Tyrosine-Selective Functionalization for Bio-Orthogonal Cross-Linking of Engineered Protein Hydrogels

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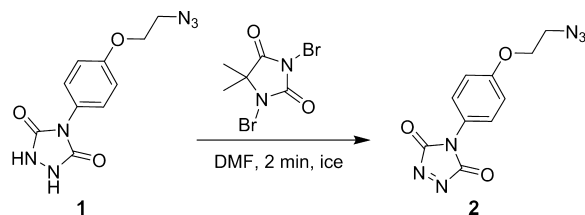
Ⓢ Supporting Information

ABSTRACT: Engineered protein hydrogels have shown promise as artificial extracellular matrix materials for the 3D culture of stem cells due to the ability to decouple hydrogel biochemistry and mechanics. The modular design of these proteins allows for incorporation of various bioactive sequences to regulate cellular behavior. However, the chemistry used to cross-link the proteins into hydrogels can limit what bioactive sequences can be incorporated, in order to prevent nonspecific cross-linking within the bioactive region. Bio-orthogonal cross-linking chemistries may allow for the incorporation of any arbitrary bioactive sequence, but site-selective and scalable incorporation of bio-orthogonal reactive groups such as azides that do not rely on commonly used amine-reactive chemistry is often challenging. In response, we have optimized the reaction of an azide-bearing 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) with engineered elastin-like proteins (ELPs) to selectively azide-functionalize tyrosine residues within the proteins. The PTAD-azide functionalized ELPs cross-link with bicyclononyne (BCN) functionalized ELPs via the strain-promoted azide−alkyne cycloaddition (SPAAC) reaction to form hydrogels. Human mesenchymal stem cells and murine neural progenitor cells encapsulated within these hydrogels remain highly viable and maintain their phenotypes in culture. Tyrosine-specific modification may expand the number of bioactive sequences that can be designed into protein-engineered materials by permitting incorporation of lysine-containing sequences without concern for nonspecific cross-linking.
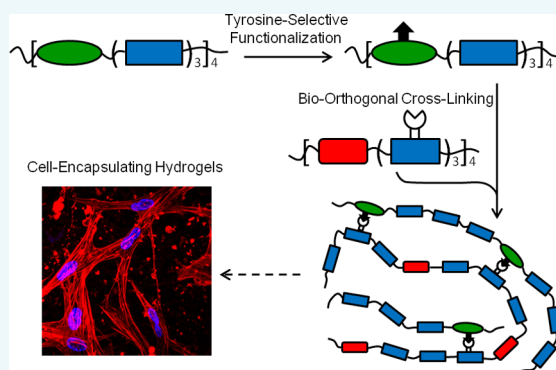
Hydrogels are commonly used as cell-encapsulating materials to study cell−matrix interactions and to serve as scaffolds for tissue engineering applications.[1−3] A variety of synthetic and naturally derived materials are routinely employed to generate cell-encapsulating gels, such as poly-(ethylene glycol) (PEG), collagen, and alginate.[1] While these materials are highly cell-compatible, each has potential drawbacks. Synthetics like PEG are bioinert and must be further functionalized with bioactive molecules to permit cellular behaviors such as spreading and migration.[4−6] On the other hand, collagen is naturally cell-adhesive and degradable, but can suffer from batch-to-batch variability and exhibits limited tuning of mechanical properties.[7,8] To overcome these limitations of commonly used hydrogel materials, protein

Scheme 1. PTAD Oxidation

engineering has been employed to generate hydrogels with the precise control over material chemistry afforded by synthetics and the cell-interactive properties of many naturally derived materials.[9]

Elastin-like proteins (ELPs) are a class of protein-engineered materials that are well-suited for use as cell-encapsulating hydrogels. ELPs can be designed as modular proteins that consist of alternating elastin-like and variable domains.[10] The elastin-like domain is based on the elastin-like VPGXG motif and confers elastic mechanical properties on hydrogels prepared from these materials.[11] The guest "X" residue can be any amino acid other than proline, permitting incorporation of various chemical functionalities to this domain, such as primary amines via lysine residues to facilitate cross-linking.[12] The variable domain facilitates incorporation of bioactive sequences to regulate cellular behaviors such as adhesion and matrix degradation.[10,13] A significant advantage of the modular design of ELPs is the ability to decouple tuning of matrix stiffness from access to bioactive cues by restricting cross-
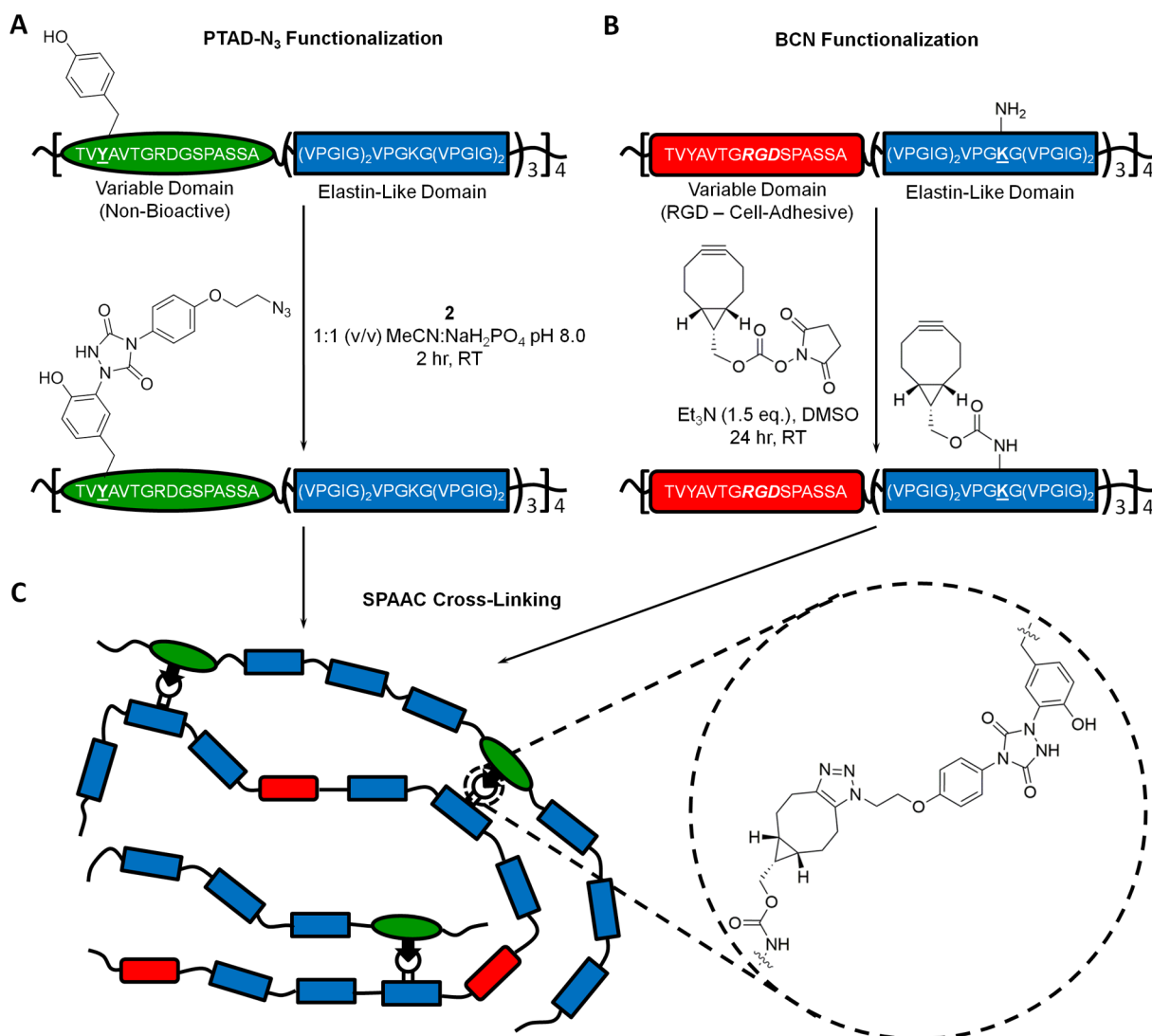
Figure 1. PTAD-N₃ functionalization of ELP for bio-orthogonal cross-linking. (A) Modification of ELP tyrosine residues with PTAD (structure **2**). (B) Modification of cell-adhesive RGD-ELP with BCN. (C) Mixing PTAD-ELP with BCN-ELP results in bio-orthogonal cross-linking via the SPAAC reaction.

linking sites to the elastin-like domain. However, in versions of ELP that rely on amine-reactive cross-linkers to form hydrogel networks, the variable domain is precluded from containing any lysine residues, or else cross-links will form at the bioactive site and potentially block the ability of the cells to interact with this domain. Common bioactive motifs, including the laminin-derived, cell-adhesive sequence IKVAV,[14] the vascular endothelial growth factor mimicking QK peptide (LTWQ-ELYQLKYKGI),[15] and the bone morphogenetic protein-2 mimicking knuckle epitope peptide (KIPKASSVPTELSAIST-LYL),[16] contain lysines and could thus not be incorporated into ELP hydrogels generated using existing amine-reactive chemistries. One potential strategy would be to take advantage of sulfhydryl-based chemistries using cysteine residues engineered into the proteins. However, the ability of the cysteine-containing proteins to cross-link via disulfide bridge formation makes purifying and manipulating such proteins difficult. An alternative strategy would be to use cross-linking chemistries that are not found in biological systems, thereby permitting incorporation of an arbitrary bioactive sequence into the variable domain of the proteins.

We recently reported the design of elastin-like protein hydrogels that cross-link via the bio-orthogonal strain-promoted azide–alkyne cycloaddition (SPAAC) reaction.[17] Due to the bio-orthogonal nature of the cross-linking reaction, gelation of these materials is highly specific, resulting in high viability and phenotypic maintenance of encapsulated cells.[17] This bio-orthogonality also provides the possibility of designing the bioactive site to contain any arbitrary amino acid sequence, because the cross-linking reaction will not interfere with any naturally occurring functional groups. However, the ELPs still must be functionalized with the azide and bicyclononyne (BCN) reaction partners post-expression. In our previous system, this was accomplished by diazo transfer or N-hydroxysuccinamide (NHS) ester-mediated reactions with primary amines on lysine residues within the elastin-like domain to produce azide- or BCN-modified ELPs, respectively. Thus, the initial versions of these bio-orthogonally cross-linked materials still rely on amine-reactive chemistry and would prevent the implementation of lysine-containing bioactive sequences.
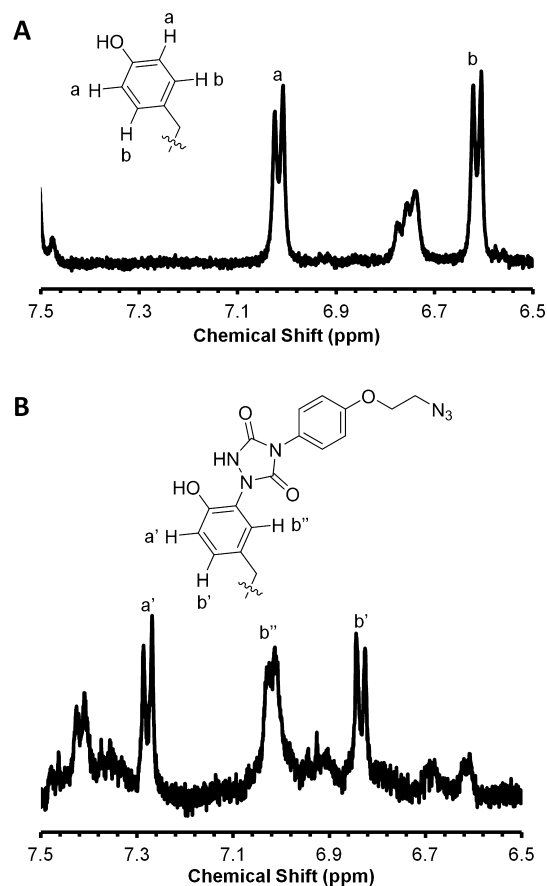
Figure 2. ^{1}H NMR to confirm PTAD functionalization of ELP tyrosines. ^{1}H NMR spectra of (A) unmodified ELP and (B) PTAD-functionalized ELP.

Table 1. Optimizing PTAD Modification of ELP

buffer	solvent	molar ratio PTAD:tyrosine	% tyrosine conversion	modified tyrosines/ELP
Tris	--	4:1	a	a
Tris	DMF	1:1	36%	1.5
Tris	DMF	2:1	54%	2.1
Tris	DMF	4:1	62%	2.5
Phosphate	--	4:1	a	a
Phosphate	DMF	4:1	81%	3.2
Phosphate	MeCN	1:1	25%	1.0
Phosphate	MeCN	2:1	60%	2.4
Phosphate	MeCN	4:1	89%	3.5

[a]Insoluble precipitate formed during PTAD modification reaction.

In order to design a system that would permit the incorporation of lysine-containing bioactive sequences in the variable domain of the ELPs, we sought to employ a chemistry that will specifically functionalize tyrosine residues with azide moieties. Previously, Barbas and colleagues demonstrated selective tyrosine modification via an ene-type reaction using 4-phenyl-3H-1,2,4-triazoline-3,5(4H)-diones (PTADs).[18,19] Here, we employ a commercially available, azide-bearing PTAD, 4-(4-(2-azidoethoxy)phenyl)-1,2,4-triazolidine-3,5-dione (**1**), to functionalize tyrosine residues in ELPs with azides for SPAAC-mediated cross-linking into hydrogels. Oxidation of **1** with 1,3-dibromo-5,5-dimethylhydantoin affords activated PTAD **2** (Scheme 1), which can participate in ene-type reactions with tyrosine side chains.[19]

To demonstrate selective azide incorporation without lysine modification, we reacted tyrosine residues found within the variable region of a nonbioactive ELP with PTAD **2** (Figure 1A). Barbas and colleagues previously demonstrated that the use of Tris buffers in the PTAD modification reaction limited the nonspecific reaction of an isocyanate decomposition product of the oxidized PTAD with primary amines on the proteins.[19] Thus, we first chose to run the ELP modification reaction in 50 mM Tris buffer (pH 7.5). However, addition of PTAD **2** to a solution of ELP in Tris buffer resulted in precipitation of the protein from solution. In order to maintain the solubility of the ELP during the PTAD reaction, a mixed solvent system consisting of a 1:1 volumetric mixture of 50 mM Tris buffer and DMF was used. The ELP remained soluble throughout the reaction following the addition of PTAD **2** at molar ratios from 1:1 to 4:1 PTAD:tyrosine. The extent of tyrosine modification was assessed using ^{1}H NMR (Figure 2, Figure S1). A 4-fold excess of PTAD relative to tyrosine resulted in modification of ~60% of tyrosine residues (Table 1). Work by Barbas and colleagues also demonstrated that PTAD modification was most efficient in phosphate buffers,[18] so we additionally ran the reaction in 100 mM phosphate buffer (pH 8.0) and a 1:1 volumetric mixture of 100 mM phosphate buffer and DMF. As with the pure Tris buffer, the pure phosphate buffer also resulted in ELP precipitation, while the mixed phosphate/DMF system maintained ELP solubility throughout the reaction. Using the mixed solvent system, a 4-fold excess of PTAD resulted in a modification of ~80% of tyrosine residues, which was a noticeable improvement over the Tris buffered condition (Table 1). This increase in reaction efficiency was not due to pH differences between the two buffers employed by Barbas and colleagues in their previous studies,[18,19] as there was no difference in tyrosine functionalization when the PTAD reaction was run at pH 7.5 versus pH 8.0 in a phosphate buffered system (Table S1).

While the phosphate buffer/DMF solvent mixture improved the efficiency of tyrosine functionalization, the removal of the Tris buffer may increase undesired side reactions with primary amines present in the protein. To assess the extent of cross-reactivity with primary amines, the presence of free amines post-modification was measured via the 2,4,6-trinitrobenzene-sulfonic acid (TNBSA) assay.[20] Consistent with previously published results,[18] PTAD modification in Tris-buffered systems resulted in no significant decrease in the number of free amines per ELP polymer (Figure 3A). However, carrying out the PTAD modification reaction in mixed phosphate buffer/DMF resulted in a ~22% decrease in the number of free amines per ELP. We hypothesized that this was likely due to reaction of an isocyanate decomposition product of the activated PTAD with primary amines in the ELP (Figure 3B).[19] The evolution of gas observed upon addition of PTAD **2** to the reaction mixture is consistent with decomposition of the PTAD into an amine-reactive isocyanate species.[19] ^{1}H NMR confirmed that PTAD modification in phosphate buffer/DMF exhibited nonspecific reaction with the primary amines of the lysine residues present in the ELP (Figure 3C). Thus, while the phosphate buffer/DMF system facilitated reasonably high tyrosine modification efficiency, it also permitted significant side reactions with primary amines, limiting the utility of this procedure in selective tyrosine functionalization.

To optimize both tyrosine functionalization efficiency and selectivity in a single system, we considered the use of polar aprotic solvents other than DMF in the reaction mixture.
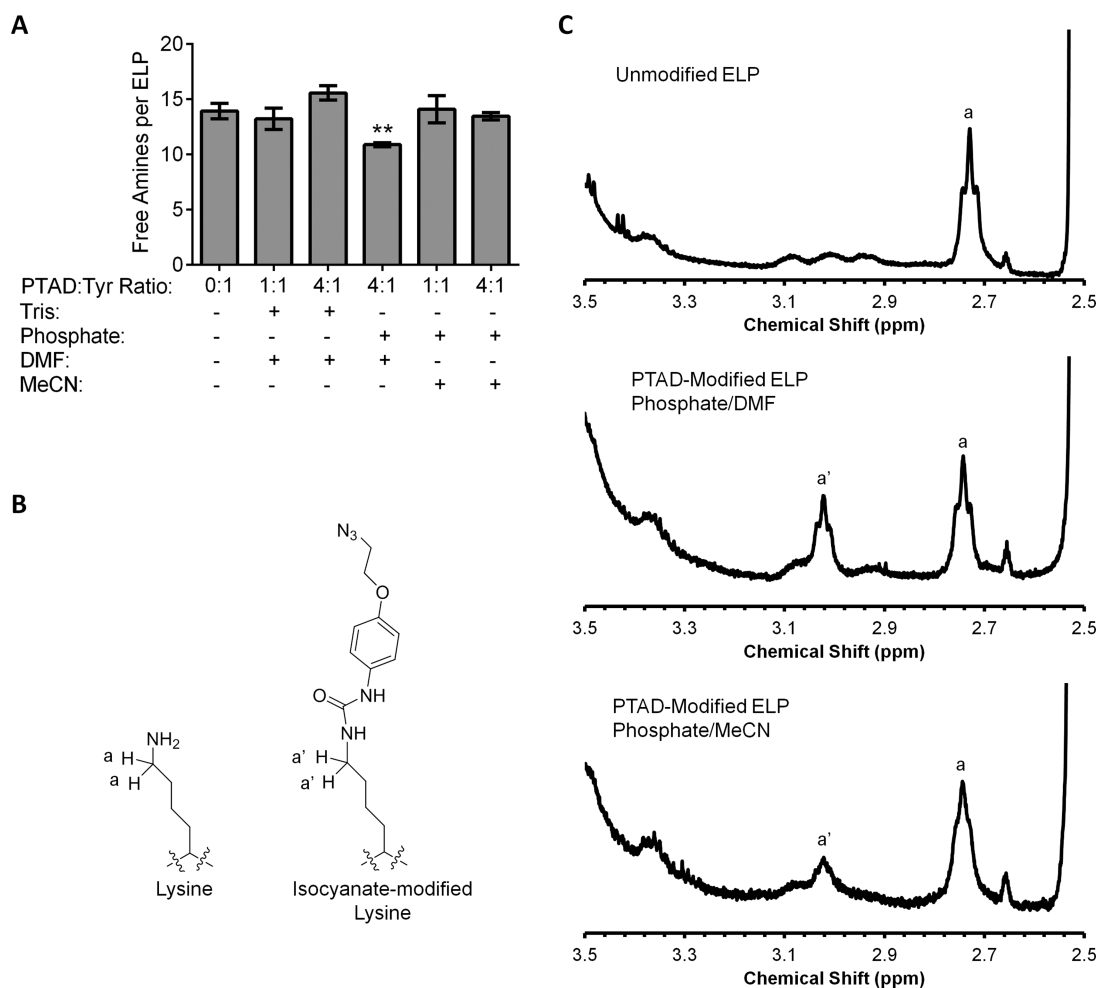
Figure 3. Optimizing solvent conditions to minimize lysine cross-reactivity. (A) Quantification of free amines present on ELPs following PTAD reaction under various solvent conditions. Only reaction in phosphate buffer/DMF exhibited significant cross-reactivity with amines present on the ELPs. **$p < 0.01$ relative to unmodified ELP, one-way ANOVA with Bonferroni post-hoc test. (B) Decomposition of PTAD **2** can generate a reactive isocyanate moiety that reacts with primary amines present on lysines in the ELP sequence. (C) ^{1}H NMR spectra reveal significant lysine modification following PTAD reaction in phosphate buffer/DMF, but only minimal lysine modification in phosphate buffer/acetonitrile.

Barbas and colleagues previously reported successful PTAD functionalization in phosphate buffer/acetonitrile (MeCN) mixtures.[18] ELP was dissolved in a 1:1 volumetric mixture of 100 mM phosphate buffer and acetonitrile, and PTAD **2** was added to a solution of ELP at molar ratios from 1:1 to 4:1 PTAD:tyrosine. A 4:1 PTAD:Tyr ratio resulted in ∼90% modification of the tyrosine residues present in the ELP, an improvement relative to the phosphate buffer/DMF system (Table 1). Furthermore, the phosphate buffer/acetonitrile system was significantly more selective for tyrosines over primary amines. No difference in the number of free amines per ELP polymer relative to unmodified ELP was detected by TNBSA assay following PTAD reaction in phosphate buffer/acetonitrile (Figure 3A). Furthermore, ^{1}H NMR suggested that the large majority of lysine residue primary amines remain unreacted post-PTAD functionalization (Figure 3C). Noticeably less gas evolution was observed in the phosphate/acetonitrile system, suggesting that PTAD decomposition was less prevalent under these reaction conditions. Using PTAD-modified ELPs produced by the optimized reaction conditions, the presence of azide groups in the PTAD-modified ELP for use in SPAAC-mediated cross-linking was confirmed by FT-IR (Figure S2). These results indicate that PTAD-functionalization

in mixed phosphate buffer/acetonitrile is an effective route for selective introduction of azides via tyrosine residues.

In order to facilitate SPAAC-mediated cross-linking of ELPs into hydrogels, a second ELP component functionalized with BCN was prepared by reacting ELP with BCN-NHS, based on our previously published procedure[17] (Figure 1B,C). The molar ratio of BCN:ELP was varied to afford an average of three lysine residues functionalized with BCN per ELP polymer.[17] To permit cell adhesion and spreading within the gels, the variable region within the BCN-modified ELP was designed to contain an extended RGD integrin-binding sequence derived from human fibronectin.[10] Azide-PTAD-functionalized ELP and BCN-functionalized RGD-ELP were separately dissolved in phosphate-buffered saline (PBS) to a concentration of 5% (w/v). Upon mixing in a 1:1 volumetric ratio, gelation occurs within seconds, and the resulting gels reach their plateau modulus within minutes ($\tau_{1/2}$ <1 min) (Figure 4A). Consistent with previous results, the SPAAC-cross-linked hydrogels exhibit predominantly elastic mechanical properties, as assessed by oscillatory rheology (Figure 4B). Hydrogel stiffness can be tuned by varying the polymer content of the hydrogels from 3% to 5% to 10% (w/v), resulting in hydrogels with storage moduli of ∼430 Pa, ∼1200 Pa, and ∼2500 Pa, respectively (Figure 4B).
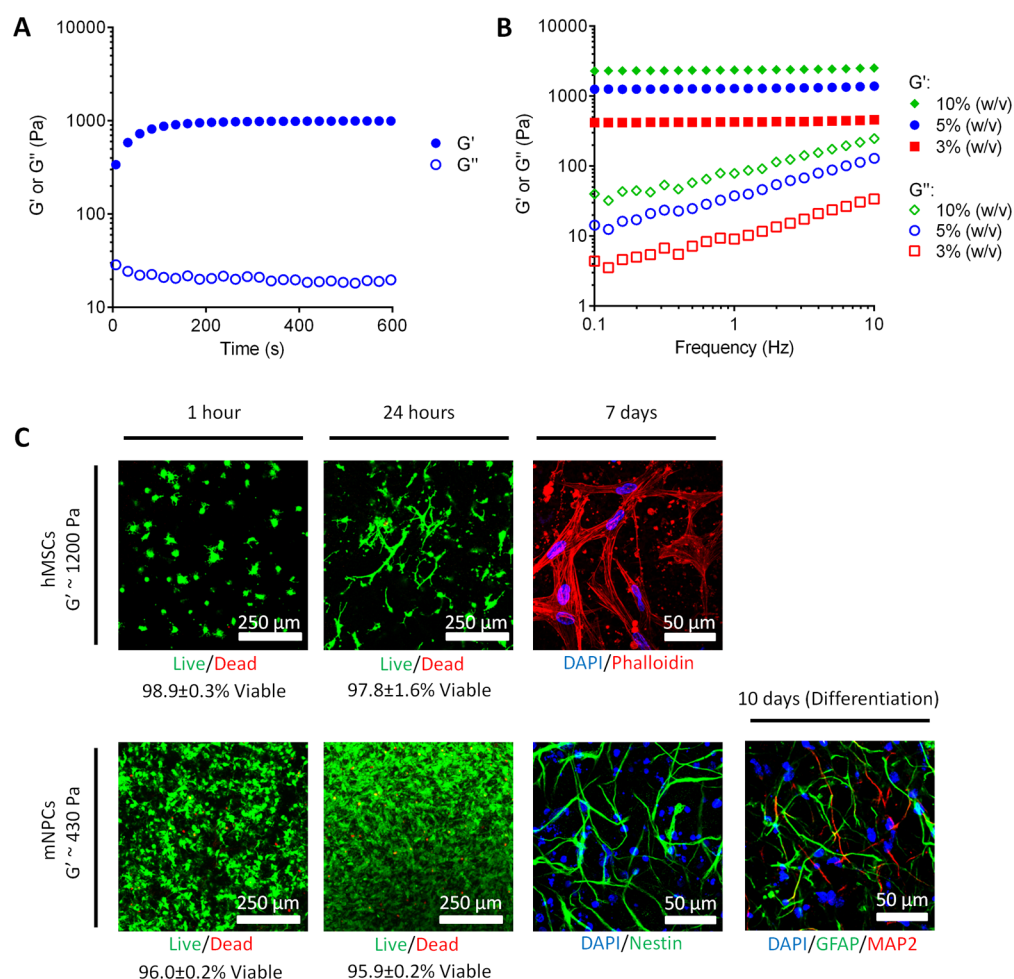
Figure 4. PTAD-azide functionalized ELP facilitates SPACC-mediated cross-linking into hydrogels and supports stem cell culture. (A) Gelation time sweep showing storage (G') and loss (G'') moduli during SPAAC cross-linking of PTAD-ELP and BCN-ELP. (B) G' and G'' of SPAAC-cross-linked ELP gels during a frequency sweep at a fixed strain of 1%, varying the polymer content of the hydrogels. (C) Confocal micrographs of hMSCs and mNPCs cultured within PTAD-functionalized, SPAAC-cross-linked ELP hydrogels. Live/dead staining indicated high viability for both cell types 1 and 24 h post-encapsulation. After 7 days in culture, hMSCs exhibit a characteristic spread morphology with actin stress fibers visualized by phalloidin staining (red), and mNPCs maintain expression of the neural stemness marker Nestin (green). After 10 days in culture, mNPCs treated with mixed differentiation medium stain positive for the astrocyte marker GFAP (green) and the neuron marker MAP2 (red). In the 7 and 10 day images, DAPI was included as a nuclear counterstain (blue).

The ability to control the mechanical and biochemical properties of ELP hydrogels makes them attractive platforms for the 3D culture of stem cells.[17,21] To confirm that SPAAC-cross-linked ELP gels produced from PTAD-functionalized proteins support the culture of stem cells, human mesenchymal stem cells (hMSCs) and murine neural progenitor cells (mNPCs) were encapsulated in ELP hydrogels with G' ~1200 Pa and ~430 Pa, respectively. These hydrogel formulations were chosen because their storage moduli fall within the range of stiffness measured for bone marrow[22] and brain tissue,[23,24] respectively. Viability of the encapsulated hMSCs and mNPCs was assessed via a live/dead cytotoxicity assay. Acute viability (~1 h post-encapsulation) was ~99% for hMSCs and ~96% for mNPCs, consistent with the SPAAC cross-linking reaction proceeding by a bio-orthogonal and biocompatible mechanism (Figure 4C). The cells remained highly viable within the hydrogels 24 h post-encapsulation, with ~98% viability for the hMSCs and ~96% viability for the mNPCs (Figure 4C). To ensure that the hydrogels support the long-term culture and phenotypic maintenance of the encapsulated cells, the hMSCs and mNPCs were cultured

within the PTAD-functionalized, SPAAC-cross-linked ELP hydrogels for 7 days. After 1 week in culture, the hMSCs exhibited a characteristic spread, spindle-shaped morphology with discernible actin stress fibers (Figure 4C). These results are consistent with previous results demonstrating hMSC spreading in ELP hydrogels presenting the RGD cell-adhesion site.[17] Additionally, mNPCs retained expression of the neural stemness marker Nestin after 1 week in culture, suggesting that the encapsulated mNPCs maintained their stem cell phenotype (Figure 4C). To further confirm maintenance of NPC stemness in the hydrogels, mNPCs were treated with mixed differentiation medium 4 days post-encapsulation to assess the differentiation capacity of the cells. Following 6 days of differentiation (10 days total culture in hydrogels), a mix of cells expressing the astrocytic marker glial fibrillary acidic protein (GFAP) and the neuronal marker microtubule-associated protein 2 (MAP2) were identified within the hydrogels (Figure 4C). Thus, mNPCs retain the capacity to differentiate into neurons and astrocytes following encapsulation. Taken together, these results indicate that PTAD-functionalized, SPAAC-cross-linked ELP hydrogels support

the culture and phenotypic maintenance of hMSCs and mNPCs.

We have demonstrated that PTAD-modification of tyrosines is an effective means for selectively functionalizing ELPs with azide groups at amino acid residues other than lysine. The resulting azide-functionalized proteins participate in bio-orthogonal SPAAC cross-linking to form cell-encapsulating hydrogels. An alternative method to incorporate the SPAAC cross-linking reactive groups into engineered proteins is the use of noncanonical amino acids in genetically modified organisms. While this technique has resulted in successful introduction of azide and BCN reaction partners,[25,26] the noncanonical amino acid starting materials and techniques used to incorporated these amino acids significantly increases the cost and decreases the yield of the engineered proteins. Such costs may be acceptable for therapeutic proteins that exhibit efficacy using relatively small amounts of protein, but the costs would become prohibitively expensive when considering the much greater amount of protein necessary for applications involving cell-encapsulating hydrogels. Selective chemical modification of the proteins post-expression provides a lower cost alternative that can still generate materials with tightly controlled biochemical and material properties. Looking forward, use of the PTAD tyrosine modification reaction to selectively introduce azide functionality to ELPs may permit combinatorial screening of cell adhesive ligands, including the lysine-containing IKVAV motif derived from laminin, to optimize the biochemical functionality of these materials to enhance cell viability[27] or neuronal differentiation and growth.[28]

CONCLUSION

Bio-orthogonally cross-linked, engineered protein hydrogels are a promising platform for stem cell encapsulation for regenerative medicine applications due to the ability to precisely tune the biochemical and mechanical properties of the hydrogels. We have introduced PTAD functionalization of tyrosine residues as a means for site-selective introduction of azides into ELPs for bio-orthogonal SPAAC cross-linkings into hydrogels. Under optimized reaction conditions, the PTAD modification reaction exhibited efficient and selective tyrosine functionalization, leaving primary amines free to participate in other reactions or cell−matrix interactions. PTAD-functionalized, SPAAC-cross-linked ELP hydrogels cross-link rapidly and support the viability and phenotypic maintenance of encapsulated hMSCs and mNPCs. Azide introduction via PTAD modification may thus allow for the development of engineered protein hydrogels with lysine-containing bioactive sequences.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00720.

> Detailed experimental procedures. PTAD-tyrosine reaction efficiency quantification via ^{1}H NMR (Figure S1). FT-IR spectra for azide incorporation in PTAD-modified ELP (Figure S2). Purity analysis of ELPs by SDS-PAGE and Western blot (Figure S3). Strain sweep for PTAD-functionalized, SPAAC-cross-linked ELP hydrogel (Figure S4). Varying pH in phosphate-buffered PTAD reaction (Table S1). (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

BCN, bicyclo[6.1.0]non-4-yne; ELP, elastin-like protein; hMSC, human mesenchymal stem cell; mNPC, murine neural progenitor cell; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; RGD-ELP, elastin-like protein containing the arginine-glycine-aspartic acid cell-adhesion sequence; SPAAC, strain-promoted azide−alkyne cycloaddition

REFERENCES

(1) Lee, K. Y., and Mooney, D. J. (2001) Hydrogels for tissue engineering. *Chem. Rev. 101*, 1869−1879.

(2) Mooney, D. J., and Vandenburgh, H. (2008) Cell delivery mechanisms for tissue repair. *Cell Stem Cell 2*, 205−211.

(3) Ratner, B. D., and Bryant, S. J. (2004) Biomaterials: Where we have been and where we are going. *Annu. Rev. Biomed. Eng. 6*, 41−75.

(4) Yang, F., Williams, C. G., Wang, D.-a., Lee, H., Manson, P. N., and Elisseeff, J. (2005) The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells. *Biomaterials 26*, 5991−5998.

(5) Burdick, J. A., and Anseth, K. S. (2002) Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials 23*, 4315−4323.

(6) Lutolf, M. P., Raeber, G. P., Zisch, A. H., Tirelli, N., and Hubbell, J. A. (2003) Cell-responsive synthetic hydrogels. *Adv. Mater. 15*, 888−892.

(7) Kharkar, P. M., Kiick, K. L., and Kloxin, A. M. (2013) Designing degradable hydrogels for orthogonal control of cell microenvironments. *Chem. Soc. Rev. 42*, 7335−7372.

(8) Little, L., Healy, K. E., and Schaffer, D. (2008) Engineering biomaterials for synthetic neural stem cell microenvironments. *Chem. Rev. 108*, 1787−1796.

(9) Romano, N. H., Sengupta, D., Chung, C., and Heilshorn, S. C. (2011) Protein-engineered biomaterials: Nanoscale mimics of the extracellular matrix. *Biochim. Biophys. Acta, Gen. Subj. 1810*, 339−349.

(10) Straley, K. S., and Heilshorn, S. C. (2009) Independent tuning of multiple biomaterial properties using protein engineering. *Soft Matter 5*, 114−124.

(11) Urry, D. W. (1997) Physical chemistry of biological free energy transduction as demonstrated by elastic protein-based polymers. *J. Phys. Chem. B 101*, 11007−11028.

(12) Di Zio, K., and Tirrell, D. (2003) Mechanical properties of artificia; protein matrices engineered for control of cell and tissue behavior. *Macromolecules 36*, 1553−1558.

(13) Straley, K. S., and Heilshorn, S. C. (2009) Dynamic, 3D-pattern formation within enzyme-responsive hydrogels. *Adv. Mater. 21*, 4148−4152.

(14) Tashiro, K.-i., Sephel, G. C., Weeks, B., Sasaki, M., Martin, G. R., Kleinman, H. K., and Yamada, Y. (1989) A synthetic peptide containing the IKVAV sequence from the a chain of laminin mediates cell attachment, migration, and neurite outgrowth. *J. Biol. Chem. 264*, 16174−16182.

(15) D'Andrea, L. D., Iaccarino, G., Fattorusso, R., Sorriento, D., Carannante, C., Capasso, D., Trimarco, B., and Pedone, C. (2005) Targeting angiogenesis: Structural characterization and biological properties of a de novo engineered VEGF mimicking peptide. *Proc. Natl. Acad. Sci. U. S. A. 102*, 14215−14220.

(16) Saito, A., Suzuki, Y., Ogata, S.-i., Ohtsuki, C., and Tanihara, M. (2003) Activation of osteo-progenitor cells by a novel synthetic peptide derived from the bone morphogenetic protein-2 knuckle epitope. *Biochim. Biophys. Acta, Proteins Proteomics 1651*, 60−67.

(17) Madl, C. M., Katz, L. M., and Heilshorn, S. C. (2016) Bio-orthogonally crosslinked, engineered protein hydrogels with tunable mechanics and biochemistry for cell encapsulation. *Adv. Funct. Mater. 26*, 3612−3620.

(18) Ban, H., Gavrilyuk, J., and Barbas, C. F., III. (2010) Tyrosine bioconjugation through aqueous ene-type reactions: A click-like reaction for tyrosine. *J. Am. Chem. Soc. 132*, 1523−1525.

(19) Ban, H., Nagano, M., Gavrilyuk, J., Hakamata, W., Inokuma, T., and Barbas, C. F., III. (2013) Facile and stabile linkages through tyrosine: Bioconjugation strategies with the tyrosine-click reaction. *Bioconjugate Chem. 24*, 520−532.

(20) Raphel, J., Parisi-Amon, A., and Heilshorn, S. C. (2012) Photoreactive elastin-like proteins for use as versatile bioactive materials and surface coatings. *J. Mater. Chem. 22*, 19429−19437.

(21) Chung, C., Anderson, E., Pera, R. R., Pruitt, B. L., and Heilshorn, S. C. (2012) Hydrogel crosslinking density regulates temporal contractility of human embryonic stem cell-derived cardiomyocytes in 3D cultures. *Soft Matter 8*, 10141−10148.

(22) Jansen, L. E., Birch, N. P., Schiffman, J. D., Crosby, A. J., and Peyton, S. R. (2015) Mechanics of intact bone marrow. *J. Mech. Behav. Biomed. Mater. 50*, 299−307.

(23) Gefen, A., and Margulies, S. S. (2004) Are in vivo and in situ brain tissues mechanically similar? *J. Biomech. 37*, 1339−1352.

(24) Taylor, Z., and Miller, K. (2004) Reassessment of brain elasticity for analysis of biomechanisms of hydrocephalus. *J. Biomech. 37*, 1263−1269.

(25) Link, A. J., Vink, M. K. S., Agard, N. J., Prescher, J. A., Bertozzi, C. R., and Tirrell, D. A. (2006) Discovery of aminoacyl-tRNA synthetase activity through cell-surface display of noncanonical amino acids. *Proc. Natl. Acad. Sci. U. S. A. 103*, 10180−10185.

(26) Borrmann, A., Milles, S., Plass, T., Dommerholt, J., Verkade, J. M. M., Wießler, M., Schultz, C., van Hest, J. C. M., van Delft, F. L., and Lemke, E. A. (2012) Genetic encoding of a bicyclo[6.1.0]nonyne-charged amino acid enables fast cellular protein imaging by metal-free ligation. *ChemBioChem 13*, 2094−2099.

(27) Jongpaiboonkit, L., King, W. J., and Murphy, W. L. (2009) Screening for 3D environments that support human mesenchymal stem cell viability using hydrogel arrays. *Tissue Eng., Part A 15*, 343−353.

(28) Lam, J., Carmichael, S. T., Lowry, W. E., and Segura, T. (2015) Hydrogel design of experiments methodology to optimize hydrogel for IPSC-NPC culture. *Adv. Healthcare Mater. 4*, 534−539.