Video Article

Synthesis of an Intein-mediated Artificial Protein Hydrogel

Miguel A. Ramirez[1], Zhilei Chen[1,2]

[1]Artie McFerrin Department of Chemical Engineering, Texas A&M University, College Station

[2]Department of Microbial and Molecular Pathogenesis, Texas A&M Health Science Center, Texas A&M University, College Station

Correspondence to: Zhilei Chen at Zchen4@tamu.edu

URL: https://www.jove.com/video/51202
DOI: doi:10.3791/51202

Keywords: Bioengineering, Issue 83, split-intein, self-assembly, shear-thinning, enzyme, immobilization, organic synthesis

Date Published: 1/27/2014

Citation: Ramirez, M.A., Chen, Z. Synthesis of an Intein-mediated Artificial Protein Hydrogel. *J. Vis. Exp.* (83), e51202, doi:10.3791/51202 (2014).

Abstract

We present the synthesis of a highly stable protein hydrogel mediated by a split-intein-catalyzed protein *trans*-splicing reaction. The building blocks of this hydrogel are two protein block-copolymers each containing a subunit of a trimeric protein that serves as a crosslinker and one half of a split intein. A highly hydrophilic random coil is inserted into one of the block-copolymers for water retention. Mixing of the two protein block copolymers triggers an intein *trans*-splicing reaction, yielding a polypeptide unit with crosslinkers at either end that rapidly self-assembles into a hydrogel. This hydrogel is very stable under both acidic and basic conditions, at temperatures up to 50 °C, and in organic solvents. The hydrogel rapidly reforms after shear-induced rupture. Incorporation of a "docking station peptide" into the hydrogel building block enables convenient incorporation of "docking protein"-tagged target proteins. The hydrogel is compatible with tissue culture growth media, supports the diffusion of 20 kDa molecules, and enables the immobilization of bioactive globular proteins. The application of the intein-mediated protein hydrogel as an organic-solvent-compatible biocatalyst was demonstrated by encapsulating the horseradish peroxidase enzyme and corroborating its activity.

Video Link

The video component of this article can be found at https://www.jove.com/video/51202/

Introduction

Hydrogels made entirely of proteins carry the potential to significantly advance fields as diverse as tissue engineering, drug delivery and biofabrication[1]. They offer advantages over traditional synthetic polymer hydrogels including biocompatibility and the potential to noninvasively support the incorporation of bioactive globular proteins.

In this work, we describe the development of a novel protein hydrogel formed via a split-intein-mediated protein trans-splicing reaction and its application as a protein immobilization scaffold (**Figure 1**). The building blocks for this hydrogel are two protein block-copolymers each comprising the N- or C-terminal fragment of a split intein (IN and IC) and a subunit of a multimeric crosslinker protein. The DnaE intein from *Nostoc punctiforme* (Npu) was used as the split intein[2,3] and a small trimeric protein (12 kDa) CutA from *Pyrococcus horikoshii* was used as the crosslinker protein[4,5]. Different crosslinkers are joined through intein catalyzed trans-splicing reaction, leading to the formation of a highly crosslinked protein network (hydrogel). Npu intein was chosen because of its fast reaction kinetics ($t_{1/2}$ = 63 sec) and high trans-splicing yield (close to 80%)[2,3]. The CutA protein was chosen as the crosslinker due to its high stability. CutA trimers have a denaturation temperature of near 150 °C and retain trimeric quaternary structure in solutions containing as much as 5 M guanidine hydrochloride [4,6]. Since subunit exchange between different crosslinkers is a major contributor of the physical hydrogel surface erosion[7], the very strong inter subunit interaction in CutA should discourage such subunit exchanges, leading to a more stable hydrogel. One of these building blocks also contains a highly hydrophilic peptide S-fragment as the mid-block to facilitate water retention[8].

Mixing of the two hydrogel building blocks initiates a *trans*-splicing reaction between the IN and IC intein fragments, generating a longer polypeptide chain with crosslinkers at both terminals. Crosslinkers from multiple such molecular units interact with each other, forming a highly crosslinked hydrogel network (**Figure 1A**). A specific "docking station peptide" (DSP) is incorporated into one of the hydrogel building blocks to facilitate stable immobilization of a "docking protein" (DP)-tagged target protein into the hydrogel. The use of a split intein to mediate the hydrogel assembly not only provides additional flexibility for protein hydrogel synthesis, but also enables high-density, uniform loading of the target protein throughout the entire hydrogel, as the target proteins are loaded prior to hydrogel formation.

The intein-mediated protein hydrogel is highly stable in aqueous solution with little-to-no detectable erosion after 3 months at room temperature. Stability is retained in a wide range of pHs (6-10) and temperatures (4-50 °C), and the hydrogel is also compatible with organic solvents. This hydrogel is used for the immobilization of two globular proteins: the green fluorescent protein (GFP) and the horseradish peroxidase (HRP). Hydrogel entrapping the latter protein is used to perform biocatalysis in an organic solvent.

Copyright © 2014 Journal of Visualized Experiments

Protocol

1. Plasmid Construction

NOTE: All genes were amplified under standard PCR reactions using Phusion High-Fidelity DNA Polymerase per the manufacturer's specifications. Primers used for cloning have been described previously[9]. All constructs are listed in **Table 1**.

1. To generate CutA-NpuN (**N, Table 1**):
    1. PCR amplify CutA and NpuN genes from plasmids pET30-CutA-Tip1[10] and KanR-IntRBS-NpuNC-CFN[11], respectively, using the appropriate primers.
    2. Digest these fragments with the appropriate restriction enzymes and sequentially insert these fragments into the pET26b vector between the T7 promoter and a C-terminal 6xHistidine tag to generate **N** (**Figure 2A**).

2. To generate NpuC-S-CutA (**C, Table 1**):
    1. PCR amplify NpuC, CutA and S fragment $[AG_3(PEG)]_{10}$ from plasmid KanR-IntRBS-NpuNC-CFN[11], pET30-CutA-Tip1[10] and pQE9 AC_{10}Atrp[12], respectively, using the appropriate primers.
    2. Digest these fragments with the appropriate restriction enzymes and sequentially insert these fragments into the pET26b vector between T7 promoter and a C-terminal 6xHistidine to generate **C** (**Figure 2B**).

3. To generate NpuC-S-$SH3_{lig}$-CutA (**C**-$SH3_{lig}$, **Table 1**):
    1. PCR amplify CutA using primers containing a $SH3_{lig}$ (PPPALPPKRRR) and a flexible linker $(GGGGS)_2$ to generate fragment $SH3_{lig}$-CutA.
    2. Replace the CutA gene from **C** with fragment $SH3_{lig}$-CutA.

4. To generate SH3-GFP (**Table 1**):
    1. Amplify the SH3 gene from plasmid pJD757[13] using the appropriate primers.
    2. Fuse this fragment to the GFP gene and insert it into the pET26b vector between the T7 promoter (**Figure 2C**) and a C-terminal 6xHistidine tag.

2. Protein Expression

1. Transform 50 µl of chemically competent *Escherichia coli* BL21(DE3) with the appropriate expression plasmid.
2. After transformation, serially dilute these cells, and plate them on Luria-Bertani (LB)/agar plates containing 50 µg/ml kanamycin.
3. Incubate plates containing transformed cells at 37 °C for ~15 hr.
4. After incubation, pick a plate that contains 50-100 colonies and resuspend all colonies in 5 ml of LB broth.
5. Transfer suspension to 1 L LB broth containing kanamycin (50 µg/ml) and grow cells at 37 °C with shaking at 250 rpm. Monitor the absorbance at 600 nm (OD_{600}). Grow culture until OD_{600} ~0.8.
    1. For **C** and **C**-$SH3_{lig}$, induce protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture (1 mM final concentration) and incubate the culture at 37 °C for 4 hr while shaking at 250 rpm.
    2. For **N** and SH3-GFP, cool the culture to ~18 °C by immersing the culture flask in an ice water bath for ~5 min. Induce protein expression by adding IPTG to the culture (1mM final concentration) and incubate the culture at 18 °C for 14-18 hr while shaking at 250 rpm.

6. After protein expression, centrifuge the culture at 6,000 x g for 20 min at 4 °C to collect the pellet. Store cell pellet at -80 °C until use.

3. Protein Purification

1. Purification of **N** (denaturing conditions)
    1. Resuspend cell pellets in Buffer A (**Table 2**) at 10 ml/g of wet pellet.
    2. Immerse the pellet suspension in an ice-water bath and disrupt cells by sonication (Amp 10, with 1 sec pulse and 6 sec pause for 1 min).
    3. Centrifuge the lysate at 16,000 x g for 20 min at 4 °C.
    4. Discard the supernatant. Resuspend the pellet in Buffer DA (containing 8 M urea) and centrifuge the suspension at 16,000 x g for 20 min at 4 °C.
    5. Pass the supernatant through a 5 ml Ni-nitrilotriacetic acid (NTA) column previously equilibrated with buffer DA.
    6. Wash column with 30 ml of Buffer DA supplemented with 45 mM imidazole. Elute purified protein using 20 ml of Buffer DA supplemented with 150 mM imidazole.
    7. Reduce the urea concentration in the protein sample to <1 mM by either one of the following methods given in 3.1.7.1 or 3.1.7.2:
        1. Dialyze protein in DPBS buffer (**Table 2**) at 4 °C overnight using tubes with <20 kDa cutoff.
        2. Centrifuge purified protein in a 30 kDa ultra-filtration spin column at 2,800 x g, 4 °C until the volume is less than 1 ml. Add 14 ml DPBS buffer to the column to dilute the protein sample. Repeat the centrifugation/dilution steps three more times.
    8. After buffer exchange, add dithiothreitol (DTT) to the purified protein (final 2 mM) and concentrate protein to ~100 mg/ml by centrifugation through a 30 kDa ultra-filtration spin column at 2,800 x g, 4 °C.
    9. Aliquot the concentrated protein and store at -80 °C until use.

Copyright © 2014 Journal of Visualized Experiments

2. Purification of **C** and **C**-$SH3_{lig}$ (native condition)
    1. Resuspend cell pellets in Buffer B (pH 6.0) (**Table 2**) supplemented with 1x protease inhibitor cocktail at 10 ml/g of wet pellet. Use acidic buffer to minimize proteolytic degradation of the target protein.
    2. Disrupt cell suspension by sonication as described in 3.1.2. Centrifuge the lysate at 16,000 x g for 20 min at 4 °C and keep the supernatant.
    3. Pass the soluble lysate through a 5-ml Ni-NTA column previously equilibrated with buffer B.
    4. Wash column with Buffer B supplemented with 45 mM imidazole, and elute the target protein in 20 ml of Buffer B supplemented with 150 mM imidazole.
    5. For **C**, skip to step 3.2.6. For **C**-$SH3_{lig}$, carry out an additional ion-exchange purification step to remove partially degraded protein as given in steps 3.2.5.1 to 3.2.5.3
        1. Reduce NaCl concentration in **C**-$SH3_{lig}$ to <1 mM following the procedure described in 3.1.7.
        2. Load the target protein onto a 5 ml anion exchanger beaded agarose matrix column previously equilibrated with sodium phosphate buffer (50 mM, pH 7.0).
        3. Elute target protein from the column by running a gradient from a solution containing 10 mM Tris-HCl pH 8.0 buffer to a solution containing the same buffer supplemented with 1 M NaCl. Take samples during protein elution and pool samples with the highest purity based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
    6. Buffer exchange the purified protein into DPBS buffer, as described in 3.1.7.
    7. Add DTT to the purified protein (final concentration 2 mM) and concentrate the protein to ~100 mg/ml using a 30 kDa ultra-filtration spin column as described in 3.1.8. Aliquot and store concentrated protein at -80 °C until use.

3. Purification of SH3-GFP
    1. Resuspend cell pellets using Buffer A at 10 ml/g of wet pellet.
    2. Disrupt pellet suspension by sonication as described in 3.1.2.
    3. Centrifuge the lysate at 16,000 x g for 20 min at 4 °C and collect the supernatant.
    4. Pass the supernatant (soluble lysate) through a 5-ml Ni-NTA column previously equilibrated with Buffer A.
    5. Wash column with 30 ml of Buffer A supplemented with 45 mM imidazole. Elute purified protein using 20 ml of Buffer A supplemented with 150 mM imidazole.
    6. Buffer exchange the purified protein into DPBS buffer using an approach similar to that described in 3.1.7 and concentrate the protein to ~150 mg/ml using a 30 kDa ultra-filtration spin column as described in 3.1.8.
    7. Aliquot and store purified protein at -80 °C until use.

4. SDS-PAGE analysis of purified samples containing CutA
    1. Dilute each purified protein in double-distilled water to reduce the concentration of NaCl to ~1 mM. At this NaCl concentration, most of the CutA trimer proteins run as monomers on the SDS-PAGE gels.
    2. Mix samples with 2x SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 20% Glycerol, 10% w/v SDS, 0.1 % w/v bromo-phenol blue, 2% β-mercaptoethanol), incubated at 95 °C for 5 min.
    3. Load the samples onto a 12% SDS-PAGE gel. Carry out electrophoresis at a constant voltage of 200 V for ~50 min.
    4. Observe protein in the gels by staining with Coomassie brilliant blue R250 following the standard protocols (**Figure 1C**).

4. Hydrogel Formation

NOTE: the sample hydrogels made in this study contain 1.6 mM of each hydrogel building block unless noted otherwise. This protein concentration yields a soft and stable hydrogel. CAUTION: Sodium azide (NaN_3) is added to the hydrogel to a final concentration of 0.5% w/v to prevent bacterial contamination. NaN_3 is highly toxic and must be handled with extreme care as indicated in the Material Safety Data Sheet.

1. Calculate the volume for each of the concentrated proteins needed to achieve a final concentration of 1.6 mM in a 100 µl sample hydrogel.
   For example:
   Concentration of **N**: 100 mg/ml
   Molecular weight of **N**: 26.3 kDa (refer to **Table 1**)
   Desired Volume: 100 µl Desired Concentration: 1.6 mM

$$moles = molarity * volume = 1.6x10^{-3}\frac{moles}{L} * 100x10^{-6}L = 1.6x10^{-7}moles$$

$$mass = moles * molecular\ weight = 1.6x10^{-7}moles * 26.3x10^{3}\frac{g}{mol}$$
$$= 4.2x10^{-3}g$$

$$volume = \frac{mass}{concentration} = \frac{4.2x10^{-3}g}{100\frac{g}{L}} = 4.2x10^{-5}L = 42\mu L$$

2. To make a 100 µl hydrogel (1.6 mM), mix **C** (*x* µl, volume calculated according to 4.1) with 5% NaN_3 (10 µl), 100 mM DTT (5 µl) and **N** (*y* µl, volume calculated according to 4.1) inside a 2 ml glass vial.
3. Add DPBS buffer ((85 - *x* - *y*) µl) to the vial to achieve a final volume of 100 µl, and manually mix all the components via a swirling motion using a pipette tip. Note: The solution becomes very viscous upon mixing.
4. Centrifuge the mixture for 2 min at 8,000 x g to remove the air bubbles.

5. Incubate the mixture at room temperature overnight to allow the intein *trans*-splicing reaction to reach completion. Confirm hydrogel formation by turning tube upside down. The proteins will not flow if a hydrogel is formed.
6. Estimate the intein *trans*-splicing yield by checking samples (0.5 µl each) collected before step 4.2 and after step 4.5 on a SDS-PAGE gel, as described in 3.4 (**Figure 1C**).

5. Immobilization of GFP via Docking Protein (DP) and Docking Station Peptide (DSP) Interaction

1. To make a 50 µl GFP-functionalized hydrogel (1.2 mM), combine **C**-$SH3_{lig}$ (x µl, calculated according to 4.1) and SH3-GFP (*y* µl, calculated according to 4.1) at 1:1 molar ratio in a 1.7 ml microcentrifuge tube and incubate the mixture at room temperature for 30 min.
2. Add 5% NaN_3 (5 µl), 100 mM DTT (2.5 µl), (42.5 - *x* - *y*) µl DPBS to the same tube. Add **N** (*y* µl, calculated according to 4.1) to achieve a 1:1 molar ratio of **N** and **C**-$SH3_{lig}$. Mix the sample by using a pipette tip by a swirling motion.
3. Centrifuge the mixture at 8,000 x g for 2 min and incubate the mixture at room temperature overnight in the dark. A hydrogel encapsulating SH3-GFP forms during incubation.

6. Use of 1.6 mM Hydrogel as an Immobilization Scaffold for Enzymatic Reaction in Organic Solvent

1. Use the HRP as a model enzyme. Prepare a stock solution of HRP (28 mg/ml or 0.63 mM) in DPBS.
2. To make a 30 µl hydrogel (1.6 mM) entrapping HRP, combine **C** (x µl, calculated according to 4.1) with HRP (2 µl), 5% NaN_3 (3 µl) and DTT (1.5 µl of 100 mM) inside a 1.7 ml centrifuge tube.
3. Add **N** (*y* µl, calculated according to 4.1) and DPBS (23.5 - *x* - *y*) µl. Mix with a pipette tip with a swirling motion.
4. Centrifuge the mixture at 8,000 x g for 2 min and incubate at room temperature overnight.
   CAUTION: the regents used for the following activity assay are highly toxic. Use specific safety recommendations by the corresponding Material Safety Data Sheets.
5. For enzymatic reaction, submerge the hydrogel in 1 ml of reaction cocktail containing *N*,*N*-dimethyl-*p*-phenylene diamine (5.8 mM), phenol (5.8 mM) and *tert*-butyl hydroperoxide (2.9 mM) in *n*-heptane[14]. Manually disrupt the gel using a pipette tip to increase the contact surface area of the hydrogel and the solvent.
6. Detect HRP product, an indophenol-type dye, by measuring the optical absorbance of samples taken at different times at 546 nm in a plate reader (**Figure 5**).

Representative Results

A schematic for intein-mediated protein hydrogel formation is presented in **Figure 1A**. The building blocks of the hydrogel are the protein copolymers CutA-NpuN (N) and NpuC-S-CutA(C) (**Figure 1A**, **Table 1**). NpuN/C are the N-/C-fragments of the naturally split DnaE intein from Nostoc punctiforme (Npu). CutA is a stable trimeric protein from *Pyrococcus horikoshii*[4,5]. Mixing of purified N and C in the presence of the reducing agent DTT induces the formation of a third protein — the ligated product (J: CutA-S-CutA) (**Figures 1A** and **1C**). Individually, the hydrogel building blocks N and C exist as viscous fluids (**Figure 1B**). Mixing of N and C yields a transparent semi-solid material that is retained on the bottom of a glass vial after inversion, indicative of the formation of a hydrogel[15,16] (**Figure 1B**)[18,19].

This intein-mediated protein hydrogel (1.6 mM) exhibits high solution stability. There is little-to-no loss of crosslinked hydrogel scaffold after 21 days at 22 °C in DPBS buffer, as the total amount of protein released into the DPBS buffer only slightly exceeds the theoretical amount of the spliced intein from the hydrogel (assuming 100% intein *trans*-splicing efficiency) (**Figure 3A**). Densitometry revealed that, during hydrogel formation, *trans*-splicing reactions were ~80% efficient (**Figure 1C**). SDS-PAGE gel analysis showed that only trace amounts of the *trans*-spliced product were present in the hydrogel's surrounding buffer (**Figure 3B**, band J), confirming that loss of the crosslinked hydrogel scaffold to erosion is minimal. The main protein present in the hydrogel's surrounding buffer is the spliced out intein. No visible signs of erosion were observed in an undisturbed hydrogel submerged in aqueous solution at room temperature for over 3 months (**Figure 3A** inlet). The hydrogel is also highly stable at 37 °C (**Figure 3C**) and in both acidic and basic buffers (**Figure 3D**).

To facilitate protein immobilization, a pair of protein and its peptide ligand was used to dock proteins of interest into the hydrogel scaffold. We chose the SH3 protein, a Src homology 3 domain from the adaptor protein CRK, as the docking protein (DP) for fusion to a protein of interest, and its ligand ($SH3_{lig}$) as the docking station peptide (DSP) for incorporation into the hydrogel scaffold. This interaction pair was chosen because of the relatively small molecular size (56 aa for SH3 and 11 aa for $SH3_{lig}$) and high affinity (k_d = 0.1 µM)[17,18]. $SH3_{lig}$ was inserted between NpuC and CutA to form **C**-$SH3_{lig}$ (**Table 1**). The SH3 protein was fused to the N-terminus of a model target globular protein, green fluorescent protein (GFP) to form SH3-GFP. The process described in the protocol (Section 5, **Figure 4A**) yields a hydrogel containing 1.2 mM *trans*-spliced hydrogel backbone building blocks and 1.2 mM GFP. The GFP-containing hydrogel exhibited a similar stability to the hydrogel lacking GFP (**Figure 4B**) with ~35% total protein loss after 21 days in DPBS buffer. Most of the proteins present in the erosion buffer were the cleaved inteins. The leaching rate of SH3-GFP from a hydrogel containing the $SH3_{lig}$ is ~30% after 3 weeks, significantly smaller than that from a hydrogel lacking the $SH3_{lig}$ (>70% protein loss in the same period of time, **Figure 4C**). The immobilized SH3-GFP in the hydrogel glows under UV light. As seen in **Figure 4D**, hydrogel containing the docking station peptide $SH3_{lig}$ retains most of the GFP fluorescence after 3 weeks while the hydrogel lacking $SH3_{lig}$ becomes essentially nonfluorescent. It is expected that the use of a higher affinity DP/DSP pair can further reduce the leaching rate of the immobilized protein.

In this experiment, GFP was used as the target protein; however, any DP-tagged protein can be conveniently immobilized into this hydrogel. Thus, the intein-mediated protein hydrogel should provide a general scaffold for protein immobilization. The density of immobilized GFP

demonstrate in this work is ~33 mol% of the hydrogel. A higher immobilization density can potentially be achieved when multiple DSP are incorporated into the hydrogel building block.

Next, the HRP enzyme was incorporated into the protein hydrogel to demonstrate its ability to support biocatalysis in organic solvents. Enzyme activity was measured by monitoring the oxidative coupling of *N*,*N*-dimethyl-*p*-phenylene diamine and phenol with *tert*-butyl hydroperoxide over time[14]. The hypothesis was that the hydrated environment of the hydrogel will protect the attached enzyme from the denaturing effect of the organic solvent. Hydrogel containing 0.042 mM HRP was immersed in *n*-heptane containing the substrates. After immersing in organic solvent, the HRP-containing hydrogel was manually disrupted into small clusters to increase the hydrophilic-hydrophobic interface area (**Figure 5B**). Hydrogel-incorporated HRP effectively catalyzed the rapid oxidation reaction, giving rise to a colorimetric product (**Figure 5C**, triangles). The product accumulation follows a linear slope, indicating little-to-no enzyme inactivation during the experiment. The control reaction with HRP dissolved directly in the organic reaction cocktail exhibited negligible catalytic activity due to enzyme denaturation (data not shown). HRP dissolved in DPBS first followed by addition to the organic solvent was able to catalyze the conversion but at a much reduced reaction rate (**Figure 5C**, circles). The low conversion rate of enzymes dissolved in DPBS is likely due to the small interfacial area between the DPBS and the organic solvent, which limits the rate of substrate/product diffusion. Incorporation of the highly hydrophilic **S** fragment in the hydrogel backbone, which effectively 'locks' the water inside the hydrogel and prevents the organic solvent to access the hydrogel interior, enables the hydrogel to withstand the denaturing effect of organic solvent. These results indicate that the intein-mediated protein hydrogel can be an effective scaffold for enzymatic reactions in organic solvent.

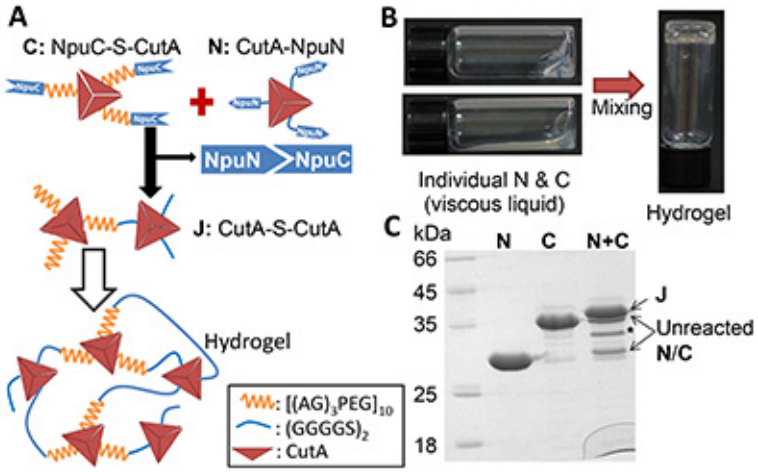

Figure 1. Intein-mediated protein hydrogel. (**A**) Schematics of intein *trans*-splicing reaction that triggers the formation of an extended protein chain (**J**) with crosslinker proteins at both termini. Crosslinker proteins from multiple **J** protein chains noncovalently associate and, upon intein-mediated protein ligation, induce the formation of a highly cross-linked protein network with hydrogel properties. NpuN/C: intein N-/C-fragment. (**B**) Mixing of purified **N** and **C** (8.3% w/v) leads to the formation of a highly crosslinked hydrogel network (1.6 mM **J**). (**C**) SDS-PAGE analysis of purified **N** and **C** building blocks before and after mixing. "N+C" corresponds to a sample taken directly from a 1.6 mM hydrogel. "*" denotes an intein C-terminal cleavage side reaction product. Intensity of each band was quantified using the 'trace module in the "Quantity One" software. Band intensity was divided by the protein molecular weight to obtain the molar equivalent. *Trans*-splicing efficiency (~80%) was calculated from the amount of the product **J** and the unreacted **N**/**C** in the same lane. Reprinted with permission from Journal of American Chemical Society (doi:10.1021/ja401075s). Click here to view larger image.

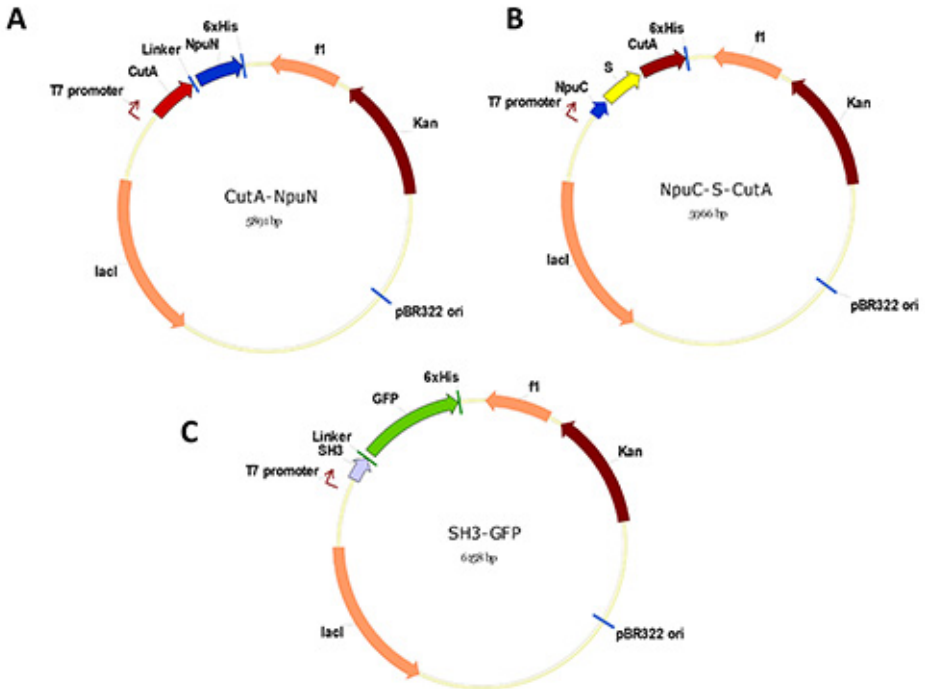

Figure 2. Plasmid maps of protein constructs. (**A**) CutA-NpuN, (**B**) NpuC-S-CutA and (**C**) SH3-GFP (**Table 1**) were cloned into pET26b vector under the control of the T7 promoter. Click here to view larger image.

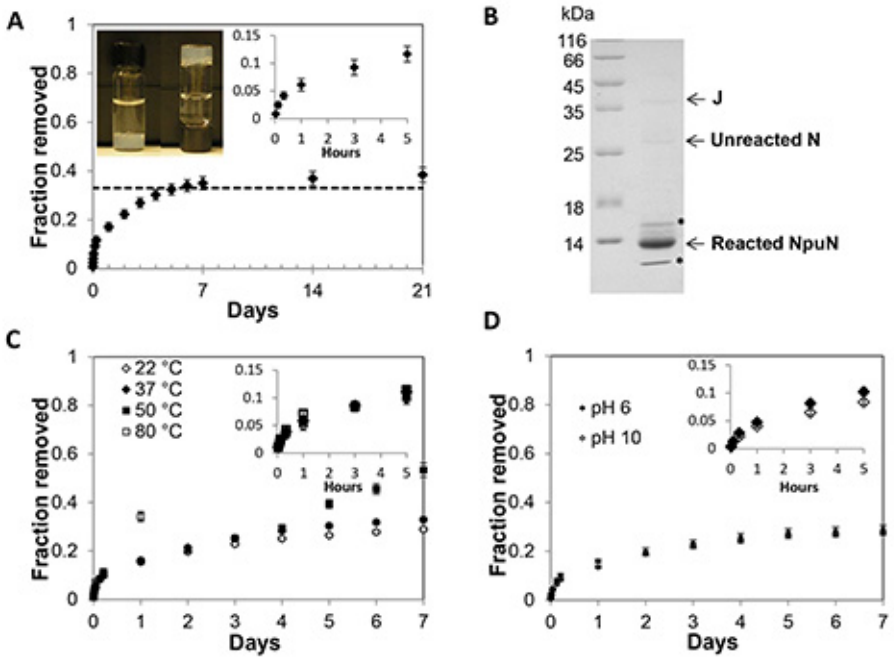

Figure 3. Stability of intein-mediated protein hydrogels in DPBS. (**A**) Erosion profile of a 1.6 mM hydrogel at 22 °C. Dotted line represents the theoretical mass of the cleaved inteins. The inlet shows an undisturbed hydrogel in DPBS after 3 months at room temperature. (**B**) SDS-PAGE analysis of hydrogel's surrounding buffer. All the samples of the buffer in which the hydrogel was immersed in (**A**) were pooled (total 7.5 ml) and concentrated 75-fold via ultrafiltration through a 10 kDa membrane prior to gel loading. **J**: intein *trans*-spliced product. **N**: unreacted CutA-NpuN. **NpuN**: spliced out N-intein fragment. Unreacted **C** and spliced out NpuC are not visible in the gel due to the small quantity and small size (4 kDa), respectively. The asterisk denotes unidentified bands. (**C**) Erosion profile of hydrogel incubated at different temperatures. (**D**) Erosion profile of hydrogels incubated at two different pHs. Reprinted with permission from Journal of American Chemical Society (doi:10.1021/ja401075s). Click here to view larger image.

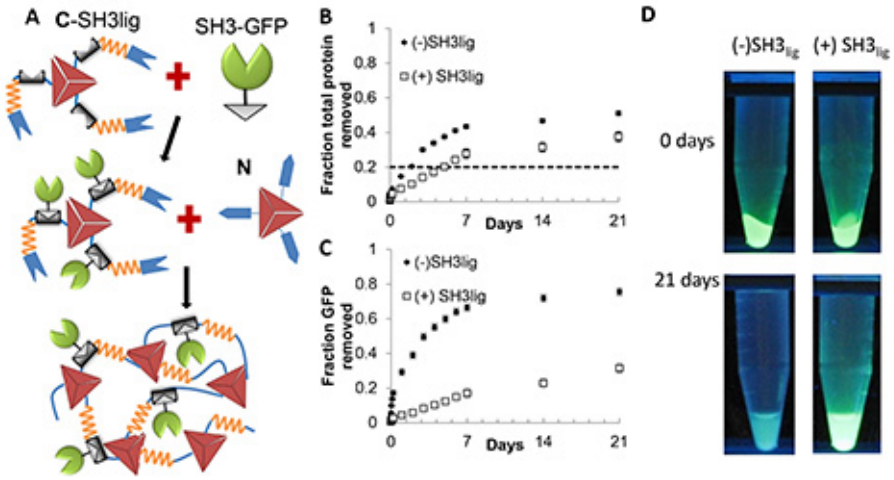

Figure 4. Intein-mediated hydrogel supports the immobilization of functional globular proteins.(**A**) Schematic of protein immobilization using GFP as a model globular protein. The DSP-containing hydrogel building block is first mixed with DP-fused target protein for target protein loading onto the building block. The complementary intein fragment-containing hydrogel building block is added to the mixture yielding a hydrogel with immobilized GFP. (**B**) Total protein erosion profile of hydrogel containing 1:1 molar ratio of SH3-GFP. Dotted line represents the theoretical mass of the spliced out inteins. The error bars represent the standard deviation of 2 independent experiments. (**C**) Leaching profile of SH3-GFP from hydrogel with and without the DSP. (**D**) Images of GFP containing hydrogels under UV exposure immediately after hydrogel formation and after 21 days. Reprinted with permission from Journal of American Chemical Society (doi:10.1021/ja401075s). Click here to view larger image.

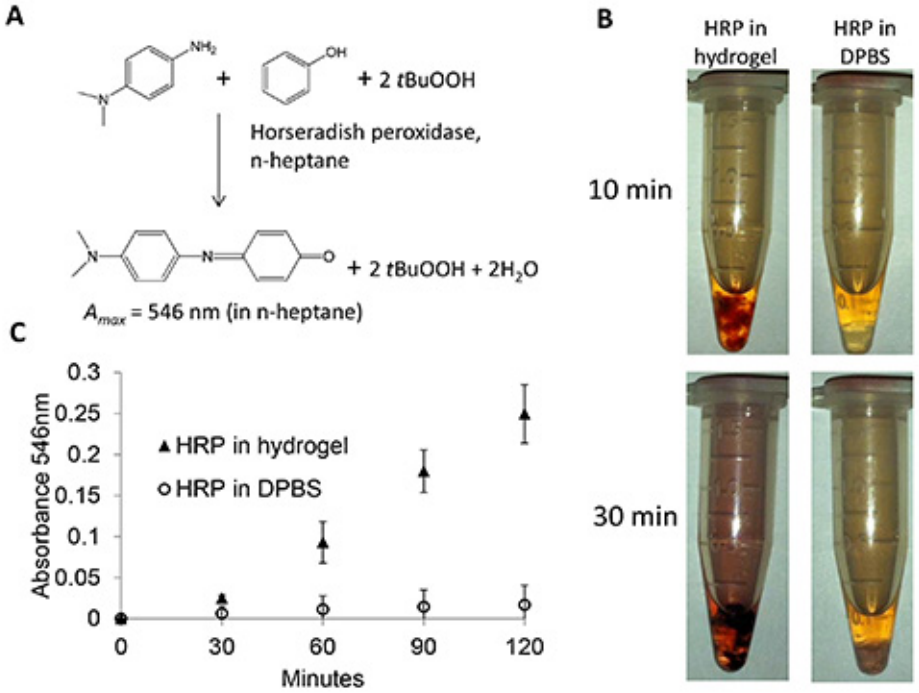

Figure 5. Horseradish peroxidase (HRP)-entrapped intein-triggered protein hydrogel facilitates HRP-catalyzed reaction in organic solvent n-heptane. (**A**) Model reaction catalyzed by HRP in organic solvents. (**B**) Photograph of hydrogels containing encapsulated HRP after being disrupted into small fragments and incubated in a reaction cocktail for 10 min and 30 min (left) and control experiment with the same amount of nonimmobilized HRP in DPBS buffer (right). (**C**) Product formation was monitored by absorbance at 546 nm. Adapted with permission from Journal of American Chemical Society (doi:10.1021/ja401075s). Click here to view larger image.

Table 1. Protein constructs used in this study.

Short Name	Protein Sequence	MW (kDa)
CutA-NpuN (**N**)	CutA-EAC-$(GGGGS)_2$-AS-NpuN-HHHHHH	26.3

NpuC-S-CutA (**C**)	NpuC-CFNKLYRDPMG- $[(AG)_3PEG]_{10}$-ARMPYV-CutA-HHHHHH	26.1
NpuC-S-$SH3_{lig}$-CutA (**C**-$SH3_{lig}$)	NpuC-CFNKLYRDPMG- $[(AG)_3PEG]_{10}$-ARMPYVGS-PPPALPPKRRR-$(GGGGS)_2$-AS-CutA-HHHHHH	28.3
SH3-GFP	SH3-KL-$(GGGGS)_2$-AS-GFP-HHHHHH	34.5

Reprinted with permission from Journal of American Chemical Society (doi:10.1021/ja401075s).

Table 2. Buffer compositions.

Buffer A	500 mM NaCl, 10 mM Tris-HCl, pH 8.0
Buffer DA	500 mM NaCl, 8 M Urea, 10 mM Tris-HCl, pH 8.0
Buffer B	500 mM NaCl, 50 mM NaPOi, pH 6.0
DPBS	Dulbecco's PBS, 137.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4

Reprinted with permission from Journal of American Chemical Society (doi:10.1021/ja401075s).

Discussion

In this work, we demonstrated the synthesis of a highly stable intein-mediated protein hydrogel. The use of a split intein enables the hydrogel to be conditionally formed in response to the mixing of two liquid-phase components. Specifically, the split intein covalently links two liquid-phase building blocks via a *trans*-splicing reaction, yielding a polypeptide unit flanked by crosslinking units that in turn self assembles into a hydrogel. The mixing-induced formation of the hydrogel bypasses technical difficulties in the synthesis of single-component protein hydrogels where gel-mediated clogging of chromatographic purification columns can occur. Furthermore, the hydrogel forms under physiological conditions without the need for any extraneous chemical inducers and/or physical triggers.

The intein-mediated hydrogel retains high stability in both acidic and basic buffers, and at physiological temperature. Hydrogels can form with as little as 0.8 mM of each individual building block (data not shown), but higher protein concentrations (~1.6 mM) yield hydrogels with better mechanical stability. The hydrogel's high stability is attributed to the use of 1) a very stable trimeric protein, CutA, as a crosslinker, and 2) intein to covalently join different crosslinkers. Densitometric analysis of SDS-PAGE gels showed that ~80% of the input proteins successfully underwent the split intein-catalyzed *trans*-splicing reaction.

For hydrogel formation, individual building blocks are first concentrated to ~100 mg/ml. A reducing agent, such as DTT, needs to be present during the protein concentration step to prevent the formation of intermolecular disulfide bonds. In the absence of reducing agent, concentrated individual hydrogel building block can sometimes form hydrogel-like material. However, this gel-like material dissolves rapidly when immersed in a buffer and/or in the presence of reducing agent.

To obtain a hydrogel with the maximum stability, it is important to ensure that the molar ratio of the two hydrogel building blocks is 1:1. Any excess building block will not be crosslinked and can affect the hydrogel's mechanical properties. Concentrated protein needs to be aliquoted and stored individually to minimize repeated freeze-thaw cycles.

We also demonstrated the use of the intein-mediated protein hydrogel as an organic-solvent-compatible biocatalyst. Specifically, hydrogels incorporating the enzyme HRP were immersed in *n*-heptane containing the substrate and successful conversion of substrate to product was observed. The protein hydrogel serves as an effective scaffold for biocatalysis in organic solvent likely due to the protection from organic-solvent-mediated denaturation offered by the highly hydrated environment of the hydrogel.

One of the limitations of this enzyme immobilization technology is the need to fuse target protein with a docking peptide (DP). This modification may affect the activity and solubility of some target protein and thus require case-by-case optimization of the DP-tagged protein constructs. In addition, a small amount of tagged proteins leached out of the docking station peptide (DSP)-containing hydrogel after 1 week. The stability of the immobilized protein is affected by the affinity of the DSP/DP pair. To achieve better immobilization efficiency, a higher affinity DSP/DP pair will be needed. Finally, this hydrogel exhibits relatively weak mechanical property due to the low plateau storage modulus (100-200 kPa)[9]. The plateau storage modulus is influenced by the structures of both the crosslinker and the midblock[19]. In the current hydrogel, a trimer protein was used as the crosslinker. The use of crosslinker protein with a higher multimeric state can potentially increase the hydrogel plateau storage modulus and its mechanical property.

In summary, we report the synthesis and characterization of a new protein hydrogel that conditionally forms upon the mixing of two liquid-phase protein building blocks, each containing one half of a split intein. Intein-mediated protein hydrogels represent a promising new material with potential applications in synthetic enzymatic reactions, organic synthesis, injectable drug delivery, and tissue engineering.

Copyright © 2014 Journal of Visualized Experiments

Disclosures

No competing financial interests exist.

Acknowledgements

The authors would like to acknowledge Dr. David Tirrell (Caltech) for his kind gift of the plasmid pQE9 AC_{10}Atrp[12], Dr. Tom Muir (Princeton University) for his kind gift of the plasmid KanR-IntRBS-NpuNC-CFN[11], Dr. Takehisa Matsuda (Kanazawa Institute of Technology, Hakusan, Ishikawa, Japan) for his kind gift of the plasmid pET30-CutA-Tip1[10], and Dr. Jay D. Keasling (UC Berkley) for his kind gift of the plasmid pJD757[13]. This work was supported in part by the National Science Foundation CAREER, US Air force YIP and Norman Hackman Advanced Research Program.

References

1. Banta, S., Wheeldon, I. R. & Blenner, M. Protein Engineering in the Development of Functional Hydrogels. *Ann. Rev. Biomed. Eng.* **12**, 167-186, doi:10.1146/annurev-bioeng-070909-105334 (2010).
2. Iwai, H., Zuger, S., Jin, J. & Tam, P. H. Highly efficient protein trans-splicing by a naturally split DnaE intein from *Nostoc punctiforme*. *FEBS Lett.* **580**, 1853-1858, doi:10.1016/j.febslet.2006.02.045 (2006).
3. Zettler, J., Schutz, V. & Mootz, H. D. The naturally split *Npu* DnaE intein exhibits an extraordinarily high rate in the protein trans-splicing reaction. *FEBS Lett.* **583**, 909-914, doi:10.1016/j.febslet.2009.02.003 (2009).
4. Tanaka, Y. *et al.* Structural implications for heavy metal-induced reversible assembly and aggregation of a protein: the case of *Pyrococcus horikoshii* CutA. *FEBS Lett.* **556**, 167-174, doi:S0014579303014029 [pii] (2004).
5. Sawano, M. *et al.* Thermodynamic basis for the stabilities of three CutA1s from *Pyrococcus horikoshii*,*Thermus thermophilus*, and *Oryza sativa*, with unusually high denaturation temperatures. *Biochemistry.* **47**, 721-730, doi:10.1021/bi701761m (2008).
6. Tanaka, T. *et al.* Hyper-thermostability of CutA1 protein, with a denaturation temperature of nearly 150 degrees C. *FEBS Lett.* **580**, 4224-4230, doi:S0014-5793(06)00807-6 [pii] 10.1016/j.febslet.2006.06.084 (2006).
7. Shen, W., Zhang, K., Kornfield, J. A. & Tirrell, D. A. Tuning the erosion rate of artificial protein hydrogels through control of network topology. *Nat. Mater.* **5**, 153-158, doi:nmat1573 [pii] 10.1038/nmat1573 (2006).
8. McGrath, K. P., Fournier, M. J., Mason, T. L. & Tirrell, D. A. Genetically directed syntheses of new polymeric materials. Expression of artificial genes encoding proteins with repeating -(AlaGly)3ProGluGly- elements. *J. Am. Chem. Soc.* **114**, 727-733, doi:10.1021/ja00028a048 (1992).
9. Ramirez, M., Guan, D., Ugaz, V. & Chen, Z. Intein-triggered artificial protein hydrogels that support the immobilization of bioactive proteins. *J. Am. Chem. Soc.* **135**, 5290-5293, doi:10.1021/ja401075s (2013).
10. Ito, F. *et al.* Reversible hydrogel formation driven by protein-peptide-specific interaction and chondrocyte entrapment. *Biomaterials.* **31**, 58-66, doi:S0142-9612(09)00956-9 [pii] 10.1016/j.biomaterials.2009.09.026 (2010).
11. Lockless, S. W. & Muir, T. W. Traceless protein splicing utilizing evolved split inteins. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 10999-11004, doi:10.1073/pnas.0902964106 (2009).
12. Shen, W., Lammertink, R. G. H., Sakata, J. K., Kornfield, J. A. & Tirrell, D. A. Assembly of an artificial protein hydrogel through leucine zipper aggregation and disulfide bond formation. *Macromolecules.* **38**, 3909-3916, doi:10.1021/Ma048348s (2005).
13. Dueber, J. E. *et al.* Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* **27**, 753-U107, doi:10.1038/Nbt.1557 (2009).
14. Bruns, N. & Tiller, J. C. Amphiphilic network as nanoreactor for enzymes in organic solvents. *Nano Lett.* **5**, 45-48, doi:10.1021/Nl048413b (2005).
15. Das, D. *et al.* Water gelation of an amino acid-based amphiphile. *Chem. Eur. J.* **12**, 5068-5074, doi:10.1002/chem.200501638 (2006).
16. Cao, Y. & Li, H. Engineering tandem modular protein based reversible hydrogels. *Chem. Commun.* 4144-4146, doi:10.1039/b806684a (2008).
17. Wu, X. *et al.* Structural basis for the specific interaction of lysine-containing proline-rich peptides with the N-terminal SH3 domain of c-Crk. *Structure.* **3**, 215-226 (1995).
18. Nguyen, J. T., Turck, C. W., Cohen, F. E., Zuckermann, R. N. & Lim, W. A. Exploiting the basis of proline recognition by SH3 and WW domains: Design of n-substituted inhibitors. *Science.* **282**, 2088-2092 (1998).
19. Olsen, B. D., Kornfield, J. A. & Tirrell, D. A. Yielding Behavior in Injectable Hydrogels from Telechelic Proteins. *Macromolecules.* **43**, 9094-9099, doi:10.1021/Ma101434a (2010).

Copyright © 2014 Journal of Visualized Experiments