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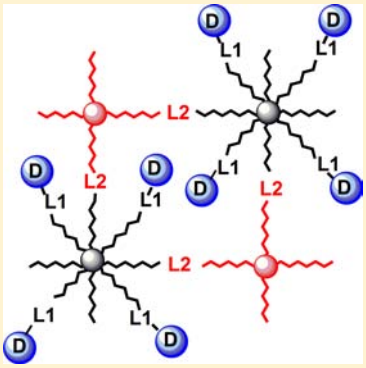
Biodegradable Tetra-PEG Hydrogels as Carriers for a Releasable Drug Delivery System

Jeff Henise, Brian R. Hearn, Gary W. Ashley, and Daniel V. Santi*

ProLynx, 455 Mission Bay Boulevard South, Suite 145, San Francisco, California 94158, United States

Supporting Information

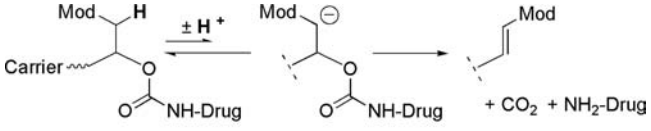
ABSTRACT: We have developed an approach to prepare drug-releasing Tetra-PEG hydrogels with exactly four cross-links per monomer. The gels contain two cleavable β-eliminative linkers: one for drug attachment that releases the drug at a predictable rate, and one with a longer half-life placed in each cross-link 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 β-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.[1,2] 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 β-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 β-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.[1] 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 ≤1 week in humans. Since we are interested in half-life extension to weeks or months, we developed a DDS using our β-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.[2]

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.[3−5] 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.

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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

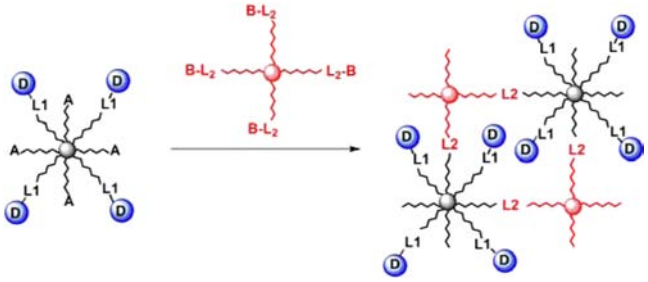

Figure 1. Formation of a hydrogel from an 8-arm PEG having four drugs **D** tethered via **L1** linkers and four connecting groups, **A**, by reaction with four **B** connecting groups of a 4-arm PEG containing cleavable cross-linkers **L2**.

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

In our first-generation hydrogel DDS,[2] an average of four of eight cyclooctyne (CO) end groups of $PEG_{40\ kDa}$-$(CO)_8$ were randomly tethered to a drug via linkers L1, Figure 2; the remaining CO end groups were reacted with 4-arm $PEG_{20\ kDa}$-$(NH\text{-}L2(Mod)\text{-}N_3)_4$ (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 $PEG_{20\ kDa}$-$(NH_2)_4$ with a $^{\alpha}N,^{\epsilon}N$-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, $PEG_{20\ kDa}$-$(NH\text{-}L2(Mod)\text{-}B)_4$, 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 $[A\text{-}Lys(X)\text{-}NH]_4$-$PEG_{20\ kDa}$ to give prepolymer A, and A + B to cross-link prepolymer A with a 4-armed prepolymer B, $PEG_{20\ kDa}$-$(NH\text{-}L2(Mod)\text{-}B)_4$. 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),[7] monofluorocyclooctyne (MFCO),[8] and aryl-less cyclooctyne (ALO),[9] which vary over a ~1000-fold range in reaction rates with aliphatic azides.[10] 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;[11] 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 dieneophile.[11] The rates of oxime formation can vary greatly with pH and the concentration of aniline catalyst used.[12,13]

Heterobifunctional Prepolymer Synthesis. While this work was in progress, we found that commercially available $PEG_{20\ kDa}$-$(NH_2)_4$ could contain up to ~15% impurities having 1- to 3 amine groups per PEG (SI Text I). 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 $PEG_{20\ kDa}$-$(NH_2)_{1-3}$ are anticipated to interfere with the polymer network of ideal Tetra-PEG gels; such impurities

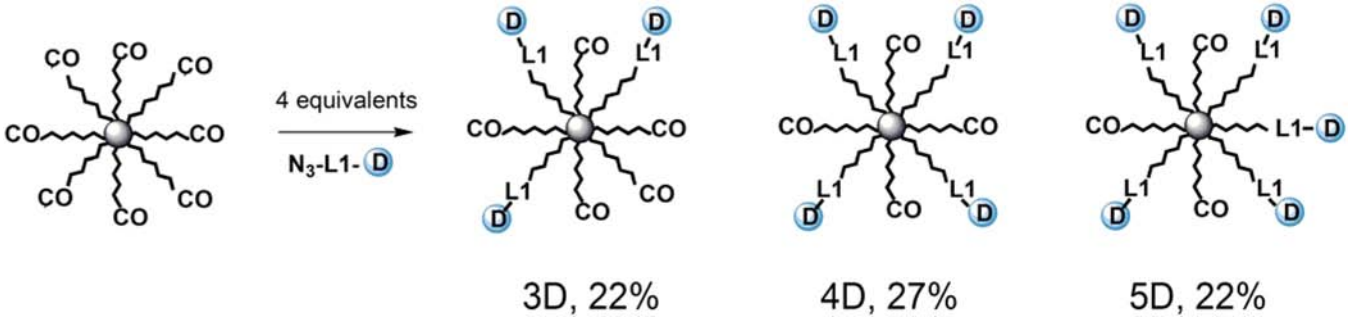

Figure 2. First generation random loading of a Tetra-PEG hydrogel precursor; only 27% of the product PEG prepolymers are expected to contain exactly 4 free CO groups.

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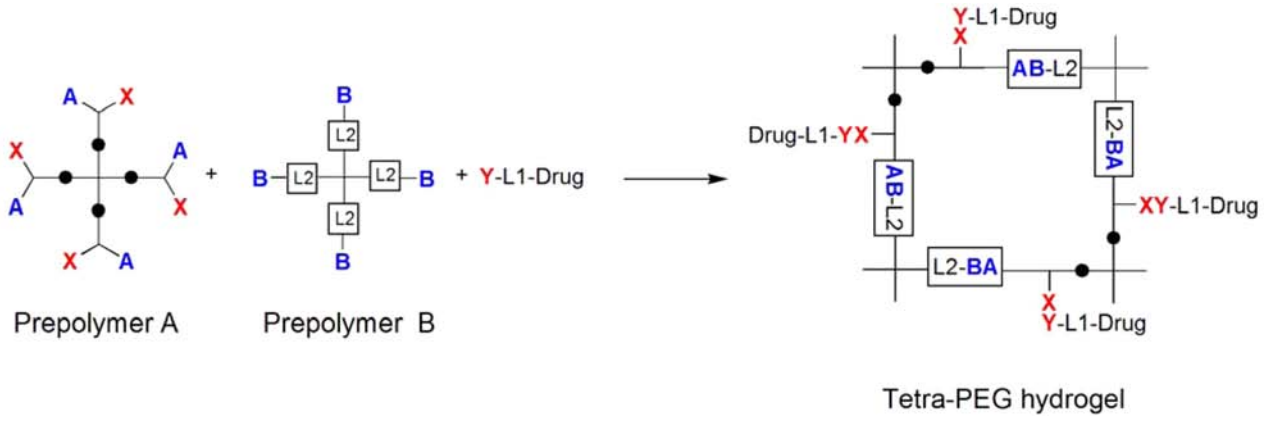

Figure 3. Second-generation hydrogel–drug conjugates. Prepolymer A contains four **X** end-groups that couple with **Y** for attachment of a drug having a cleavable linker, **L1**, and four **A** end-groups that react with **B** groups of prepolymer B to form a Tetra-PEG gel.

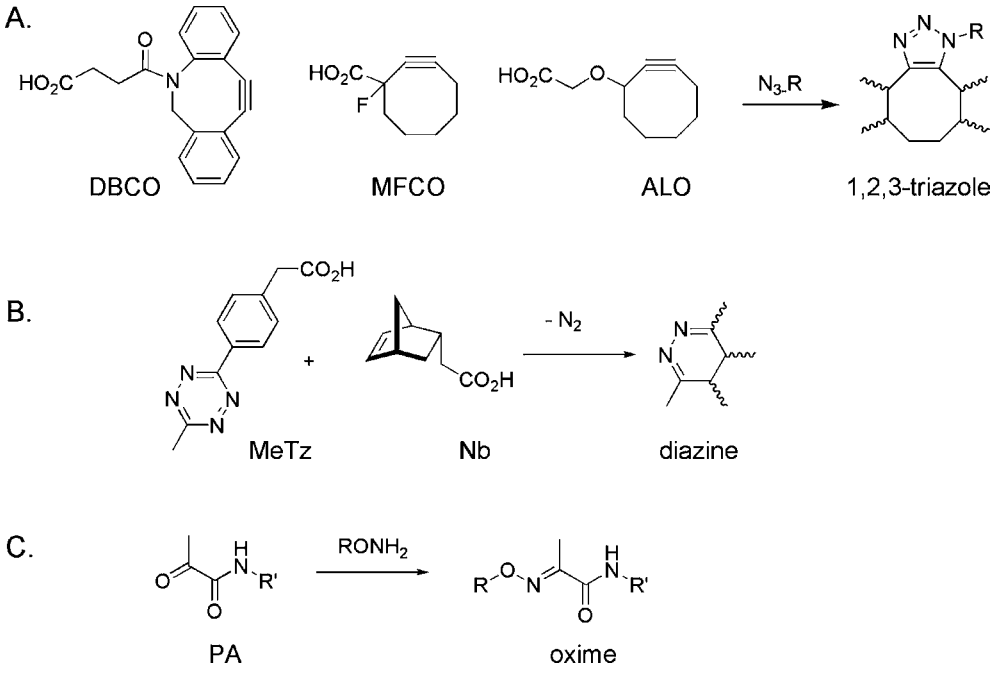

Figure 4. A. Cyclooctynes used in SPAAC reactions. B. Methyltetrazine (MeTz) and Norbornene (Nb) used in IEDDA. C. Alkoxyamine and pyruvamide (PA) used in oxime formation.

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[a]	R-N_3	triazole	2.0[b]	1.4
MFCO[a]	R-N_3	triazole	0.25	18
ALO[a]	R-N_3	triazole	0.010	560
Nb	MeTz	diazine	0.091[c]	60
$RONH_2$	pyruvamide	oxime	[d]	
$RONH_2$	–NHCOPhCH=O	oxime	[d]	7[e]

[a]For aliphatic azides in water; reported rates in MeCN are about 10-fold slower. [b]Excellent agreement with reported rate of 2.1 M^{-1} s^{-1} in water;[14] rates with DBCO are consistently ~5-fold faster (~10 M^{-1} s^{-1}) when the azide contained DEAC. [c]The rate can be modified by using alternative tetrazines;[11] however, faster reacting tetrazines are inconveniently less stable. [d]Dependent on pH and catalyst. [e]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]$_4$-PEG$_{20\ kDa}$, **7** (Scheme 2). PEG$_{20\ kDa}$-(NH$_2$)$_4$ 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]$_4$-PEG$_{20\ kDa}$, **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]$_4$-PEG$_{20\ kDa}$ **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]$_4$-PEG$_{20\ kDa}$, **7**. The intermediate [H-Lys-(DBCO)-NH]$_4$-PEG$_{20\ kDa}$ 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

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Scheme 2

PEG_{20KDa}-$(NH_2)_4$ ⟶ [Fmoc-Lys(Boc)-NH]$_4$-PEG_{20kDa} (**4**) ⟶ [Fmoc-Lys(H)-NH]$_4$-PEG_{20kDa} (**5**)

[Fmoc-Lys(DBCO)-NH]$_4$-PEG_{20kDa} (**6**) ⟶ [H-Lys(DBCO)-NH]$_4$-PEG_{20kDa} (**7**) ⟶ [R-Lys(DBCO)-NH]$_4$-PEG_{20kDa} (**8**)

Scheme 3

PEG_{20kDa}-$(NH_2)_4$ ⟶ PEG_{20kDa}-[NH-L2(Mod)-N_3]$_4$ (**9**)

PEG_{20kDa}-[NH-L2(Mod)-NHBoc]$_4$ (**10**) ⟶ PEG_{20kDa}-[NH-L2(Mod)-NH_2]$_4$ (**11**) ⟶ PEG_{20kDa}-[NH-L2(Mod)-R]$_4$ (**12**)

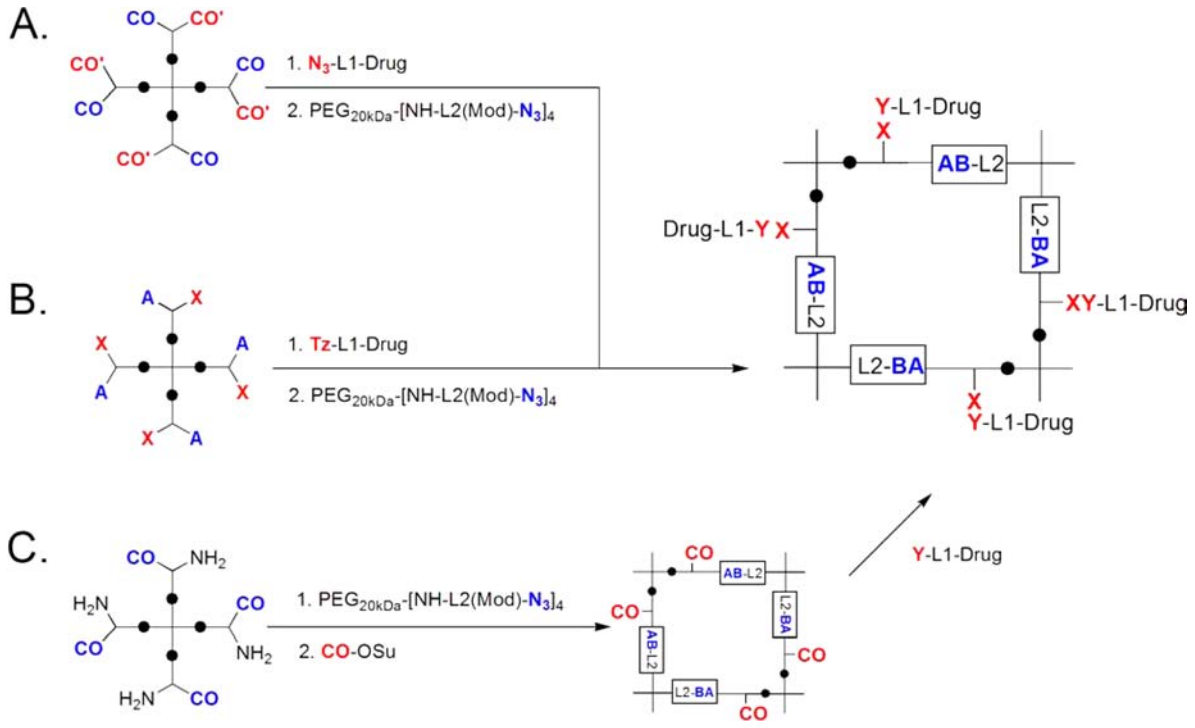

Figure 5. Different formats for Tetra-PEG hydrogel DDSs. A. Analogous connecting groups (CO and CO′) with different reaction rates. B. Two completely orthogonal connecting groups (A and X). C Amino-hydrogel formation, followed by activation with a connecting group, CO, then loading of the preformed gel with a releasable drug.

prepolymers, **8**, poised for connection with Y-L1-Drug and for polymerization with $PEG_{20\ kDa}$-(NH-L2(Mod)-B)$_4$. Interestingly, in most of these reactions detection of intermediates proceeding to product could be followed by HPLC. After the aforementioned acylations, reactions were treated with 20 mM Ac_2O or Ac-HSE to cap residual free amines and prevent subsequent reactions.

Prepolymer B. Starting with $PEG_{20\ kDa}$-$(NH_2)_4$ and N_3-L2-OCO_2Su, we prepared prepolymers $PEG_{20\ kDa}$-[NH-L2(Mod)-N_3]$_4$ (**9**) as described,[2] and shown in Scheme 3. Alternatively (Scheme 3), PEG_{20KDa}-$(NH_2)_4$ was acylated with BocNH-L2-OCO_2Su to give $PEG_{20\ kDa}$-[NH-L2(Mod)-NHBoc]$_4$, **10**. This intermediate was unblocked to give the amino-prepolymer, $PEG_{20\ kDa}$-[NH-L2(Mod)-NH_2]$_4$, **11**, a key common precursor for acylation by any of the bioorthogonal coupling groups used here to give **12**. In the present work, we used the $PhSO_2$-modulator[1] for most L2 linkers so we could compare the effects of other differences in the polymers on cross-link cleavage in gels formed with these prepolymers.

Formation of Hydrogels. In typical preparations, solutions of each of the two prepolymers were mixed in equal molar equivalents along with sufficient buffer (pH 5 to 7.4) to give a 5% w/v solution and placed in 9 × 1 mm^2 disk molds to polymerize.[2] Generally, such gels had the capacity to link 5 μmol drug/mL gel. Table 1 shows the gel times of hydrogel formation using various cross-link connection chemistry. Using DBCO and azide connecting groups, we could continuously monitor the disappearance of DBCO absorbance concomitant with gel formation (SI Text II). The calculated second-order rate constants for initial gel formation were similar to those of solution-phase reactions, and the time required for ∼50% DBCO consumption was closely related to the observed gelation time.

Formats for Drug-Bound Tetra-PEG Hydrogel Formation. We developed three general formats to prepare

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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.[15] 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]$_4$-PEG$_{20\ kDa}$ 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_3-L1-drug, and the remaining CO groups were used for polymerization with prepolymer B, PEG$_{20\ kDa}$-(NH-L2(Mod)-N$_3$)$_4$. 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]$_4$-PEG$_{20\ kDa}$ would be consumed, and 98.5% would react using [ALO-Lys(DBCO)-NH]$_4$-PEG$_{20\ kDa}$. 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_3) (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$_{20\ kDa}$-(NH-DBCO)$_4$ or [Nb-Lys(DBCO)-NH]$_4$-PEG$_{20\ kDa}$ was titrated with 4 equiv of azide, exactly 1 mol of DBCO was consumed per mole of azide. Likewise, for [ALO-Lys(DBCO)-NH]$_4$-PEG$_{20\ kDa}$ ≥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]$_4$-PEG$_{20\ kDa}$, 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 $^{\alpha}$N-azido-L1[1] to the DBCO end-groups of [MFCO-Lys(DBCO)-NH]$_4$-PEG$_{20\ kDa}$. After polymerization with a 4-arm azido-PEG$_{20\ kDa}$ prepolymer B, the hydrogel had 4.0 μmol (17 mg) of tethered peptide/mL gel and released the drug with the expected cleavage rate.

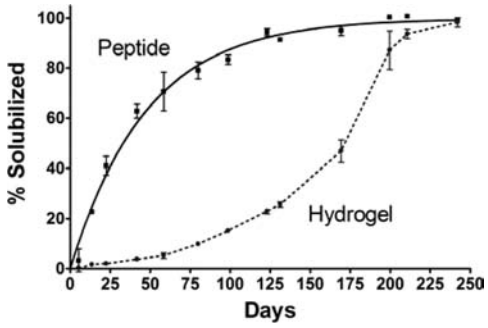


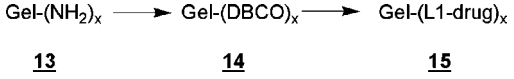
Figure 6. Release of exenatide from a hydrogel formed from [MFCO-Lys(DBCO)-NH]$_4$-PEG$_{20\ kDa}$ prepolymer A and PEG$_{20\ kDa}$-[NH-L2(CN)-N$_3$]$_4$ prepolymer B. Peptide release and degelation were recorded at pH 9.4 and extrapolated to pH 7.4 using $t_{RG,pH7.4} = t_{RG,pH9.4}(10^{pH9.4\text{-}pH7.4})$ (1,2); peptide (−■−) and hydrogel solubilization (−●−).

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]$_4$-PEG$_{20\ kDa}$ contains four Lys groups in which one amine connects a DBCO for SPAAC while the other contains the orthogonal Nb group for IEDDA.[11] Karver et al.[14] 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,[16] and we have also examined that approach in this study. The IEDDA reaction produces N_2 which we have observed to cause trapped bubbles in the gel when used for polymerization. The gel bubbles can be circumvented by polymerizating 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]$_4$-PEG$_{20\ kDa}$ followed by polymerization with a PEG$_{20\ kDa}$-[NH-L2(Mod)-N$_3$]$_4$ 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]$_4$-PEG$_{20\ kDa}$ prepolymer was polymerized with PEG$_{20\ kDa}$-[NH-L2(Mod)-N$_3$]$_4$ 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_3-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.

Scheme 4



Using this format, we attached 22 kDa N_3-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]$_4$-PEG$_{20\ kDa}$ and PEG$_{20\ kDa}$-[NH-L2(Mod)-N$_3$]$_4$ 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-aminooxyalkyl 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.[2]

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Table 2. Degradation of Hydrogels Prepared with β-Eliminative Linkers Containing the $PhSO_2$-pK_a Modulator in Cross-Links

entry	prepolymer A	cap/erosion probe[a]	prepolymer B	cross-link	t_{RG}[d] (h)	AUC $0.7t_{RG}$
1	$[Nb\text{-}Lys(DBCO)\text{-}NH]_4\text{-}PEG_{20\ kDa}$	none	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$DBCO/N_3$	96 ± 6.7	4.6 ± 0.3
2	$[MFCO\text{-}Lys(DBCO)\text{-}NH]4\text{-}PEG_{20\ kDa}$	none	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$MFCO/N_3$	100 ± 2.8	5.2 ± 0.3
3	$PEG_{20\ kDa}\text{-}(NH\text{-}DBCO)_4$	none	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$DBCO/N_3$	76.0 ± 3.0	4.9 ± 0.6
4	$PEG_{40\ kDa}\text{-}(NH\text{-}DBCO)_8$	$PEG7\text{-}N_3$	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$DBCO/N_3$	85.5 ± 3.5	6.5 ± 0.9
5	$PEG_{40\ kDa}\text{-}(NH\text{-}DBCO)_8$	$PEG7\text{-}N_3$[b]	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$DBCO/N_3$	70 ± 4.0	7.6 ± 0.5
6	$[MFCO\text{-}Lys(DBCO)\text{-}NH]_4\text{-}PEG_{20\ kDa}$	$PEG7\text{-}N_3/DEAC\text{-}N_3$[c]	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$MFCO/N_3$	163 ± 5.3	4.1 ± 0.1
7	$[Nb\text{-}Lys(DBCO)\text{-}NH]_4\text{-}PEG_{20\ kDa}$	$PEG7\text{-}N_3/DEAC\text{-}N_3$[c]	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}MeTz)_4$	Nb/Tz	174 ± 7.2	4.0 ± 0.3
8	$[Nb\text{-}Lys(DBCO)\text{-}NH]_4\text{-}PEG_{20\ kDa}$	$MeTz\text{-}COOH/DEAC\text{-}MeTz$[c]	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$	$DBCO/N_3$	216 ± 26	3.3 ± 0.5
9	$PEG_{40\ kDa}\text{-}(NH\text{-}DBCO)_8$	$PEG7\text{-}N_3/DEAC\text{-}N_3$[c]	$PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhS0_2)\text{-}N_{3)4}$	$DBCO/N_3$	118 ± 8	5.1 ± 0.5

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

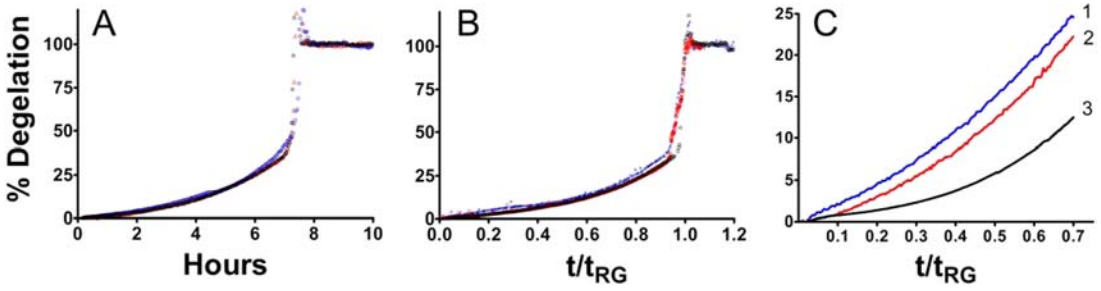

Figure 7. Degelation curves of hydrogels with the $PhSO_2$-modulator in cleavable cross-links at pH 8.4, 37 °C. A. Reproducibility of the degelation assay using a gel made from $PEG_{20\ kDa}\text{-}(NH\text{-}DBCO)_4$ and $PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$ assayed on three different 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 $AUC_{0.7}$ curves for a structured gel, C3, (Table 2, entry 8) and randomly formed gels prepared from $PEG_{40\ kDa}\text{-}(NH\text{-}DBCO)_8$ and $PEG_{20\ kDa}\text{-}(NH\text{-}L2(PhSO_2)\text{-}N_3)_4$ 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 $PEG7\text{-}N_3$, and stability of the triazole product was monitored by HPLC at A_{254}. At either pH 5.4 (4 °C) or pH 7.4 (4 and 37 °C), ≤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, ≤5% cleavage occurred indicating that $t_{1/2}$ was ≤1 yr. At pH 7.4, 37 °C, 8% cleavage was observed at 37 days, indicating a $t_{1/2}$ of ∼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.[12] However, if an aldehyde or ketone is included as a trap for the liberated alkoxyamine, oxime hydrolysis is in effect irreversible.[17] 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 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.[2] 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 (ε_{261} 6600 M^{-1} cm^{-1}) 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

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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 $PEG_{20\ kDa}$-(NH-L2($PhSO_2$)-N_3)$_4$ and $PEG_{20\ kDa}$-(NH-DBCO)$_4$ (Table 2, entry 3), the intrarun variation of the reverse gelation time (t_{RG}) was ±6% SD (n = 4), and interday variation (Figure 7A) was ±2% SD (n = 3).

Using the automated method for gel degradation analysis, we monitored degradation of different gels containing the same $PhSO_2$-modulator in the cleavable cross-linker,[2] 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} ($AUC_{0.7}$). After that point, release of large fragments compromised reproducibility. As shown in Table 2, the $AUC_{0.7}$ for the gels developed in the present study are quite similar at ~3.3% to 5.2% × t (entries 1, 2, and 6−8). We also prepared randomly formed gels by first capping 8-arm $PEG_{40\ kDa}$-(NH-DBCO)$_8$ with 4 equiv PEG7-N_3 followed by polymerization with the $PEG_{20\ kDa}$-(NH-L2($PhSO_2$)-N_3)$_4$ (Table 2, entry 4), or by polymerizing 8-arm $PEG_{40\ kDa}$-(DBCO)$_8$ with 1.0 equiv of $PEG_{20\ kDa}$(NH-L2($PhSO_2$)-N_3)$_4$ followed by capping with PEG7-N_3 (Table 2, entry 5). The $AUC_{0.7}$ 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 ~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 ~30% (Table 2, entries 1, 2 vs 3), and with two other modulators (−CN, O(CH_2CH_2)$_2$$NSO_2$−) in the cross-linker, a tethered exenatide increased the t_{RG} by ~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_a of the acidic C−H bond required for β-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_2 bubbles. The t_{RG} of the gel with bubbles was ~8% longer than those without, and $AUC_{0.7}$ was decreased 15%. Compared to SPACC cross-linked gels, the MeTz-Nb cross-linked gels showed both a slightly reduced t_{RG} and $AUC_{0.7}$.

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_{free}/D_{ttl}) released from the intact hydrogel or gel fragments is given by eq 1

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

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.

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

This shows that D_{free}/D_{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_{free}/D_{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_{free}/D_{ttl} can be empirically adjusted by modifying the t_{RG}.

DISCUSSION

We recently described the use of cleavable β-eliminative linkers both to attach drugs to hydrogels for predictable drug release, and to cross-link the polymers for tunable control of biodegradation rates.[2] Our drug-releasing gels were initially prepared by randomly attaching 4 equiv of linker-drug to an 8-arm $PEG_{40\ kDa}$, and using the remaining arms to cross-link the gel to a 4-arm $PEG_{20\ kDa}$. 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 $PEG_{20\ kDa}$-$(NH_2)_4$ 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 $PEG_{20\ kDa}$-$(NH_2)_4$, 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.

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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 aminooxy-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[18]—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 $PEG_{20\ kDa}$ 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.[2] Gels cast at 5% PEG have a capacity of 5 μmol 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 μmol/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 ($D_{free}/D_{ttl} = 1-0.5^{t_{RG}/t_{1/2,LI}}$). 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 t1/2 of cross-link-cleavage is ≥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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: Daniel.V.Santi@prolynxllc.com. Phone: 415 552 5306.

Notes

The authors declare no competing financial interest.

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■ 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, $[Fmoc\text{-}Lys(Boc)\text{-}NH]_4\text{-}PEG_{20\ kDa}$ repre-

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sents a four-armed tetra-amino-PEG with each or its arms connected to the C-terminus of Lys containing an α-Fmoc and ε-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, $PEG_{20\ kDa}$-[NH-L2(Mod)-N_3]$_4$ is a 20 kDa 4-arm amino PEG containing an azido-alkyl linker, L2, activated by a pK_a 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 $PEG_{20\ kDa}$-$(NH_2)_4$ (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 $PEG_{20\ kDa}$-(NH-DBCO)$_4$ and $PEG_{20\ kDa}$-[NH-L2(MePhSO2)-N_3]$_4$. After precipitation with MTBE HPLC analysis (308 nm) showed 11% tri-substituted in the unpurified sample, and 3.3% in the purified $PEG_{20\ kDa}$-(NH-DBCO)$_4$. Hydrogels were prepared from the purified and unpurified prepolymers. Degradation analysis at pH 9.3, 37 °C, showed similar $AUC_{0.7}$ values, but a 9% increase in t_{RG} (143 vs 157 min at pH 9.4).

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