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Phosphopantetheinyl Transferase-Catalyzed Formation of Bioactive Hydrogels for Tissue Engineering

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Mild and highly selective cross-linking chemistries are crucial for the performance of state-of-the-art hydrogel biomaterials.[1] Since these materials often need to be cross-linked in the presence of cells or biomolecules, such as is the case in three-dimensional (3D) in vitro cell culture applications or in tissue engineering in vivo, the lack of selectivity in gel cross-linking invariably results in significant cell death or biomolecule inactivation. Consequently, a variety of chemical schemes have been exploited to overcome these complexities, including selective covalent chemical reactions such as Michael-type addition[2] or click chemistry,[3] native chemical ligation,[4] or supramolecular cross-linking.[1,5,6] Surprisingly, apart from transglutaminase- and oxidoreductase-mediated cross-linking,[7−11] enzymatic reactions have not been utilized for the formation of covalently cross-linked hydrogels, restricting the impact of this material's class in clinical tissue engineering.

We reasoned that one such reaction, the phosphopantetheinyl transferase (PPTase) mediated modification of carrier proteins (CPs) by the covalent attachment of the 4-phosphopantetheine moiety of coenzyme A (CoA) to a serine residue (Figure 1a), could be well suited for the formation and functionalization of polymer hydrogels. PPTase plays a key role in the biosynthesis of natural products including fatty acids, polyketides, and nonribosomal peptides.[12] PPTase-mediated cross-linking is covalent, fast, and highly specific. Moreover, CPs are relatively small (generally <85 residues), and peptides as short as 11 amino acids have been identified that mimic CP function,[13] making PPTase cross-linking an ideal target for biomaterials engineering. Indeed, PPTases have already been successfully utilized for the site-specific protein labeling with small-molecule probes including biotin and various fluorophores, which has resulted in powerful technology platforms such as multicolor live cell imaging to elucidate protein translocation in real time,[14,15] or functional protein microarrays.[16]

Here we report for the first time the PPTase-catalyzed formation of polymer hydrogels, as well as the selective covalent modification of hydrogels with bioactive peptide ligands in one step. Hydrogels were formed from two building blocks, namely CoA-functionalized poly(ethylene glycol) (PEG) macromers (Figure 1b) and an engineered apo-acyl carrier protein (ACP) dimer, ACP_ACP (Figure 1c), containing two phosphopante-theinylation sites. Cross-linking was mediated through the action of the surfactin synthetase (Sfp) from *Bacillus subtilis*,[12,17] a 16.2 kDa enzyme belonging to the PPTase family. Sfp was produced in *E.coli* as described.[18] We obtained it in a yield of 46.3 mg/L (shake-flask culture) and >95% purity using His-tag. For simplicity we refer to Sfp here as "PPTase".

We first explored in solution PPTase-catalyzed conjugation of ACP by hydrophilic PEG. Two CoA-functionalized model PEGs were synthesized, starting from either linear methoxy-PEG-vinylsulfone (mPEG-VS, mol. weight 5 kDa) or branched PEG-vinylsulfone (8-armPEG-VS, mol. weight 40 kDa). 8-armPEG-VS was selected because it was expected to be conducive for cross-linking into solid 3D hydrogels. The free thiol of CoA was first attached to the VS termini of these PEGs via Michael-type addition to form the conjugates 1-PEG-CoA and 8-PEG-CoA, respectively (Figure 1b and Figure S1, Supporting Information (SI)). ^{1}H NMR analyses indicated that the reactions were complete as judged by the absence of characteristic VS peaks upon addition of CoA (Figure S2a and b, SI).

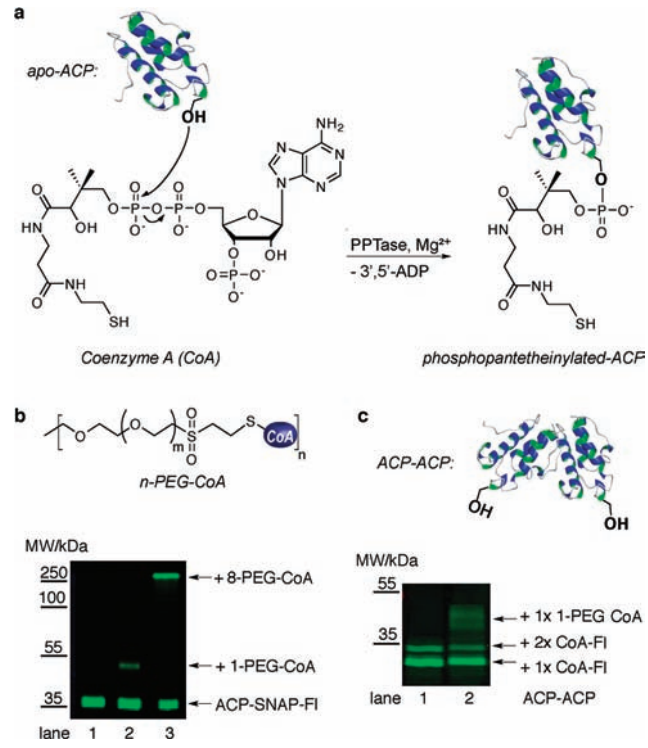

Figure 1. (a) General mechanism of PPTase-catalyzed transfer of phosphopantethein prostetic group of CoA to the serine residue of ACP; (b) linear (1-PEG-CoA; 5 kDa) and branched PEG-CoA (8-PEG-CoA; 40 kDa) reacted with a fluorescent ACP fusion protein (ACP-SNAP-Fl, 34 kDa). SDS-PAGE and laser-based in-gel fluorescence scanning show corresponding molecular weight shifts. (c) ACP_ACP reacted with CoA-Fl (∼1 kDa) (lane 1) or both CoA-Fl and 1-PEG-CoA (lane 2).

Next, PPTase-catalyzed cross-linking of CoA-modified PEGs onto ACP was qualitatively assessed by SDS page (Figure 1b and c). A fluorescein (Fl)-labeled fusion protein ACP-SNAP was chosen for detecting cross-linking, as SNAP can be readily tagged with a fluorescent marker.[19] Upon mixing of ACP-SNAP-Fl and CoA-modified PEGs and in the presence of PPTase, a marked shift in molecular weight was detected on SDS-PAGE (Figure 1b), demonstrating successful bioconjugation.

To form hydrogel networks from 8-PEG-CoA via PPTase catalysis, the linker protein ACP_ACP was designed and produced

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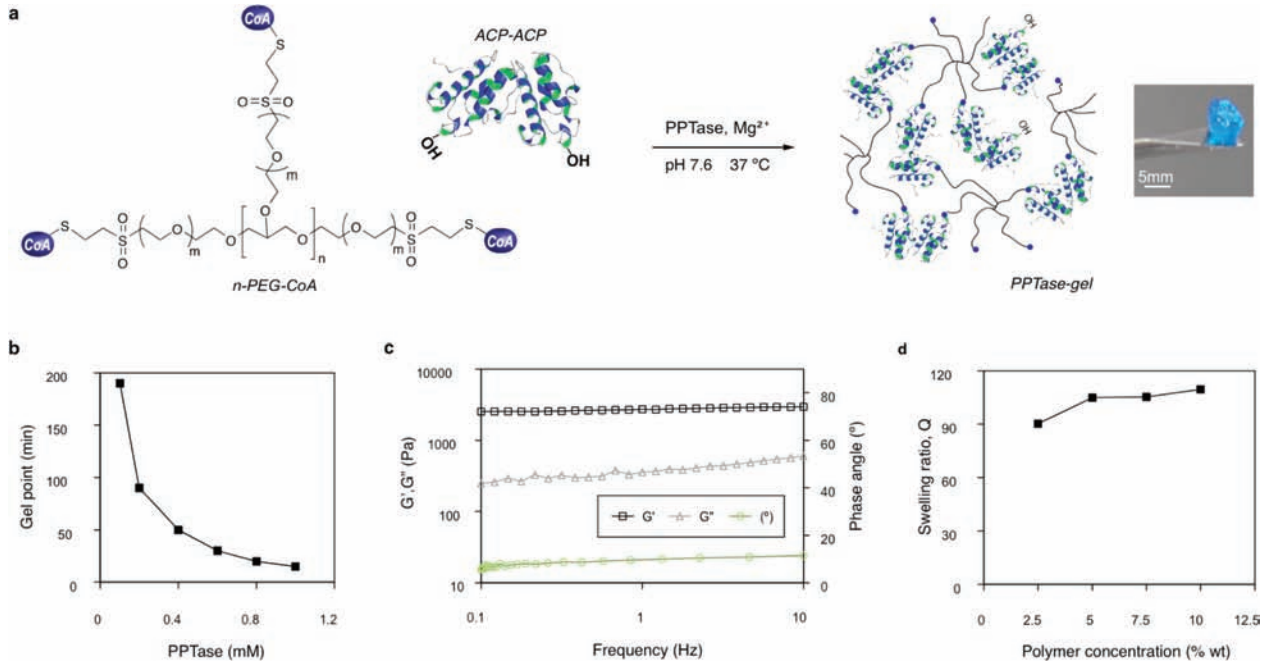

Figure 2. (a) Scheme of PPTase-mediated cross-linking of PEG hydrogels and example of obtained 5% PPTase-hydrogel (stained in blue) after swelling in water; (b) assessment of gelation kinetics reveals a marked dependence of the gel point on enzyme concentration; (c) rheometry experiments (frequency sweep) indicate the presence of a cross-linked elastic polymer network having viscoelastic properties that are nearly independent of the frequency and $G' > G''$; (d) gel swelling ratio Q increases slightly as a function of the polymer precursor concentration.

through overexpression in *E.coli* (design and procedure are described in the SI; Figure S4). The dual functionality of ACP_ACP was confirmed by reaction with CoA-fluorescein (CoA-Fl) and 1-PEG-CoA (Figure 1c).

Next, hybrid polymer gels were created by mixing two stoichiometrically balanced liquid precursors containing ACP_ACP and 8-PEG-CoA (Figure 2). At 37 °C and neutral pH in a Hepes buffer containing 10 mM $MgCl_2$ and at the highest concentration of PPTase tested (1.0 mM), a 5% (w/v) precursor solution gelled within ca. 15 min which is relatively slow compared to existing enzymatically cross-linked gels (e.g., refs 7−11). As expected, at lower PPTase concentration an increase in the gel point was measured (Figure 2b). The efficiencies of the Sfp-catalyzed modification of related carrier proteins ($k_{cat}/K_m = 0.2-2.5\ \mu M^{-1}\ min^{-1}$)[13,20] are comparable to that of transglutaminase FXIIIa ($k_{cat}/K_m = 0.56\ \mu M^{-1}\ min^{-1}$)[8] which we have previously used for cross-linking of PEG-based gels.[9] However, the molecular architecture of gel precursors might also influence gelation kinetics. Follow-up experiments to optimize gel cross-linking are ongoing. Importantly, all negative control conditions (e.g., lack of one gel component or PPTase, imbalanced stoichoimetry) failed in forming hydrogels (Table S1). Therefore, gel formation occurred solely through specific, PPTase-mediated cross-linking.

Small strain shear rheometry on fully cross-linked hydrogels revealed a frequency dependence of G' and G'' that is typical of elastic, covalently cross-linked polymer gels, with an elastic modulus ($G' = 2.3$ kPa, at a frequency of 1 Hz) that was higher than the loss modulus ($G'' = 0.6$ kPa, at a frequency of 1 Hz) (Figure 2c).[21] When immersed overnight in water, the gels swelled considerably and reached an equilibrium swelling ratio Q of ca. 110 (Figure 2d), slightly increasing with precursor concentration between 2.5 and 10%.

To expand the functionality of these PPTase-cross-linked semisynthetic hydrogels, we explored the possibility of incorporating a biologically active peptide as a pendant motif into the network (Figure S6 in SI). The prototypical cell-adhesion peptide RGDS, derived from a cell attachment site of fibronectin, was chosen as an integrin-binding cell adhesion ligand of the otherwise nonspecifically adhesive gel. To render RGDS susceptible for PPTase-catalyzed cross-linking, CoA was conjugated to the N-terminus of the peptide using NHS-PEG-VS, a heterofunctional PEG linker (Figure S5). The resulting CoA-RGDS conjugate was expected to be reactive toward ACP_ACP during network formation. To demonstrate this, we qualitatively investigated the cellular response to RGDS-modified hydrogels in a 2D in vitro cell adhesion assay. Addition of 50 μM CoA-RGDS rendered PPTase hydrogels adhesive for primary fibroblasts. After 2 h of culture, cell spreading to a spindle-shaped morphology was apparent (Figure S6b). In contrast, the negative control lacking RGDS did not enable cell spreading. Thus, apart from hydrogel cross-linking, PPTase can also be utilized for the biofunctionalization of synthetic hydrogels in the same "one-pot" reaction.

Finally, we assessed the biological performance of the novel gels using a more stringent 3D in vitro cell assay. Primary mouse fibroblasts were directly encapsulated in gels by conducting the PPTase-mediated cross-linking in the presence of cells. Importantly, cell viability after 3D encapsulation was ca. 95% and not significantly different from the viability of cells encapsulated within matrices formed by selective Michael-type addition[21] (Figure S7). This demonstrates the excellent specificity and mild character of PPTase-mediated gelation. We tracked individual, 3D encapsulated cells by time-lapse microscopy over 4 days and found that RGDS-tethered gels supported the migration of single cells over long distances (Figure 3c, d). Control gels lacking the RGDS signal did not support extensive 3D migration. This was apparent from representative track plots of single cells in both gel types (Figure 3c), as well as a quantification of the 3D cell migration rates (Figure 3d; representative time-lapse movies can be found in the SI).

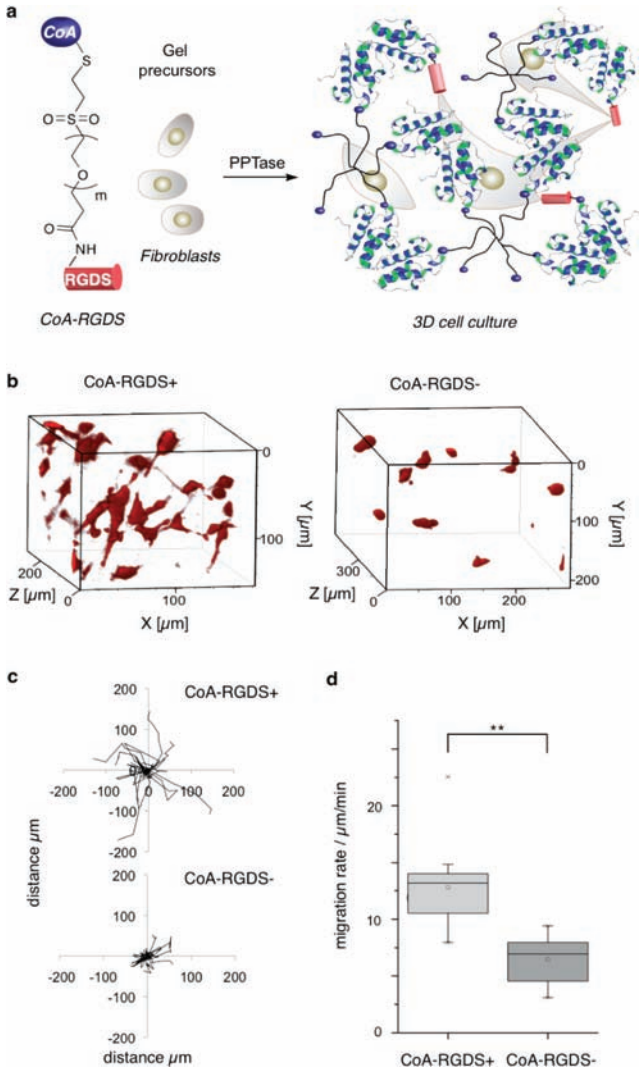

Figure 3. (a) 3D-encapsulation of primary fibroblasts in PPTase-cross-linked and RGDS-modified gels. (b) Comparison of 3D cell culture in the presence or absence of RGDS at day 4 (3D reconstruction of confocal images acquired through the full 400 μm thickness of the gel). (c−d) 3D cell migration behavior: Single-cell migration tracks and the migration rate are significantly different upon gel modification with RGDS (**$p < 0.001$).

In conclusion, we demonstrate that PPTase, an enzyme previously explored in biotechnology for example for specific protein-labeling,[14] is well suited to catalyze selective hydrogel formation and modification with bioactive moieties. The small size of the PPTase we used here, its high expression yields, ease of purification, and very high specificity makes us optimistic that the PPTase gel system is an attractive alternative to gels cross-linked for example *via* transglutaminases,[7−9] much larger enzymes that are very difficult to recombinantly produce. The resulting gels possess promising biological characteristics (Figures 3, S6−8 in SI). A replacement of the ACP_ACP linker protein by short peptide analogs[13] is possible (Mosiewicz and Lutolf, unpublished) and should further enhance gel characteristics, for example toward faster cross-linking. We therefore envision a wealth of useful applications of PPTase gels in cell biology and tissue engineering.

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Supporting Information Available: Detailed procedures and results on the following: PEG-CoA, ACP_ACP, CoA-RGDS design, synthesis and characterization, hydrogel formation and characterization, 2D and 3D cell culture and characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Lutolf, M. P.; Hubbell, J. A. *Nat. Biotechnol.* **2005**, *23*, 47–55.
(2) Lutolf, M. P.; Tirelli, N.; Cerritelli, S.; Cavalli, L.; Hubbell, J. A. *Bioconjugate Chem.* **2001**, *12*, 1051–6.
(3) DeForest, C. A.; Polizzotti, B. D.; Anseth, K. S. *Nat. Mater.* **2009**, *8*, 659–664.
(4) Hu, B. H.; Su, J.; Messersmith, P. B. *Biomacromolecules* **2009**, *10*, 2194–2200.
(5) Toledano, S.; Williams, R. J.; Jayawarna, V.; Ulijn, R. V. *J. Am. Chem. Soc.* **2006**, *128*, 1070–1.
(6) Yang, Z. M.; Liang, G. L.; Xu, B. *Soft Matter* **2007**, *3*, 515–520.
(7) Sperinde, J. J.; Griffith, L. G. *Macromolecules* **1997**, *30*, 5255–5264.
(8) Hu, B. H.; Messersmith, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 14298–14299.
(9) Ehrbar, M.; Rizzi, S. C.; Schoenmakers, R.; Hubbell, J. A.; Weber, F. E.; Lutolf, M. P. *Biomacromolecules* **2007**, *8*, 3000–3007.
(10) Chen, T. H.; Embree, H. D.; Brown, E. M.; Taylor, M. M.; Payne, G. F. *Biomaterials* **2003**, *24*, 2831–2841.
(11) Kurisawa, M.; Chung, J. E.; Yang, Y. Y.; Gao, S. J.; Uyama, H. *Chem. Commun.* **2005**, 4312–4314.
(12) Lambalot, R. H.; Gehring, A. M.; Flugel, R. S.; Zuber, P.; LaCelle, M.; Marahiel, M. A.; Reid, R.; Khosla, C.; Walsh, C. T. *Chem. Biol.* **1996**, *3*, 923–936.
(13) Zhou, Z.; Cironi, P.; Lin, A. J.; Xu, Y.; Hrvatin, S.; Golan, D. E.; Silver, P. A.; Walsh, C. T.; Yin, J. *ACS Chem. Biol.* **2007**, *2*, 337–46.
(14) Vivero-Pol, L.; George, N.; Krumm, H.; Johnsson, K.; Johnsson, N. *J. Am. Chem. Soc.* **2005**, *127*, 12770–12771.
(15) George, N.; Pick, H.; Vogel, H.; Johnsson, N.; Johnsson, K. *J. Am. Chem. Soc.* **2004**, *126*, 8896–8897.
(16) Wong, L. S.; Thirlway, J.; Micklefield, J. *J. Am. Chem. Soc.* **2008**, *130*, 12456–64.
(17) Quadri, L. E. N.; Weinreb, P. H.; Lei, M.; Nakano, M. M.; Zuber, P.; Walsh, C. T. *Biochemistry* **1998**, *37*, 1585–1595.
(18) Yin, J.; Lin, A. J.; Golan, D. E.; Walsh, C. T. *Nat. Protoc.* **2006**, *1*, 280–285.
(19) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86–89.
(20) Yin, J.; Straight, P. D.; McLoughlin, S. M.; Zhou, Z.; Lin, A. J.; Golan, D. E.; Kelleher, N. L.; Kolter, R.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 15815–15820.
(21) Lutolf, M. P.; Hubbell, J. A. *Biomacromolecules* **2003**, *4*, 713–722.

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