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Simultaneous Orthogonal Dual-Click Approach to Tough, *in-Situ*-Forming Hydrogels for Cell Encapsulation

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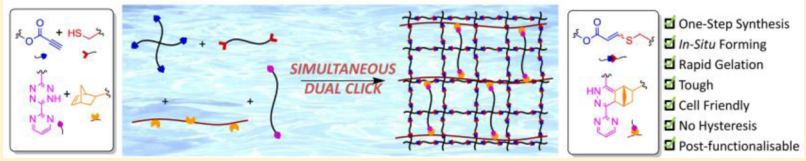
Supporting Information



ABSTRACT: The use of tough hydrogels as biomaterials is limited as a consequence of time-consuming fabrication techniques, toxic starting materials, and large strain hysteresis under deformation. Herein, we report the simultaneous application of nucleophilic thiol-yne and inverse electron-demand Diels–Alder additions to independently create two interpenetrating networks in a simple one-step procedure. The resultant hydrogels display compressive stresses of 14–15 MPa at 98% compression without fracture or hysteresis upon repeated load. The hydrogel networks can be spatially and temporally postfunctionalized via radical thiylation and/or inverse electron-demand Diels–Alder addition to residual functional groups within the network. Furthermore, gelation occurs rapidly under physiological conditions, enabling encapsulation of human cells.

INTRODUCTION

The wide-ranging family of "click" reactions is fast becoming an essential tool in materials chemistry.[1−3] While many such reactions are applicable in both organic and aqueous media, a subset have been demonstrated to work efficiently in biological media and in the presence of living cells. Such bioorthogonal click chemistries not only have been applied in cell labeling,[4] imaging,[5] and cell surface modification,[6] but also offer new opportunities in the fabrication of cell-encapsulated hydrogels.[7−9] Several bioorthogonal click chemistries have been applied in this way including Michael addition reactions,[10−12] radical thiol-ene/yne reactions,[11,13−18] reaction of an azide with a ring-strained alkyne (SPAAC)[19−23] or an activated alkyne,[24,25] the oxime click reaction with glutaraldehyde,[26,27] Diels–Alder[28,29] and inverse electron-demand Diels–Alder additions,[30] and the tetrazole-alkene click reaction.[31] Despite the efficiency of many of these reactions, the hydrogels that result commonly display low mechanical stability.

In biological systems, soft tissues usually consist of a dense fiber matrix embedded in a soft elastic network[32−34] which in turn provides the high strength necessary for biomechanical support and stability. This composited structural formation can be applied to build high strength hydrogels such as double network (DN) hydrogels which are the strongest synthetic soft materials with a water content >90 wt %.[35−39] However, the high mechanical strength of multicomponent DN hydrogels is currently most commonly realized by multistep fabrication processes which generally involve free radical polymerization, swelling, diffusion, and second free radical polymerization. These fabrication techniques have several limitations: (i) they are time-consuming; (ii) it is difficult to control the molar ratio of the components and thus the reproducibility of the hydrogel mechanical properties; (iii) hydrogels with complex shapes are difficult to prepare; and (iv) the free-radical polymerization processes lead to a high degree of heterogeneity, and thus the gels display large hysteresis.[40,41] Although recent attempts have been made to prepare high strength DN hydrogels in a one-pot synthesis by combining ionic and covalent cross-linking processes,[40,42−45] the procedures still require high temperature processing,[40] long preparation time,[43−45] and cross-linking via free radical polymerization.[35,37,39,40,42−45] The use of toxic acrylamide starting materials in all these DN gels prevents their use as extracellular matrices for 3D cell culture and the potential regeneration of living organs.

Here we report the novel, simultaneous application of two orthogonal click chemistries to prepare DN hydrogels under physiological conditions that maintain the high mechanical strength and composited structural formation of other tough hydrogels but are also able to encapsulate human cells with

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excellent viability. These materials can be formed *in situ* using a simple one-step method which enables them to be molded as required. Furthermore, the novel DN hydrogels display high compressive and tensile stresses without fracture or hysteresis upon repeated load and have been shown to be spatially and temporally postfunctionalized using residual functional groups within the network. So far, no other hydrogel materials including nanocomposite hydrogels[46−49] or slip-link gels[50] have been shown to possess all of these features. In turn, our novel approach provides a critical step forward toward the realization of a versatile hydrogel platform for use in a wide range of applications, including tissue engineering.

RESULTS AND DISCUSSION

Our novel approach to the one-step preparation of DN hydrogels uses two orthogonal click chemistries: nucleophilic thiol-alkyne addition and tetrazine-norbornene (Tz-Nb) inverse-electron demand Diels−Alder cycloaddition for dual cross-linking to simultaneously form a dense network and a loose network (Figure 1). Importantly, these reactions occurred

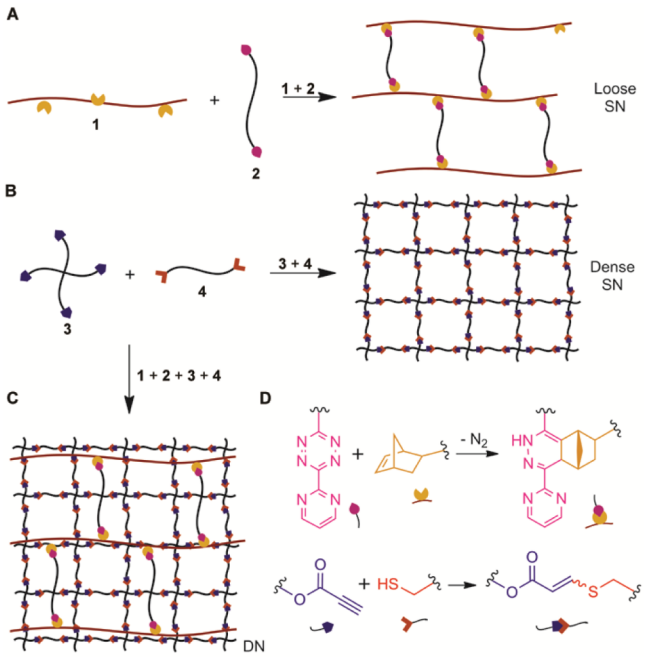

Figure 1. Schematic of DN hydrogel fabrication. (A) Loose network formed by norbornene-tetrazine addition. (B) Dense network formed by nucleophilic thiol-yne addition. (C) DN gel formed by mixing all 4 components. (D) Tetrazine-norbornene and nucleophilic thiol-yne addition chemistries used for cross-linking.

under physiologically relevant conditions, namely at pH 7.4 in both phosphate-buffered saline (PBS) solution and cell culture media (α-minimum essential medium (MEM) solution). The Tz-Nb reaction was shown recently to be effective chemistry for preparation of injectable hydrogels capable of 3D cell encapsulation[30] while the nucleophilic thiol-yne addition was reported to proceed efficiently in pH 7.4 PBS solution.[51,52] Our investigation of gel formation by Tz-Nb cross-linking showed large pores with a diameter of ca. 100 μm were formed within the gel structure (Supporting Information Figure S1); this was not observed in hydrogels cross-linked by the thiol-yne reaction and was assumed to be due to the release of the nitrogen side-product from the Tz-Nb reation. While formation of a small amount of nitrogen is harmless in biological systems, the presence of nitrogen bubbles within the resultant hydrogel (Supporting Information Figure S1A) will ultimately affect its mechanical integrity under swelling and compression/stretching. We therefore chose the Tz-Nb cross-linking for the formation of the loose network, which is composed of a norbornene-functionalized chitosan (**1**) and a linear polyethylene glycol (PEG)-ditetrazine (**2**), and optimized the concentration of the tetrazine functional groups in the mixture so that no visible nitrogen bubbles formed in the hydrogels. The dense network was therefore constructed from cross-linking a 4-arm PEG-tetraalkyne (**3**) and a linear PEG-dithiol (**4**). We were able to prepare all the precursors with functional groups via one-step modifications of the readily available biocompatible polymers including PEG and chitosan.

The DN gels were formed rapidly in either PBS pH 7.4 or MEM solutions at ca. 90 wt % water content within 3 min after mixing the precursor solutions. This simple preparation procedure allows the hydrogels to form in any mold having desirable shape and to potentially be used as injectable materials. The gelation kinetics followed by rheology (Supporting Information Figure S2) revealed that the gelation times, i.e., the time when the storage moduli reach equilibrium values, of both SNs were quite similar (ca. 5 min), but more rapid gelation in PBS pH 7.4 was observed for the formation of the DN, with complete gelation at ca. 3 min. The DN gel also had a higher storage modulus compared to either SN gel at the point of complete gelation. The resultant DN gel had a compressive stress of 15.56 ± 0.51 MPa at ca. 98% compression without fracture, while the compressive stress of the dense network was 2.49 ± 0.77 MPa and the compressive stress value of the loose SN was only 0.13 ± 0.02 kPa (Figure 2A). In addition, the DN hydrogel could be stretched to ca. 6 times its initial length and displayed a maximum tensile strength of 220 ± 14 kPa while the dense SN gel could only stretch to 1.5 times its original length with a maximum tensile strength of 44 ± 6 kPa (Figure 2B). The high mechanical strengths of our DN hydrogels are comparable to those of tough DN hydrogels reported previously; importantly, however, our hydrogels are prepared in a single step rather than through several polymerization and purification steps.[35−37,45] DN hydrogels prepared in α-MEM solution showed similar mechanical properties to hydrogels prepared in PBS pH 7.4 which suggests that the cross-linking is unaffected by the components present in the α-MEM culture medium. Notably, the DN gels reported herein can undergo cyclic compression at 95% compression [Figure 2C and Supporting Information Movie S1 (ja511681s_si_001)] and extension to 5.5 times their original length [Supporting Information Figure S3 and Movie S2 (ja511681s_si_002)] without hysteresis which indicates a very high level of elasticity. This is completely different from the conventional DN hydrogels that are prepared by free radical cross-linking which undergo large strain hysteresis after the first compressive loading and unloading cycle.[41] The high degree of recovery after deformation in our hydrogels can be attributed to the high degree of homogeneity in the hydrogel structure which was formed through step-growth polymerization[53,54] in contrast to the heterogeneous free radical DN hydrogels, formed through addition polymerization. The fast and complete recovery under continuous stress observed in our hydrogels is highly important for application as matrices for the repair of load-bearing soft tissues where the materials are constantly subjected to rapid deformation and release.

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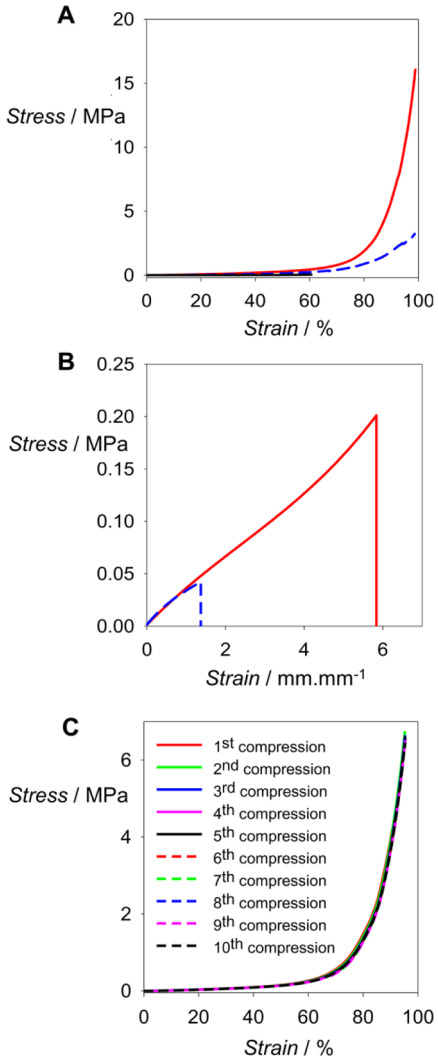

Figure 2. (A) Representative compression curves of dense SN gel (dash blue line), loose SN gel (black line), and DN gel (red line). (B) Representative elongation curves of dense SN gel (dash blue line) and DN gel (red line). (C) Cyclic compression test of DN gel undergoing 10 cycles of compression to 95% strain.

The high strength of our DN hydrogels could only be realized by having all 4 precursors in the mixture; any 3-component mixture resulted in hydrogels with a mechanical strength similar to that of the SN hydrogels. In addition, when only chitosan-norbornene (**1**) was mixed with the dense SN gel precursor mixture (**3** + **4**), a cloudy gel resulted which was a consequence of the chitosan precipitating out of the gel (Supporting Information Figure S4C). Similarly, when PEG-ditetrazine (**2**) was mixed with the dense SN precursors (**3** + **4**), it remained intact, indicated by the retention of the intense pink coloration of the gel (Supporting Information Figure S4B). The loose SN (**1** + **2**) did not form when either one component of the dense SN (**3** or **4**) was present in the precursor mixture. The DN hydrogel prepared by mixing all 4 components is transparent and does not retain the pink color from the tetrazine functional group (Supporting Information Figure S4D). These observations suggest that both networks are necessary to embed the chitosan within the DN hydrogel and are an essential aspect of the enhancement in hydrogel mechanical strength. Further examination of the morphology of the hydrogels by cryo-SEM (Supporting Information Figure S5) revealed that the DN hydrogel contains a morphology that most closely resembles the highly packed small pore regions of the dense SN hydrogel, although these are likely to be intertwined with larger porous structures observed in the morphology of the loose SN hydrogel.

The ability to readily tune the chemical and physical properties of the precursors presents an easy-to-use and versatile hydrogel platform. For example, the chitosan-norbornene precursor can be replaced by other norbornene-functionalized polysaccharides, such as alginate or dextran, in addition to the synthetic norbornene-functionalized poly(vinyl alcohol) which all yield DN hydrogels with comparable mechanical behaviors (Supporting Information Figure S6). In addition, such material substitutions allow the ready tuning of the physical properties of the hydrogel materials. Chitosan and alginate, being inherently cationic and anionic, respectively, on the basis of the pH of the environments also induce pH responsive swelling of the resultant DN hydrogels (Figure 3A).

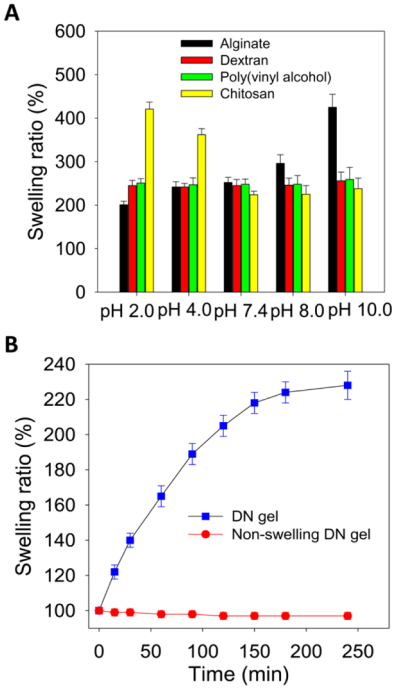

Figure 3. (A) Swelling of hydrogels with different components in the loose network while keeping the same dense PEG network at ambient temperature and different pH. (B) Swelling kinetics of DN hydrogels with PEG dense network (blue square) and thermally responsive PEG−PPG−PEG (red circle) network at 37 °C. Hydrogels were first prepared at ambient temperature with a water content of 90 wt % before being allowed to swell in deionized water at 37 °C.

This can be seen by the higher swelling ratio of the DN hydrogel containing chitosan in acidic pH (swelling ratio of 425 ± 15 at pH 2) compared to neutral and basic pH (swelling ratio of 259 ± 14). In contrast, DN hydrogels containing alginate swelled more in a basic environment (swelling ratio of 421 ± 8 at pH 10) compared to neutral and acidic pH (swelling ratio of 251 ± 5). Further modification of the dithiol component in the dense SN (**4**) enabled ready modification of the swelling and degradation properties of the DN hydrogels. Replacing this component with a thermoresponsive PEG−poly(propylene glycol)−PEG−dithiol resulted in a DN hydrogel that was thermally responsive and as such did not swell at elevated

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temperature due to the shrinkage of thermoresponsive segment[55](Figure 3B) thus retaining not only its water content at 37 °C but also its mechanical properties. The swelling and subsequent reduction in mechanical strength is a common phenomenon in most hydrogels including the high strength DN gels.[35,38,40] Therefore, suppression of the swelling maintains the mechanical strength of the hydrogels even when exposed to aqueous solutions. Furthermore, the degradability of the DN hydrogel can be controlled by selective incorporation of the ester functionality in the PEG-dithiol precursor. In particular, we replaced the ester group in the PEG-dithiol precursor with ether groups (Supporting Information Figure S7) to produce a DN hydrogel that displayed greater stability against hydrolytic degradation as demonstrated by no additional swelling once equilibrium was reached.

The very low cross-linking density that is required to form the loose SN leaves residual unreacted norbornene groups within the DN hydrogel that can be used for postfunctionalization of the material. To demonstrate this, we carried out postfunctionalization of hydrogels by radical thiol-ene addition with a fluorescent BODIBY-SH dye and Tz-Nb addition with a tetrazine-functionalized-biotin which can be subsequently bound to a fluorescently labeled streptavidin. The strong green and red colors observed under fluorescence microscopy imaging demonstrated the success of these approaches (Supporting Information Figure S8A,B). Notably, radical thiol-ene conjugation was not successful on the dense SN hydrogel, confirming that, as previously reported,[52] the thioether product from thiol-yne addition is stable against second thiol addition by photoinitiation and that the Nb functionality is the main reactive group toward radical thiylation. Photoconjugation of the DN hydrogel via radical thiylation also allows spatially controlled addition.[19] For example, the hydrogel can be photopatterned with BODIBY-SH using a TEM grid as the photomask to allow dye attachment only to the UV exposed area (Figure 4A and Supporting Information Figure S8C). Additionally, biotin conjugation via Tz-Nb can be carried out on the photopatterned hydrogel to allow dual functionalization (Supporting Information Figure S8C) of the same gel which in turn demonstrates the temporal control over postfunctionalization of our DN hydrogel.

The bioorthogonalilty of both click additions applied for cross-linking of these tough hydrogel materials also enabled the encapsulation and culture of human cells in our DN hydrogels. Human mesenchymal stem cells (hMSCs) were used as a model cell type because of their extensive utilization in tissue engineering applications. Uniform cell distribution and excellent cell viability (>99%; Figure 4B) was confirmed throughout the DN hydrogels using confocal microscopy on live/dead stained 3D-cultured hMSCs 48 h postencapsulation, thus demonstrating the cytocompatibility of the dual cross-linking reactions. This experiment shows that extra bioactive factors (e.g., growth factors) capable of controlling or directing cell phenotype and function can be attached to our DN hydrogel under physiological conditions in a temporally controlled manner.

CONCLUSION

We have demonstrated that the novel application of orthogonal click reactions results in a simple approach to the preparation of high strength DN hydrogels under physiological conditions from polymer precursors with easily accessed synthetic functionalities. The resulting DN hydrogels have high mechanical strengths and are suitable to be used as a versatile biocompatible platform, across a range of applications, including in (stem) cell studies and tissue engineering/regenerative medicine.

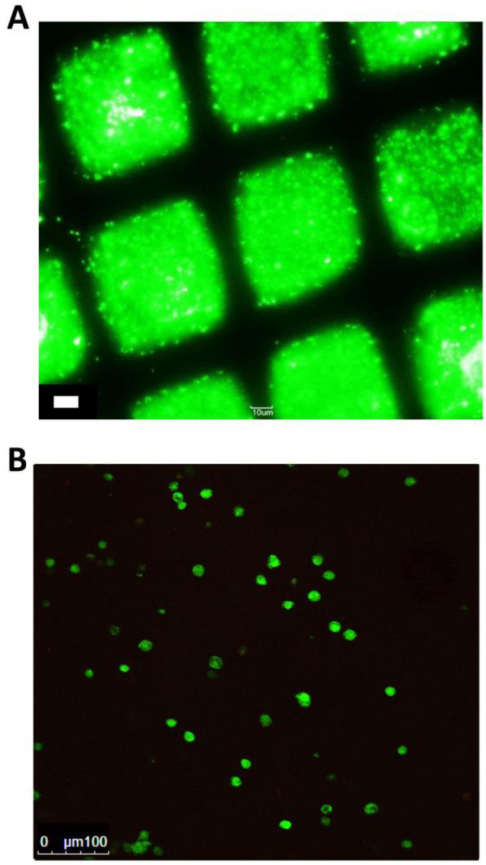

Figure 4. (A) DN hydrogel after postgelation photopatterning with BODIBY-SH by radical thiylation; scale bar =10 μm. (B) Representative live/dead image of hMSCs 48 h postencapsulation (green cell = alive, red cell = dead); scale bar = 100 μm.

ASSOCIATED CONTENT

Supporting Information

Experimental details, additional data, spectra, and movies of DN hydrogels. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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