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Original Article

Site-specific and effective immobilization of proteins by *Npu* DnaE split-intein mediated protein trans-splicing reaction

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Abstract Immobilized proteins on solid supports provide a useful platform for various biological assays including proteomics research, protein-protein interaction studies, and diagnosis. In fabricating protein chips, proteins should be immobilized to maintain activity and specificity towards their targets. Here, we describe an approach to attach a protein of interest onto a solid support through a covalent bond as well as *in situ* monitoring of protein immobilization and subsequent binding assays. Our system utilized self-assembled monolayers of alkanethiolates on gold as biologically inert solid substrates, and *Nostoc punctiforme* DnaE split-inteins were used to mediate biospecific interactions and covalently conjugate proteins on the solid support. Use of a gold substrate enabled *in situ* monitoring of the protein-protein interactions using surface plasmon resonance spectroscopy. This approach provides a flexible method for further protein immobilization applications.

Keywords: Split-intein, Protein chip, Protein immobilization

Introduction

Protein biochips have become a useful platform for disease diagnosis, drug screening, and protein profiling[1-5]. To fabricate well-functioning biochips, proteins must be immobilized onto solid supports and retain their native conformation to maintain their biological activity and target specificity. Current strategies for protein immobilization include non-covalent adsorption of proteins[6,7], covalent cross-linking of proteins[8], and affinity-tag based immobilization of proteins[9]. As these methods often suffer from limitations such as non-specific adsorption of proteins, denaturation of immobilized proteins, presence of linker proteins, incompatibility with quantitative analysis, and lack of reproducibility, an efficient and effective method to immobilize proteins is required[10,11].

In this study, we designed an approach to immobilize protein probes onto an inert self-assembled monolayer (SAM) utilizing split-intein mediated protein trans-splicing reaction (PTS) (Figure 1). Split-intein mediated protein immobilization was demonstrated earlier by using *Synechocystis* sp. (*Ssp*) DnaE split-inteins[12]. While the previous study showed an unprecedented example of traceless immobilization of proteins, the slower kinetics of PTS mediated by *Ssp* DnaE intein was a drawback for applications[13-15]. Here, we adopted an engineered *Nostoc punctiforme (Npu)* DnaE split-intein[16,17]. This system offers two distinctive advantages over previous *Ssp* DnaE split-intein based approaches. First, *Npu* intein mediates a faster PTS for effective attachment of proteins[13] and, second, as the engineered C-intein (eI_C) is considerably shorter compared to wild-type C-inteins (wI_C)[16,17], the preparation of C-intein (I_C) becomes easier to make this approach more practical[18]. Additionally, the use of SAMs on gold enabled real-time monitoring of protein immobilization as well as target binding[19].

Our approach consists of the following three steps. The first step involves the formation of mixed alkanethiolate SAMs on a gold substrate[19,20]. The mixed

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SAMs were comprised of 99% tri(ethylene)glycol-terminated alkanethiols to make a biologically inert substrate and 1% acetylene-terminated alkanethiolates to mediate a bio-specific interaction with an N-intein (I_N) fused protein of interest[21]. Second, the I_C fragment was conjugated to SAMs through an azide-alkyne cycloaddition reaction, namely, Click Chemistry[22,23]. Relatively small I_C peptides were prepared by Fmoc-based sold-phase peptide synthesis, and a non-native azide functional group was introduced to the synthetic peptide. The copper catalyzed azide-alkyne Click Chemistry reaction was used for easy and selective assembly of two small molecules[24]. Finally, the protein fused to I_N was flowed to the I_C presenting SAM, and the immobilization was monitored in real-time using surface plasmon resonance (SPR) spectroscopy.

In this paper, we demonstrated that probe proteins were selectively immobilized on a gold substrate using a pair of engineered split-intein mediated PTS reaction. The immobilization of proteins proceeded with rapid kinetics and immobilized probes maintained specific target binding efficiency. Additionally, the probe proteins are immobilized in a uniform orientation onto the gold surface without a linker protein, as the split-inteins mediated a bio-specific interaction and excised themselves out into solution. Protein binding was determined using quantitative SPR monitoring[25].

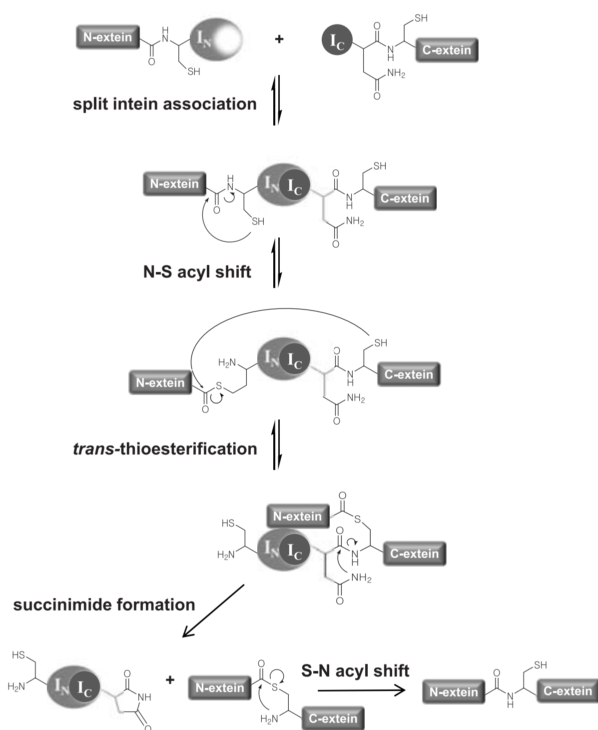

Figure 1. Mechanism of split-intein mediated PTS. A pair of split-inteins binds to each other to form a functionally active domain that can mediate the PTS reaction. The first step involves an N to S acyl shift at the first residue of the intein sequence and the N-extein domain is transferred to the side chain SH group of a cysteine residue. Subsequently, the entire N-extein is transferred to another conserved cysteine at the N-terminal of C-extein, i.e. trans-thioesterification. Then, the cyclization reaction of the Asp at the C-terminal of intein leads to excision of the full intein with C-terminal succinimide derivative. In the final step, a stable amide bond is formed between the N-extein and C-extein after an S to N acyl shift.

Results and Discussion

Preparation of the I_N fused model protein and the I_C peptide

We designed an approach to immobilize a protein of interest to a solid support based on split-intein mediated PTS as described earlier[12]. In brief, the protein of interest was genetically fused to I_N and expressed using a bacterial expression system. The C-terminal fragment was chemically synthesized to carry a nonnative functional group, and this functional group was used to conjugate the synthetic I_C to the solid support presenting the complementary functional group.

The split-intein sequences were obtained from the

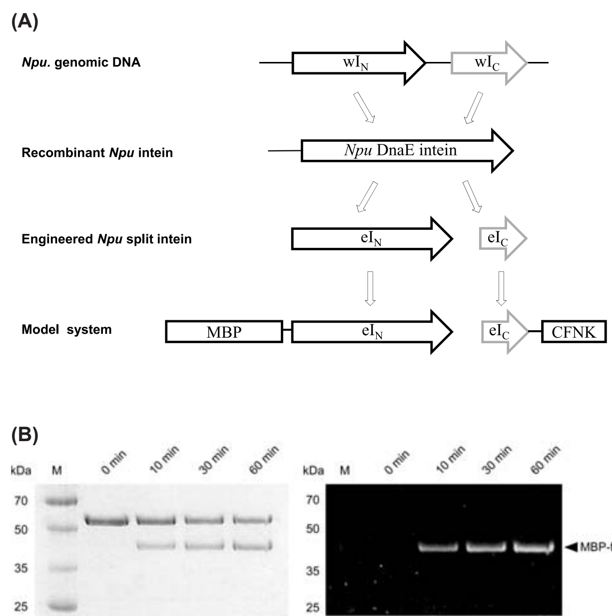

Figure 2. Generation of model systems and *in vitro* PTS. (A) Engineered split-inteins were generated by using *Nostoc* punctiforme ATCC29133 and used to generate model protein MBP-I_N. eI_C was synthetically prepared to carry CFNK on C-terminus and analyzed by using HPLC and MS. (B) PTS of MBP-I_N and fluorophore carrying I_C was monitored by SDS-PAGE analysis.

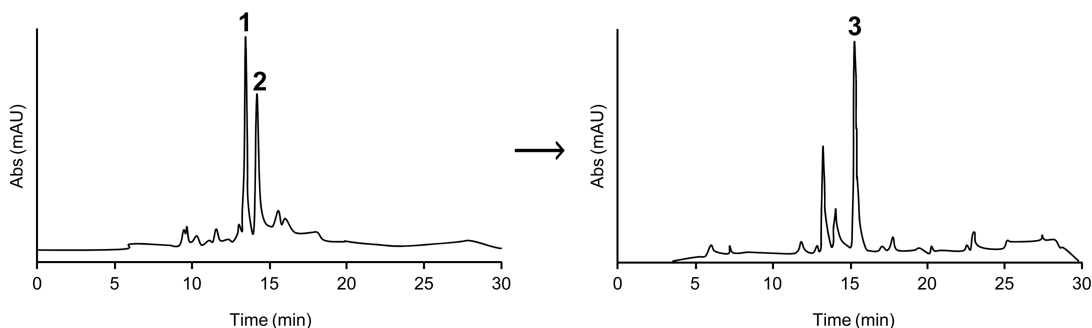

Figure 3. HPLC analysis of Click Chemistry. Click Chemistry between alkyne-EG3-thiol and azide conjugated I_C was monitored by using HPLC at a wavelength of 280 nm (1, Azide conjugated I_C; 2, alkyne-EG3-thiol; 3, Cyclic adduct of 1 and 2).

DNA of *Nostoc punctiforme* PCC73102 (*Npu*) and fused in frame to the 137 amino acids of the *Npu* intein (Figure 2A). The engineered I_N sequence[16] (123 amino acids) was polymerase chain reaction (PCR) amplified using the generated whole *Npu* intein as a template and was inserted on the C-terminus of the maltose binding protein (MBP). MBP was used as a model protein to investigate the immobilization approach, as it allowed efficient soluble expression of fusion proteins, easy purification using amylose resin, and could be detected using commercially available antibodies. The C-terminal intein (14 amino acids), carrying a short extein sequence of Cys-Phe-Asn and a Lys, was chemically synthesized using Fmoc-based solid phase peptide synthesis (SPPS)[26]. An extein sequence was introduced to ensure PTS activity, and Lys was used to conjugate an azide functional group to an ε-amine group. The site-selective introduction of an azide functional group was achieved using orthogonal 1-(4,4-dimethyl2,6-dioxocyclohex1ylidene)-ethyl (Dde) side-chain protected lysine. The eI_C had considerably smaller size compared to the 35 amino acids of the wI_C and was synthesized in higher yields.

Protein trans-splicing reaction in solution

Next, we performed PTS in solution using the prepared MBP-I_N and synthetic I_C. Fluorescein isothiocyanate (FITC) was introduced to the C-terminal lysine side chain of I_C to analyze the progress of the reaction using fluorescence imaging after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The PTS was carried out at 37°C in the presence of 1 mM Tris(2-carboxyethyl)phosphine (TCEP) to suppress formation of disulfide bonds in the synthetic peptide. The expected splicing products were produced as fluorescein-labeled MBP and observed using Coomassie Brilliant Blue staining and fluorescent imaging under UV light (Figure 2B).

Characterization of click chemistry

In our approach, I_C needs to be introduced to SAMs through a selective reaction, and the thiol functional group on Cys must be available for the PTS reaction. We chose Click Chemistry to site-selectively conjugate the I_C to the SAMs to satisfy both requirements. 6-Azidohexanoic acid was synthesized and coupled to the C-terminal lysine residue through *O*-benzotriazole-*N,N,N′,N′*-tetramethyluroniumhexafluorophosphate (HBTU) activation of carboxylic acid. Azido-I_C was mixed with acetylene-terminated alkanethiol in the presence of Cu^{2+} and the progress of the reaction was analyzed by high performance liquid chromatography (HPLC). As the reaction progressed, the formation of cyclic adduct was monitored at a retention time of 15.5 min (Figure 3).

Preparation of SAMs, immobilization of proteins, and binding assays

Mixed SAMs were prepared by immersing a freshly prepared gold coated SPR sensor chip in an ethanolic solution of 1% alkyne-terminated alkanethiol and 99% tri(ethylene)glycol-terminated alkanethiol (Figure 4). A tri(ethylene)glycol group was introduced to prevent nonspecific adsorption of proteins, and the alkyne group provided a chemical handle to selectively immobilize I_C using Click Chemistry. SAM was incubated in an azido-I_C solution overnight to conjugate with I_C.

The MBP-I_N was allowed to flow over the SPR sensor chip with I_C, and the change in SPR angle was monitored. A significant increase in RU value was observed upon immediate addition of MBP-I_N and then an unexpected decrease in the RU value was observed during continuous flow of MBP-I_N. The initial increase was probably due to rapid binding of I_N to the surface-attached I_C, and the subsequent decrease was likely due to the progress of PTS. As *Npu* intein

is a 15 kDa protein, a decrease in the RU value was observed when this sizable protein was excised from the surface. After immobilization of MBP-I_N, phosphate buffered saline (PBS) was flowed over the SPR sensor chip to remove any unbound protein. The anti-MBP antibody showed selective binding to the MBP immobilized substrate. After removing the excess antibody with PBS, the difference in RU revealed effective binding of antibody to the MBP immobilized sensor chip (Figure 5A). In a control experiment, the anti-MBP antibody was flowed over a sensor chip with I_C. The increase in RU was monitored during the flow of antibody but the RU value decreased to baseline after the PBS wash (Figure 5B). This result suggests that the immobilized proteins maintained selective binding to the target, and that the I_C presenting SAMs are inert to non-specific target binding.

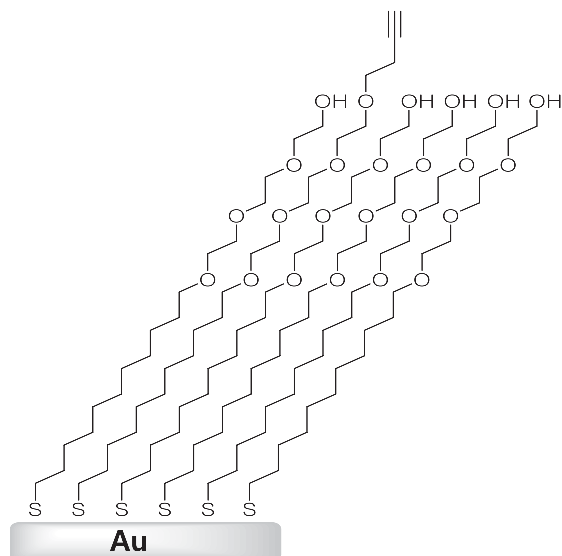

Figure 4. Structure of mixed SAMs. Mixed SAMs were formed to present acetylene group and tri(ethyleneglycol) group. Acetylene was introduced to carry about Click Chemistry reaction with azide functional group and tri(ethyleneglycol) group was used to make substrate inert to non-specific binding of random proteins.

Conclusions

While there are many protein immobilization methods available, many of them result in denaturation of immobilized proteins. Denatured proteins can cause non-specific adsorption of random protein to generate false positive signal. Also, large-scale linker proteins are often needed for specific immobilization and these can mediate unwanted interactions in the course of assays. Here we showed an efficient method to immobilize proteins using engineered *Npu* split intein-mediated trans-splicing. The protein of interest was immobilized in a uniform orientation on the surface of an SPR sensor chip through a specific interaction. The fast PTS enabled real-time monitoring of protein im-

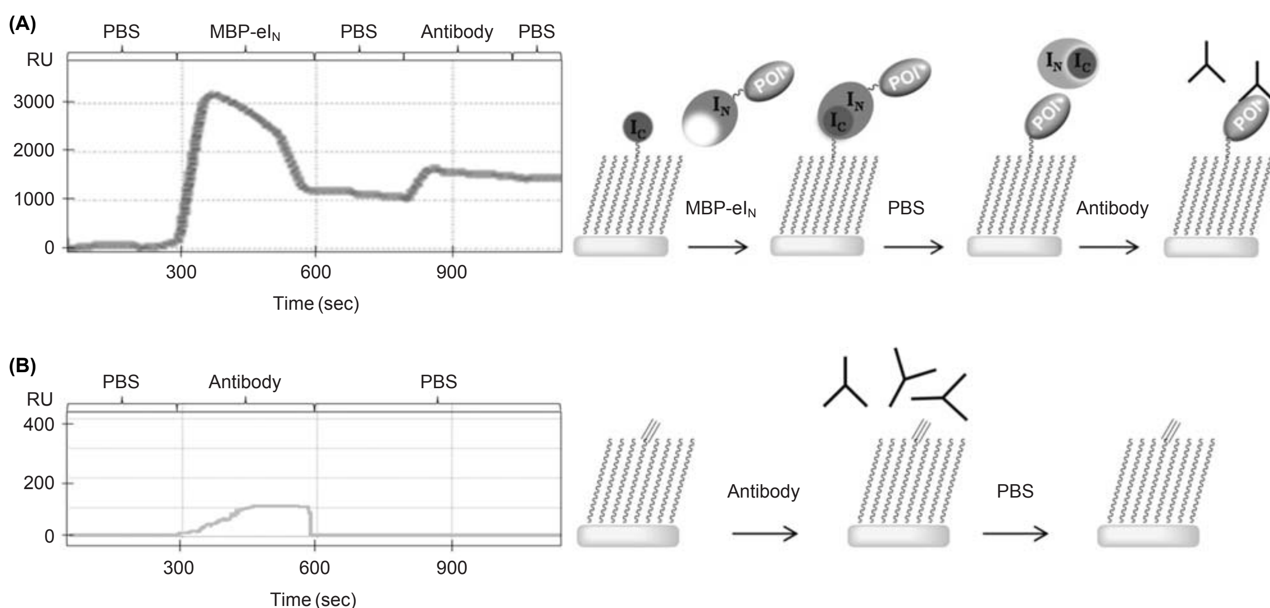

Figure 5. SPR analysis of MBP-I_N immobilization and binding assay. (A) MBP-I_N was immobilized to an SPR sensor chip presenting I_C. Immobilized MBP retained its binding affinity to anti-MBP antibody. (B) Anti-MBP antibody did not bind to an SPR sensor chip presenting I_C when no MBP was present.

mobilization and the use of eI_C made synthetic preparation of components easy for practical applications. Importantly, our approach provided traceless and covalent immobilization of probe proteins without a linker protein, and SPR-based detection provided a platform for qualitative and quantitative assay as well as real-time monitoring.

Materials and Methods

General procedures

The Plasmid DNA Mini-Prep kit and all restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA) and Elpis Biotech (Daejeon, Korea) The PCR kit and gel extraction product kit came from Real Biotech Corp (Taipei, Taiwan). All oligomers were commercially synthesized by Bioneer (Daejeon, Korea). Isopropyl-β-d-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO, USA). Protected amino acids and resins for SPPS were purchased from NovaBiochem (Bad Soden, Germany) or AnaSpec (San Jose, CA, USA). General chemicals were of the best grade available and supplied by Sigma-Aldrich (St Louis, MO, USA). The bacterial expression vector pET28a and expression host *Escherichia coli* strain BL21 (DE3) were purchased from Novagen (Madison, WI, USA). Plasmid pMAL-c2 and amylose resin was obtained from New England Biolab. The gold chip was purchased from Micobiomed (Sungnam, Korea). Analytical HPLC was performed on a Waters 2796 Bioseparations Module instrument with a Waters 2487 dual λ absorbance detector (Waters Corp., Bedford, MA, USA) using Vydac C8 and C18 columns (5 μm, 4.6 × 150 mm) (Vydac, Hesperia, CA, USA) at a flow rate of 1 mL/min. All runs used linear gradients of 0.1% aqueous formic acid (solvent A) *vs*. 90% acetonitrile (MeCN) in H_2O with 0.1% formic acid (solvent B) or 0.1% aqueous TFA (solvent C) *vs*. MeCN with 0.1% TFA (solvent D). SPR was performed using a MiCo SPR nano instrument (MiCoBioMed, Seongnam, Korea) using the GCP sensor chip. Protein samples were analyzed on 12% polyacrylamide gels and were stained with Coomassie Brilliant Blue R.

Construction of recombinant DNA

All DNA constructs were prepared using a general cloning strategy and sequences were identified by DNA sequencing.

His6-wI$_N$, pJDH011
The wI_N coding sequence was amplified by PCR using total DNA from *Npu* (strain ATCC 29133/PCC 73102) as a template and a pair of oligo-nucleotide primers (5′-GGT CGC CAT ATG TGT TTA AGC TAT-3′ and 5′-CAA ATT GTC GAC CCG CAT-3′; restriction sites are indicated by underlines). The resulting PCR product was digested and inserted into the pET28a vector using *Nde* I and *Sal* I restriction sites to create the pJDH011 plasmid, which encodes His6-wI_N.

Npu DnaE whole intein (pJDH012)
The wI_C coding sequence was amplified by PCR using total DNA from *Npu* (strain ATCC 29133/PCC 73102) as a template with a pair of oligo-nucleotide primers (5′-TAA GTC GAC AAT TTG CCG AAT ATC AAA ATA GCC-3′ and 5′-TCC CGT CTC GAG ATT AGA AGC TAT-3′). The resulting PCR product was digested and inserted into the pJDH011 vector using the *Sal* I and *Xho* I restriction sites to create pJDH012 plasmid, which encodes His6-wI_N.

MBP-eI$_N$ (pJDH015)
The eI_N coding sequence was amplified by PCR using the pJDH012 plasmid as the template and a pair of oligo-nucleotide primers (5′-GAT ATA GAA TTC TGT TTA AGC TAT GAA AC-3′ and 5′-GCA AA AAG CTT TTA GCG CTC AAC-3′). The resulting PCR product was digested and inserted into the pMAL-c2 vector using the *EcoR* I and *Hind* III restriction sites to create the pJDH015 plasmid, which encodes MBP-eI_N.

Bacterial expression and purification of MBP-eI$_N$

E. coli BL21(DE3) cells were transformed with pJD H015. Expression was carried out in Luria-Bertani medium at 37°C. The cells were grown to an $OD_{600\,nm}$ of 0.6, and protein expression was induced by adding IPTG to a final concentration of 0.5 mM, and the cells were harvested by centrifugation after 6 h. The cell pellet was resuspended in bacterial lysis buffer (100 mM sodium phosphate, 250 mM NaCl, 1 mM EDTA, and 1 mM PMSF, pH 7.5) and lysed by sonication. The lysate was clarified by centrifugation at 15,000 rpm for 30 min. The clarified supernatant containing MBP-eI_N was incubated with amylose resin (New England Biolabs) at 4°C for 1 h with gentle shaking. The amylose resin was washed with 30 mL of column buffer (100 mM sodium phosphate, 250 mM NaCl, and 1 mM EDTA, pH 7.5) containing 0.1% Triton X-100. The MBP-eI_N protein was eluted with trans-splicing buffer (50 mM Tris·HCl, 350 mM NaCl, 1 mM EDTA, 1 mM TCEP, and 10% (v/v) glycerol, pH 7.0) containing 10 mM maltose. Pure protein was quantified by UV spectrophotometry (ε_{280}=82280 M^{-1} cm^{-1}) and used immediately for *in vitro* trans-splicing experiments.

Solid phase peptide synthesis

Peptides were synthesized on a 0.5 mmol scale on Rink Amide AM resin LL according to the HBTU activation protocol for Fmoc SPPS. Fmoc protected amino acids with acid labile side chain protecting groups were used for the synthesis except the Lys18 residues, which were incorporated as Fmoc-Lys(Dde)-OH. The Fmoc-Lys (Dde)-OH was used for orthogonal deprotection of the ε-amine functional group and the Dde group was removed by treatment with 2% hydrazine in DMF for 3 min four times after chain assembly. Then, the DMF solution of FITC (4 equiv.) and DIPEA (8 equiv.) or HBTU-activated 6-azidohexanoic acid was added to the resin, and the coupling reaction was carried out for 3 h in the dark to generate fluorescence I_C or azido-I_C. The peptide was cleaved from the resin and the side chain functional groups were deprotected by treating them with cleavage cocktail, i.e., TFA : EtSH : H_2O : triisopropylsilane (TIS)=90 : 5 : 2.5 : 2.5 v/v) for 3 h at room temperature. The resin was filtered, and the peptide was precipitated in cold Et_2O. The crude material was dissolved in a minimal amount of H_2O : MeCN (2 : 1 v/v). The crude peptide was purified by HPLC using a linear gradient of 0 to 70% B over 30 min.

In solution protein trans-splicing assay

The purified fluorescein I_C peptide and I_N fusion proteins were combined in freshly prepared trans-splicing buffer (50 mM Tris/HCl, 350 mM NaCl, 1 mM EDTA, 1 mM TCEP, and 10% glycerol (v/v) pH 7.0) to final concentrations of 10 μM and 2 μM, respectively. The trans-splicing reactions were performed at 37°C with shaking. The reaction was then quenched by dilution in SDS-PAGE sample buffer containing 20% (v/v) 2-mercaptoethanol before the SDS-PAGE analysis. The splicing product was observed as a Coomassie Blue staining band and a fluorescent band under UV light at 310-360 nm.

Synthesis of 6-azidohexanoic acid

6-Bromohexanoic acid (25.6 mmol) and sodium azide (128 mmol) were dissolved in DMSO and stirred at room temperature for 18 h. The reaction mixture was then dissolved in CH_2Cl_2 and washed with 0.1 N aq. HCl. The organic layer was dried over anhydrous $NaSO_4$, filtered, and concentrated under reduced pressure to give the 6-azidohexanoic acid as oil.

SPR analysis

GCP sensor chips were carefully cleaned with Piranha solution (H_2SO_4 : H_2O_2=3 : 1), rinsed with deionized water, and dried under nitrogen gas. To generate mixed SAMs on the gold surface, the chip was immersed in ethanolic solution containing the acetylene-terminated alkanethiol and tri(ethyleneglycol)-terminated alkanethiol mixture (1 : 9) overnight, rinsed with ethanol, and dried under nitrogen gas. The SAM coated sensor chip was then immersed in a PBS solution of $CuSO_4$ (1 mM), L-ascorbic acid (2 mM), and excess azide-I_C for 6 h to carry out Click Chemistry. The I_C presenting sensor chip was mounted on a chip holder in SPR, and the protein solutions and buffers were flown at 300 μL/min.

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