ASSOCIATED CONTENT

Tetrakis (hydroxylmethyl) phosphonium chloride as a covalent crosslinking agent for cell encapsulation within protein-based hydrogels

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DETAILED METHODOLOGY

Tetrakis(hydroxylmethyl)phosphonium chloride (THPC)-amine reaction characterization. The presence of formaldehyde in the Mannich-type reaction was detected using a colorimetric formaldehyde assay based on a carbazole reaction[1]. Briefly, 100 μl of THPC of varying concentrations (0 to 0.43 M) was combined with 3 ml of concentrated sulfuric acid in borosilicate test tubes. 100 μl of 0.1% carbazole in ethanol was then added to the test tube, and allowed to incubate at room temperature for 90 minutes. Absorbance was read at 649 nm. For THPC reaction with lysine, glycine, proline, and cysteine, amino acids were combined in a 10:1 molar ratio with 0.43 M THPC, mixed, and allowed to incubate at room temperature for 20 minutes. The colorimetric assay was then performed on the samples as mentioned above. Amino acid samples without the addition of THPC were used as negative controls. ^{31}P NMR spectroscopy was used to confirm chemical coupling of THPC with free amines. Each amino acid was reacted at a 1:1 molar ratio with THPC in deuterium water at room temperature for 20 minutes. The reaction mixture was then diluted in approximately 10-fold in deuterium water

prior to ^{31}P NMR spectroscopy. NMR data was collected on a 400 MHz Varian NMR spectrometer (Department of Chemistry, NMR Facility). The extent of the THPC-amine reaction was characterized using a 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay according to manufacturer instructions, using a standard curve generated from un-reacted lysine.

Elastin-like protein expression and purification. Elastin-like protein (ELP) was expressed and purified using standard recombinant protein technology as previously reported[2-3]. Protein sequences were cloned into pET15b plasmids using traditional recombinant techniques, expressed in *Escherichia coli*, BL21(DE3), for 3-5 hrs, and purified using an inverse temperature-cycling process. The ELP was then lyophilized and stored at 4°C until use.

ELP hydrogel characterization. To demonstrate the ability of THPC to tune hydrogel properties, solutions of ELP (5 wt% in phosphate-buffered saline) were crosslinked using various stoichiometric ratios of THPC to protein reactive groups. For gelation time, time sweeps were performed at an oscillatory stress of 4 Pa at room temperature, sampling every 10 seconds for 30 minutes on an ARG2 rheometer. Exploiting the fixed stress setup, the gelation point can be determined from the sample strain curve over time[4]. With gelation, the sample strain rapidly decreases due to an increase in modulus. The gelation time was defined as the time at which the sample strain curve reached an inflection point, as a good estimate of gelation point. For hydrogel mechanical characterization, samples were allowed to crosslink in situ on the rheometer. Hydrogels were incubated for 10 min at room temperature followed by 10 min at 37°C prior to conducting strain and frequency sweeps. Strain sweeps were performed from 0.1 to 100% strain at 1 Hz, where 0.7% strain was determined to be within the linear viscoelastic

regime (not shown). The frequency dependency of the storage modulus (G') for the ELP hydrogels was determined in the range of 0.1 to 40 Hz using a constant 0.7% strain. A cone geometry was used for all rheological testing.

Cell compatibility. Mouse embryonic stem cells with a GFP-tagged α-myosin heavy chain reporter[5] were cultured on 0.25% gelatin-coated tissue culture polystyrene in Glasgow minimum essential medium, supplemented with knockout serum replacement and leukemia inhibitory factor. Differentiation was induced by embryoid body formation via the hanging drop technique[6-7] in fetal bovine serum supplemented medium for 2 days. Embryoid bodies were removed from hanging drops and immediately encapsulated in 5 wt% ELP hydrogels of varying crosslinker to protein reactive group stoichiometry (0.5:1, 1:1 and 2:1) with 1 embryoid body per 2.5 μl gel (2-mm diameter and 0.5-mm height). Hydrogels were formed by incubating at room temperature for 10 minutes followed by 37°C for 10 minutes. Live/dead cytotoxicity assay (Invitrogen) was used to assess cell viability immediately after encapsulation and after 7 days of culture. Percent viability was determined by creating a max projection and quantifying pixel area of both live and dead stains. To show that THPC crosslinking supported the retention of cell function, encapsulated embryoid bodies were also monitored for cardiomyocyte differentiation through visualization of the GFP-tagged α-myosin heavy chain reporter[5]. Samples were fixed in 4% paraformaldehyde overnight, stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei visualization, and imaged on a Leica SPE confocal microscope.

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-crosslinked 3 wt% ELP hydrogels, with 1 DRG per 15.5 μl gel (5-mm diameter, 0.5-mm height) using the same crosslinking procedure as the mouse embryoid body encapsulation. Encapsulated DRGs were cultured in Dulbecco's modified eagle medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 50 ng/ml nerve growth factor (NGF). After 7 days of culture, DRGs stained for neuronal and glial markers (β-tubulin and S100, respectively). Hydrogels were fixed overnight in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 in PBS for 30 minutes, and blocked with 5% bovine serum albumin (BSA) and 0.5% Triton X-100 in PBS for 90 minutes. Samples were then incubated overnight with primary antibody (mouse anti-beta-tubulin, 1:500; rabbit anti-S100, 1:400; diluted in 2.5% BSA and 0.5% Triton X-100 in PBS), rinsed 3 times in PBS at 60 minutes each, and incubated with secondary antibodies (goat anti-mouse, 1:500; goat anti-rabbit, 1:500) with Hoescht (1:2500) overnight. Images were collected using a Leica SPE confocal.

SUPPLEMENTARY FIGURES

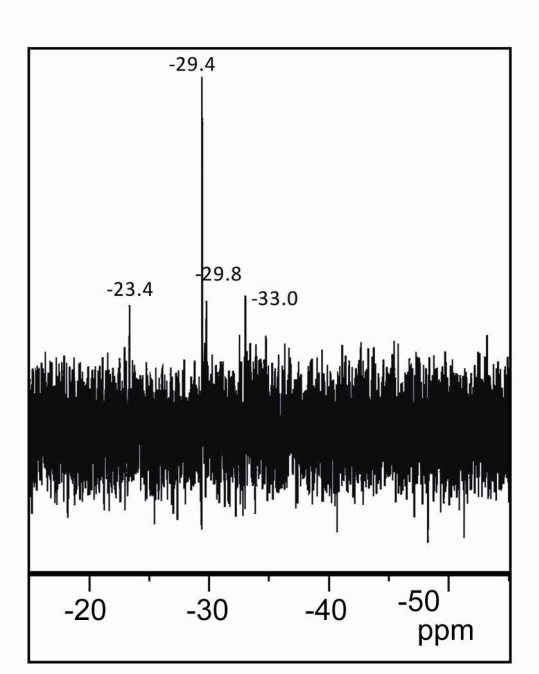

Figure S1. A zoomed in image of ^{31}P NMR chemical shifts of the THPC-Lys reaction for better visualization of the peaks in the range of -20 to -50 ppm.

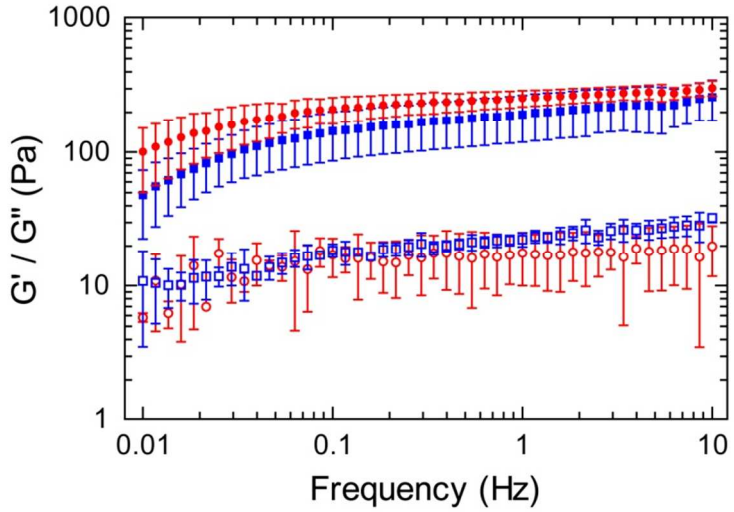

Figure S2. Frequency dependency of the storage (G', closed) and loss moduli (G'', open) of 2.4mg/ml collagen gels with (red circles) or without (blue squares) the addition of 200 μM THPC over 0.01 to 10 Hz at 0.3 Pa stress, which was determined to be within the linear

viscoelastic regime. Collagen gels are traditionally formed via physical interactions. The addition of THPC significantly stiffens ($p < 0.05$, paired t-test) the gel by introducing covalent crosslinks in addition to the already existing physical interactions of the hydrogel network.

SUPPLEMENTARY REFERENCES

1. Taylor, K. A., A colorimetric formaldehyde assay. *Appl. Biochem. Biotechnol.* **1997,** 68, (1-2), 81-93.

2. Chung, C.; Anderson, E.; Pera, R. R.; Pruitt, B. L.; Heilshorn, S. C., Hydrogel crosslinking density regulates temporal contractility of human embryonic stem cell-derived cardiomyocytes in 3D cultures. *Soft Matter* **2012,** 8, (39), 10141-10148.

3. Straley, K. S.; Heilshorn, S. C., Independent tuning of multiple biomaterial properties using protein engineering. *Soft Matter* **2009,** 5, (1), 114-124.

4. Bonino, C. A.; Samorezov, J. E.; Jeon, O.; Alsberg, E.; Khan, S. A., Real-time in situ rheology of alginate hydrogel photocrosslinking. *Soft Matter* **2011,** 7, (24), 11510-11517.

5. Takahashi, T.; Lord, B.; Schulze, P. C.; Fryer, R. M.; Sarang, S. S.; Gullans, S. R.; Lee, R. T., Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. *Circulation* **2003,** 107, (14), 1912-1916.

6. Dang, S. M.; Kyba, M.; Perlingeiro, R.; Daley, G. Q.; Zandstra, P. W., Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol. Bioeng.* **2002,** 78, (4), 442-453.

7. Keller, G. M., In-vitro differentiation of embryonic stem-cells. *Curr. Opin. Cell Biol.* **1995,** 7, (6), 862-869.