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Facile Synthesis of Rapidly Degrading PEG-Based Thiol-Norbornene Hydrogels

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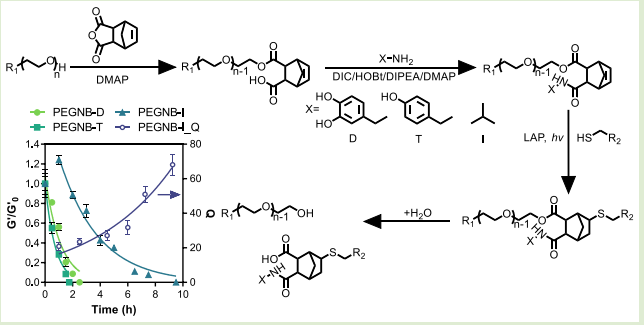
ABSTRACT: An alternate synthesis route was developed to prepare norbornene-functionalized poly(ethylene glycol) (PEG) from reacting multiarm PEG with carbic anhydride. The macromer, $PEGNB_{CA}$, permits photo-cross-linking of thiol-norbornene hydrogels with kinetics comparable to conventional PEGNB macromer. In addition, $PEGNB_{CA}$ provides an additional carboxylate group for further conjugation with amine-bearing molecules. Interestingly, $PEGNB_{CA}$ thiol-norbornene hydrogels are highly susceptible to hydrolytic degradation through enhanced ester hydrolysis. The ester linkage is further weakened after the secondary conjugation, resulting in extremely rapid degradation of PEGNB hydrogels. More importantly, the degradation can be readily adjusted via tuning macromer compositions, with complete degradation time ranging from hours to weeks. The $PEGNB_{CA}$ hydrogels are also highly cytocompatible toward various cell types, providing opportunities for future applications in tissue engineering and advanced biofabrication.



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Poly(ethylene glycol) (PEG)-based hydrogels are widely used in tissue engineering and biofabrication for their excellent biocompatibility,[1] adaptable cross-linking mechanisms, and readily tunable physiochemical properties. PEG-based hydrogels are particularly desirable in tissue regeneration,[2,3] controlled release,[4] and biosensor applications.[5] Conjugation of functional groups (e.g., acrylate, norbornene, vinylsulfone, maleimide) on the termini of PEG chains is necessary to permit their cross-linking into hydrogels. On the other hand, labile motifs (e.g., poly(lactic acid), PLA)[6] are routinely copolymerized with functionalized PEG to render the otherwise stable network hydrolytically labile. Alternatively, hydrolytic degradation of PEG-based hydrogels may be engineered by synthesizing ester-containing cross-linkers with different hydrolytic susceptibility, such as groups with steric hindrance, local hydrophobicity, and electron-withdrawing moieties.[3,7]

Among the various cross-linking chemistries, PEG-based hydrogels fabricated by orthogonal thiol-norbornene photopolymerization are increasingly used in drug delivery and tissue engineering applications.[8] In particular, multiarm PEG-norbornene (PEGNB) can be cross-linked into an idealized network using a multifunctional thiol cross-linker through a radical-medicated thiol-norbornene reaction initiated by ultraviolet light,[9−12] visible light,[13,14] or enzymatic reaction.[15] We and others have prepared biomimetic PEG-based thiol-norbornene hydrogels for a variety of biomedical applications.[16−20] In all prior studies, utilizing PEGNB macromers, NB group functionalization was typically achieved between 5-norbornene-2-carboxylic acid (NB-acid) and either PEG hydroxyls (i.e., esterification) or PEG amines (i.e., carbodiimide chemistry).[21] The former reaction produces ester linkages (i.e., PEGeNB), whereas the later yields stable amide bonds (i.e., PEGaNB). Compared with hydrogels cross-linked by PEGaNB, the more hydrolytically labile PEGeNB (referring to PEGNB thereafter) hydrogels improved survival, proliferation, and spreading of human mesenchymal stem cells (hMSCs).

To functionalize hydrolytically labile PEGNB (Figure 1A, X = H) through Steglich esterification,[22] the current synthesis requires that NB-acid being activated with *N*,*N*′-dicyclohexylcarbodiimide (DCC) to form the *O*-acylisourea intermediate (Figure 1A-I). The intermediate is filtered into PEG-hydroxyls in the presence of pyridine and 4-dimethylaminopyridine (DMAP),[9] followed by an overnight reaction and precipitation in cold ether. To maximize the degree of substitution (DS), multiple efforts were developed, such as extended refluxing PEG in toluene to remove excess bond water, blanketing the reaction with inert gas, conducting back-to-back reactions, and limiting the reaction to smaller batches (<5 g). Unfortunately,

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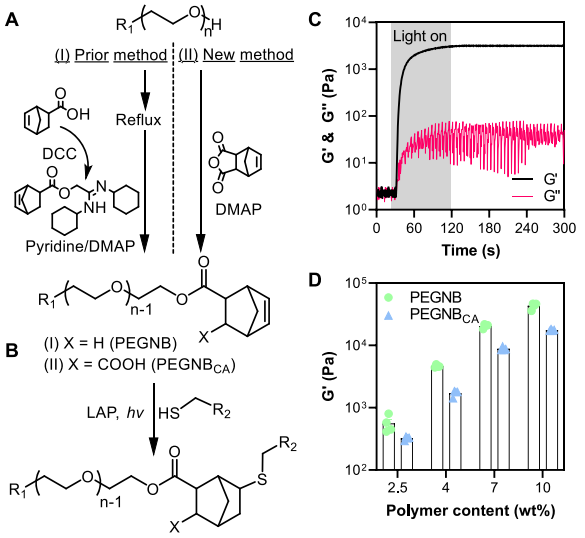


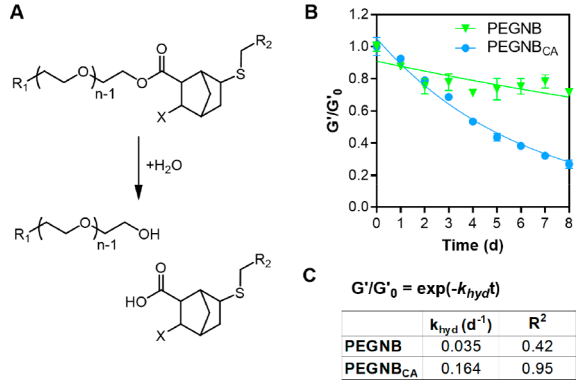
Figure 1. Synthesis and characterization of PEGNB$_{CA}$ hydrogels. (A) Reaction and purification routes for the synthesis of norbornene-functionalized PEG. I: X = H (PEGNB); II: X = COOH (PEGNB$_{CA}$). (B) Light and radical initiated thiol-norbornene gelation by lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). (C) In situ photorheometry of 2.5 wt % PEGNB$_{CA}$ with DTT (R = 1). (D) Shear moduli of hydrogels cross-linked by PEGNB or PEGNB$_{CA}$ with DTT. PI: 1 mM LAP, 2 min 365 nm light exposure (5 mW/cm^2).

none of these lengthy and laborious steps (~5 days) eliminates the pungent and extremely unpleasant odor of NB-acid. Stringent containment efforts are taken during and post-synthesis to minimize the unpleasant order of NB-acid. While PEGNB macromers are commercially available, they are pricy and, hence, not a sustainable option. These disadvantages significantly limit the widespread usage of this powerful and diverse macromer.

We sought to circumvent the cumbersome steps in the current PEGNB synthesis by adopting an alternative reaction protocol using carbic anhydride (CA), an odorless norbornene derivative containing diacid anhydride group (Figure 1A-II). CA is chosen as we have previously used it to synthesize gelatin-modified norbornene (e.g., GelNB).[23,24] In this work, we established a user-friendly CA-based synthesis protocol for introducing norbornene to multiarm PEG-hydroxyls. The reaction was catalyzed by DMAP[25] in tetrahydrofuran (THF) (Figure 1A-II), affording a macromer PEGNB$_{CA}$ (Figure 1A, X = COOH). Importantly, no odor containment effort was needed for the PEGNB$_{CA}$ synthesis, and the method shortened the overall synthesis time from 5 days to 2 days. We verified high NB substitution on PEGNB$_{CA}$ via quantifying thiol consumption using thiol-norbornene reaction with cysteine (Cys; Figure S1). Moreover, the PEGNB$_{CA}$ macromer contains an additional carboxylic acid moiety, which may be exploited for subsequent conjugation of functional groups via standard carbodiimide chemistry.

We tested thiol-norbornene photogelation (Figure 1B) using the PEGNB$_{CA}$ and dithiothreitol (DTT) at a stoichiometric ratio (i.e., $R_{thiol/ene}$ = 1). The gelation kinetics as evaluated by in situ photorheometry revealed a rapid gel point (~10 s) typical to the UV-initiated thiol-norbornene gelation (Figure 1C). However, compared with hydrogels cross-linked by conventional PEGNB, the shear moduli (G') of the hydrogels cross-linked by PEGNB$_{CA}$ were consistently lower at equivalent polymer contents (Figure 1D), suggesting a lower reaction efficiency of PEGNB$_{CA}$ toward thiols. The reduced thiol-norbornene reaction efficiency of PEGNB$_{CA}$ may be a result from the additional carboxylic acid group that reduces the local pH value. This is consistent with a recent report by Colak et al., where it was demonstrated that thiol-norbornene reaction efficiency was lower with a carbic anhydride derivative functionalized with hexane-1,6-diamine.[26] Nonetheless, this protocol offers a greener and more user-friendly route to the synthesis of norbornene-functionalized PEG for orthogonal thiol-norbornene hydrogel cross-linking.

Ester-linked PEGNB hydrogels are known to undergo slow and predictable hydrolytic degradation (Figure 2A).[9] Indeed, 4



	k_{hyd} (d^{-1})	R^2
PEGNB	0.035	0.42
PEGNB$_{CA}$	0.164	0.95

Figure 2. Hydrolytic degradation of PEGNB/PEGNB$_{CA}$ hydrogels. (A) Schematic of ester bond hydrolysis. (B) Characterization of hydrogel shear moduli changes (i.e., G'/G'_0) as a function of time. (C) Parameters of pseudo-first order hydrolytic degradation kinetics.

wt % of PEGNB-DTT hydrogels degraded partially (G'/G'_0 ~ 25%) after 8 days of incubation (Figure 2B), a result in agreement with our earlier work.[9] To our surprise, however, hydrogels cross-linked by the PEGNB$_{CA}$ degraded much faster than the PEGNB counterpart (Figures 2B and S3). After further analysis of the hydrolytic degradation kinetics with a pseudo-first order degradation kinetics (G'/G'_0 = exp($-k_{hyd}t$)),[9] we obtained a hydrolysis kinetic rate constant (k_{hyd}) of 0.035 d^{-1} and 0.164 d^{-1} for PEGNB and PEGNB$_{CA}$ gels, respectively (Figure 2C). As the conventional thiol-norbornene hydrogels did not degrade sufficiently in the first few days, the fitting for PEGNB gel was not ideal (R^2 = 0.42). However, the k_{hyd} value (0.035 d^{-1}) was within the range of previous results with longer term degradation (i.e., k_{hyd} = 0.02−0.07 d^{-1}).[9] The R^2 value for curve fitting of PEGNB$_{CA}$ degradation, on the other hand, reached over 0.95, indicating that the degradation of PEGNB$_{CA}$ gels was a result of ester bond hydrolysis.[27] Compared with PEGNB-DTT hydrogels, PEGNB$_{CA}$-DTT hydrogels degraded much faster and reached complete degradation within 2 weeks (data not shown). In addition to the moduli change, the degradation was also tracked by the swelling ratio (Q; Figure S3). In particular, the swelling ratio of PEGNB-DTT and PEGNB$_{CA}$-DTT hydrogels was comparable (Q ~ 30) on day 1. However, the swelling ratio of PEGNB$_{CA}$-DTT hydrogels continuously increased while that of PEGNB-DTT hydrogels remained relatively unchanged during the 8 days incubation course. The accelerated hydrolysis of PEGNB$_{CA}$ gels was likely due to the presence of an additional carboxylic acid group that destabilizes the ester bonds. The faster degradation kinetics of

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PEGNB$_{CA}$ hydrogels present opportunities for future applications when controllable hydrogel degradation is desired.

PEG-based thiol-norbornene hydrogels are known for their high cytocompatibility for in situ cell encapsulation.[8,12,16,28] The PEGNB$_{CA}$ hydrogels also showed excellent cytocompatibility, as exemplified by the encapsulation of human induced pluripotent stem cells (hiPSC), dental pulp stem cells (DPSC), and PANC-1 human pancreatic cancer cells (Figure 3). All

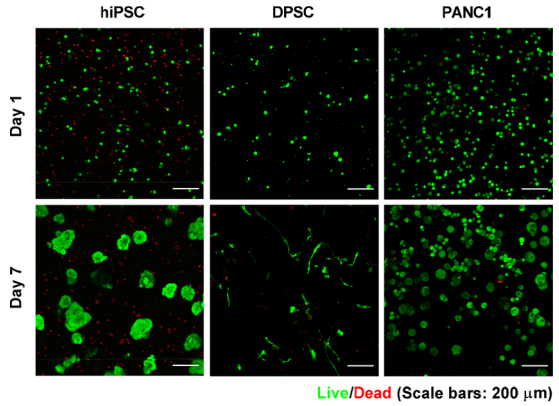
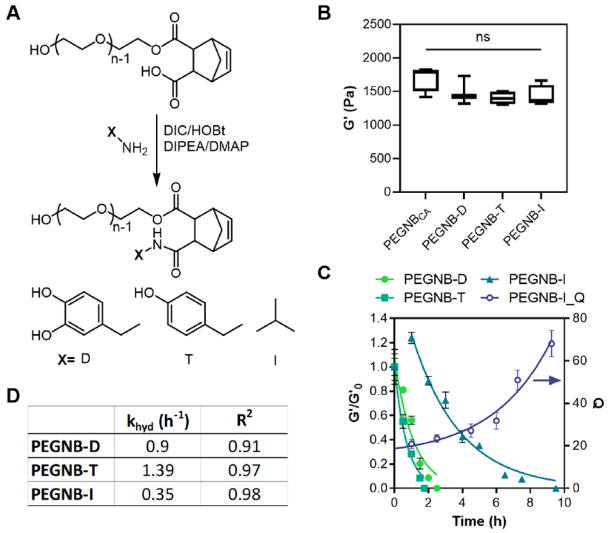


Figure 3. Live/dead staining and confocal imaging of cell-laden PEGNB$_{CA}$-peptide hydrogels.

hydrogels were cross-linked by matrix metalloproteinase (MMP)-sensitive peptide linker (i.e., KCGPQG*IWGQCK, *cleavage site) to permit cell-mediated matrix remodeling and immobilized with 1 mM RGDS peptide to support cell survival. Live/Dead staining results showed that very limited number of dead cells were visible for all cell types following encapsulation. After 7 days of in vitro culture, hiPSC and PANC-1 cells proliferated to form multicellular spheroids, while DPSC exhibited spreading morphology, indicating extensive cell-mediated matrix cleavage. These results have established PEGNB$_{CA}$ hydrogels as an attractive alternative material for in situ encapsulation of a variety of cells.

In addition to demonstrating an improvement in synthesis procedures and high cytocompatibility, the PEGNB$_{CA}$ affords a carboxylic acid moiety amenable for secondary conjugation with amine-bearing molecules (Figure 4A, PEGNB-X). The conversion of these X conjugates (e.g., L-dopamine (D), tyramine (T), or isopropylamine (I), Figure 4A) reached 83–100% depending on the reactants, as calculated from ^{1}H NMR spectra (Figure S2), using a protocol modified from Zhang et al.[29] These demonstrated X conjugates did not affect cross-linking efficiency of PEGNB-X-DTT hydrogels, as demonstrated by comparable G'_0 to that of the PEGNB$_{CA}$-DTT hydrogels (Figure 4B).

We next asked if the secondary conjugation would affect the hydrolytic degradation of the hydrogels. Surprisingly, the ester linkage is further weakened by the second conjugation. After overnight swelling at 37 °C, all PEGNB-X-DTT hydrogels completely degraded, suggesting that the secondary conjugation substantially decreased stability of the ester bonds. In a previous study, Fairbanks et al. reported a similar norbornene functionalized macromer (mPEG5KNorbA).[30] The additional carboxylic group was further reacted with 5-norbornene-2-methylamine, yielding two norbornene groups for forming miktoarm star copolymers. However, the study did not explore the application of this macromer for hydrogel fabrication, nor did it examine hydrolysis kinetics of the ester linkages.



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	k_{hyd} (h^{-1})	R^2
PEGNB-D	0.9	0.91
PEGNB-T	1.39	0.97
PEGNB-I	0.35	0.98

Figure 4. Cross-linking and degradation of PEGNB, PEGNB$_{CA}$, and PEGNB-X hydrogels. (A) Schematic of PEGNB-X synthesis. (B) Initial shear moduli of hydrogels cross-linked by 4 wt % PEGNB$_{CA}$, PEGNB-D, PEGNB-T, and PEGNB-I with DTT. (C) Degradation of PEGNB-X hydrogels at 37 °C by shear moduli changes (G'/G'_0) and swelling ratio (Q). Curve fittings represent pseudo first order ester hydrolysis kinetics. (D) Parameters of pseudo-first order hydrolytic degradation kinetics. All hydrogels were cross-linked by DTT (R = 1) with 1 mM LAP and 2 min 365 nm light exposure (5 mW/cm^2).

Nevertheless, PEGNB-Xs and PEGNB-X hydrogels behaved quite differently in aqueous environment. We determined the degradation rate of PEGNB-D, PEGNB-T, and PEGNB-I hydrogels (Figure 4C). At 37 °C, PEGNB-D and PEGNB-T hydrogels fully degraded within 2 h, while PEGNB-I hydrogels degraded in about 10 h (Figure 4C). It is worth noting that the three sets of gels were cross-linked to have similar initial shear moduli (G'_0 ~ 1.5 kPa, Figure 4B). Hydrogels cross-linked by PEGNB-D and PEGNB-T degraded extremely fast, with k_{hyd} = 0.9 and 1.39 h^{-1}, respectively (Figure 4D). These represented ~50- to ~200-fold faster degradation rate than that with PEGNB$_{CA}$ hydrogels (Figure 2C. k_{hyd} = 0.164 d^{-1} or 0.0068 h^{-1}). Interestingly, compared with the T or D conjugate, I conjugation slowed gel degradation considerably (Figure 4C,D, k_{hyd} = 0.35 h^{-1}). Note that the first data point of PEGNB-I was neglected in the exponential fitting, since G' slightly increased in the first hour of incubation, potentially due to intermolecular hydrophobic interactions between isopropyl moieties. Therefore, we measured the swelling ratio (Q) of these hydrogels to gain insight into their degradation behavior. There was limited increase in swelling ratio in the first 2 h, after which the swelling increased substantially (Figure 4C). The limited initial swelling and increase in the initial G' could be attributed to the hydrophobic interactions between isopropyl groups. Nonetheless, the degradation of PEGNB-I hydrogels could still be described by a pseudo-first order degradation kinetics (R^2 = 0.98. Figure 4D). Furthermore, the hydrolytic degradation of PEGNB-X hydrogels appeared to be temperature-dependent and the degradation rates were reduced at room temperature (Figure S4).

Besides L-dopamine, tyramine, and isopropylamine, we also conjugated longer aliphatic amines, such as hexylamine and stearamine, as well as aromatic benzylamine to PEGNB$_{CA}$. However, the conjugation of these long or bulky hydrophobic amines were not as successful and the norbornene concen-

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tration was drastically decreased after switching the dialysis solvent from methanol to water (data not shown). As a result, hydrogel cross-linking using PEGNB-hexylamine, PEGNB-stearamine, and PEGNB-benzylamine was not feasible since the norbornene moiety on these PEGNB-X was not sufficiently high (data not shown).

The facile cross-linking and rapid degradation of the PEGNB-X hydrogels offer an opportunity to tune hydrogel degradation precisely by cross-linking with different cross-linkers and mixing PEGNB-X with $PEGNB_{CA}$ while fixing the total PEGNB content (Figure 5). It is well-known that thiol−

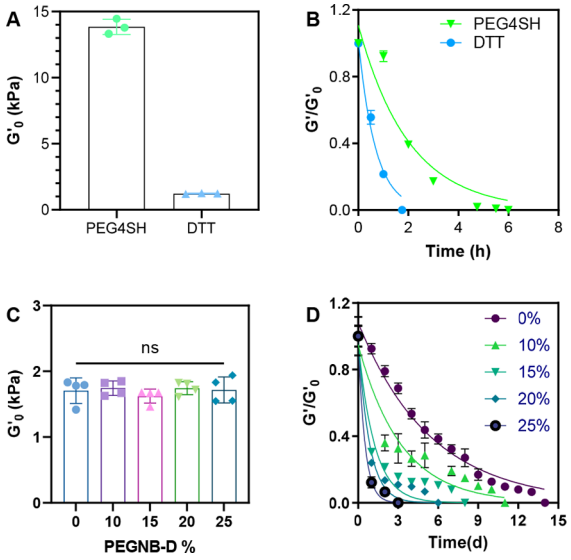

Figure 5. Controlling degradation of PEGNB hydrogels via cross-linking with different cross-linkers and mixing in PEGNB-D. (A) Initial shear moduli and degradation (B) of 4 wt % PEGNB-D cross-linked with DTT and 4 arms-thiolated PEG (PEG4SH). (C) Initial shear moduli and (D) degradation of soft (4 wt %) $PEGNB_{CA}$/PEGNB-D-DTT hydrogels with different PEGNB-D content. Curve fittings represent pseudo first order ester hydrolysis kinetics and listed in Tables S3 and S4. Hydrogels were cross-linked with either DTT (R = 1) or PEG4SH (R = 1) with 1 mM LAP and 2 min 365 nm light exposure (5 mW/cm^2).

ene hydrogel cross-linking efficiency and hydrolytic degradation kinetics are both significantly affected by the functionality and structure of the cross-linkers.[31] To demonstrate this, we compared the cross-linking and degradation of PEGNB-D hydrogels cross-linked by either tetra-functional (i.e., PEG4SH) or bifunctional thiol (i.e., DTT). As expected, at an identical PEGNB-D macromer content (i.e., 4 wt %), hydrogels cross-linked by PEG4SH were considerably stiffer (G'_0 ∼ 14 kPa) than that formed with DTT (G'_0 ∼ 1.6 kPa, Figure 5A). While these two sets of gels were cross-linked with an identical amount of norbornene ester bonds, the degradation kinetics were significantly different, with PEG4SH-cross-linked gels degraded roughly 3 times slower than that with DTT (Figure 5B, Table S3). These results implied that the degradation of the hydrogels was primarily governed by the bulk hydrogel properties (e.g., swelling, cross-linking density), rather than merey by the kinetics of ester bond hydrolysis.

In addition to tuning hydrogel degradation by varying the functionality of the cross-linker, the degradation can be further regulated by mixing PEGNB-D with $PEGNB_{CA}$ at different ratios during hydrogel fabrication. At a fixed total PEGNB content (e.g., 4 or 10 wt %), we showed that the inclusion of PEGNB-D did not alter hydrogel initial cross-linking (G'_0 ∼ 1.6 and 17 kPa for 4 wt % (soft) and 10 wt % (stiff) hydrogels, respectively, Figures 5C and S5A). However, the degradation kinetics were decoupled from the degree of network connectivity (Figures 5D and S5B). For soft hydrogels, the degradation period ranged from 3 to 14 days (Figure 5B). Notably, there was a significant drop in G' early on (1−2 days) when PEGNB-D was included. For soft hydrogels incorporated with more than 25% PEGNB-D, complete gel degradation occurred overnight (data not shown). These results implied that the rapid ester hydrolysis of PEGNB-D quickly loosened the hydrogel network, leading to accelerated degradation in the nearby $PEGNB_{CA}$ macromer. Similar degradation was observed in stiff hydrogels. With these highly cross-linked networks, the degradation was further prolonged to 25 days (Figure S5B). Interestingly, stiff hydrogels still completed degradation overnight when PEGNB-D was higher than 50%. During this extended incubation, hydrogels notably swelled and discolored, likely due to oxidation of the residual dopamine (Figure S6). The cytocompatibility of soft $PEGNB_{CA}$/PEGNB-D hydrogels at different PEGNB-D and $PEGNB_{CA}$ ratios were further demonstrated by encapsulation of PANC-1 cells (Figures S7 and S8). It is worth noting that the hydrogels with higher than 25% PEGNB-D degraded overnight following encapsulation. Hence, cell viability staining was performed right after the encapsulation. For 25% PEGNB-D hydrogels, the cell viability was tracked over 3 days as the gels degraded in a slower rate. In all conditions, PEGNB-D hydrogels demonstrated exceptional cytocompatibility. Overall, this study established that it is possible to decouple the initial hydrogel moduli with their degradation rate, a unique feature not easily achievable by conventional covalent hydrogels.

In summary, we present a robust and user-friendly protocol to synthesize norbornene-functionalized PEG via using carbic anhydride. The method eliminates the use of an extremely pungent odor of norbornene acid. The additional carboxylic group of $PEGNB_{CA}$ was further conjugated with amino-moieties, giving rise to PEGNB-X macromers. $PEGNB_{CA}$ and PEGNB-X were readily cross-linked by dithiols into thiol-norbornene hydrogels liable to accelerated yet tunable hydrolytic degradation. The facile synthesis and cross-linking, excellent cytocompatibility, and fast yet highly tunable degradation of $PEGNB_{CA}$ and PEGNB-X hydrogels provide opportunities in future tissue engineering applications. Furthermore, these hydrogels preserve all benefits of thiol-norbornene photogelation, including the spatiotemporally tunable cross-linking. This property has the potential in advanced biofabrication since the highly cytocompatible PEGNB-X hydrogels can be exploited as sacrificial hydrogels to control cell distribution by means of microfluidics and 3D bioprinting.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmacrolett.1c00056.

Experimental section, norbornene substitution ratio quantification, and PEGNB-R ^{1}H NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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