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Research Article

Modulating Viscoelasticity, Stiffness, and Degradation of Synthetic Cellular Niches via Stoichiometric Tuning of Covalent versus Dynamic Noncovalent Cross-Linking

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S *Supporting Information*

ABSTRACT: Viscoelasticity, stiffness, and degradation of tissue matrices regulate cell behavior, yet predictive synergistic tuning of these properties in synthetic cellular niches remains elusive. We hypothesize that reversible physical cross-linking can be quantitatively introduced to synthetic hydrogels to accelerate stress relaxation and enhance network stiffness, while strategic placement of isolated labile linkages near cross-linking sites can predict hydrogel degradation, both of which are essential for creating adaptive cellular niches. To test these hypotheses, chondrocytes were encapsulated in hydrogels formed by biorthogonal covalent and noncovalent physical cross-linking of a pair of hydrophilic building blocks. The stiffer and more viscoelastic hydrogels with DBCO−DBCO physical cross-links facilitated proliferation and chondrogenic ECM deposition of encapsulated cells by dissipating stress imposed by expanding cell mass/ECM via dynamic disruption/reformation of physical cross-links. Degradation of labile linkages near covalent cross-linkers further facilitated cell proliferation and timed cell release while maintaining chondrogenic phenotype. This work presents new chemical tools for engineering permissive synthetic niches for cell encapsulation, 3D expansion, and release.
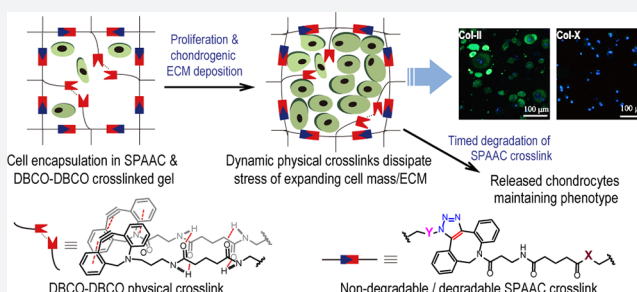
INTRODUCTION

Hydrogels have long been used to encapsulate/deliver stem cells or primary cells to promote tissue regeneration.[1−7] It is increasingly appreciated that hydrogel matrix viscoelasticity, stiffness, and degradation, like essential covalent and non-covalent molecular interactions that define the physicochemical cues of native extracellular matrix (ECM),[8] modulate cell−hydrogel interactions and consequently cell proliferation, morphogenesis, differentiation, and phenotypical matrix deposition.[9−12] Natural protein and polysaccharide-based hydrogels (e.g., MatrigelTM, alginate)[8] have long been exploited for cell encapsulations due to their cytocompatibility and high viscoelasticity imposed by extensive physical interactions among entangled macromolecular chains. Recently, the role of viscoelasticity of alginate-based hydrogels on the cellular behavior of encapsulated adult stem and primary cells[13−15] was probed by modulating the molecular weight of alginate, the degree of Ca^{2+}-induced physical cross-linking, and the introduction of covalently tethered poly(ethylene glycol) chains, demonstrating the benefit of faster stress relaxing for alleviating the elastic stress to encapsulated cells. However, general difficulties in achieving regiospecific and stoichiometrically controlled chemical modification of natural polymers impede predictive and reproducible tuning of their biophysical, biomechanical, and degradative properties. This limitation, combined with batch-to-batch variation in their matrix compositions, residue animal-derived components, and/or

risks for contamination/immunogenicity, presents significant hurdles to their regulatory approval and clinical translation.

Unlike natural biopolymer-based hydrogels, wholly synthetic hydrogels can be prepared free of biocontaminants, with their chemical, mechanical, and degradative properties prospectively and reproducibly tuned by salient selection of building blocks and methods of cross-linking.[16,17] The use of wholly synthetic hydrogels for cell encapsulation, however, has been largely limited to investigating the impact of stiffness of covalently cross-linked hydrogels (e.g., photo-cross-linking of polymetha-crylates; "click" cross-linking of poly(ethylene glycol) (PEG)) on the fate of encapsulated stem/progenitor cells[18−20] or the matrix deposition of encapsulated primary cells such as chondrocytes *in vitro*.[21,22] Modulation of stiffness of these hydrogels was mainly accomplished by altering the degree of covalent cross-linking or polymer weight fractions, which did not address the negative impact of high elastic stress imposed by these hydrogel networks on the metabolism of encapsulated cells. Meanwhile, introduction of degradability to elastic covalently cross-linked hydrogel network to promote encapsulated ECM deposition, cell migration, and cell release has been accomplished by incorporation of hydrolytically degradable polylactide segments[23,24] or substrates of degradative enzymes.[20,25−28] These methods of modulating hydrogel
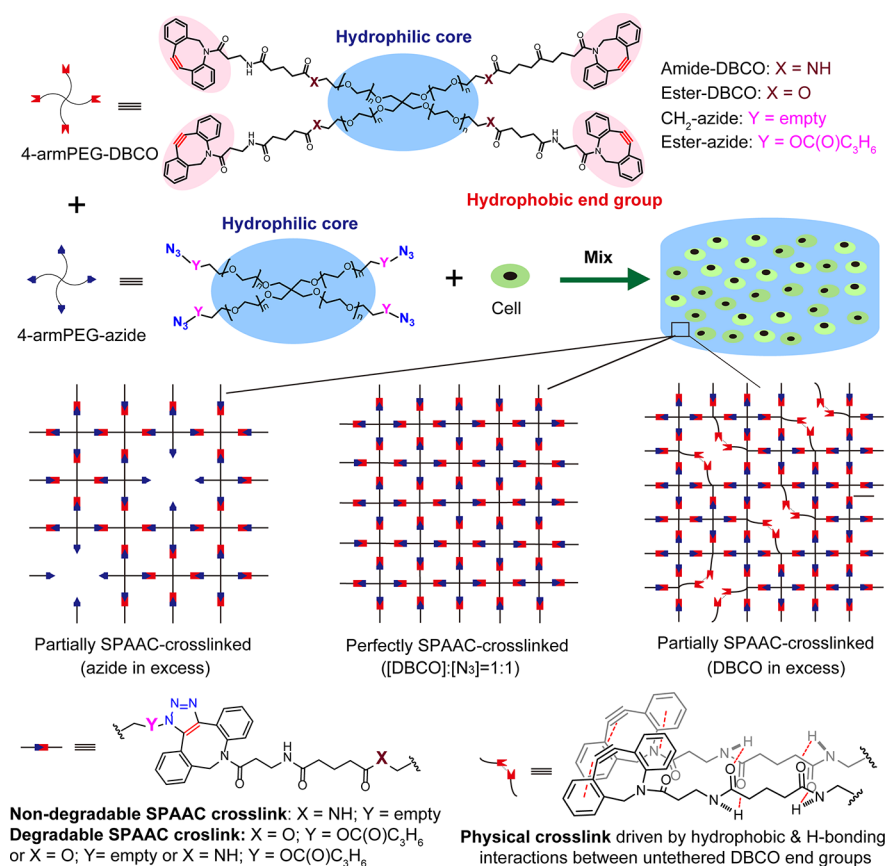
Figure 1. Depiction of cell encapsulation by ClickGels with varying degrees of SPAAC cross-links and physical cross-links formed by mixing nondegradable or degradable azide- and DBCO-terminated 4-armPEG macromer building blocks in various ratios.

degradability are often not predictive in nature (e.g., kinetics of hydrolytic degradation of hydrophobic segments highly dependent on network structure; metalloprotease substrates tend to exhibit different sensitivity to enzyme isoforms) require customized substrate design (e.g., tissue-specific enzymatic degradation substrates), or generate significant inflammatory acidic degradation products. Overall, it remains a significant challenge to develop wholly synthetic hydrogels where their matrix viscoelasticity, stiffness, and degradative properties can be quantitatively, predictively, and synergistically tuned by using a small set of well-defined building blocks for facile cell encapsulation, 3D cell expansion, and timed release.

We hypothesize that these challenges may be addressed by well-structured hydrophilic synthetic hydrogels where the matrix stiffness and viscoelasticity are dictated by tunable ratios of biorthogonal covalent vs noncovalent physical cross-linking of a pair of cytocompatible building blocks while the degradation is predictively controlled by the strategic placement of isolated labile linkages near the covalent cross-linking site. The dynamic disruption/reformation of physical cross-links and controlled degradation near covalent cross-links are both expected to help dissipate the local stress imposed by proliferating cells and their ECM secretion. Strain-promoted alkyne–azide cycloaddition (SPAAC)[29−32] is a biorthogonal, chemoselective covalent "click" conjugation. It can proceed efficiently under physiological conditions free of catalysts, irradiation, or heat, between reactants functionalized with azides and cyclooctynes, which are not present in native cellular/tissue environment. It has been utilized for the preparation of covalently cross-linked hydrogels for cell

encapsulation.[33−35] We recently demonstrated that, by SPAAC-cross-linking 4-armed poly(ethylene glycol)-tetra-dibenzocyclooctyl (4-armPEG-DBCO) and 4-armed poly-(ethylene glycol)-tetra-azide (4-armPEG-azide) with a single labile or stable linkage near the azide or DBCO group at an overall ratio of $[DBCO]:[N_3] = 1:1$ (Figure 1), cytocompatible ClickGels with predictive degradation over a broad range could be prepared.[33] The strategic placement of a single labile ester linkage at either side of the SPAAC cross-link (X = O; Y= OC(O)−C_3H_6, Figure 1; distinct K_d's for hydrolysis at X vs Y site) within a well-structured hydrophilic network enables prediction of the ClickGel disintegration based on first-order hydrolytic cleavage kinetics.[33] Prospective tuning of the ClickGel disintegration rate from days to months, precisely matching theoretical predictions, was accomplished. Herein, we propose that hydrophobic DBCO end groups, when left untethered by mixing the reacting building blocks in mismatched $[DBCO]:[N_3]$ ratios, could form dynamic DBCO−DBCO physical cross-links driven by hydrophobic and H-bonding interactions (Figure 1, bottom, $[DBCO]:[N_3] > 1$). This system would allow us to quantitatively tune matrix viscoelasticity and stiffness by conveniently altering the ratio of the reacting macromer building blocks (the degrees of covalent SPAAC vs noncovalent DBCO−DBCO cross-linking), and thus is uniquely suited for testing our hypotheses.

To examine whether precisely tuning of matrix viscoelasticity, stiffness, and degradative property may translate into improved 3D synthetic cellular niches, chondrocyte encapsulation/release is chosen as the proof-of-concept. Matrix assisted chondrocyte delivery is a vital clinical treatment
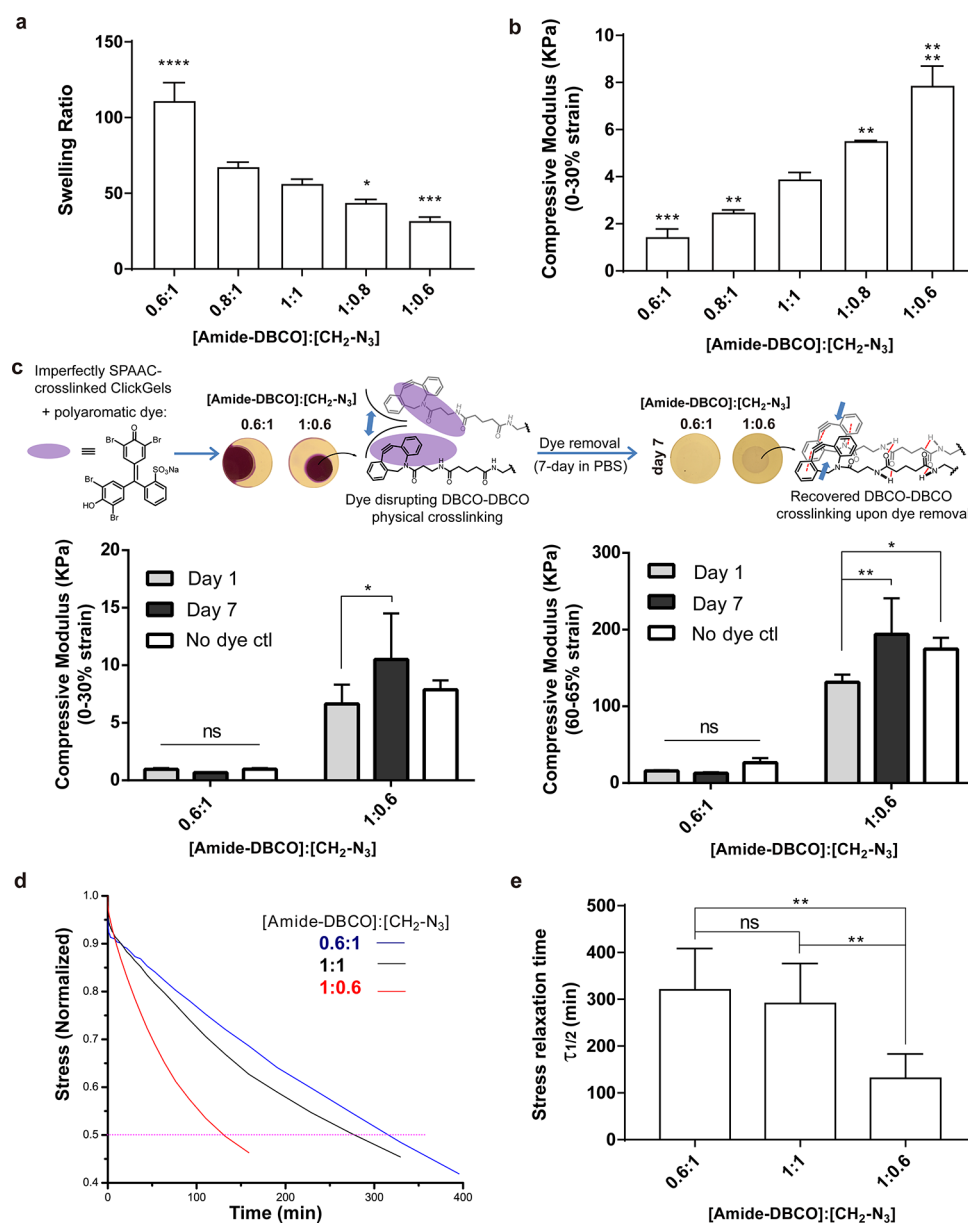
Figure 2. Nondegradable ClickGels (5% w/v) cross-linked between mismatched ratios of DBCO- and N_3-terminated macromers exhibit tunable swelling behavior, stiffness, and viscoelasticity, with stiffer ClickGels formed with excess DBCO-terminated macromers exhibiting faster stress relaxation. (a) Swelling ratio. (b) Compressive moduli (0−30% strain) of ClickGels cross-linked between varying ratios of DBCO- and N_3-terminated macromers. (c) Compressive modulus changes (0−30% and 60−65% stain ranges) upon the addition and removal of polyaromatic dye (bromophenol blue sodium salt). (d) Representative stress relaxation profiles of ClickGels composed of varying ratios of DBCO- and N_3-terminated macromers. (e) Stress relaxation time ($\tau_{1/2}$) of varying ratios of DBCO- and N_3-terminated macromers. ns: $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *****$p < 0.0001$ (a, b: one-way ANOVA with Dunnett's multiple comparisons vs the 1:1 formulation. c, e: two-way and one-way ANOVA with Tukey's multiple comparisons, respectively).

option for articular cartilage lesion, which is known for limited self-repair and regenerative capability due to its avascular, aneural nature and a dense ECM that impedes autologous cell migration.[36] An improved chondrocyte encapsulation, 3D expansion, and *in vivo* delivery strategy will benefit osteoarthritic patients suffering from focal cartilage lesions. Specifically, we first test the hypothesis that, by altering the stoichiometric ratios of 4-armPEG-DBCO and 4-armPEG-azide, ClickGels with varying compressive stiffness and viscoelasticity/stress relaxation can be prepared, wherein the proliferation and phenotypical ECM deposition of encapsulated chondrocytes directly correlate with the degree of dynamic physical cross-linking. We then tested the hypothesis

that the unique degradation control of this platform could further encourage 3D cell expansion and ECM deposition, and enable timed release of encapsulated cells with retained chondrogenic phenotype. We chose immature murine chondrocytes (iMACs) for initial screening to identify suitable SPAAC-to-physical cross-link ratios at given polymer contents that ensure long-term viability and chondrogenic phenotype of encapsulated cells. We then validate the applicability of the ClickGels for the long-term encapsulation of human articular chondrocytes (hACs) and timed cell release.

■ RESULTS AND DISCUSSION

By altering the molar ratios of nondegradable 4-armPEG-azide (Y = empty) to nondegradable 4-armPEG-amide-DBCO (X = NH) mixed in PBS (5% w/v polymer content) from 1:0.6 to 0.6:1, we prepared partially or perfectly ([DBCO]:[N$_3$] = 1) SPAAC-cross-linked nondegradable ClickGels (Figure 1) in gelling kinetics (2−5 min) suitable for cell encapsulation. Quantitative azide-DBCO coupling in the perfectly SPAAC-cross-linked ClickGel was previously validated by the complete conversion of azide and DBCO end groups into SPAAC cross-links by FTIR and UV/vis characterizations.[37] The partially SPAAC-cross-linked ClickGels formed with an excess of 4-armPEG-azide ([DBCO]:[N$_3$] < 1) exhibited much higher swelling ratios (Figure 2a) and significantly weaker compressive moduli (Figure 2b) than the perfectly SPAAC-cross-linked ClickGel. The observed 2-fold increase in swelling ratio and 2-fold decrease in compressive stiffness (at the 0−30% strain range) when [DBCO]:[azide] changed from 1:1 to 0.6:1 are attributed to the reduced SPAAC covalent cross-links, and thus a more loosely tethered 3D network. By contrast, partially SPAAC-cross-linked ClickGels formed with an excess of 4-armPEG-DBCO macromers exhibited significantly higher compressive moduli and lower swelling ratios compared to the perfectly SPAAC-cross-linked ClickGel (Figure 2a,b). We attribute the ∼2-fold decrease in swelling ratio and 2-fold increase in compressive stiffness to the increasing physical cross-links between untethered DBCO's when [DBCO]:[N$_3$] changed from 1:1 to 1:0.6. These physical cross-links, presumably driven by a combination of hydrophobic interaction between the tricycles of DBCO's and H-bonding interactions between adjacent amide linkages (Figure 1), were robust enough to overcome the reduction in covalent SPAAC cross-links, resulting in a mechanically strengthened 3D network. The mechanical contribution of DBCO−DBCO physical cross-links within the imperfectly SPAAC-cross-linked system (e.g., [DBCO]:[N$_3$] = 1:0.6) was validated by the reduction in compressive moduli upon addition of polyaromatic dye to disrupt the DBCO−DBCO interaction (day 1, Figure 2c). Furthermore, the ability of untethered DBCO groups to reform physical cross-links was made evident by the restoration of the compressive moduli of the hydrogel upon dye removal (after 7 day equilibration in PBS) to the level of ClickGels without dye treatment (Figure 2c), supporting the dynamic/reversible nature of the DBCO−DBCO cross-links. By contrast, the addition and removal of the polyaromatic dye to and from the imperfectly SPAAC-cross-linked ClickGels with azides in excess (e.g., [DBCO]:[N$_3$] = 0.6:1) did not cause significant perturbations in their stiffness (Figure 2c), supporting negligible physical cross-links between untethered azide-terminated chains. Further demonstrating the robustness of DBCO−DBCO physical cross-links was the observation that gelling occurred at a mismatched ratio as drastic as [DBCO]:[N$_3$] = 1:0.3, with the resulting ClickGel possessing only 30% covalent SPAAC cross-links but 70% DBCO−DBCO physical cross-links stiffer than those formed at [DBCO]:[N$_3$] = 0.6:1 and 0.8:1 (Supporting Information, Figure S1). By contrast, formulations with a significant fraction of excess azide end groups ([DBCO]:[N$_3$] = 0.3:1, 0.4:1, or 0.5:1) could barely gel into network with sufficient integrity due to lack of physical cross-linking among excess azide groups, despite the 30−50% of covalent SPAAC cross-links.

Viscoelastic hydrogels are attractive for cell encapsulation due to their ability to better accommodate cell spreading, migration, proliferation, and matrix deposition through more effective/faster stress relaxation.[15,38−40] Incorporating ionic bonds,[41−43] reversible covalent (e.g., hydrazone) bonds,[40] as well as untethered polymer chains have been exploited as methods for expediting stress relaxation.[13−15] Here we show that engineering the reversible DBCO−DBCO physical cross-links into the wholly synthetic network translated into significantly faster stress relaxation in these stiffer hydrogels (Figure 2d,e). Specifically, stress relaxation ($\tau_{1/2}$) in hydrogels formed with excess DBCO-terminated macromers (e.g., [DBCO]:[N$_3$] = 1:0.6, 131 ± 52 min) was significantly faster than that in hydrogels with 100% SPAAC-cross-linking ([DBCO]:[N$_3$] = 1:1, 291 ± 85 min) or those formed with excess azide-terminated macromers ([DBCO]:[N$_3$] = 0.6:1, 320 ± 88 min). These data support that dynamic DBCO−DBCO physical cross-links (their breakage and reformation) are more effective in dissipating energy than any potential physical interactions between the SPAAC cross-links or the untethered azide-terminated PEG arms in the weaker ClickGels. There is likely some level of hydrophobic interactions among the triazole moieties of the SPAAC cross-links, the disruption of which may have expedited energy dissipation to a degree comparable to that due to the mobility of untethered PEG-azide arm (no statistically significant difference in $\tau_{1/2}$, [DBCO]:[N$_3$] = 1:1 vs [DBCO]:[N$_3$] = 0.6:1; Figure 2e).

Cartilage is a viscoelastic tissue[44] with complex dynamic mechanical properties that change throughout development.[45,46] Previous work suggests that chondrocytes proliferate better within low-stiffness hydrogels,[2,47] although minimal stiffness requirement to support their 3D proliferation without compromising their chondrogenic phenotype remains to be established. Chaudhuri et al. recently showed that faster relaxing hydrogels, with viscoelasticity modulated independent of stiffness and matrix degradability, better promote chondrocyte ECM secretion.[13] Here, the ability to strengthen ClickGel while expediting stress relaxation via precise tuning of the degrees of reversible DBCO−DBCO physical vs covalent SPAAC cross-linking by mismatching the ratio of DBCO- vs N$_3$-terminated macromers at a given overall polymer content offers an exciting opportunity to interrogate the impact of these synergistic biomechanical cues on the cellular behavior of encapsulated chondrocytes. Accordingly, we isolated iMACs, which can undergo robust cartilage matrix protein syntheses in expansion media (EM), for encapsulation in nondegradable ClickGels with varying degrees of SPAAC/physical cross-linking and evaluated for their viability/proliferation and matrix syntheses over 8 weeks. We first screened for an appropriate initial iMACs encapsulation density (100 000−1 000 000 cells per 25 μL) in perfectly SPAAC-cross-linked nondegradable ClickGel (5% w/v) (Supporting Information, Note A1 and Figure S2a). Whereas iMACs were able to proliferate within the first 3−4 weeks at all encapsulation densities examined, the 250 000 per gel initial encapsulation density resulted in the most viable cells by 8 weeks (Supporting Information, Figure S2b) while maintaining the expression of chondrogenic markers type II collagen and aggrecan (Supporting Information, Figure S2c). As polymer content is also known to affect the mechanical properties of cross-linked hydrogels, we then investigated whether and how modulating ClickGel polymer content affect the cellular
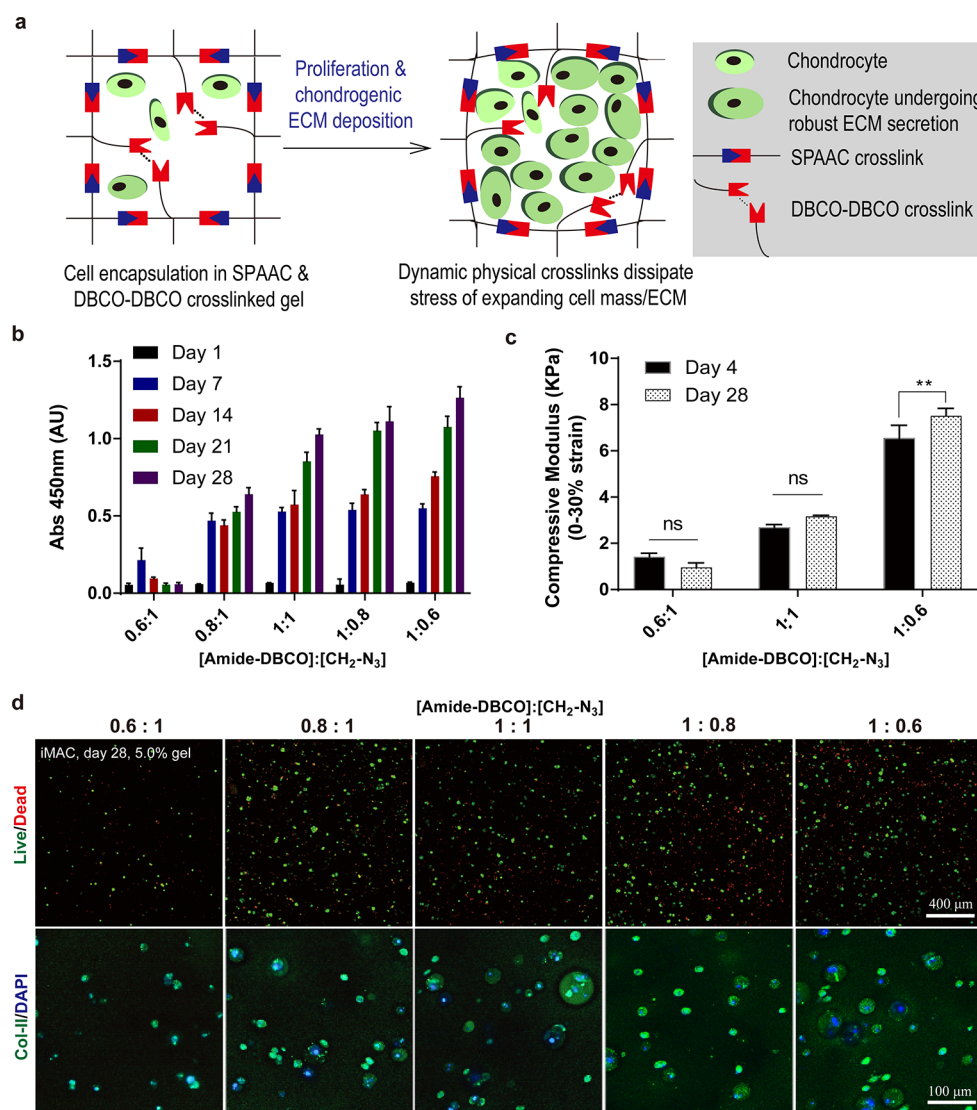
Figure 3. Stiffer nondegradable ClickGels (5% w/v) with dynamic DBCO−DBCO physical cross-links better accommodate the proliferation and chondrogenic ECM deposition of encapsulated iMACs over time. (a) Depiction of reversible formation of DBCO−DBCO cross-links helping dissipate stress imposed by the expanding cell mass ECM deposition by encapsulated cells. (b) Viability of encapsulated iMACs over time. (c) Temporal changes in compressive moduli (0−30% strain) of iMAC-laden ClickGels over 28 day culture. (d) Live (green)/dead (red) staining and type II collagen (green)/DAPI (blue) immunofluorescent staining of iMAC-laden ClickGels after 28 day culture. 250 000 iMACs were encapsulated in 25 μL of 5% w/v nondegradable ClickGels of varying macromer ratios and cultured in expansion media. ns: $p > 0.05$; **$p < 0.01$ (two-way ANOVA with Sidak's multiple comparisons).

behavior of encapsulated iMACs (Supporting Information, Note A2). As expected, increasing and decreasing polymer content of ClickGels resulted in proportional enhancement and reduction in their compressive moduli (Figure S3a), but the benefit of increasing polymer contents on early cell proliferation (first week) was only manifested in the much weaker imperfectly SPAAC-cross-linked ClickGels with excess untethered azide chains ([DBCO]:[N_3] = 0.6:1) (Figure S3b). In both perfectly SPAAC-cross-linked ClickGels and the stiffer and more viscoelastic ClickGels with DBCO−DBCO cross-linking, reducing polymer content from 5% to 2.5% w/v (compressive moduli from 3.9 to 2.5 kPa and from 7.9 to 3.3 kPa, respectively) negatively impacted both the proliferation and long-term viability of encapsulated iMACs (Figure S3c,d). There was no significant benefit for increasing polymer content from 5% to 10% w/v in these ClickGels. This observation suggests that once the stiffness of a synthetic niche falls within

a suitable range, further increasing the stiffness at the cost of increasing polymer content (which could negatively impact nutrient/waste transport in and out of the 3D network) may not be beneficial. It is worth noting that despite the similar compressive moduli of 10% w/v perfectly SPAAC-cross-linked (7.5 kPa) vs 5% w/v partially SPAAC-cross-linked and DBCO−DBCO strengthened more viscoelastic (7.9 kPa) ClickGels, the latter better supported sustained proliferation and viability of encapsulated iMACs over 4 weeks. Overall, these experiments reveal ∼3 kPa as a likely lower threshold for the ClickGel system below which the encapsulated iMACs could not undergo sustained proliferation. Beyond this threshold, introducing dynamic DBCO−DBCO physical cross-links is advantageous to polymer content increases as a means to improve the stiffness of the chondrogenic cellular niche.
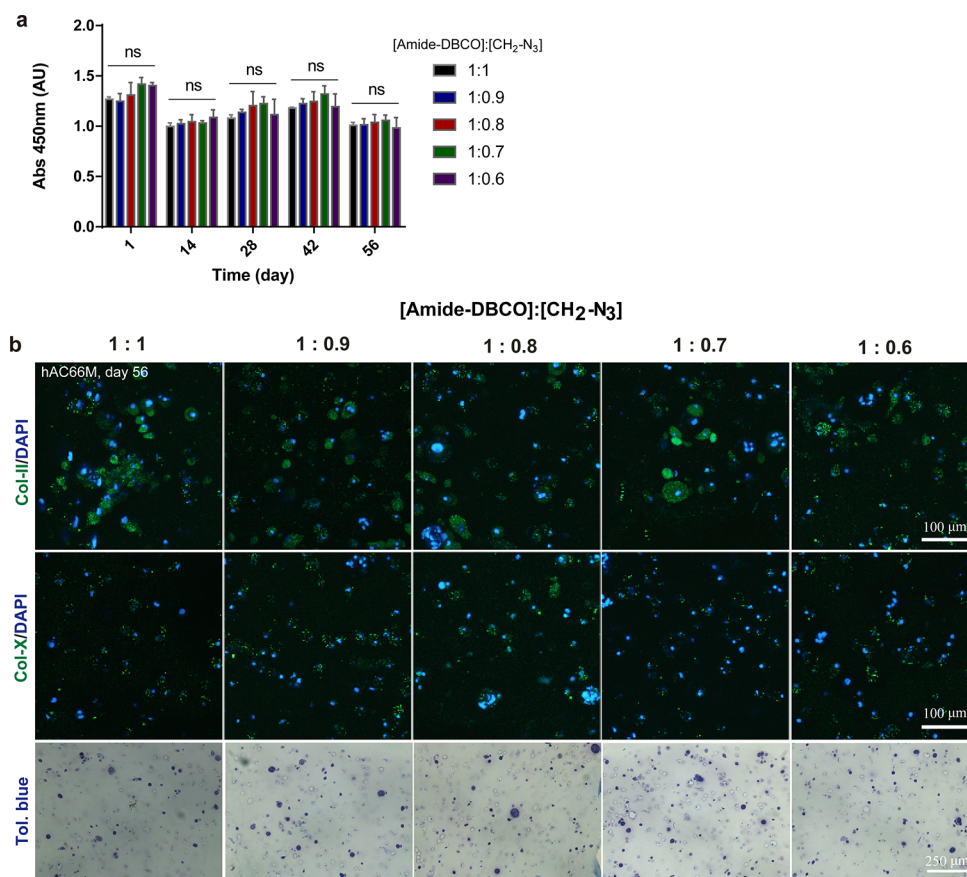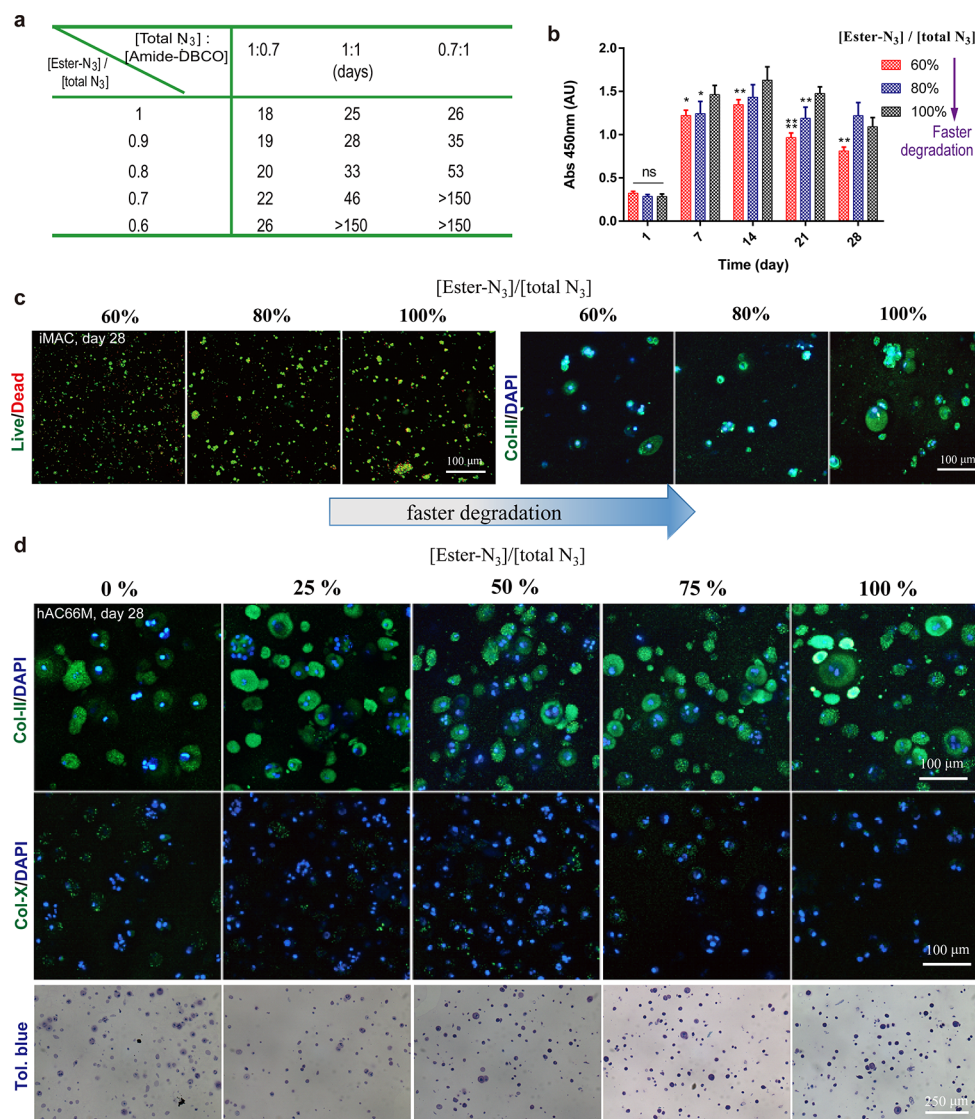
Figure 4. Human chondrocytes encapsulated in nondegradable ClickGels maintain long-term viability and chondrocyte phenotype regardless of the ratio of DBCO- and N_3-terminated macromers. (a) Viability of encapsulated human chondrocytes as a function of ClickGel composition over 56 days. (b) Type II collagen (green)/DAPI (blue), type X collagen (green)/DAPI (blue) immunofluorescent staining, and toluidine blue (for GAG) staining of human chondrocyte-laden ClickGels of varying compositions on day 56 of chondrogenic culture. 500 000 human chondrocytes were encapsulated in 25 μL of 5% w/v nondegradable ClickGels of varying macromer ratios and cultured in chondrogenic media (high-glucose DMEM, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 1% insulin-transferrin-selenous acid mixture, 100 nM dexamethasone and 10 ng/mL TGF-β3). ns: $p > 0.05$ (two-way ANOVA with Tukey's multiple comparisons vs the 1:1 formulation at a given time).

Using the optimized initial cell encapsulation density (25 000 cells/25 μL gel) and polymer content of ClickGel (5% w/v), we tested the hypothesis that stiffer ClickGels with dynamic DBCO−DBCO physical cross-links ([DBCO]:[N_3] > 1) constitute a more adaptive/permissive niche environment for cell proliferation and ECM deposition (Figure 3a). Indeed, the stiffer and more viscoelastic ClickGel strengthened by DBCO−DBCO cross-links supported better cell proliferation of the encapsulated iMACs over 4 weeks in EM compared to those encapsulated in the perfectly SPAAC-cross-linked ClickGel (Figure 3b), accompanied by more robust type II collagen expression (Figure 3d). By contrast, in the absence of DBCO−DBCO physical cross-links, iMACs encapsulated within the weaker and less viscoelastic ClickGels ([DBCO]:[N_3] < 1) exhibited poorer cell proliferation and viability beyond the first week, with the ClickGel with the most untethered azide-terminated PEG arms ([DBCO]:[N_3] = 0.6:1) being the least favorable 3D environment for iMACs. Consistent with these observations, only the stiffer and more viscoelastic iMAC-laden ClickGel ([DBCO]:[N_3] = 1:0.6) exhibited statistically significant enhancement in stiffness, presumably due to more robust ECM deposition, after 4 week culture in EM (Figure 3c).

Using the 5% w/v perfectly SPAAC-cross-linked and the stiffer and more viscoelastic ClickGels containing various fractions of DBCO−DBCO physical cross-links ([DBCO]: [N_3] = 1:1, 1:0.9, 1:0.8, 1:0.7, or 1:0.6), we then validated the general applicability of the system for long-term encapsulation of human articular chondrocytes. The encapsulated hACs (500 000 cells per 25 μL of gel) remained viable in all formulations examined over the 8 week culture in low-serum chondrogenic media (which promotes chondrogenic matrix synthesis rather than cell proliferation), supporting that nutrients/waste could readily penetrate in and out of these hydrophilic 3D network at this cell encapsulation density (Figure 4a). Equally important, toluidine blue staining and immunofluorescent staining revealed robust deposition of GAG and type II collagen secretions by hACs encapsulated in all ClickGel formulations examined, with minimal type X collagen detected by week 8 (Figure 4b; note that the hACs were isolated from the relatively healthy portion of discarded osteoarthritic joint tissues). The successful encapsulation of hACs by ClickGels and their extended *in vitro* culture within these 3D synthetic niches without compromised viability or chondrogenic phenotype point to promising utilities for *ex vivo* cartilage tissue engineering applications.

For cartilage tissue regeneration *in vivo*, it is critical to also demonstrate the feasibility of tuning the degradative properties of ClickGels to promote the proliferation and chondrogenic matrix deposition of encapsulated cells as the hydrogel

Figure 5. ClickGel degradation enhances proliferation and chondrogenic ECM depositions of encapsulated iMACs and human chondrocytes. (a) Disintegration time of ClickGels (5% w/v) formed between nonlabile DBCO-terminated macromer and a mixture of nonlabile and labile azide-terminated macromers upon incubation in expansion media. (b) Viability of iMACs encapsulated within perfectly SPAAC-cross-linked ClickGels with varying degradability (250 000/25 μL gel) in expansion media over time. (c) Live (green)/dead (red) staining and type II collagen (green)/ DAPI (blue) immunofluorescent staining of iMAC-laden ClickGels with varying degradability on day 28 of culture in expansion media. (d) Type II collagen (green)/DAPI (blue), type X collagen (green)/DAPI (blue) immunofluorescent staining, and toluidine blue (for GAG) staining of human chondrocyte-laden ClickGels with varying degradability in chondrogenic media. ns: $p > 0.05$; *$p < 0.05$; **$p < 0.01$; *****$p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons vs the 100% gel).

degrades and ensure that cells released upon hydrogel disintegration maintain their phenotypes. Synchronizing the rate of synthetic niche degradation with that of the neotissue integration could preserve the overall mechanical integrity of the cell-laden construct throughout the dynamic guided tissue regeneration process.[48] Too fast of a degradation will compromise the stiffness necessary for maintaining active metabolism of encapsulated cells while too slow of a degradation will impede ECM (GAG and collagen fibrils are macromolecules of microns in size[23]) integration and eventual replacement of the synthetic niche by regenerated neotissue. By mixing labile 4-armPEG-ester-azide and stable 4-armPEG-azide macromers in varying ratios with the stable 4-armPEG-amide-DBCO while keeping [DBCO]:[total N₃] = 0.7:1, 1:1, or 1:0.7, 5% w/v ClickGels with varying degrees of SPAAC and DBCO−DBCO physical cross-linking were prepared.

These ClickGels disintegrated in 18−53 days or remained intact >150 days upon incubation in EM (Figure 5a). The weaker partially SPAAC-cross-linked ClickGels (azide in excess) with the highest labile 4-armPEG-ester-azide fraction (100%) disintegrated the fastest while the much stiffer and more viscoelastic ClickGels with DBCO−DBCO physical cross-links containing the least fraction of labile 4-armPEG-ester-azide (60%) disintegrated the slowest. In a conventional degradable hydrogel system, it is generally believed that partially covalently cross-linked network will undergo more rapid degradation. Here, we show that a partially covalently cross-linked ClickGel with excess untethered DBCO moieties can in fact strengthen the network via the dynamic DBCO−DBCO physical cross-linking and slow the degradation. The hydrophobicity around the DBCO−DBCO physical cross-links combined with the more densely packed network has likely
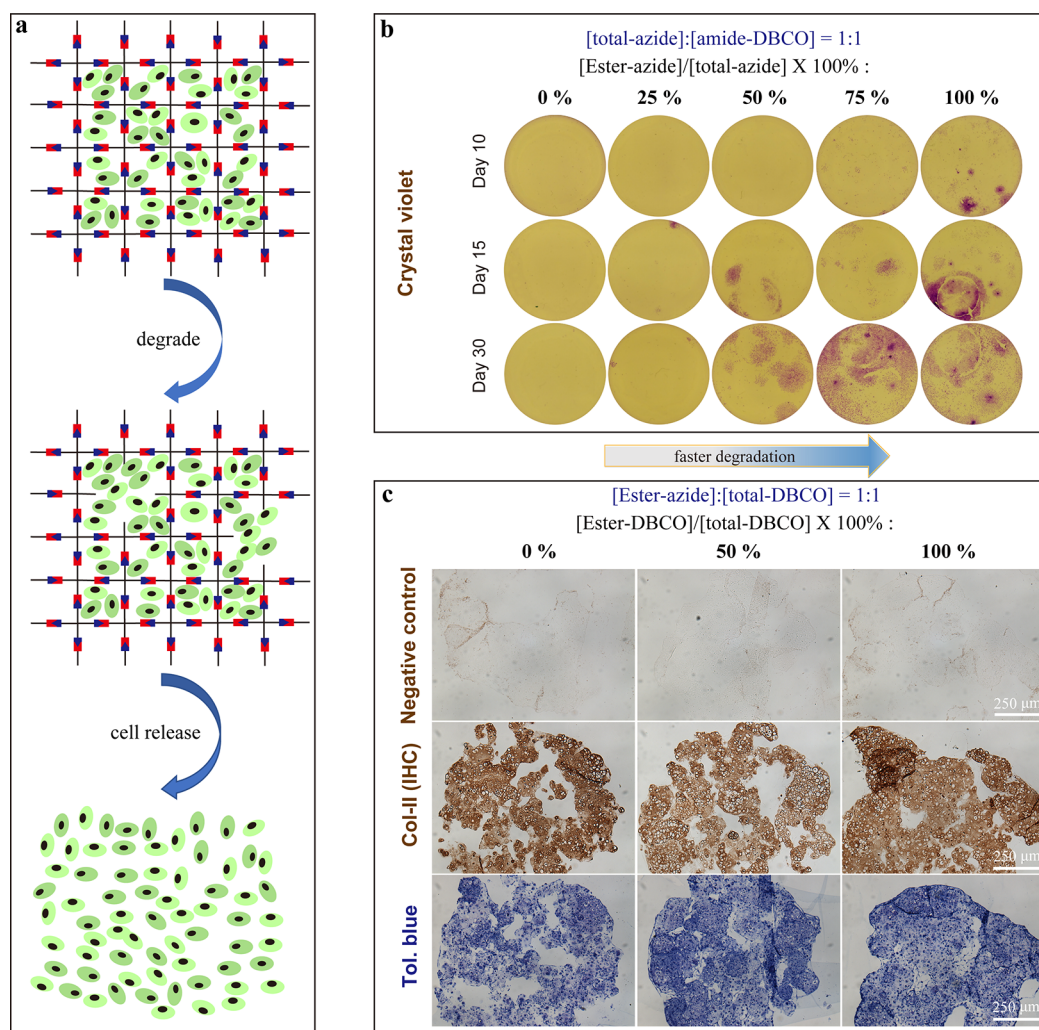
Figure 6. Degradable ClickGels (5% w/v, perfectly SPAAC-cross-linked) enable timed release of encapsulated iMACs with retained chondrogenic phenotype. (a) Depiction of ClickGel network disintegration as a result of hydrolysis of labile SPAAC cross-links and the release of encapsulated cells. (b) Crystal violet staining of cells released from ClickGels with varying degradability over different culture duration in expansion media. (c) GAG and type II collagen staining of the pellet of released iMACs. Upon complete disintegration of the degradable ClickGel, released iMACs were pelleted and cultured in expansion media for 10 days prior to staining.

slowed free water penetration to the labile ester linkage near the SPAAC cross-links. At any given degree of SPAAC/ physical cross-linking, the disintegration rate expectedly accelerated with the increasing fractions of labile 4-armPEG-ester-azide macromer.[33] In contrast to tuning the molecular weight of degradable polymer chains, covalent cross-linking contents, or overall polymer fractions as the means of altering degradable hydrogel degradation rates,[24,49] the prospective tuning of ClickGel degradation rate by facile adjustment of the ratio of a pair of building blocks containing a single labile linkage (without altering overall polymer content) avoids excessive immunogenic acidic degradation products or poor nutrient transport associated with high polymer fractions.

The iMACs encapsulated in faster-degrading ClickGels better proliferated and maintained their viability throughout 4 weeks (Figure 5b), consistent with the higher fraction of live cells in faster-degrading ClickGels at a given degree of SPAAC-cross-linking as revealed by live/dead staining (Supporting Information, Figure S4). The faster-degrading ClickGels also supported more robust type II collagen secretion by the encapsulated iMACs (Figure 5c). It is worth noting that the

cell-laden construct with 100% labile 4-armPEG-ester-azide did not fully disintegrate on day 28 (although some viable cells already release from the weakening gel). The slightly slower disintegration compared to the cell-free construct (which disintegrated in 25 days in EM) is likely due to the high cell mass impeding free water penetration to some extent. When the perfectly SPAAC-cross-linked 5% w/v ClickGel with increasing fractions of labile 4-armPEG-ester-azide was used to encapsulate hACs, more robust GAG and type II collagen secretions by encapsulated hACs were observed in all degradable formulations after 4 weeks in chondrogenic culture (Figure 5d). The expression of hypertrophy marker type X collagen by hACs was not observed in the faster-degrading formulations (75% and 100% 4-armPEG-ester-azide). These observations support that degrading synthetic niches are more conducive to chondrogenic matrix deposition by encapsulated chondrocytes in general.

Finally, we examined the tunable release of chondrocytes from degradable ClickGels and whether the released cells maintain their chondrogenic phenotype, which are critical for matrix assisted autologous chondrocyte implantation applica-

tions.[50] It has been well-documented that chondrocytes tend to lose their chondrogenic phenotype in monolayer cultures with increasing passages.[51] Thus, chondrocyte proliferation within a degradable ClickGel niche and their subsequent release without compromising chondrogenic phenotype (Figure 6a) could provide a promising solution. Crystal violet staining was used to monitor the iMACs released (and adhered to the culture plate) from perfectly SPAAC-cross-linked ClickGels with varying fractions of labile 4-armPEG-ester-azide over 30 day culture in EM (Figure 6b). On day 10, only the ClickGel formed with 100% labile 4-armPEG-ester-azide released a small number of iMACs. On day 15, this faster-degrading ClickGel released a bulk content of encapsulated cells while the ones containing 75%, 50%, or 25% labile 4-armPEG-ester-azide began to release. On day 30, the fastest-degrading ClickGel released the remaining iMACs whereas the one containing 75% labile ester-N_3 linkages started to release the bulk content of its encapsulated cells. These observations support a positive correlation between the content of labile ester linkages within the ClickGel (gel disintegration rate) and the cell release rate. When varying ratios of labile 4-armPEG-ester-DBCO (100%, 50%, 0%) and stable 4-armPEG-amide-DBCO were mixed with 100% labile 4-armPEG-ester-N_3 to encapsulate iMACs, we observed the same correlation between labile ester linkage fractions and the cell release rate, although the cell-laden constructs fully disintegrated more rapidly, on days 25, 42, and 56, respectively. The accelerated degradation is expected given the faster hydrolysis kinetics of the ester-DBCO linkage compared to ester-azide linkage.[33]

The iMACs released from these ClickGels were then pelleted and cultured in EM for another 10 days before being stained for phenotypical chondrogenic markers. Robust expression of type II collagen and GAG was observed with these iMAC pellets (Figure 6c), supporting that these cells maintained their chondrogenic phenotype throughout their encapsulation within the degradable ClickGels and upon their release.

■ CONCLUSION

This study presents a new tool for precisely tuning hydrogel stiffness and matrix viscoelasticity via the explicit control over the degree of covalent SPAAC cross-linking vs dynamic physical cross-links between untethered end groups of biorthogonal macromer building blocks. Conventional methods of modulating synthetic hydrogel stiffness and degradation by altering polymer fractions or covalent cross-linking degrees could be detrimental to the proliferation and long-term viability of encapsulated cells. Modulation of viscoelasticity of natural polymers such as alginate-based hydrogels often involves adjusting molecular weights of alginate, degrees of Ca^{2+}-cross-linking, and the covalent attachment of other polymer tethers.[14] By contrast, with only 2 pairs of wholly synthetic designer building blocks (4-armPEG end-functionalized with DBCO or azide via stable or labile linkers), here we demonstrate that ClickGels with a broad range of stiffness, viscoelasticity, and degradative properties could be prepared by simply altering their mixing ratio along with cells of interest.

Our strategy of introducing robust yet dynamic physical cross-links between untethered DBCO end groups at the expense of the reduction in covalent SPAAC cross-links resulted in the enhancement in both ClickGel stiffness and network viscoelasticity, enabling more robust proliferation and matrix deposition of encapsulated chondrocytes. On the other

hand, increasing untethered azide-functionalized PEG arms at the expense of reduced SPAAC cross-linking degree resulted in reduced hydrogel stiffness but not significantly altered viscoelasticity, which was found to be unfavorable for chondrocyte proliferation. Our elucidation of how the dynamic disruption and reformation of DBCO—DBCO physical cross-linking translates into expedited stress relaxation in ClickGel opens new possibilities of engineering permissive cellular niches with an even broader range of viscoelasticity through stoichiometric incorporation of noncovalent molecular interactions varying in nature (e.g., electrostatic, H-bonding, van der Waals, $\pi-\pi$, or ligand-reception interactions) and strength (e.g., isolated vs clustered interactions).

Furthermore, we demonstrated that controlled degradation can be implemented in ClickGels to achieve predictable disintegration over a broad range without generating excessive immunogenic acidic degradation products. This was accomplished by strategic placement of a single labile linkage on either side of the SPAAC cross-link. ClickGel degradation promoted both proliferation and chondrogenic matrix deposition of encapsulated mouse or human chondrocytes, and the chondrocytes released from fully disintegrated ClickGels maintained their chondrogenic phenotypes.

These properties combined make ClickGels, a wholly synthetic platform free of biological contaminants and with readily and reproducibly tunable physical and mechanical properties, uniquely suited as 3D synthetic niches for chondrocyte encapsulation, *in vitro* expansion, and timed release. It could also benefit the *ex vivo* expansion of scarcely available stem cells (e.g., hematopoietic stem cells) known to be difficult to expand/enrich via conventional 2D cultures for other cell-based therapies. More broadly speaking, the novel concept of engineering network viscoelasticity via controlled integration of dynamic physical cross-links and the strategic placement of single labile linkages near cross-linking sites provide exciting new tools for engineering 3D cellular niches and tissue models for regenerative medicine and drug discovery applications.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00170.

> Additional discussion, methods, and figures including compressive modulus vs macromer ratio, live/dead staining, type II collagen/DAPI staining, type X collagen/DAPI staining, toluidine blue staining, and viability of iMACs as a function of cell encapsulation density and polymer content / degradability of ClickGel over time (PDF)

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Notes

The authors declare no competing financial interest.

No unexpected or unusually high safety hazards were encountered.

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