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Synthesis of an intein-mediated artificial protein hydrogel

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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 re-forms 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. We demonstrate the application of an intein-mediated protein hydrogel functionalized with an enzyme, horseradish peroxidase, as an organic-solvent-compatible biocatalyst.
Author Comments:	
Additional Information:	
Question	**Response**

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Dear Editor,

Please find enclosed a revised protocol entitled “Synthesis of an intein-mediated artificial protein hydrogel”. We modified the protocol according to the editorial review comments. All revised texts are shown in red color. Our point-by-point response to the editorial comments is also attached.

We hope the revised protocol would be suitable for publication in *JoVE*.

Sincerely,

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Key words: split-intein, self-assembly, shear-thinning, enzyme, immobilization, organic synthesis

Short Abstract: We present the synthesis of a split-intein-mediated protein hydrogel. The building blocks of this hydrogel are two protein copolymers each containing a subunit of a trimeric protein that serves as a crosslinker and one half of a split intein. Mixing of the two protein copolymers triggers an intein *trans*-splicing reaction, yielding a polypeptide unit that self-assembles into a hydrogel. This hydrogel is highly pH- and temperature-stable, compatible with organic solvents, and easily incorporates functional globular proteins.

Long 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 re-forms 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.

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 non-invasively 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 (I_N and I_C) 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 s) 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 I_N and I_C 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.

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.

1.1 To generate CutA-NpuN (**N,** Table 1):

1.1.1 PCR amplify CutA and NpuN genes from plasmids pET30-CutA-Tip1[10] and KanR-IntRBS-NpuNC-CFN[11], respectively, using the appropriate primers.

1.1.2 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).

1.2 To generate NpuC-S-CutA (**C,** Table 1):

1.2.1 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.

1.2.2 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).

1.3 To generate NpuC-S-$SH3_{lig}$-CutA (**C**-$SH3_{lig}$, Table 1):

1.3.1 PCR amplify CutA using primers containing a SH3lig (PPPALPPKRRR) and a flexible linker $(GGGGS)_2$ to generate fragment $SH3_{lig}$-CutA.

1.3.2 Replace the CutA gene from **C** with fragment $SH3_{lig}$-CutA.

1.4 To generate SH3-GFP (Table 1):

1.4.1 Amplify the SH3 gene from plasmid pJD757[13] using the appropriate primers.

1.4.2 Fuse this fragment to the GFP gene and insert it into the pET26b vector between the T7 promoter (Figure 3C) and a C-terminal 6xHistidine tag.

2 Protein expression

2.1 Transform 50 µL of chemically competent *Escherichia coli* BL21(DE3) with the appropriate expression plasmid.

2.2 After transformation, serially dilute these cells, and plate them on Luria-Bertani (LB)/agar plates containing 50 µg/mL kanamycin.

2.3 Incubate plates containing transformed cells at 37 °C for ~15 hours.

2.4 After incubation, pick a plate that contains 50-100 colonies and resuspend all colonies in 5 mL of LB broth.

2.5 Transfer suspension to 1L 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.

2.5.1 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 hours while shaking at 250 rpm.

2.5.2 For **N** and SH3-GFP, cool the culture to ~18 °C by immersing the culture flask in an ice water bath for ~5 minutes. Induce protein expression by adding IPTG to the culture (1mM final concentration) and incubate the culture at 18 °C for 14-18 hours while shaking at 250 rpm.

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

3 Protein purification

3.1 Purification of **N** (denaturing conditions)

3.1.1 Resuspend cell pellets in Buffer A (Table 2) at 10 mL per gram of wet pellet.

3.1.2 Immerse the pellet suspension in an ice-water bath and disrupt cells by sonication (Amp 10, with 1 sec pulse 6 sec pause for 1 min).

3.1.3 Centrifuge the lysate at 16,000 x g for 20 minutes at 4 °C.

3.1.4 Discard the supernatant. Resuspend the pellet in Buffer DA (containing 8 M urea) and centrifuge the suspension at 16,000 *x g* for 20 minutes at 4 ˚C.

3.1.5 Pass the supernatant through a 5-mL Ni-nitrilotriacetic acid (NTA) column previously equilibrated with buffer DA.

3.1.6 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.

3.1.7 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:

3.1.7.1 Dialyze protein in DPBS buffer (Table 2) at 4 °C overnight using tubes with < 20kDa cutoff.

3.1.7.2 Centrifuge purified protein in a 30-kDa ultra-filtration spin column at 2800 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.

3.1.8 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 2800 x g, 4 °C.

3.1.9 Aliquot the concentrated protein and store at -80 °C until use.

3.2 Purification of **C** and **C**-$SH3_{lig}$ (native condition)

3.2.1 Resuspend cell pellets in Buffer B (pH 6.0) (Table 2) supplemented with 1x protease inhibitor cocktail at 10 mL per gram of wet pellet. Use acidic buffer to minimize proteolytic degradation of the target protein.

3.2.2 Disrupt cell suspension by sonication as described in 3.1.2. Centrifuge the lysate at 16,000 x g for 20 minutes at 4 °C and keep the supernatant.

3.2.3 Pass the soluble lysate through a 5-mL Ni-NTA column previously equilibrated with buffer B.

3.2.4 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.

3.2.5 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

3.2.5.1 Reduce NaCl concentration in **C**-$SH3_{lig}$ to <1 mM following the procedure described in 3.1.7.

3.2.5.2 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).

3.2.5.3 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).

3.2.6 Buffer exchange the purified protein into DPBS buffer, as described in 3.1.7.

3.2.7 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.

3.3 Purification of SH3-GFP

3.3.1 Resuspend cell pellets using Buffer A at 10 mL per gram of wet pellet.

3.3.2 Disrupt pellet suspension by sonication as described in 3.1.2.

3.3.3 Centrifuge the lysate at 16,000 x g for 20 minutes at 4 °C and collect the supernatant.

3.3.4 Pass the supernatant (soluble lysate) through a 5-mL Ni-NTA column previously equilibrated with Buffer A.

3.3.5 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.

3.3.6 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.

3.3.7 Aliquot and store purified protein at -80 °C until use.

3.4 SDS-PAGE analysis of purified samples containing CutA

3.4.1 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.

3.4.2 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 minutes.

3.4.3 Load the samples onto a 12% SDS-PAGE gel. Carry out electrophoresis at a constant voltage of 200 V for ~50 minutes.

3.4.4 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.

4.1 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

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

$$mass = moles * molecular\ weight = 1.6x10^{-7}\, moles * 26.3x10^{3}\frac{gram}{moles} = 4.2x10^{-3}\, gram\,.$$

$$volume = \frac{mass}{concentration} = \frac{4.2x10^{-3}\, gram}{100\frac{gram}{liter}} = 4.2x10^{-5}\, liter = 42\,\mu L\,.$$

4.2 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.

4.3 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.

4.4 Centrifuge the mixture for 2 minutes at 8,000 *x g* to remove the air bubbles.

4.5 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.

4.6 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

5.1 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 minutes.

5.2 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.

5.3 Centrifuge the mixture at 8,000 *x g* for 2 minutes 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.

6.1 Use the HRP as a model enzyme. Prepare a stock solution of HRP (28 mg/mL or 0.63 mM) in DPBS.

6.2 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.

6.3 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.

6.4 Centrifuge the mixture at 8,000 *x g* for 2 minutes 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.

6.5 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.

6.6 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).

7. 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-*Npu*N (**N**) and *Npu*C-S-CutA(**C**) (Figure 1A, Table 1). *Npu*N/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) (Figure 1A, C). 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 **J**) 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 non-fluorescent. 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.

Figures

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 non-covalently 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)

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.

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)

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)

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 minutes and 30 minutes (left) and control experiment with the same amount of non-immobilized 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)

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.

Disclosures

No competing financial interests exist.

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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.

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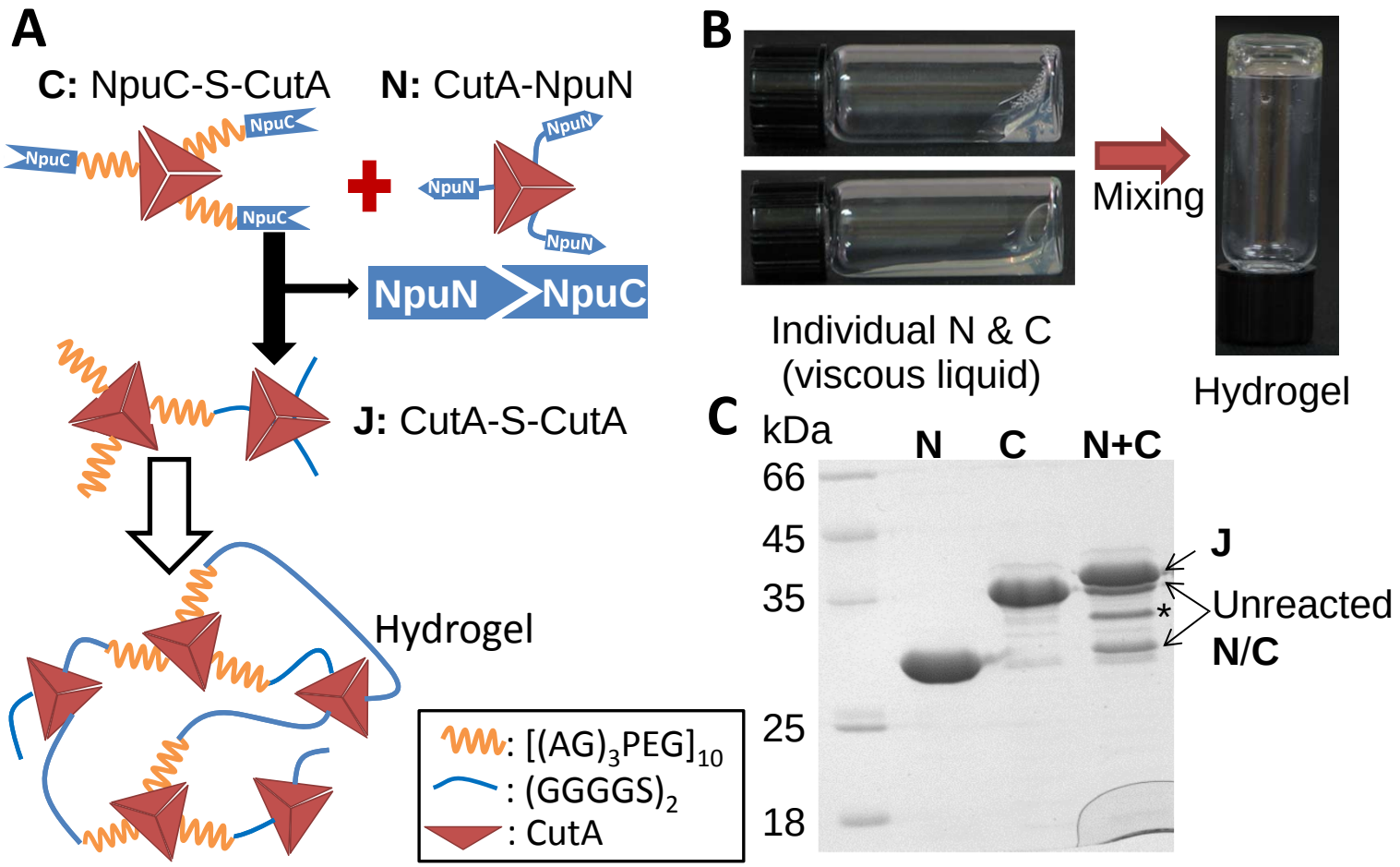

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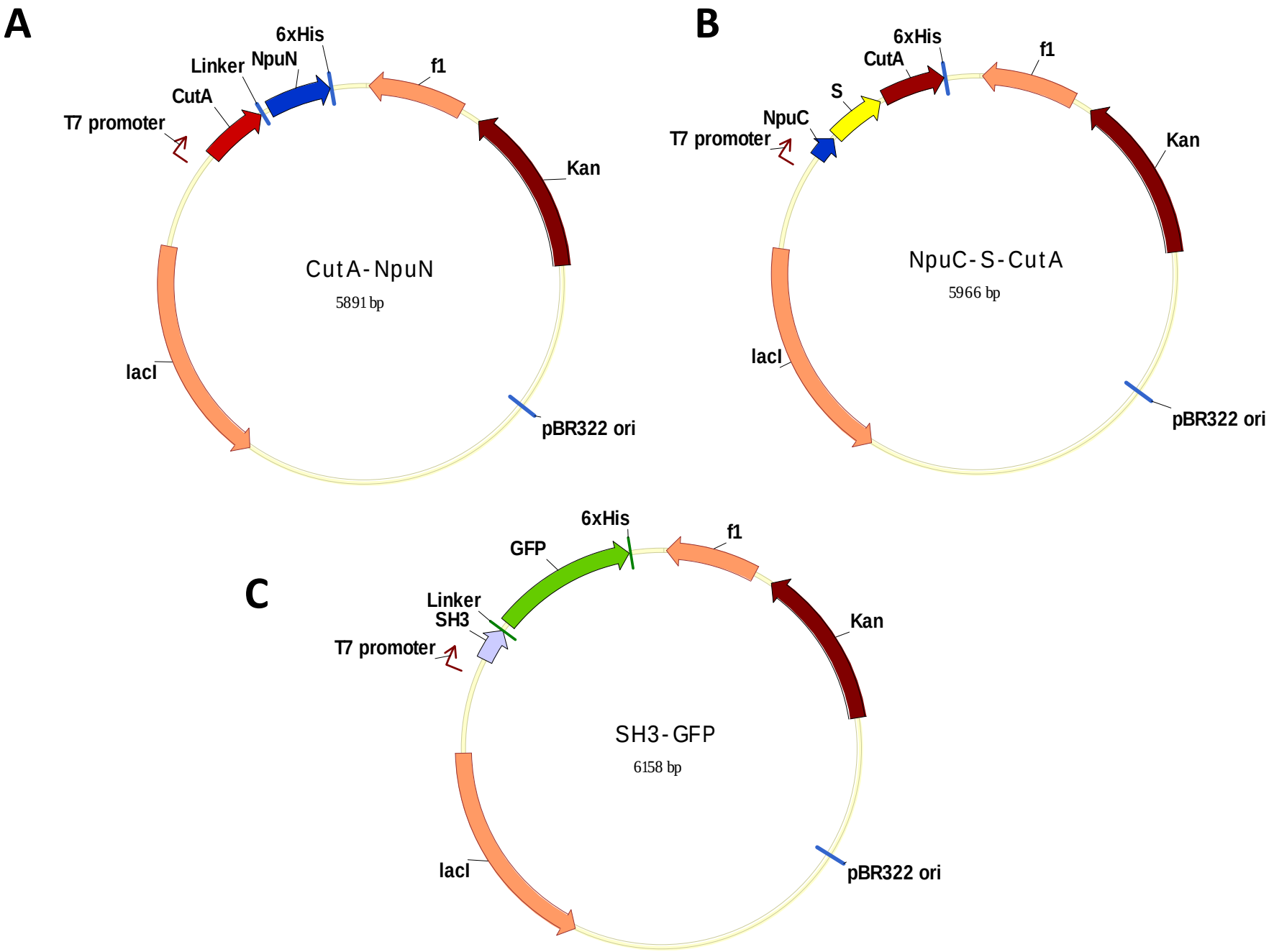

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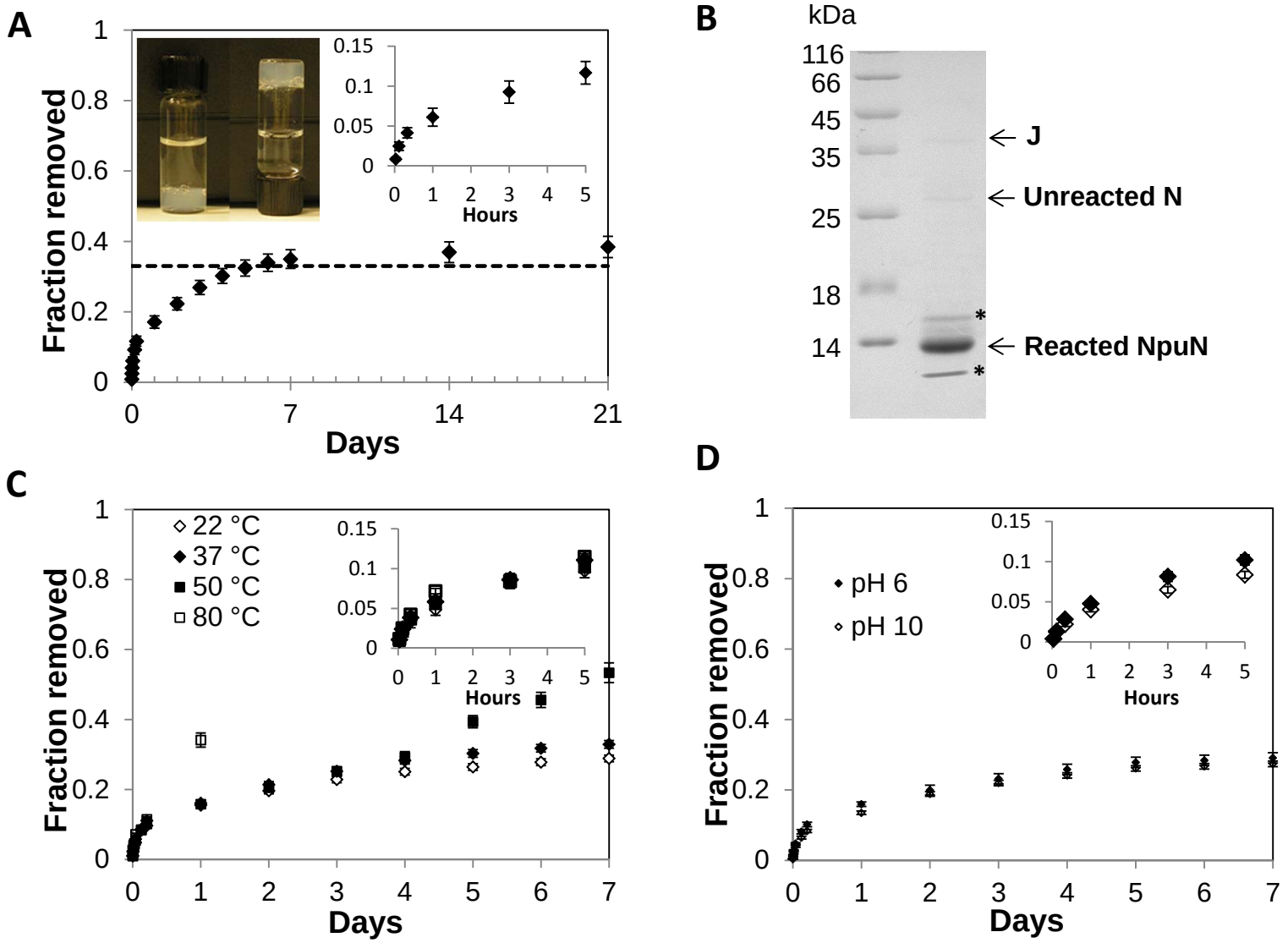

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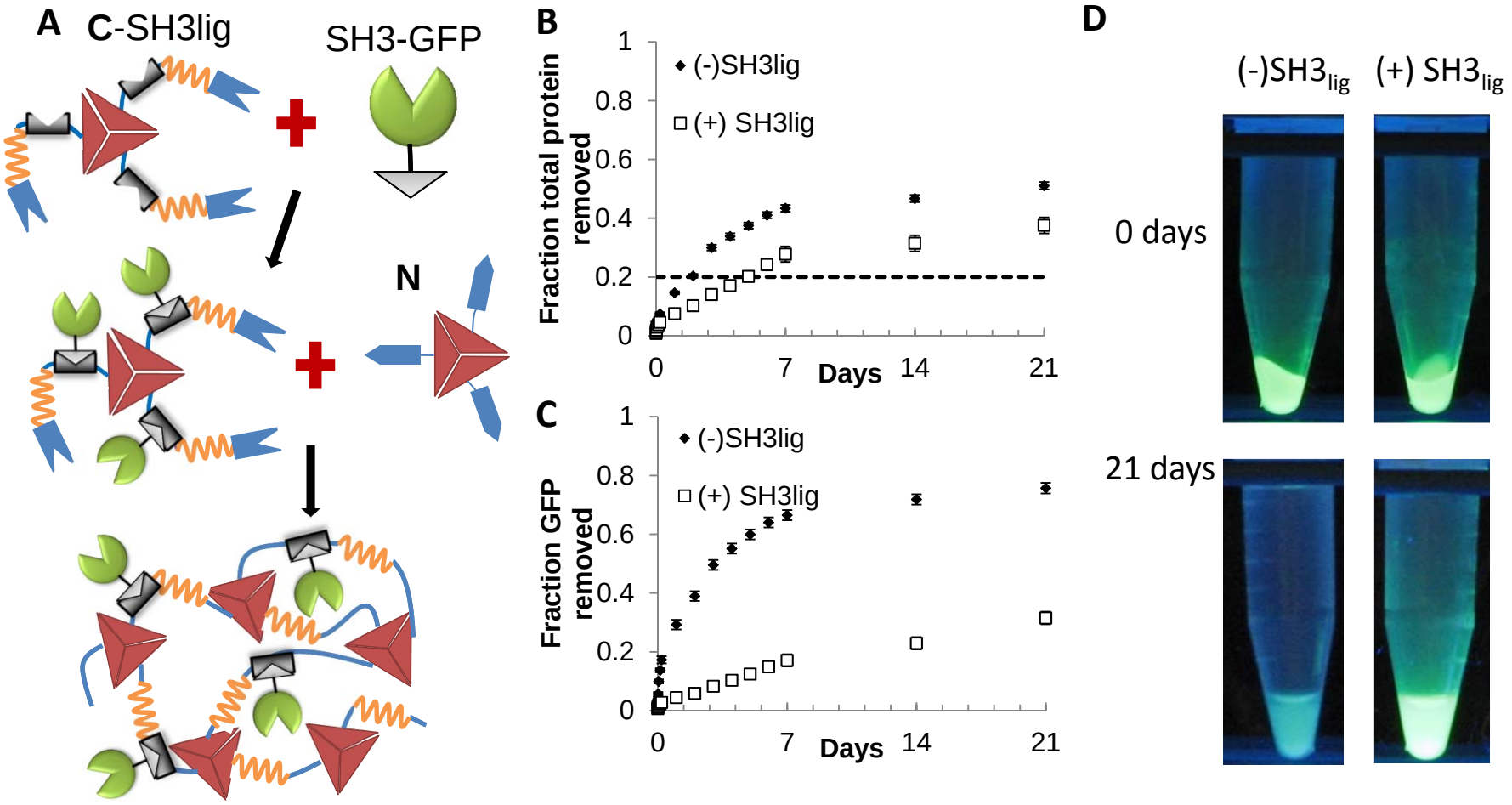

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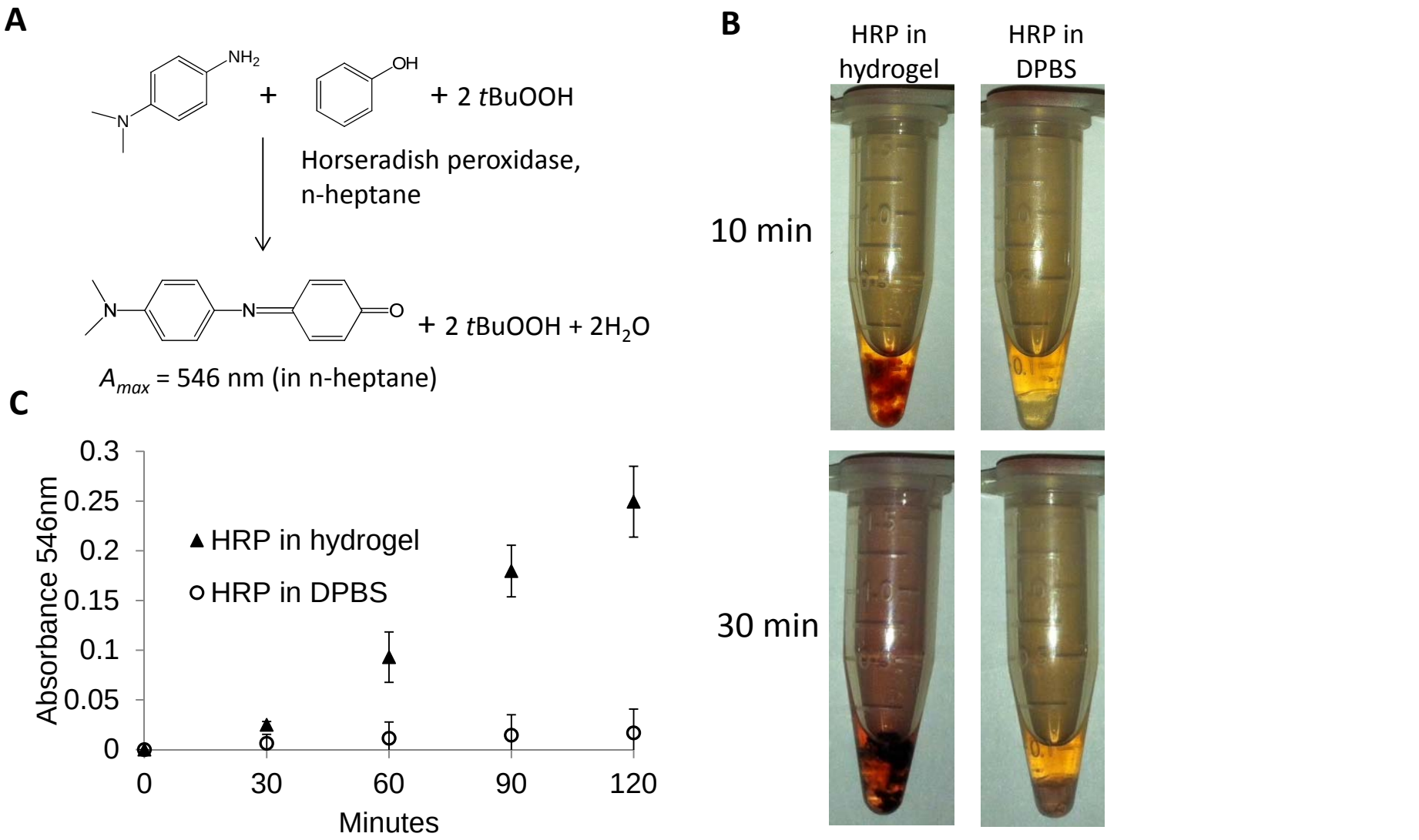

Figure 5.

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Table I. Protein constructs used in this stud

Short Name
CutA-*Npu* N (**N**)
Npu C-S-CutA (**C**)
NpuC-S-$SH3_{lig}$-CutA (**C**-$SH3_{lig}$)
SH3-GFP

**Italic* : $SH3_{lig}$ sequence

dy

Protein Sequence
CutA-EAC-$(GGGGS)_2$-AS-NpuN-HHHHHH
NpuC-CFNKLYRDPMG- $[(AG)_3PEG]_{10}$-ARMPYV-CutA-HHHHHH
NpuC-CFNKLYRDPMG- $[(AG)_3PEG]_{10}$-ARMPYVGS-*PPPALPPKRRR* -$(GGGGS)_2$-AS-CutA-HHHHHH
SH3-KL-$(GGGGS)_2$-AS-GFP-HHHHHH

MW (kDa)
26.3
26.1
28.3
34.5

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Table 2. Buffer compositions

Buffer A
Buffer DA
Buffer B
50 mM Phosphate Buffer pH 7.0

500 mM NaCl, 10 mM Tris-HCl, pH 8.0
500 mM NaCl, 8 M Urea, 10 mM Tris-HCl, pH 8.0
500 mM NaCl, 50 mM NaPOi, pH 6.0
30.5 mM Na_2HPO_4, 19.5 mM NaH_2PO_4, pH 7.0

*Excel Spreadsheet- Table of Materials/Equipment

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Name of Material	Company	Catalog Number	Comments/Description
Phusion High Fidelity DNA polymerase	New England BioLabs	M0530S	
Competent Escherichia coli BL21 (DE3)	New England BioLabs	C2527I	
Luria Bertani	VWR	90003-350	
Bacto Agar Media	VWR	90000-760	
kanamycin sulfate	VWR	97061-602	
IPTG	VWR	EM-5820	
Imidazole	VWR	EM-5720	
Urea	VWR	EM-9510	
Dithiothreitol (DTT)	Fisher	BP172-5	
Protease Inhibitor cocktail	Roche Applied Science	11836153001	
DPBS	VWR	82020-066	
Brilliant Blue R	Acros Organics	A0297990	
Sodium Azide	Fisher	AC190380050	Caution, highly toxic
Horseradish peroxidase	Sigma	P8125-5KU	
N ,*N* -dimethyl-*p* -phenylene diamine	Fisher	AC408460250	Caution, highly toxic
phenol	Fisher	AC149340500	Caution, highly toxic
tert -butyl hydroperoxide	Fisher	AC180340050	Caution, highly toxic
n-heptane	Acros Organics	120340010	

Equipment	Company	Catalog Number	Comments/Description
Shaker/Incubator	Fisher Scientific	Max Q 6000	
Centrifuge	Sorvall	RC 6	
Sonicator	QSonica	Misonix 200	
Ultrafiltration Tubes	Amicon Ultra	UFC903024	
Ni Sepharose High Performance HisTrap co	GE Healthcare Life Sciences	17-5248-01	
HiTrap SP Sepharose FF ion exchange colum	GE Healthcare Life Sciences	17-5156-01	
Plate reader	Molecular Devices	SpectraMax Gemini EM	

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The text has been edited throughout to limit the use of “we”.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this manuscript the authors describe a method to produce protein-based hydrogels that can be used to immobilize enzymes for biocatalysis. The hydrogels are made by physically mixing two separate components. Upon mixing the split intein that is a terminal functional group of both components undergoes a splicing reaction to initiate gellation. The resulting gels are stable in solution and can support the immobilization and entrapment of enzymes.

Major Concerns:
There are no major concerns.

Minor Concerns:
Please address the following minor concerns.

Introduction: The sentence beginning "Since many physical hydrogel..." is worded poorly and is confusing. Please edit/modify.

We modified the sentence as follows:

Page 3, Line 63-66

“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.”

3.1.7.2: 3 repeats at 1 mL diluted in 14 mL will not reduce 8M urea to
This step is modified as follows:

"Add 14 mL DPBS buffer to the column to dilute the protein sample. Repeat the centrifugation/dilution steps three more times."

4.2: There appears to be a typo

This step is modified as follows:

"4.2 To make a 100 µL hydrogel (1.6 mM), mix C (x µL, volume calculated according to 4.1) with 5% NaN3 (10 µL), 100 mM DTT (5 µL) and N (y µL, volume calculated according to 4.1) inside a 2 mL glass vial."

4.6: How big of a gel sample is used?

0.5 µL.

This information is included in the text:

"4.6 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)."

6.2: HRP-functionalized is misleading as the HRP is only entrapped in the hydrogel network

We changed "HRP-functionalized hydrogel (1.6 mM)" to "hydrogel (1.6 mM) entrapping HPR…"

The modified sentence is shown below:

"6.2 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% NaN3 (3 µL) and DTT (1.5 µL of 100 mM) inside a 1.7 mL centrifuge tube."

Page 3, line 81:

Hydrogel entrapping the latter protein is used to perform biocatalysis in an organic solvent.

Caption for Figure 5:

Figure 5. Horseradish peroxidase (HRP)-entrapped intein-triggered protein hydrogel facilitates HRP-catalyzed reaction in organic solvent n-heptane

7. Representative Results:

The adjective "extremely" used to describe the stability of the hydrogels is unjustified- the data demonstrate up to 30% fraction removed.

The vast majority of the 30% fraction removed corresponds to the spliced out intein (Figure 3A). Only trace amount of crosslinked hydrogel building block is present in the supernatant. In addition, small air bubbles that were trapped at the hydrogel surface remained after 3 months in solution, indicating that this hydrogel exhibits little to no surface erosion. Nevertheless, “extremely” sounds a little overstating and we removed this word. The modified sentence is shown below:

Page 10 , Line 342

“This intein-mediated protein hydrogel (1.6 mM J) exhibits high solution stability.”

In the following sentence- "into only" ?

It should be “into the DPBS buffer only slightly…”
page 10, lane 344

The modified full sentence is shown below:

“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).”

Figure 1. Caption: "+" should read "*"
The caption was corrected as suggested:

“(**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. Reprinted with permission from Journal of American Chemical Society (DOI 10.1021/ja401075s)”

Discussion: There is an extra "we" in the first line of the second last paragraph.
This typo was corrected as follows:

We also demonstrated the use of the intein-mediated protein hydrogel as an organic-solvent-compatible biocatalyst.

Additional Comments to Authors:
N/A

Reviewer #2:

Manuscript Summary:

This study focuses on the synthesis of split-intein-medicated protein hydrogels and demonstrates the application of this new method with the enzyme horseradish peroxidase. This paper is extremely well written and concise. The paper does a nice job explaining how the method works and demonstrating its usefulness. The experimental technique employed by the authors is solid. Only a few minor revisions are suggested.

Major Concerns:

None.

Minor Concerns:

Page 2, Line38, add a comma after 50°C in the long abstract

Correction was made as suggested.

Page 3, Line 59, change "extraordinarily quick " to "fast"

Correction was made as suggested:

page 3, line 60:

Npu intein was chosen because of its fast reaction kinetics (t1/2 = 63 s) and high trans-splicing yield (close to 80%).

Page 3, Line 60, change "very high trans-splicing" to "high trans-splicing"

correction was made as suggested:

page 3, line 60:

Npu intein was chosen because of its fast reaction kinetics (t1/2 = 63 s) and high trans-splicing yield (close to 80%).

Page 3, Line 63, define GuHCl

“Guanidine hydrochloride” was inserted in the paragraph.

Page 3, Line 63, change "many" to "most"

This sentence was modified as follows:

Page 3, Line 63 - 66:

“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.”

Page 3, Line 80, change "solvent" to "solvents"

Correction was made as suggested.

The modified sentence is shown below:

Page 3, line 78 -80:

“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.”

Page 4, Line 87, change "per manufacture's specifications" to "per the manufacturer's specifications"

Correction was made as suggested.

Page 4, Line 87:

“: All genes were amplified under standard PCR reactions using Phusion High-Fidelity DNA Polymerase per the manufacture’s specifications”

Page 4, Line 102, change "pet30-CutA-Tip1" to "pET30-CutA-Tip1"
Correction was made as suggested in the following sentences:

1.2.1 PCR amplify NpuC, CutA and S fragment [AG3(PEG)]10 from plasmid KanR-IntRBS-NpuNC-CFN, pET30-CutA-Tip1 and pQE9 AC10Atrp, respectively, using the appropriate primers.

1.2.1 PCR amplify NpuC, CutA and S fragment [AG3(PEG)]10 from plasmid KanR-IntRBS-NpuNC-CFN11, pET30-CutA-Tip110 and pQE9 AC10Atrp12, respectively, using the appropriate primers.

Page 5, Line 134, add a space between 5mL

Correction was made as suggested.
Page 5, Line 134:

2.4 After incubation, pick a plate that contains 50-100 colonies and resuspend all colonies in 5 mL of LB broth.

Page 5, Line 137 add a space between 600nm (check spacing in the rest of the paper for other units)

Corrections were made as suggested:

Page 5, Line 137:

Transfer suspension to 1L 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}).

Page 5, Line 141, change "to achieve a final concentration of 1 mM" to "(1 mM final concentration)"

Correction was made as suggested.

Page5, Line 141:

2.5.1 For C and C-SH3lig, induce protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture to 1 mM final concentration and incubate the culture at 37 ˚C for 4 hours while shaking at 250 rpm.

Page 5, Line 145, change "to achieve a final concentration of 1 mM" to "(1 mM final concentration)"

Correction was made as suggested.

Page 5, Line 144-146:

2.5.2 For N and SH3-GFP, cool the culture to ~18 °C by immersing the culture flask in an ice water bath for ~5 minutes. Induce protein expression by adding IPTG to the culture to 1mM final concentration and incubate the culture at 18 °C for 14-18 hours while shaking at 250 rpm.

Page 6, Line 210, be more specific about the phosphate buffer, NaPOi?

NaPOi stands for sodium phosphate.

The sentence was modified:

Page 8, line 208-209

“3.2.5.2 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).”

Page 8, Line 277, sentence starts with "o...", think this should be "To...."

We corrected this mistake. The modified sentence is shown below:

“4.2 To make a 100 µL hydrogel (1.6 mM), mix C (x µL, volume calculated according to 4.1) with 5% NaN3 (10 µL), 100 mM DTT (5 µL) and N (y µL, volume calculated according to 4.1) inside a 2 mL glass vial.”

Page 10, Line 375, change to "protein; however, in theory any DP-tagged protein could be conveniently..."

This sentence was modified as suggested:

Page 10, Line 373-374:

“In this experiment, GFP was used as the target protein; however, any DP-tagged protein can be conveniently immobilized into this hydrogel.”

Page 11, Line 395, change to "in DPBS is likely due..."

This sentence was modified as suggested:

Page 11, Line 392:

“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.”

Page 13, Line 423, change "pet26b" to "pET26b"
Correction was made as suggested.

Page 2, line 92

1.1.1 PCR amplify CutA and NpuN genes from plasmids pET30-CutA-Tip110 and KanR-IntRBS-NpuNC-CFN11, respectively, using the appropriate primers.

Page 2, line 102

1.2.1 PCR amplify NpuC, CutA and S fragment [AG3(PEG)]10 from plasmid KanR-IntRBS-NpuNC-CFN11, pET30-CutA-Tip110 and pQE9 AC10Atrp12, respectively, using the appropriate primers.

Page 12, Line 415:

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.

Page 16, line 505

Page 17, Reference, some of the references are abbreviated and some are not, please follow JOVE reference standards

References were fixed to meet JOVE standards.

Figure 3A, 3C, and 3D, should be rescaled, most of the data is at the bottom of the plots

We modified Figure 3A,C and D to include inlet graphs depicting the erosion profiles of the first 5 hours.

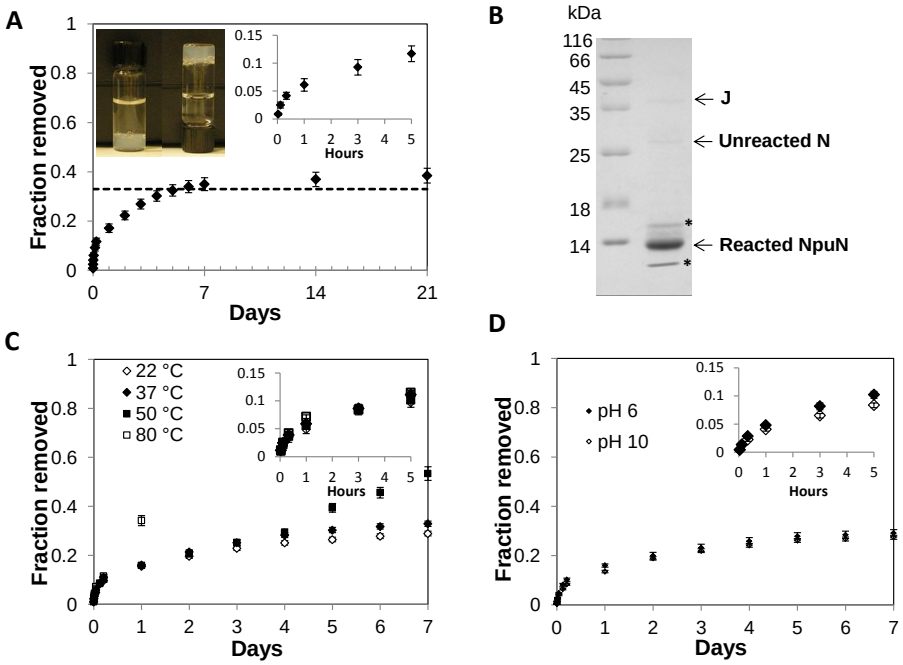

Figure 3.

Figure 4B, 4C, align the labels

Figure 4D, change the figure to match Figure 5B for consistency (time labels on the side)

We modified figure 4 as requested.

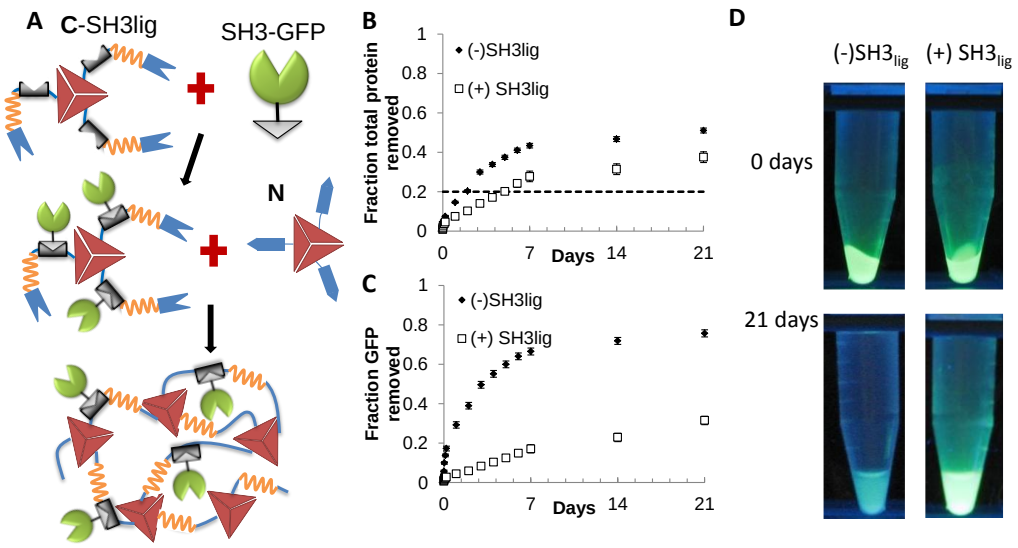

Figure 4.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This manuscript entitled "Synthesis of an intein-mediated artificial protein hydrogel" reports the synthesis of a split-intein-mediated protein hydrogel and gives its application for the immobilization. The manuscript is well organized and is fairly well written except for minor corrections as stated below:

Major Concerns:
N/A

Minor Concerns:
1. Page 3, line 81 (and throughout the manuscript). The abbreviation should be spelled out when it is mentioned for the first time.

GFP was spelled out as suggested.

Page 3, line 80-81 is the first mention of GFP where it's spelled out:

"This hydrogel is used for the immobilization of two globular proteins: the green fluorescent protein (GFP) and the horseradish peroxidase"

Subsequent mentions of GFP are in the abbreviated form.

2. Page 8 and 9. There are spelling mistakes such as pipet for pipette. It needs proofreading throughout the manuscript.

We corrected these mistakes

Page 8, line 283

4.3 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.

Page 9, line 326

"Manually disrupt the gel using a pipette tip to increase the contact surface area of the hydrogel and the solvent."

3. Page 17. Scientific names of organisms should be italicized. For example, Nostoc punctiforme, Pyrococcus horikoshii, Thermus thermophilus, and Oryza sativa.

We modified the font of the scientific names of the organisms to italic.

Page 3, line 56

"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]."

Page 10, line 336

"*Npu*N/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]."

Page 16, line 519

Iwai, H., Zuger, S., Jin, J. & Tam, P. H. Highly efficient protein trans-splicing by a naturally split DnaE intein from *Nostoc punctiforme*. FEBS letters 580, 1853-1858,

Page 16, line 521

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 letters 583, 909-914, doi:10.1016/j.febslet.2009.02.003 (2009).

Page 16, line 525

Tanaka, Y. et al. Structural implications for heavy metal-induced reversible assembly and aggregation of a protein: the case of *Pyrococcus horikoshii* CutA. FEBS letters 556, 167-174, doi:S0014579303014029 [pii] (2004).

Page 16, line 527

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).

4. Table 2. The author have mentioned buffers DMEM, buffer E and EB1-3. But these buffers were not mentioned in any of the methods given in the manuscript. The author should provide the details of these buffers.

We modified Table 2 to include only buffers used in the main text.

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
50 mM Phosphate Buffer pH 7.0	30.5 mM Na_2HPO_4, 19.5 mM NaH_2PO_4, pH 7.0

5. Page 8, section 4.1. The symbol in the formula should be consistent.

The formulas were modified to have consistent symbols

Page 8, line 273-276

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

.

. Page 8, line 277. "o" should be removed.

The “o” should be “To”. We corrected this sentence as follows:

4.2 To make a 100 µL hydrogel (1.6 mM), mix C (x µL, volume calculated according to 4.1) with 5% NaN3 (10 µL), 100 mM DTT (5 µL) and N (y µL, volume calculated according to 4.1) inside a 2 mL glass vial.

7. Page 10, line 348. The authors mentioned that ~80% trans-splicing reactions efficiency for the figure 1C. Based on the size of N, C, and N+C, the authors can explain this figure in detail.

The *trans*-splicing efficiency was calculated from each band's intensity quantified by "Quantity One" software using the "trace" module. Band intensity was divided by the band molecular weight to obtain the molar equivalent. We included an additional sentence in the figure caption to clarify the method used:

Page 12, line 411

"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. "

8. Page 3, line 80. The authors can provide the appropriate references here.
Since GFP and HRP are very commonly used, we don't feel that a reference is needed in this part of the text.

Additional Comments to Authors:
N/A

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