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Strain-Promoted Cross-Linking of PEG-Based Hydrogels via Copper-Free Cycloaddition

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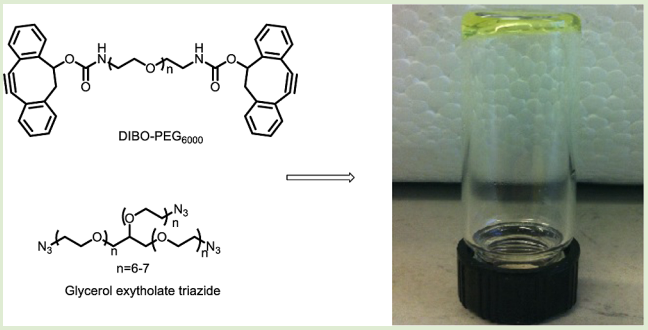
Supporting Information

ABSTRACT: The synthesis of a 4-dibenzocyclooctynol (DIBO) functionalized poly(ethylene glycol) (PEG) and fabrication of hydrogels via strain-promoted, metal-free, azide−alkyne cycloaddition is reported. The resulting hydrogel materials provide a versatile alternative to encapsulate cells that are sensitive to photochemical or chemical cross-linking mechanisms.



Polymeric hydrogels are used widely in a number of regenerative medicine applications.[1−3] Hydrogels are ideal materials for implantation because they generally introduce low levels of foreign matter into the host and afford high levels of metabolite and biomolecule transport.[4] Injectable hydrogels that form in situ hold additional promise as they are able to adapt to complicated defect sites relative to preformed hydrogels.[5] The cross-linking mechanisms in hydrogels can be either chemical or physical.[6−16] Both have advantages and disadvantages.[4,12,17] The onset of gel formation in covalently cross-linked hydrogels is typically dependent on chemical or photochemical processes to initiate network formation from monomeric precursors. Chemical methods include various click reactions,[18] thiolene additions,[7] metal-catalyzed azide−alkyne cycloadditions,[6,19] Michael additions,[20] and Diels−Alder reactions.[14] However, the use of radical initiating systems, ultraviolet (UV) light, and the presence of residual metal catalysts, organic solvents, and the incomplete conversion of the functional groups may lead to biocompatibility problems in sensitive cell types. Because many chemical cross-linkers are toxic and result in noninjectable gels, physical hydrogels are preferred for many biomedical applications. However, the assembly of polymers into physical hydrogels for cell encapsulation typically requires the use of an external trigger including a change in pH, temperature, or ionic concentration to induce gelation.[4,16,21−24] The experimental demands for each of these gelation and functionalization strategies places distinct constraints on the utility and versatility of the respective hydrogel and make direct clinical translation very difficult.

Metal-free, strain-promoted azide−alkyne "click" cycloaddition reactions have been applied to cell imaging[25−27] as well as hydrogel systems due to its highly efficient conversion, orthogonality, and biofriendly characteristics.[9,11,13] In this work, we describe the fabrication of a covalently cross-linked hydrogel via a strain-promoted cycloaddition reaction between 4-dibenzocyclooctynol (DIBO) functionalized poly(ethylene glycol) (PEG) and a three-arm glycerol exytholate triazide. The DIBO-functionalized PEG was synthesized using a 4-nitrophenyl chloroformate activated 4-dibenzocyclooctynol and poly(ethylene glycol) bis-amine. ^{1}H NMR (Supporting Information (SI)) and matrix-assisted desorption ionization time-of-flight (MALDI-TOF; Figure 1) results demonstrate the complete conversion of the amine group to dibenzocyclooctynol. Concentration control is critical to achieving complete conversion.

Glycerol exytholate triazide was synthesized using glycerol exytholate as the starting material. The hydroxyl groups were derivatized with methane sulfonyl chloride and further substituted using sodium azide in a two-step process. The desired product was purified by column chromatography and substantiated with ^{1}H NMR (SI) and MALDI-TOF (Figure 2). To demonstrate the gelation of the two precursors in aqueous solution, a DIBO-functionalized PEG was dissolved in 50 μL of ultrapure water, based on the calculation of 1:1 molar ratio for

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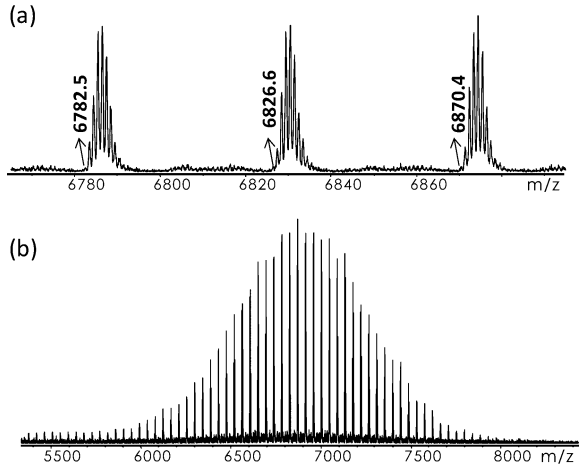


Figure 1. MALDI-TOF mass spectrum of DIBO-PEG (cationized with NaTFA); monoisotopic mass-to-charge ratios (m/z) are marked in the upper spectrum. The measured m/z values agree with the desired structure.

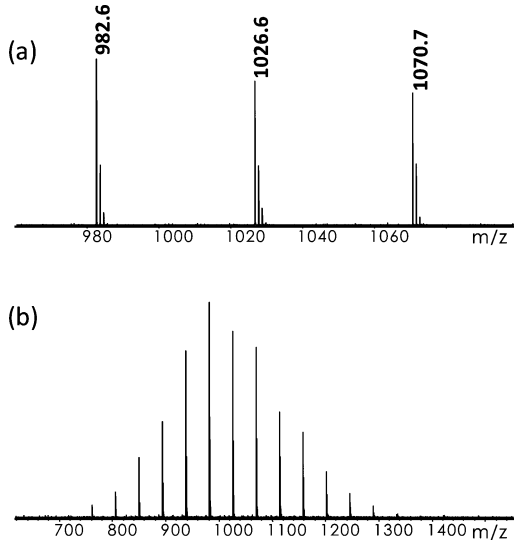
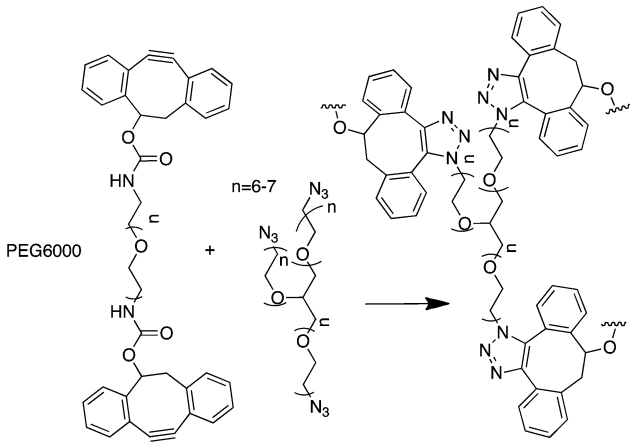


Figure 2. MALDI-TOF mass spectrum of glycerol exytholate triazide (cationized with NaTFA); monoisotopic mass-to-charge ratios (m/z) are marked in the upper spectrum. The measured m/z values agree with the desired structure.

the DIBO group and azide group; 1.1 mg of glycerol exytholate triazide is dissolved in 50 μL of ultrapure water, and these two solutions were mixed. Within 5 min, the solution gelled when agitated gently (Scheme 1). Fourier transform infrared spectroscopy (FTIR) of the freeze-dried hydrogels confirms the disappearance of the azide groups (SI).

To assess whether the gel is suitable for injectable gel applications, an oscillatory shear rheology experiment was performed to study the gelation kinetics under applied mechanical forces (Figure 3). Measurements were made at 24 °C with a TA Instruments ARES-G2 rheometer equipped with 8 mm parallel plates and using a frequency of 10 rad/s (1.6 Hz) and a strain amplitude of 10%. To maintain hydration of the hydrogel sample during the experiment, the lower plate fixture included a solvent trap filled with an aqueous solution of DIBO-PEG and glycerol exytholate triazide (1:1). The large scatter in the data at times less than 600 s is a consequence of the very low viscosity of the solution before sufficient cross-

Scheme 1. DIBO-PEG and Glycerol Exytholate Triazide Form Hydrogel Networks When Mixed in Situ. The Rate of Network Formation Can Be Influenced by the Rate and Amplitude of Strain



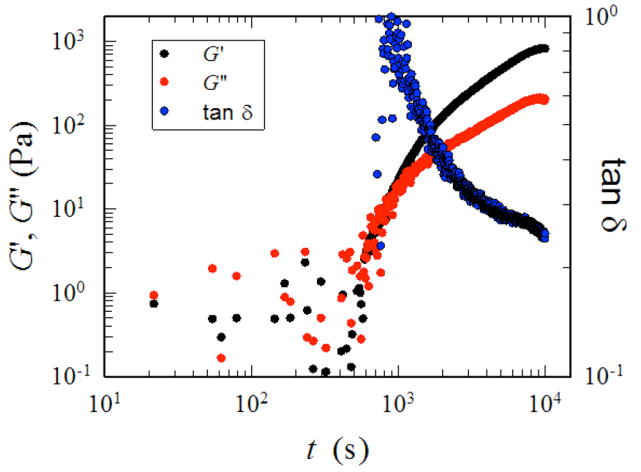


Figure 3. Modulus-time dependence of hydrogels during the process of oscillation shear.

linking was achieved. As a consequence, the torque values are too low to obtain reliable readings from the force transducer. However, after 600 s the torque magnitude was sufficiently high, and both G' (the elastic part of the complex modulus, G^*) and G'' (the viscous component of G^*) increased exponentially with time.

At the beginning of the experiment, the solution is a viscous liquid, and it is expected that $G'' > G'$. The crossover of G' and G'' near $t \sim 1000$ represents the gel point, where the cross-linking reaction has proceeded sufficiently in that the material transforms from a viscous liquid to a viscoelastic solid.[28] After about 2.5 h, the dynamic moduli reached equilibrium values, indicating that the cross-linking reaction was complete. The gel was fully hydrated throughout the experiment, and at the end of the reaction the gel contained 96.1% water and the plateau modulus, GN', was ~0.8 kPa. This corresponds to a cross-link density of 8.6 mol/m^3, which was calculated using the classical theory of rubber elasticity. The loss factor tan δ (G''/G') decreased from a value of ~1, which is typical of a viscous liquid to a value of ~0.25 at the end of the reaction, which is consistent with the formation of a viscoelastic solid (tan δ is zero for an elastic solid). This reaction was also shown to be strain-sensitive.

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Human mesenchymal stem cells (hMSCs) were used to test the influence of the hydrogel cross-linking on cell viability (Figure 4). hMSCs (passage 5, 500 000 cells, Lonza, Basel, Switzerland) were suspended in 25 μL of 20 wt % DIBO-PEG in α-minimum essential medium (MEM) basal media (Lonza). A portion of 25 μL of 2.56 wt % glycerol exytholate triazide in α-MEM basal media was added to the cell suspension. The samples were mixed via gentle pipetting and placed in a mold to solidify. After 5 min, the hydrogels were transferred by spatula to a 12-well plate and cultured in α-MEM basal media for 24 h in a 37 °C, 5% CO_2 incubator. A live–dead assay (Invitrogen, Carlsbad, CA) was performed. A sample of 1.5 μL of 1 mg/mL calcein AM and 0.1 μL of 2 mM ethidium homodimer-1 was added per milliliter of culture media, and the samples were incubated for 10 min. The samples were then washed with media and viewed on a 1 × 81× microscope (Olympus, Center Valley, PA). The encapsulation of cells and presence of media additives did not affect the gelation process. The dominant green fluorescence from live cells in the live–dead cell staining after 24 h of culture demonstrates the excellent biocompatibility of these hydrogels (89% ± 2%). Triarm molecules not converted to the azide do not gel as expected, and hMSCs mixed with the individual components do not exhibit toxicity (data not shown) under identical concentration conditions.

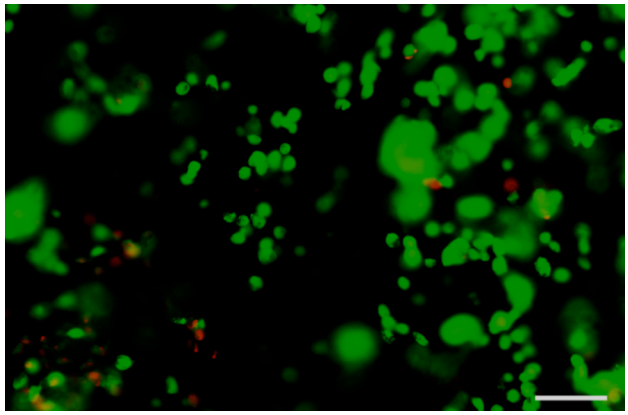

Figure 4. A live–dead assay was used to assess the viability (89% ± 2%) of human mesenchymal stem cells that were cross-linked in situ and cultured in hydrogels for 24 h. Live cells are stained green, and dead cells are stained red. The scale bar is 50 μm.

In conclusion, we have made a covalently cross-linked hydrogel that is versatile and biocompatible based on a metal free, strain-promoted azide–alkyne cycloaddition process. These results demonstrate an effective strategy to encapsulate cells within hydrogels where gelation is based on the cumulative effects of specific molecular-recognition interactions between strained cyclocotyne units and azide terminated PEG chains. The potential variability in the molecular mass of the respective components, number of branch units, and compatibility with both hMSCs and cell media provides versatility in applications where syringe injectable materials or in situ formation of hydrogels are necessary.

■ ASSOCIATED CONTENT

Supporting Information

Details about the synthesis of DIBO, DIBO-functionalized PEG, and the rheology experiments. 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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