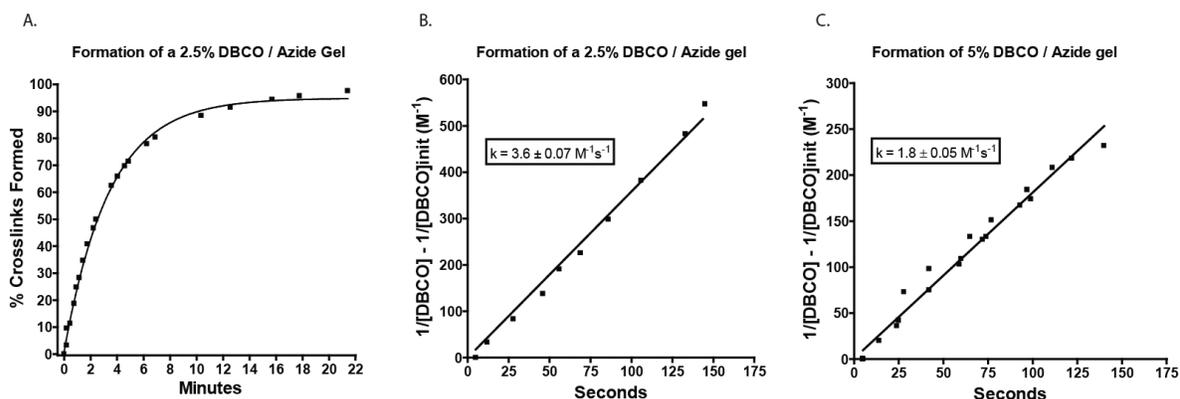
1. Selectivity of azides for DBCO over MFCO, Nb, and ALO on Prepolymer A  
Aqueous solutions of prepolymers [ALO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>, [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>, [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>, and PEG<sub>20kDa</sub>-(NH-DBCO)<sub>4</sub> containing 0.73 ± 0.05 mM DBCO were prepared. Four aliquots (0.50 mL) of each solution in cuvettes (0.2 cm path length) were individually treated with aqueous solutions (0.50 mL) containing 0.125 (0.09 mM), 0.25 (0.18 mM), 0.5 (0.37 mM), or 1.0 (0.73 mM) equivalents of O-(2-azidoethyl)heptaethylene glycol (Sigma). After 23 h the reactions were assessed for DBCO concentration by absorbance at 308 nm. Plots of the amount of DBCO consumed vs azide concentration gave the fraction of DBCO reacted, from which the extent of modification of the second functional group was calculated: % modified = (1-[(slope [R-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>)/(slope PEG<sub>20kDa</sub>-(NH-DBCO)<sub>4</sub>]) x 100).



**Figure S15.** Click reaction selectivity of O-(2-azidoethyl)heptaethylene glycol for DBCO in the presence of ALO, Nb and MFCO. Comparison of titration curves for the reaction of azide with PEG<sub>20kDa</sub>-(NH-DBCO)<sub>4</sub>, [ALO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub>, [Nb-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> and [MFCO-Lys(DBCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> revealed ≤1% modification of ALO and Nb and 19% modification of MFCO.

### 2. Rate of DBCO-azide gel crosslinking

For 2.5% PEG w/v gels, a solution of PEG<sub>20kDa</sub>-(NH-DBCO)<sub>4</sub> (0.050 mL of 5.0 mM DBCO) in water was added to a solution of PEG<sub>20kDa</sub>-[NHOCO-(CH<sub>2</sub>)<sub>6</sub>-N<sub>3</sub>]<sub>4</sub> (0.050 mL of 5.0 mM azide) in a 1 mL micro cuvette with a 0.2 cm path length. The reaction of DBCO was monitored on the 320 nm shoulder ( $\epsilon_{320} = 1304 \text{ M}^{-1}\text{cm}^{-1}$ ) and gelation occurred in ~ 150 seconds. For 5% PEG w/v gels the same procedure was used with 10 mM solutions of prepolymers; gelation occurred in ~ 90 seconds.



**Figure S16.** A. Progress curve for the DBCO/azide crosslinking reaction during formation of a 2.5% PEG hydrogel; B and C. The rate of consumption of DBCO in a hydrogel crosslinking reaction was monitored and fit to a linear second order plot. The second order rate constant for the reaction of a 2.5% PEG gel (B) was  $3.6 \text{ M}^{-1}\text{s}^{-1} \pm 0.07$  at  $22 \text{ }^\circ\text{C}$  and a 5% PEG gel (C) was  $1.8 \text{ M}^{-1}\text{s}^{-1} \pm 0.05$ .

### 3. Exenatide-triazole-hydrogel

A solution of [DBCO-Lys(MFCO)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (350 nmol, 105  $\mu\text{mol}$  DBCO end groups) in water (58.2  $\mu\text{L}$ ) was treated with a solution of exenatide-(MeSO<sub>2</sub>-linker-N<sub>3</sub>) (1.11 nmol) (1) in DMF (25.0  $\mu\text{L}$ ), followed by solutions of O-(2-azidoethyl)heptaethylene glycol (2.55  $\mu\text{L}$ , 54.6 mM, 139 nmol) in water, and 7-azido-1-(Mod)-2-heptyloxycarbonyl-AAF (Mod = MeSO<sub>2</sub>, 140 nmol) in DMF (2.46  $\mu\text{L}$ ). The mixture was allowed to stand at  $4 \text{ }^\circ\text{C}$  for 21 h. To the exenatide-loaded prepolymer A (60  $\mu\text{L}$ , 2.50 mM, 150 nmol) was added a solution of PEG<sub>20kDa</sub>-[NH-L2(CN)-N<sub>3</sub>]<sub>4</sub> (150 nmol) in 10 mM acetate, pH 4.5 (60  $\mu\text{L}$ ). The solution was gently mixed by pipetting and cast in 9 x 1 mm cylindrical disk molds. The gels were allowed to cure overnight in a humidified chamber, then washed with 2 x 5 mL 10 mM NaOAc pH 4.5, to remove unbound peptide. Exenatide release (280 nm) and degelation (495 nm) were measured by absorbance of the supernatant over time upon incubating the gels in pH 9.4 borate buffer at  $37 \text{ }^\circ\text{C}$ .

#### B. Bioorthogonal reaction pairs

1. Reactions of tetrazines with norbornene and 1-methylcyclopropene dienophiles. Pseudo-first-order reactions of tetrazines with dienophiles were obtained at ambient temperature in PBS, pH 7.4, containing 5% DMSO. Typically, 0.1 mM of the tetrazine (3) was reacted with 1 mM and 2 mM dienophile, and reaction progress was monitored by UV spectrophotometry. Absorbance loss at 520 nm was plotted vs time, and the data were fitted to a first-order rate equation. Second-order rate constants were estimated as  $k_{\text{obsd}}/[\text{dienophile}]$ .

**Table S11.** Second-order rate constants ( $\text{M}^{-1}\text{s}^{-1}$ )<sup>a</sup> for reactions of tetrazines with norbornene and 1-methylcyclopropene dienophiles.

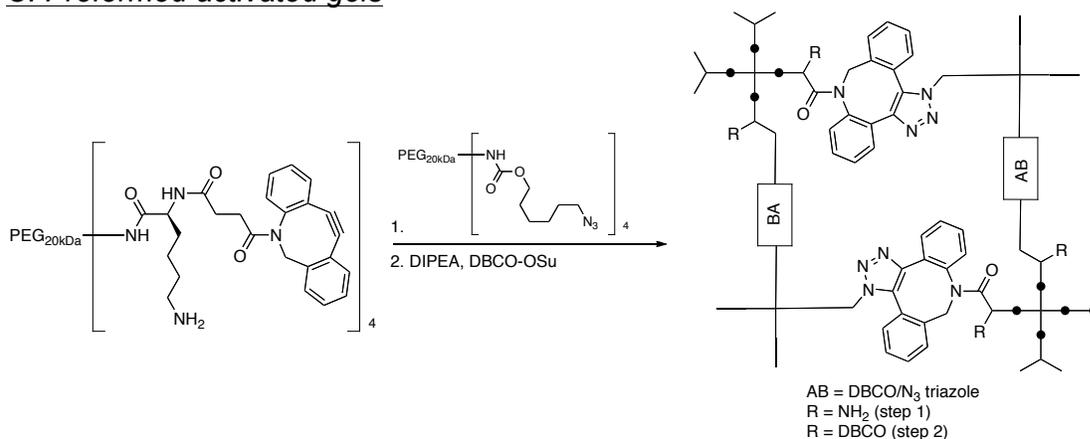
	$2.6 \pm 0.2$	$1.2 \pm 0.0$	$2.1 \pm 0.0$	$3.6 \pm 0.3$	$0.059 \pm 0.008$
	$0.091 \pm 0.02$	---	---	$0.52 \pm 0.01$	---

<sup>a</sup> Average values  $\pm$  (range/2) of duplicate measurements performed at ambient temperature at two concentrations of dienophile.

## 2. Fab-diazine-hydrogel

[DBCO-Lys(Nb)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (49  $\mu$ L of 86 mg/mL, 190 nmol PEG) was treated with a MeTz-L1-Fab (86  $\mu$ L of 0.33 mM, 28 nmol). Reaction progress was monitored by disappearance of A<sub>520</sub> and was judged to be complete within 75 min. To the Fab-loaded prepolymer A (61  $\mu$ L of 31 mg/mL, 85 nmol PEG) was added PEG<sub>20kDa</sub>-[NHOCO-(CH<sub>2</sub>)<sub>6</sub>-N<sub>3</sub>]<sub>4</sub> (11  $\mu$ L of 151 mg/mL, 85 nmol). The solution was gently mixed by pipetting and placed into a 60  $\mu$ L (9 x 1 mm) cylindrical disk mold. The gel disk was allowed to cure overnight in a humidity chamber then washed with 3 x 1 mL of 10 mM MES, pH 6.0 to remove unbound protein. Treatment of the disk at pH 9.4 caused first-order release of the Fab to give 9 mg protein/mL gel.

### C. Preformed activated gels



#### 1. (Amine)<sub>x</sub>-Hydrogel

In a 1.5 mL Eppendorf tube, water (14.1  $\mu$ L), and PEG<sub>20kDa</sub>-[NHOCO-(CH<sub>2</sub>)<sub>6</sub>-N<sub>3</sub>]<sub>4</sub> (71 nmol in 24.8  $\mu$ L water, 283 nmol azide) were successively added to [DBCO-Lys(H)-NH]<sub>4</sub>-PEG<sub>20kDa</sub> (71 nmol in 21.1  $\mu$ L water, 283 nmol DBCO). The reaction tube was capped, quickly vortexed for ~2 sec, centrifuged for ~5 sec and allowed to set overnight to form a cone-shaped 5% (w/v) hydrogel.

#### 2. (DBCO)<sub>x</sub>-Hydrogel

The 60  $\mu$ L (amine)<sub>x</sub>-hydrogel cone (283 nmol NH<sub>2</sub> groups) was successively washed for 20 min with 1 mL each of water, EtOH, and 3 x 1 mL of MeCN. The hydrogel was suspended in 0.7 mL of MeCN, and DIPEA (1  $\mu$ L, 6  $\mu$ mol) was added. After 5 min, DBCO-OSu (330 nmol in 85  $\mu$ L MeCN) was added, and the loss of DBCO absorbance in the supernatant was monitored at 308 nm. After 18 h, the concentration of DBCO in the supernatant was 83  $\mu$ M (71 nmol in 0.85 mL) indicating that 90% of the hydrogel amines were loaded with 255 nmol of DBCO. The supernatant was removed, and the hydrogel was washed with 1 mL of MeCN for 20 min. After removing the MeCN wash, the hydrogel was resuspended in 0.5 mL MeCN. DIPEA (1  $\mu$ L, 6  $\mu$ mol) and Ac<sub>2</sub>O (1  $\mu$ L, 10  $\mu$ mol) were successively added to cap unreacted amines for 20 min at room temperature. The supernatant was removed, and the hydrogel was washed with 3 x 1 mL of MeCN for 20 min each to provide a hydrogel loaded with 255 nmol DBCO.

#### 3. Human growth hormone (hGH)- triazole-hydrogel.

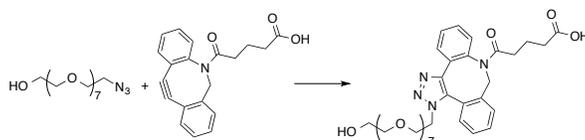
The (DBCO)<sub>x</sub>-hydrogel (60  $\mu$ L, 255 nmol DBCO), was sliced into ~10 fragments, transferred to a 1.5 mL Eppendorf tube and treated with a mixture of hGH and N<sub>3</sub>-linker-hGH, 55:45 (Mod = MeSO<sub>2</sub> 1.2 mg, 55 nmol N<sub>3</sub>-linker-hGH in a loading buffer of 1.0 mL of 10 mM Na citrate, pH 6.1, 1.9 mg/mL Tween 20). After 16 h, loss of absorbance at 280 nm (A<sub>280</sub>) indicated 0.91 mg (41

nmol, 75% input) of N<sub>3</sub>-linker-hGH had loaded onto the gel. The supernatant was replaced with an identical second portion of the hGH/N<sub>3</sub>-linker-hGH mixture. After 22.5 h, the loss of A<sub>280</sub> indicated an additional 0.82 mg (37 nmol, 69% input) of N<sub>3</sub>-L1-hGH had loaded for a total of 1.7 mg, 78 nmol, 72% overall. The supernatant was removed, and the gel was washed twice with 1 mL of the loading buffer for 20 min each. No absorbance at 280 nm was observed in the final wash, indicating that no non-covalently bound hGH remained. The β-eliminative linker was cleaved by treatment of the hGH-loaded hydrogel with 0.1 M borate, pH 9.4, containing 0.1% NaN<sub>3</sub> and 1.9 mg/mL Tween 20, resulting in the release of hGH (1.9 mg, 85 nmol; 32 mg hGH/mL gel), indicating that 33% of the DBCO moieties of the gel had coupled to N<sub>3</sub>-linker-hGH.

### III. Stability studies of connecting groups: model systems

#### A. Synthesis of models for stability studies

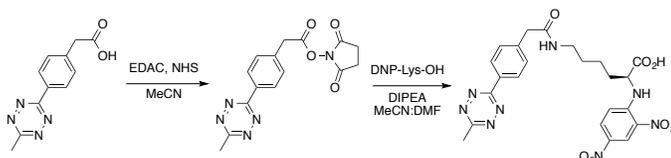
##### 1. Triazole



A solution of DBCO-acid (0.7 mg, 2 μmol) and O-(2-azidoethyl)heptaethylene glycol (2.2 μmol in 196 μL H<sub>2</sub>O) in 98 μL of methanol was incubated at ambient temperature for 2.5 h. The reaction mixture was purified on a 1 g C<sub>18</sub> BondElut cartridge, eluting with a gradient of MeOH in water, to provide the desired PEGylated DBCO-triazole.

C<sub>18</sub> HPLC purity (λ = 254 nm): >95%; MS: *m/z* = 729.3

##### 2. Diazine

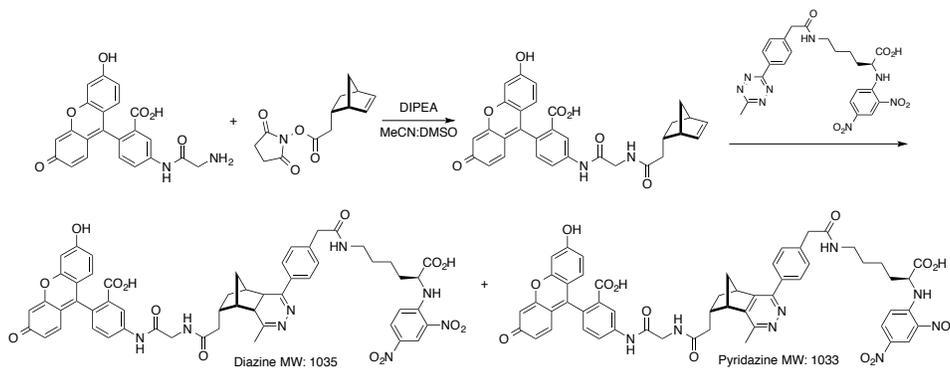


**6-Methyltetrazine-3-phenylacetic acid, NHS ester.** A solution of EDAC (28 mg, 0.14 mmol), N-hydroxysuccinimide (22 mg, 0.19 mmol), and 6-methyltetrazine-3-phenylacetic acid (22 mg, 96 μmol) (3) in 2.5 mL of MeCN was stirred at ambient temperature overnight. The reaction mixture was partitioned between 1:1 EtOAc:25% aq NaHCO<sub>3</sub>. The layers were separated, and the organic phase was successively washed with 5% aq KHSO<sub>4</sub>, water, and brine. The organic layer was dried over MgSO<sub>4</sub>, filtered, and concentrated. The crude red concentrate was purified on a silica gel column, eluting with a gradient of acetone in CH<sub>2</sub>Cl<sub>2</sub>, to provide the title compound as a pink solid.

C<sub>18</sub> HPLC (λ = 520 nm): 95.2%; MS: *m/z* = 328.0 [M+H]<sup>+</sup>

**DNP-Lys(Tetrazine)-OH.** A suspension of α-DNP-Lysine (7.8 mg, 22 μmol), succinimidyl 2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetate (6.6 mg, 20 μmol), and DIPEA (7.8 μL, 45 μmol) in 2 mL of 1:1 MeCN:DMF was stirred at ambient temperature overnight. The reaction mixture was partitioned between 1:1 EtOAc:5% aq KHSO<sub>4</sub>. The layers were separated, and the organic phase was successively washed with water (3x) and brine. The organic layer was dried over MgSO<sub>4</sub>, filtered, and concentrated. The crude material was purified on a silica gel column, eluting with a gradient of acetone in EtOAc followed by 0.1% AcOH in acetone, to provide the title compound as a red oil that solidified on standing.

C<sub>18</sub> HPLC purity ( $\lambda = 254 \text{ nm}$ ): >95%; MS:  $m/z = 525.1 \text{ [M+H]}^+$



**Norbornene-fluorescein.** A solution of norbornene acetic acid NHS ester (1.1 mg, 4.4  $\mu\text{mol}$ ), 5-(aminoacetamido)fluorescein (5.10  $\mu\text{mol}$  in 220  $\mu\text{L}$  DMSO), and DIPEA (1.6  $\mu\text{L}$ , 9.3  $\mu\text{mol}$ ) in 0.2 mL of MeCN was stirred at ambient temperature overnight. The reaction mixture was partitioned between 1:1 EtOAc:5% aq KHSO<sub>4</sub>. The layers were separated, and the organic phase was successively washed with water and brine. The organic layer was dried over MgSO<sub>4</sub>, filtered, and concentrated. The crude material was purified on a 1g C<sub>18</sub> BondElut column, eluting with a gradient of MeOH in water, to provide the title compound as a yellow solid.

C<sub>18</sub> HPLC purity ( $\lambda = 254 \text{ nm}$ ): >95%; MS:  $m/z = 539.2 \text{ [M+H]}^+$

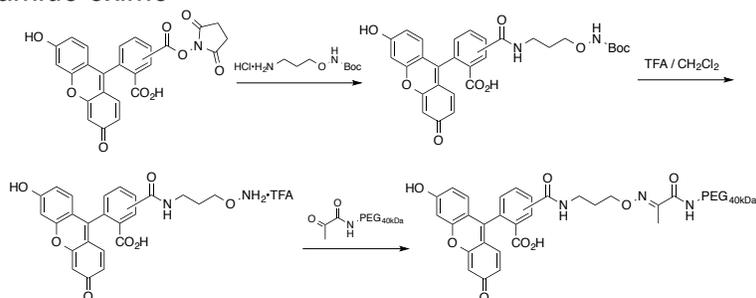
**DNP-Lys(Diazine-Fluorescein)-OH.** A solution of norbornene-fluorescein (2.91  $\mu\text{mol}$  in 260  $\mu\text{L}$  DMSO) and DNP-Lys(tetrazine)-OH (2.91  $\mu\text{mol}$  in 233  $\mu\text{L}$  MeOH) was incubated at ambient temperature for 4 d. The reaction mixture was partitioned between 1:1 EtOAc:5% aq KHSO<sub>4</sub>. The layers were separated, and the organic phase was successively washed with water and brine. Residual product was washed from the separatory funnel with acetone. The combined organic layer and acetone wash were dried over MgSO<sub>4</sub>, filtered, and concentrated to provide a crude orange/pink film. The residue was washed with 3 mL of EtOAc to remove unreacted norbornene and tetrazine starting materials, and the remaining film was purified on a 1g C<sub>18</sub> BondElut column, eluting with a gradient of MeOH in water, to provide the title compound as a yellow solid.

C<sub>18</sub> HPLC ( $\lambda = 254 \text{ nm}$ ): 2 major peaks (RV = 6.17, 6.21 mL) and 2 minor peaks (RV = 6.51, 6.60 mL) with relative area of ~ 4:4:1:2, resp.

MS:  $m/z = 1035.2 \text{ [M+H]}^+$  diazines (major HPLC peaks) and  $1033.1 \text{ [M+H]}^+$  pyridazines (minor HPLC peaks)

### 3. Oximes

#### a. pyruvamide oxime



**Fluorescein-N-Boc-propoxyamine.** A solution of succinimidyl 5(6)-carboxyfluorescein (46 mg, 0.11 mmol), *N*-(3-aminopropoxy)carbamic acid, *tert*-butyl ester·HCl (48 mg, 0.212 mmol), and

DIPEA (67  $\mu$ L, 0.39 mmol) in anhydrous DMF was stirred at ambient temperature for 4 h. The reaction mixture was partitioned between 1:1 EtOAc:5% aq KHSO<sub>4</sub>. The aqueous phase was extracted 3x with EtOAc, and the combined organic extracts were washed with water and brine. The organic layer was dried over MgSO<sub>4</sub>, filtered, and concentrated. The crude orange oil was purified on a silica gel column, eluting with a gradient of EtOAc in hexane, to provide the title compound as an orange oil.

C<sub>18</sub> HPLC purity ( $\lambda$  = 441 nm): 97%

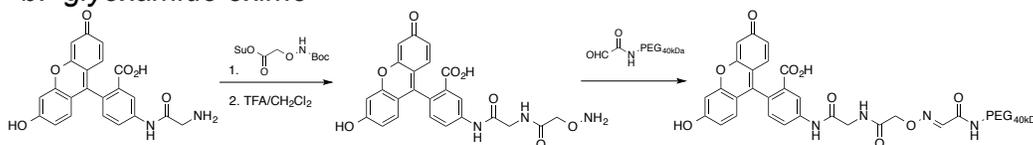
**Fluorescein-propoxyamine.** A 1 mL solution of 1:1 TFA:CH<sub>2</sub>Cl<sub>2</sub> was cooled to 0 °C in an ice-water bath then added to fluorescein-*N*-Boc-propoxyamine (34 mg, 62  $\mu$ mol). After 30 min at 0 °C, toluene (1 mL) was added, and the reaction mixture was concentrated. The crude material was purified on a 1 g C<sub>18</sub> BondElut column, eluting with a gradient of MeCN in water, 0.1% TFA, to provide the title compound as a sticky yellow solid.

C<sub>18</sub> HPLC purity ( $\lambda$  = 441 nm): 91%

**Fluorescein-PEG<sub>40kDa</sub> pyruvamide oxime.** A solution of fluorescein-propoxyamine (2.9  $\mu$ mol), PEG<sub>40kDa</sub>-pyruvamide (1.05  $\mu$ mol), and 5-methoxyanthranilic acid (10  $\mu$ mol) in 0.5 mL of 0.2 M NaOAc, pH 5.0, was incubated overnight. Oxime formation was confirmed by SEC analysis, and the reaction mixture was purified on a PD-10 column, eluting with 20 mM NaOAc, pH 5.5, to provide the desired fluorescein oxime of PEG<sub>40kDa</sub>-pyruvamide.

SEC HPLC purity ( $\lambda$  = 441 nm): >99%. RV (6.1 mL) consistent with 40kDa PEG

*b. glyoxamide oxime*



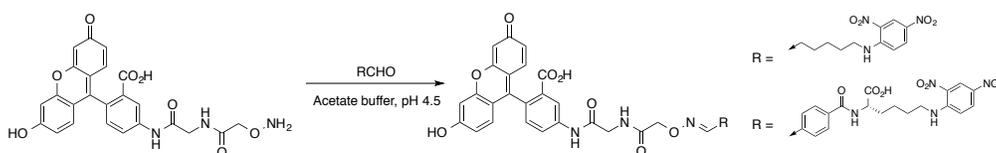
**Aminoxyacetyl-AAF.** Et<sub>3</sub>N (5.8  $\mu$ L, 4.2 mg, 42  $\mu$ mol) and 2,5-dioxopyrrolidin-1-yl 2-(*tert*-butoxycarbonylaminoxy)acetate (8.9 mg, 31  $\mu$ mol) in DMF (0.27 mL) were added to 5-(aminoacetamido)fluorescein (21  $\mu$ mol in 1.0 mL DMF). After incubating for 3 h at ambient temperature, the reaction mixture was diluted with 0.01 N HCl and extracted 4 times with EtOAc. The combined extracts were washed with water then brine and concentrated to dryness. The crude yellow residue (12 mg) was treated with 1:1 TFA:CH<sub>2</sub>Cl<sub>2</sub> at ambient temperature for 3 h, then the reaction mixture was concentrated to dryness. The crude thick syrup was dissolved in MeOH and concentrated to dryness (3x) then purified by C<sub>18</sub> RP HPLC (Peeke Scientific Hi-Q 5 $\mu$ m C<sub>18</sub> 50x20 mm column, 10 mL/min, 20-85% MeCN w/ 0.1% TFA linear gradient over 13 min, product RV = 40-45 mL). The product-containing fractions were concentrated to 3 mL to give a 4 mM solution of 5-AAF-O-NH<sub>2</sub> (12  $\mu$ mol, 57% yield).

C<sub>18</sub> HPLC purity ( $\lambda$  = 441 nm): 92%

**AAF-PEG<sub>40kDa</sub> glyoxamide oxime.** Aminoxyacetyl-AAF (65 nmol in 36  $\mu$ L MeCN) and PEG<sub>40kDa</sub>-glyoxamide (3.3 nmol in 25  $\mu$ L NaOAc, pH 5.5) were incubated overnight in the presence of 12 mM aniline. Oxime formation was confirmed by SEC analysis, then the reaction mixture was purified on a PD MidiTrap column, eluting with 20 mM NaOAc, pH 5.5, to provide the desired fluorescein oxime of PEG<sub>40kDa</sub>-glyoxamide.

SEC HPLC purity ( $\lambda$  = 441 nm): >99%. RV (6.1 mL)

*c. hexanal and benzaldehyde oximes*



**N-DNP-6-Aminoxyacetyl AAF-oxime.** A solution of aminoxyacetyl-AAF (0.25  $\mu\text{mol}$  in 100  $\mu\text{L}$ ) in DMF/ $\text{H}_2\text{O}$ /MeCN and a solution of *N*-DNP-6-aminoxyacetyl (0.25  $\mu\text{mol}$  in 25  $\mu\text{L}$  DMF) were added to 0.5 mL of 0.5 M NaOAc, pH 4.5. The mixture was kept at ambient temperature for 18 h then purified on a  $\text{C}_{18}$  BondElut column. Fractions were cooled to  $-20^\circ\text{C}$ , and the separated product-containing yellow MeCN layers were removed and diluted with EtOAc. After washing with water (4x) and brine, the organic layer was concentrated to dryness to give a yellow residue that was dissolved in MeCN.

$\text{C}_{18}$  HPLC purity ( $\lambda = 441 \text{ nm}$ ): >99%.

**4-Formylbenzoyl-Lys(DNP)- aminoxyacetyl-AAF oxime.** Synthesized from *N*-succinimidyl *p*-formylbenzoate (SFB) (4) as described above for *N*-DNP-6-aminoxyacetyl-AAF-oxime.

$\text{C}_{18}$  HPLC purity ( $\lambda = 441 \text{ nm}$ ): >99%

### B. Stability assays of model systems

Compounds were incubated in either 0.1 M NaOAc (pH 4.5 or pH 5.4) or 0.1 M HEPES (pH 7.4) at either  $4^\circ\text{C}$  or  $37^\circ\text{C}$ . Unless otherwise specified, reactions of oximes also contained 0.5 M acetone to trap the aminoxy component upon cleavage. Aliquots were periodically removed and analyzed by HPLC. Reaction progress, reported as percent reacted (% rx), was determined for DBCO triazole by integrating the area of newly formed peaks ( $\lambda = 254 \text{ nm}$ ). For norbornene diazine, the percent reacted was determined by integrating the area of newly formed peaks that possessed only fluorescein absorbance. For oximes of PEG, percent reacted in the presence of acetone was determined as the peak area ratio of fluorescein-acetone oxime:PEG fluorescein oxime, and percent reacted in the absence of acetone was determined as the ratio of peak areas for fluorescein-alkoxyamine:PEG fluorescein oxime.

**Table S12.** Aqueous stability of connecting functional groups.

	pH 4.5, $4^\circ\text{C}$		pH 5.4, $4^\circ\text{C}$		pH 7.4, $4^\circ\text{C}$		pH 7.4, $37^\circ\text{C}$	
	% rx/t <sup>a</sup>	$t_{1/2}$ , mos <sup>b</sup>	% rx/t	$t_{1/2}$ , mos	% rx/t	$t_{1/2}$ , mos	% rx/t	$t_{1/2}$ , mos
Triazole, DBCO	---	---	nd <sup>c</sup> /96 d	>32	nd/96 d	>32	nd/96 d	>32
Diazine, norbornene	---	---	nd/37 d	>12	nd/37 d	>12	8%/37 d	7.7
Oxime, pyruvamide	5%/131 d nd/131 d <sup>d</sup>	43 >43 <sup>d</sup>	5%/170 d	>56	nd/170 d	>56	nd/209 d	>69
Oxime, glyoxamide	---	---	---	---	---	---	74%/105 d	2
Oxime, hexanal	---	---	---	---	---	---	30%/114 d	6
Oxime, benzaldehyde	---	---	---	---	---	---	nd/114 d	>38

<sup>a</sup> % rx/t is % reacted measured at the last time point examined

<sup>b</sup> If the  $t_{1/2}$  value is designated as >, it is assumed that 5% reaction is an LOQ and that the cleavage reaction was first-order.

<sup>c</sup> nd = not detected

<sup>d</sup> Incubated in the absence of acetone

## IV. Hydrogel degradation

### A. Manual Assay

Plastic macro cuvettes, 2.5 -5.0 mL (BrandTech 7591, 1 cm path length) were fitted with a horizontal stainless steel mesh divider (2 mm mesh, 1 x 1 cm) to keep eroding hydrogels above the spectrophotometer light path. Gels were immersed in the upper compartment of the divided cuvettes which contained 3.0 mL of 0.1 M bicine buffer, pH 8.4. The cuvettes were covered with parafilm and then tight-fitting plastic caps (Ocean Optics 839750) to create an air-tight seal, and kept at 37 °C in a water bath. The cuvettes were inverted several times prior to measuring UV-vis absorbance of the solution at the specified wavelength, usually the absorbance maximum. Generally, measurements were made at intervals  $\leq 0.1 \times$  expected  $t_{RG}$  until ~40% degelaton, and then  $\sim 0.02 \times$  expected  $t_{RG}$  until the gel was completely dissolved. The readings of absorbance in the solution versus time were plotted.

### B. Automated Assay

The cuvettes described above containing a magnetic stir bar (VWR 58949-030) were kept in a temperature controlled (37 °C) multi-sample compartment of a spectrophotometer fitted with a magnetic stirrer (2mag-USA, cuvette mix drive 1); the compartment also contained a cuvette with an immersed temperature probe (type T thermocouple) interfaced to the computer controlling the spectrophotometer. At regular intervals of  $\sim 2$  min, a reading of absorbance and temperature were made and plotted vs time.

## Abbreviations

HSE, hydroxysuccinimide ester; DEAC, 7-diethylaminocoumarin-3-carboxylic acid; Pfp, pentafluorophenyl ester; EDAC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; MTBE, methyl tertiary butyl ether; LOQ, limit of quantitation as defined as a signal  $>3$  times the standard deviation of the background noise; EtOAc, ethyl acetate; AcOH, acetic acid; AAF, 5-(aminoacetamido)fluorescein; DNP, 2,4-dinitrophenol.

## V. References

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