Biomacromolecules

Communication

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Tetrakis(hydroxymethyl) Phosphonium Chloride as a Covalent Cross-Linking Agent for Cell Encapsulation within Protein-Based Hydrogels

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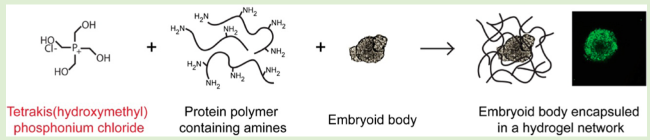
Supporting Information

ABSTRACT: Native tissues provide cells with complex, three-dimensional (3D) environments comprised of hydrated networks of extracellular matrix proteins and sugars. By mimicking the dimensionality of native tissue while deconstructing the effects of environmental parameters, protein-based hydrogels serve as attractive, in vitro platforms to investigate cell—matrix interactions. For cell encapsulation, the process of hydrogel formation through physical or covalent cross-linking must be mild and cell compatible. While many chemical cross-linkers are commercially available for hydrogel formation, only a subset are cytocompatible; therefore, the identification of new and reliable cytocompatible cross-linkers allows for greater flexibility of hydrogel design for cell encapsulation applications. Here, we introduce tetrakis(hydroxymethyl) phosphonium chloride (THPC) as an inexpensive, amine-reactive, aqueous cross-linker for 3D cell encapsulation in protein-based hydrogels. We characterize the THPC-amine reaction by demonstrating THPC's ability to react with primary and secondary amines of various amino acids. In addition, we demonstrate the utility of THPC to tune hydrogel gelation time (6.7 ± 0.2 to 27 ± 1.2 min) and mechanical properties (storage moduli ~250 Pa to ~2200 Pa) with a recombinant elastin-like protein. Lastly, we show cytocompatibility of THPC for cell encapsulation with two cell types, embryonic stem cells and neuronal cells, where cells exhibited the ability to differentiate and grow in elastin-like protein hydrogels. The primary goal of this communication is to report the identification and utility of tetrakis(hydroxymethyl) phosphonium chloride (THPC) as an inexpensive but widely applicable cross-linker for protein-based materials.



Within native tissue, cells often reside in complex, three-dimensional (3D) hydrated networks of extracellular matrix (ECM) proteins and sugars. To understand how cells respond and interact with these complex environments, 3D culture systems, developed in vitro, have been employed to recapitulate the dimensionality of native tissue while deconstructing the effects of environmental parameters. In particular, hydrogels, exhibiting high water content and tissue-like elastic properties, serve as attractive 3D cell culture platforms.[1] For cell encapsulation, the process of hydrogel formation through physical or covalent cross-linking must be mild and cell compatible, thus, limiting the number of suitable materials.

Here, we focus on protein hydrogel formation through covalent cross-linking. Protein-based materials are comprised of amino acids, the building blocks of biological systems, and can provide cellular cues through microscale biological motifs.[2] Ionizable amino acid side groups (e.g., lysine's ε-amine and cysteine's sulfhydryl) provide site specific targets for cross-linking reactions and hydrogel formation. Covalent cross-links offer precise control of hydrogel cross-linking density and allow for tunable mechanical properties that are capable of matching the stiffness of native tissues.[3,4] To date, a variety of chemical cross-linking chemistries have been utilized to form covalently cross-linked protein hydrogels. Amine-reactive *N*-hydroxysuccinimide (NHS) esters and their water-soluble analogs, sulfo-NHS esters, are commonly used homofunctional cross-linkers[5,6] that demonstrate good reactivity at physiological pH but are often susceptible to hydrolysis and degradation during the cross-linking reaction, which can lead to poor conjugation efficiency.[7] Typical gelation times for NHS ester systems are on the order of minutes to hours. Sulfhydryl groups have also been utilized to form disulfide cross-links for hydrogel formation.[8,9] However, without the addition of external oxidizers, gelation is slow, requiring several hours. Additionally, generated disulfide cross-links are sensitive to reducing agents that may be present in cell culture media. While several chemical cross-linkers are commercially available, only a subset is cytocompatible; therefore, the discovery of robust new cytocompatible cross-linkers promotes greater flexibility in hydrogel design for cell encapsulation applications.

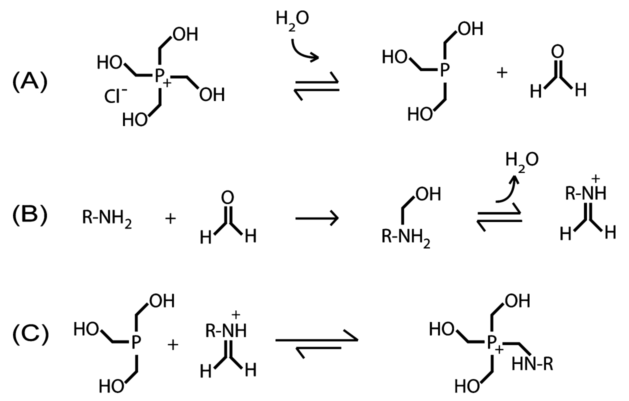
Previously, Lim et al. introduced β-[tris(hydroxylmethyl) phosphino] propionic acid (THPP) as a trifunctional cross-linker for polypeptide-based biomaterials. Through a Mannich-type condensation, THPP reacts with primary and secondary amines to form covalent cross-links in aqueous solution at physiological pH. Single-suspension fibroblasts,[10] chondrocytes,[11] and human embryoid bodies[12] were successfully encapsulated within THPP cross-linked hydrogels demonstrating cytocompatibility. Unfortunately, due to the complicated chemical synthesis of THPP, it is no longer readily available.

Received: September 28, 2012
Revised: November 12, 2012
Published: November 14, 2012

As a potential replacement for THPP, we investigated the reactivity of tetrakis(hydroxymethyl) phosphonium chloride (THPC). THPC is a phosphonium salt used as a precursor to fire-retardant materials and is readily available in aqueous solution. Due to the similarity in structure, we hypothesized that THPC could be used as a tetra-functional cross-linker for cell encapsulation in protein-based hydrogels, where THPC would react with primary and secondary amines through a Mannich-type reaction. The objective of this study was to assess THPC as a chemical cross-linker for protein-based hydrogels and demonstrate its cytocompatibility for cell encapsulation applications.

In Scheme 1, we illustrate the suggested reaction mechanism of THPC and a primary amine. The Mannich-type reaction is

Scheme 1. Suggested THPC Reaction Mechanism[a]



[a](A) Formation of formaldehyde to initiate hydroxymethyl arm replacement, (B) amine-formaldehyde reaction to yield an immonium ion in a Mannich-type reaction, and (C) phosphorus reaction with the immonium ion to complete the amine coupling.

initiated by the generation of formaldehyde (HCOH), which then reacts with the amine to yield an immonium ion. Subsequently, the THPC derivative reacts with the immonium ion to complete the hydroxymethyl arm replacement, resulting in the amine coupling. Reaction with additional amines can then occur in a similar manner with the remaining, unreacted hydroxymethyl arms.

Evidence to support a Mannich-type reaction rather than a simple condensation reaction with loss of water has been shown by others in similar reaction chemistries with hydroxymethyl phosphorus compounds.[13,14] Using a colorimetric formaldehyde detection assay based on a carbazole reaction,[15] we detected millimolar concentrations of formaldehyde in molar concentrations of THPC, where the quantity of formaldehyde detected positively correlated to THPC concentration (Figure 1A). Several amino acids (lysine, glycine, proline, and cysteine) were then individually reacted with THPC, using identical amino acid to THPC molar ratios. Formaldehyde generation was observed in all THPC reactions, with the amino acids at levels significantly greater than that of the negative controls (i.e., amino acid alone), where no THPC was present (Figure 1B). After 20 min, we observed the greatest quantity of formaldehyde generation in the reaction with lysine, which contains two primary amines, followed by glycine, which has one primary amine, and then proline, which contains only a secondary amine. The smallest quantity of formaldehyde was detected in the reaction with cysteine; thus, cysteine's sulfhydryl may interfere with the formaldehyde generation

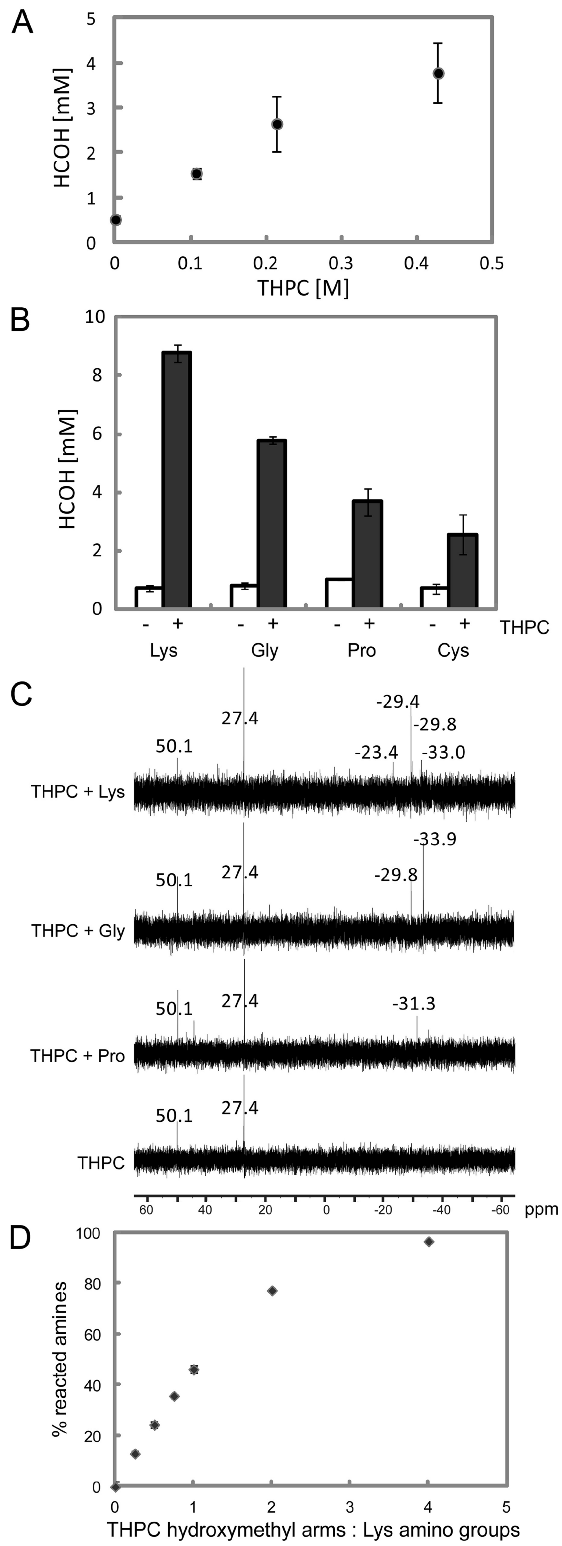


Figure 1. (A) Spontaneous formaldehyde (HCOH) generation positively correlates with THPC concentration. (B) Formaldehyde generation from various amino acids: lysine, glycine, proline, and cysteine alone (−) or reacted in the presence of 0.43 M THPC (+). (C) ^{31}P NMR of THPC alone compared to THPC reacted with proline (Pro), glycine (Gly), or lysine (Lys), where chemical shifts are referenced to 85% phosphoric acid, which is assigned a chemical shift of 0. (D) Extent of THPC-lysine reaction as a function of THPC to lysine reactive group molar ratio.

step. While these data do not provide detailed information about the kinetics of the reaction, they suggest that formaldehyde is an intermediate product in the THPC-amine reaction, which is consistent with the proposed Mannich-type reaction given in Scheme 1.

From ^{31}P NMR (Figure 1C), we see that within the THPC stock solution, the phosphorus resides in two different states, corresponding to THPC (50.1 ppm) and THPC minus a hydroxymethyl arm, or tris(hydroxymethyl)phosphine (27.4 ppm), as expected (Scheme 1A). After reaction with lysine, glycine, or proline, we observe that the phosphorus signal shifts upfield, indicating a more electron-rich state. This suggests that both primary and secondary amines react with THPC (Figure 1C). In addition, Figure 1C shows that the α-amine (N-terminal) and the ε-amine (side chain) of lysine are both reactive (Supporting Information, Figure 1), as evidenced by the different chemical upshifts when compared to the reaction with glycine, which contains only the α-amine. Relative peak intensities also suggest greater reactivity of the lysine over glycine and proline, which is in agreement with Figure 1B. The extent of the THPC-lysine reaction was characterized using a 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay to detect free amino groups (Figure 1D). We observe that amino groups are being consumed by the reaction with THPC and that, by increasing the molar ratio of THPC reactive groups to amino groups, the reaction can be driven to near completion. The amine-reactivity of THPC allows us to exploit this small molecule as a covalent cross-linker for protein-based materials. The covalent cross-links formed during the THPC-amine reaction are not susceptible to hydrolytic degradation; thus, the THPC cross-linker can be used to form stable cross-linked networks.

Previously our laboratory has reported the design and synthesis of a modular recombinant elastin-like protein (ELP) containing bioactive domains[16] with specified lysines to act as amine-reactive cross-linking sites for formation of thin films and 2D cell scaffolds (Figure 2A). To demonstrate the ability of THPC to tune the properties of 3D protein-based hydrogels, solutions of ELP (5 wt % in phosphate-buffered saline) were cross-linked with various stoichiometric ratios of THPC. Gelation time was determined using an oscillatory rheometer (ARG2, TA Instruments). By exploiting the fixed stress experimental setup, the gelation point can be determined from the sample strain curve over time. With gelation, the sample strain rapidly decreases due to an increase in modulus. Gelation time was defined as the time at which the sample strain curve reached an inflection point, providing a good estimate of the gelation point. In varying THPC to ELP protein reactive group stoichiometry, gelation time could be varied in a biphasic manner, where the fastest gelation time for a 5 wt % ELP hydrogel was 6.7 min at a 1:1 THPC to ELP reactive group molar ratio (Figure 2B). As expected, both decreases (e.g., 0.5:1, 0.75:1) and increases (e.g., 2:1, 4:1) in this ratio result in longer gelation times likely due to undersaturated or oversaturated amine reaction sites, respectively. As the amount of THPC reactive sites is increased to match the amount of amine reactive groups, the probability of the cross-linking reaction occurring is increased, resulting in faster gelation times.[17] However, when the amount of THPC reactive sites exceeds the number of amine sites, more THPC molecules may achieve single-arm binding events, consuming the free amines without forming effective cross-links, as opposed to multiple-arm binding events, which are required for cross-link formation.

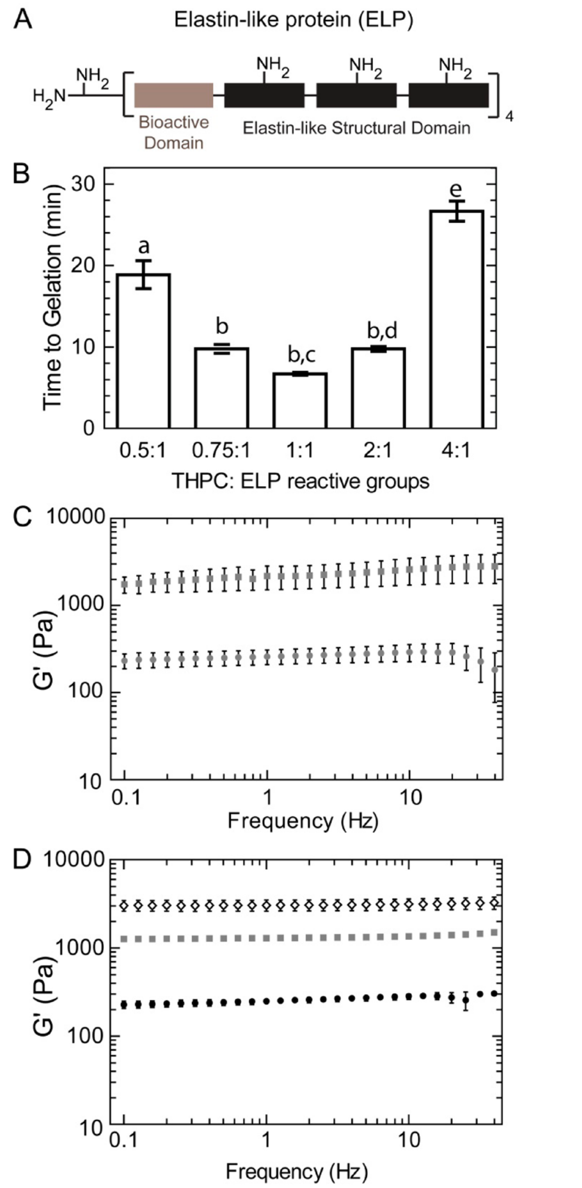


Figure 2. (A) Schematic of elastin-like protein (ELP). (B) Time to gelation of 5 wt % ELP hydrogels with various THPC/ELP reactive group molar ratios. Statistical significance is represented by letters above each column, with different letters signifying distinct statistical groups, $p < 0.05$. (C) Storage modulus, G', of 5 wt % ELP hydrogels for 0.5:1 (gray circle) and 1:1 (gray squares) cross-linking density groups during frequency sweeps at a shear strain of 0.7%, which was determined to be within the linear viscoelastic regime. (D) Storage moduli, G', of ELP hydrogels containing 3 (black circles), 5 (gray squares), and 10 (clear diamonds) wt % ELP at 1:1 cross-linking density.

Thus, as observed, longer gelation time and more compliant hydrogels are expected when the amine reactive sites are oversaturated with a stoichiometric excess of THPC reactive sites.

According to elasticity theory, elastic free-energy is dependent on the number of active polymer chains between cross-links in a network.[18] Thus, changes in hydrogel cross-linking density, tuned by varying cross-linker to protein reactive group stoichiometry, are reflected in hydrogel mechanics. The frequency dependency of the storage modulus (G') for the ELP hydrogels was determined in the range of 0.1 to 40 Hz

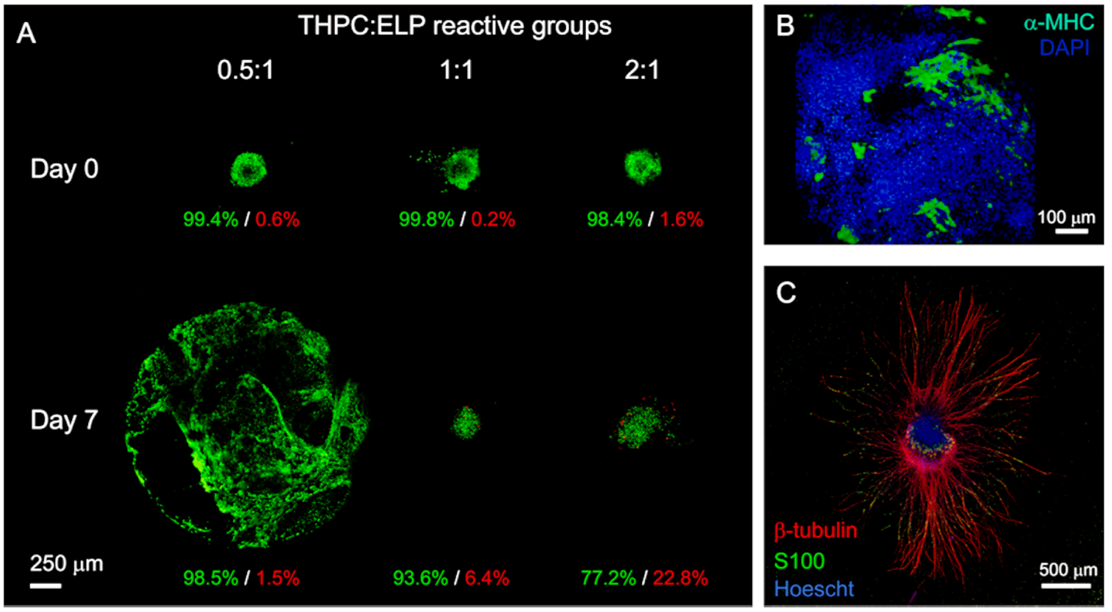


Figure 3. (A) Representative live (green)/dead (red) projections of encapsulated mouse embryoid bodies in 5 wt % ELP hydrogels (2 mm in diameter, 0.5 mm in height) of varying THPC/ELP reactive group molar ratios immediately after encapsulation and after 7 days of culture. Hydrogels with 0.5:1, 1:1, and 2:1 cross-linking density correspond to storage moduli of ~260, 2200, and 1400 Pa, respectively. Percentage of live and dead cells as quantified by pixel analysis are listed in green and red, respectively, beneath each gel. By day 7, cellular outgrowth of the lowest cross-linking density hydrogel (0.5:1) encompassed the entire hydrogel. Scale bar = 250 μm. (B) Cardiomyocyte differentiation of an encapsulated mouse embryoid body, in a 5 wt % ELP hydrogel with 0.5:1 cross-linking density, was visualized by expression of GFP-tagged α-myosin heavy chain (α-MHC) reporter (green) and counterstained with DAPI (blue) for nuclei visualization after 8 days of culture. Scale bar = 100 μm. (C) Encapsulated chick dorsal root ganglion (DRG), in a 3 wt % ELP hydrogel with 1:1 cross-linking density (modulus ~ 1500 Pa), cultured for 7 days, and stained for neuronal marker, β-tubulin (red), and glial marker, S100 (green), with Hoescht (blue) for nuclei visualization. Scale bar = 500 μm.

using a constant 0.7% strain, which was verified to be in the linear viscoelastic regime. For 5 wt % ELP hydrogels with 1:1 cross-linking density, a plateau storage modulus of ~2200 Pa was found. By decreasing the cross-linking density, using a 0.5:1 THPC to protein reactive group stoichiometry, the plateau storage modulus could be reduced to ~250 Pa (Figure 2C). Similar to other biopolymer hydrogels, increasing the protein weight percent of the ELP hydrogel while keeping the cross-linker to protein stoichiometry the same also resulted in increased hydrogel storage moduli (Figure 2D). Increasing macromer content from 3 to 5 to 10 wt % in ELP hydrogels resulted in increases in storage moduli from 300 ± 22 to 1320 ± 23 to 3090 ± 16 Pa, respectively.

In Figures 1 and 2, THPC has been shown to react with primary and secondary amines of various amino acids, where this covalent coupling reaction can be utilized to cross-link protein-based polymers containing free amines. While THPC concentration has been shown here to modulate hydrogel mechanics of a custom-designed recombinant protein, THPC can also be added to other protein-based, physical hydrogel systems, like collagen, to significantly stiffen the matrix by adding additional cross-links into the network without changing the protein concentration (Supporting Information, Figure 2).

Lastly to demonstrate THPC's compatibility for 3D cell encapsulation, mouse embryoid bodies were encapsulated in 5 wt % ELP hydrogels varying THPC cross-linker to protein reactive group ratios (0.5:1, 1:1, and 2:1). Mouse embryoid bodies were formed from embryonic stem cells through the hanging drop method.[19,20] Each embryoid body was encapsulated in a single ELP hydrogel that contained RGD, cell-adhesive bioactive domains. Hydrogels were formed by incubating at room temperature for 10 min followed by 37 °C for 10 min. Live/dead cytotoxicity assay (Invitrogen) was used to assess cell viability immediately after encapsulation and after 7 days of culture (Figure 3A). Over 98% cell viability was observed in all hydrogels on day 0 immediately after cross-linking. After one week, viability ranged from 77 to 98%, with extensive cellular outgrowth from embryoid bodies encapsulated in hydrogels using the lowest cross-linking density. The phenomenon of greater cellular outgrowth in hydrogels with lower cross-linking density agrees with cell outgrowth and spreading observations in other cell–hydrogel systems.[21,22] Even though good viability was observed under these conditions, free amines located on the cell surface are susceptible to cross-linking with THPC and may affect cell function. This is true for all covalent cross-linkers that target primary amines; thus, it is important to assess cell viability for each cross-linker, cell type, and hydrogel combination. Additionally, the THPC-amine reaction generates a formaldehyde intermediate, which at high concentrations can be cytotoxic. At the millimolar concentrations of THPC (~2.3–9.2 mM for a 5 wt % ELP hydrogel at 0.5:1 to 2:1 cross-linking density) used for cell encapsulation in these studies, only micromolar concentrations of formaldehyde may be present and do not appear to result in significant cell death. However, THPC concentration should be minimized for a desired hydrogel stiffness to minimize potential cytotoxic effects of formaldehyde.

To show that THPC cross-linking is not only cell compatible but supports the retention of cell function, encapsulated embryoid bodies were also monitored for cardiomyocyte differentiation. Using a mouse embryonic stem cell line containing a GFP-tagged α-myosin heavy chain reporter,[23] cardiomyocyte differentiation could be visualized within the

THPC-cross-linked 5 wt % ELP hydrogels (Figure 3B). As further evidence of cell compatibility and potential utility for cultures of a variety of different tissue types, dorsal root ganglia (DRGs), isolated from E9 chick embryos, were encapsulated in more compliant 1:1 THPC-cross-linked 3 wt % ELP hydrogels. After 7 days of culture, DRGs stained for neuronal and glial markers (β-tubulin and S100, respectively), demonstrating the ability of THPC-cross-linked ELP hydrogels to support neuronal axon growth into the hydrogel (Figure 3C).

■ CONCLUSION

Protein-based hydrogel systems serve as attractive in vitro systems to investigate cell response to environmental cues. The identification of new, cytocompatible cross-linkers allows for greater flexibility of hydrogel design. Here, we have introduced THPC as an inexpensive, aqueous cross-linker for 3D cell encapsulation in protein-based hydrogels. We have characterized the THPC-amine reaction, demonstrated the use of THPC in tuning hydrogel properties, and showed cytocompatibility with retention of cell growth and phenotype in an ELP hydrogel system.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods are provided. Zoomed in image of ^{31}P NMR chemical shifts for THPC-Lys reaction (Figure S1). Storage (G') and loss moduli (G'') of THPC-stiffened collagen hydrogels (Figure S2). 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.

■ ACKNOWLEDGMENTS

The authors would like to thank Andrew T. Higgs from the Sellinger lab at Stanford University for his help in ^{31}P NMR experiments, the Kamm lab from Massachusetts Institute of Technology for the generous gift of mouse embryonic stem cells with GFP-tagged α-myosin heavy chain reporter, and Debanti Sengupta and Dr. Justin Du Bois for helpful discussion about THPP reaction mechanism. The authors would also like to acknowledge funding support from AHA 10POST4190103 (C.C.), NIH NRSA F32NS076222 (K.J.L.), NSF EFRI-CBE 0735551 (S.C.H.), NIH DP2OD006477 (S.C.H.), NIH R21 AR062359 (S.C.H.), NSF DMR 0846363 (S.C.H.), Stanford Bio-X IIP5-85 (S.C.H.), and Stanford Cardiovascular Institute Younger Grant (S.C.H.).

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