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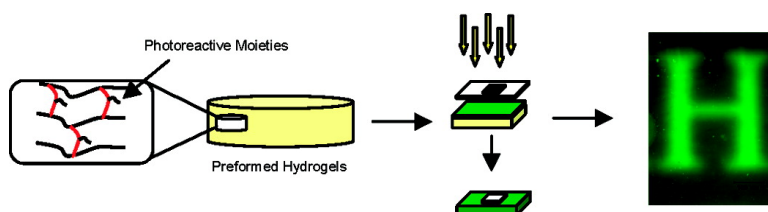
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Three-Dimensional Biochemical Patterning of Click-Based Composite Hydrogels via Thiolene Photopolymerization

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Hydrogels formed from (meth)acrylated poly(ethylene glycol) precursors are commonly used in a variety of biomedical applications ranging from tissue engineering to biosensors. While this approach has proven quite diverse, a major limitation to this approach is the heterogeneities and nonidealities that arise in the gels from the chain polymerization process, which increases the difficulty in relating the network structure to the final physical properties of the gel. Here we have exploited the specificity and fidelity of the [3+2] cycloaddition reaction to synthesize hydrogels with controlled architectures and improved mechanical properties. Moreover, we demonstrate a general approach toward the integration of multifunctional photoreactive polypeptide sequences into the network structure that provides a facile way to independently tune the 3D chemical and physical properties of the gel. Standard photolithographic techniques were used to generate a variety of two- and three-dimensional patterns as well as controlled biochemical gradients within existing preformed hydrogels.

Biomedical applications ranging from drug delivery to biosensors to tissue engineering rely on the ability to tune and control the properties of poly(ethylene glycol) (PEG) hydrogels. In many instances, researchers rely on the photoinitiated chain polymerization of (meth)acrylated PEG precursors to create gels with various material properties and pendant functionalities. While this approach has proven quite diverse, a major limitation to this synthetic route is the heterogeneities and nonidealities that arise in the PEG gels from the chain polymerization process, which influence many of the final material properties. For example, Bryant et al. has shown how these heterogeneities lead to differences in cell morphology under applied loads.¹ As a result, approaches to create ideal PEG gel structures are emerging, especially those based on highly selective and orthogonal reactions that proceed with high efficiency under a variety of mild conditions.

Recently, several reports have demonstrated the formation of hydrogels via the copper(I)-catalyzed [3+2] cycloaddition reaction between azides and terminal acetylene moieties.^{2–4} These studies were motivated by the fact that the copper-catalyzed reaction is highly specific, quantitative, and tolerant to a variety of functional groups under physiological conditions.^{5,6} In particular, Malkock et al. demonstrated that PEG hydrogels formed via this Click chemistry resulted in highly cross-linked gels with ideal structures and dramatically improved mechanical properties when compared to the traditional chain polymerized PEG-based systems.³ For instance, only 0.2% unreacted functional groups remained after gel formation, thus suggesting the efficiency of network formation and the establishment of a nearly ideal structure.³ Furthermore, the tensile stress and tensile strain of Click hydrogels were found to be approximately 34-fold and 10-fold greater than the chain polymerized PEG gels, respectively.³ The improved mechanical properties of the Click gels were attributed to the controlled nature of the cross-linking

reaction, which leads to a more even distribution of cross-link junctions. Beyond controlling network structure, Malkock also demonstrated the ability to tune the chemical and physical properties of the gels by creating small variations in the azide/acetylene ratio.³ This stoichiometric imbalance resulted in either unreacted azide or acetylene groups, which could subsequently be used to immobilize other molecules into the gel network.

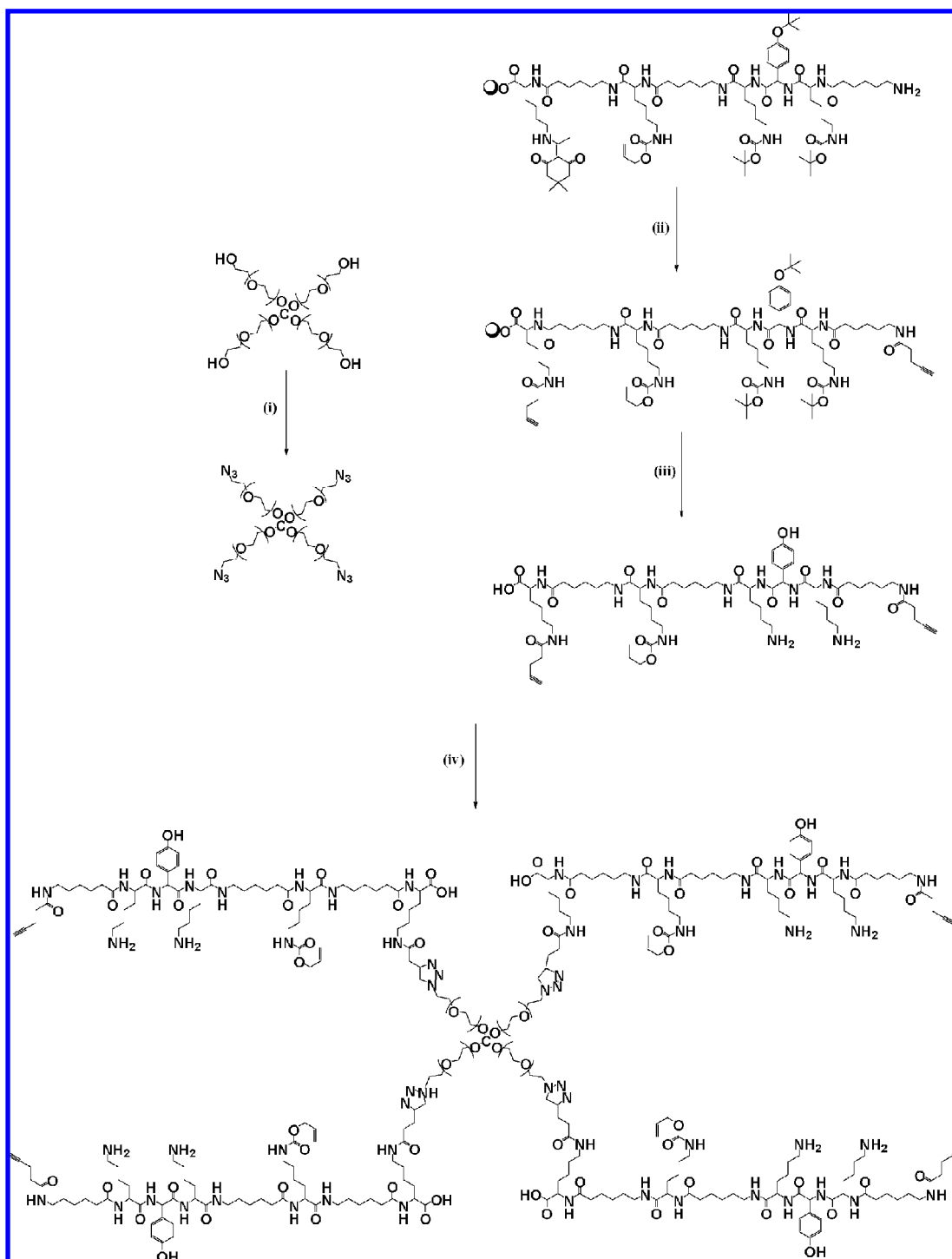
With this foundational work in mind, we sought to exploit the specificity and fidelity of the Click reaction with an orthogonal reaction to functionalize the gels with moieties of biological importance, specifically polypeptides, in an approach that would allow independent control of the network chemical and physical properties. Beyond controlling these properties homogeneously, a photochemical reaction was exploited to allow the generation of spatially complex patterns and gradients in three dimensions, as required by many biological systems. For example, West and co-workers created uniform and freeform 3D patterns within preformed poly(ethylene glycol)-diacrylate (PEGDA) hydrogels via multiple photolithographic techniques and illustrated how 3D cell migration was confined to peptide patterned channels.^{7,8} However, this strategy was based on the same synthetic approaches that lead to heterogeneous PEG gel structures and the patterning process itself further alters the base gel structure.⁹ As such, we sought to develop alternative chemistries that would permit the synthesis of well-defined cross-linked hydrogels in which one could independently control the physical and chemical properties of the material in 3D.

In this communication, we exploit the specificity and fidelity of the Click reaction to synthesize hydrogels with controlled architectures and improved mechanical properties. Uniquely, we demonstrate a general approach toward the integration of multifunctional photoreactive polypeptide sequences into the network structure that provides a facile way to independently tune the 3D chemical and physical properties of the material, which is important for applications directed at controlling cell interactions and cell function. Specifically, we utilized a tetraazide-multiarm PEG and diacetylene-functionalized allyl ester containing polypeptides to generate well-defined PEG-

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Scheme 1. Modular Approach for the Synthesis of Preformed Hydrogels via the [3+2] Cycloaddition Reaction^a

^a Reagents and conditions: (i) mesyl chloride/DCM followed by NaN_3 /DMF; (ii) 2% hydrazine monohydrate/NMP followed by treatment with 4-pentynoic acid/HATU/DIEA; (iii) treatment with TFA/TIS/water (95:2.5:2.5); (iv) CuSO_4 , ascorbic acid, azide, and acetylene (0.5:5.0:1:1) in PBS at rt.

peptide hydrogels (PEGtides). The synthesis of tetraazide-functionalized PEG and the diacetylene-functionalized allyl ester polypeptides is shown in Scheme 1.

Activation of the hydroxyl groups on the multiarm PEG (10K) with mesyl chloride in DCM followed by nucleophilic substitution with sodium azide in DMF at 80 °C produced the tetraazide functionalized PEG with a degree of substitution ranging from 90 to 95% overall yield (as determined via NMR). The synthesis of the photoreactive peptide cross-link was facilitated via standard solid-phase peptide synthesis using Fmoc protected amino acids and HBTU/HOBt coupling chemistries. The pho-

toactive component was incorporated into the peptide via the use of the commercially available Fmoc-K(Alloc)-OH amino acid. Although originally designed as an orthogonal protecting group for lysine, the alloxycarbonyl contains a vinyl functional group, which we exploited in a second polymerization mechanism for chemical modifications without influencing the ideal network structure. Specifically, a thiol-ene photocoupling reaction was used to react the alloxycarboxyl with any cysteine containing compound.^{10–13} Also, the Alloc protecting group is stable to Fmoc deprotection and TFA cleavage, making it an ideal candidate as the photoreactive component of our PEGtide

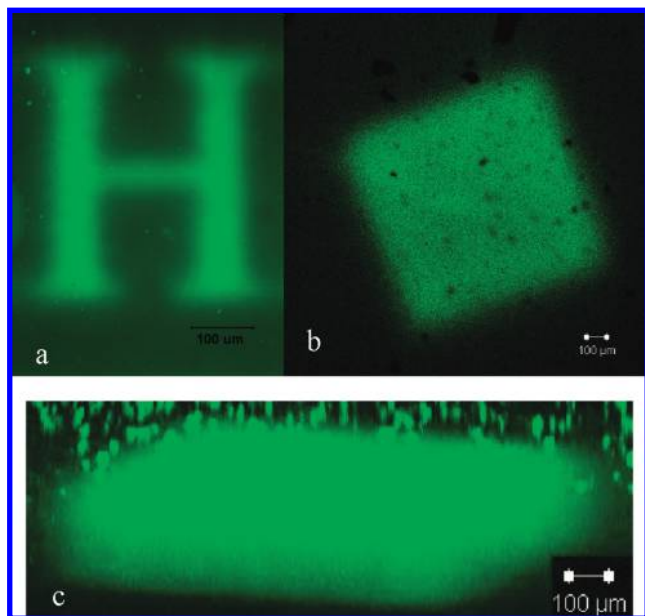


Figure 1. 3D patterns formed within preformed hydrogel networks via transparency-based photolithographic patterning. (a) Fluorescent and (b) confocal images of click-based PEGtide hydrogels patterned with fluorescently labeled RGDSC. (c) Image z-stacks, (b) 270 μm thick, were acquired at 10 μm intervals and then projected into single plane image. Scale bar is 100 μm in all images.

hydrogel. Incorporation of the diacetylene functionality was accomplished by placing Fmoc-Ahx-OH and Fmoc-K(Dde)-OH at the N- and C-termini, respectively. After the final Fmoc deprotection, the resin was treated with a solution of hydrazine monohydrate in DMF (to selectively remove the Dde protecting

group). This step exposes two primary amines, one at both termini, which were subsequently reacted with a preactivated solution of 4-pentynoic acid. Finally, the peptide was cleaved from the resin via treatment with TFA and purified via RP-HPLC to yield the final product in 60% overall yield.

Hydrogel formation was facilitated via reaction of the PEG-tetraazide with 2.0 equiv of the photoreactive cross-link at room temperature with 0.5 equiv of copper sulfate pentahydrate and 5.0 equiv of sodium ascorbate. Under these conditions, hydrogels were formed within minutes as determined via dynamic time sweep rheology experiments (Supporting Information). Additionally, hydrogel formation was shown to occur via a step-growth mechanism as predicted by the statistical gelation model for stepgrowth networks developed by Flory and Stockmayer (Supporting Information). These results suggest that the network formation is close to ideal, as suggested by Malkock et al.

The ability to tailor the 3D microenvironment within the PEGtide hydrogels was demonstrated via reaction of the Alloc moiety (tethered to the diacetylene-peptide cross-link) and a fluorescently labeled cysteine containing peptide (Alexa Fluor 488-AhxRGDSC, Supporting Information) via thiol-ene photocoupling using previously reported photolithographic methods.^{8,14} Figures 1a,b demonstrate representative 2D patterns created on PEGtide hydrogels via thiol-ene photocoupling and transparency-based photolithography. Closer inspection of the figures reveals that the feature sizes of the photomasks are preserved during the patterning process. Analysis of Figure 1b with ImageJ image analysis software (NIH, Bethesda, MD) revealed the average dimension of the photopatterned square to be approximately $1050 \pm 50 \mu\text{m}$, compared to the photomask which had an absolute dimension of 1000 μm . Furthermore, these gels support the facile diffusion of small molecules into the bulk which enables the production of 3D patterns of uniform cross

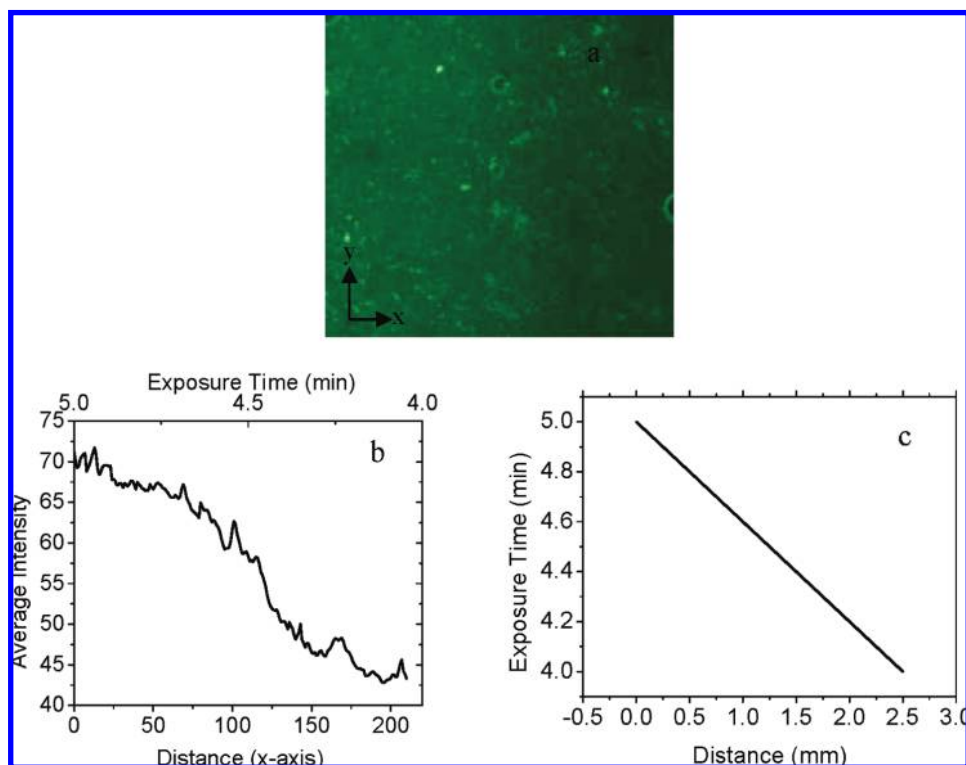


Figure 2. (a) Fluorescent microscopy image of biochemical gradient generated using Click-based PEGtide hydrogels via thiol-ene photocoupling. The length of the displayed image is approximately 2.5 mm. (b) Plot of the average intensity as a function of distance and exposure time. The data clearly demonstrates the formation of a biochemical gradient within preformed Click-based PEGtide hydrogels. (c) Exposure time as a function of distance. As is clearly evident, a longer exposure time (5 min) results in the greatest average intensity.

section that span the thickness of the hydrogel. Inspection of Figure 1c reveals that the z-dimension (thickness) of the 3D patterned region was approximately $230 \pm 15 \mu\text{m}$, as determined via ImageJ. These results clearly demonstrate the validity of the described approach toward the development of highly tailorable cross-linked networks of defined chemical and physical structure.

Cellular migration in response to biochemical gradients is perhaps one of the most widely studied biological processes and is known to play a critical role in tissue development, inflammation, wound healing, and angiogenesis.¹⁵ To demonstrate the feasibility of our approach to generate biochemical gradients with the potential of guiding cell migration, preformed hydrogels were patterned with fluorescently labeled RGDSC using a programmable linear motion stage that drove an optically opaque coverplate across the sample (Supporting Information). With the thiol-ene chemistry, gradient gels can be synthesized by creating gradients in either the reactants or the initiating light source. Figure 2a shows a fluorescent microscope image of the resultant RGDSC gradient. Analysis of the image using ImageJ clearly shows that as one travels across the x-axis (left to right) the average intensity decreases suggesting a lower degree of tethered fluorescently labeled cysteine containing peptide. To the best of our knowledge, this is the first system capable of producing well-defined biochemical gradients within ideal networks (Supporting Information).

We have presented a new approach toward the development of patterned materials via controlled two- and three-dimensional patterning within existing preformed hydrogels with controlled architectures. The use of multiple chemistries, click chemistry for gelation and thiol-ene photocoupling for complex patterning, allows one to independently tune the materials physical and biochemical properties. For instance, gelation via the Huisgen [3+2] cycloaddition reaction proceeds via a step-growth mechanism, thus resulting in a nearly ideal cross-linked network. However, varying specific parameters such as PEG molecular weight or the molar ratios of the azide to acetylene allows one to tailor the physical properties of the hydrogel to a specific application. Furthermore, the use of thiol-ene photocoupling to generate biochemical gradients is highly controlled and results in a well-defined number of conjugated moieties. Here we have demonstrated the orthogonality of the two step gelation and patterning process, which is readily amenable to many photofabrication strategies, such as two-photon absorption photolithography, to create more complex chemical patterns in three dimensions.⁷ Such investigations are ongoing and will be reported shortly.

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Abbreviations

PEG, poly(ethylene glycol)
DCM, dichloromethane
DMF, dimethylformamide
FMOC, fluorenyl-methoxy-carbonyl
HBTU, *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate
HOBt, 1-hydroxybenzotriazole hydrate
DIEA, diisopropylethylamine
Alloc, allyloxycarbonyl
TFA, trifluoroacetic acid
TIS, triisopropylsilane
NaN₃, sodium azide
HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Ahx, 6-aminohexanoic acid
Dde, 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene
RP-HPLC, reverse phase high pressure liquid chromatography

Supporting Information Available. Synthesis of select compounds. General procedures for hydrogel formation, photopatterning, and characterization of network structure. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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