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Split Spy0128 as a Potent Scaffold for Protein Cross-Linking and Immobilization

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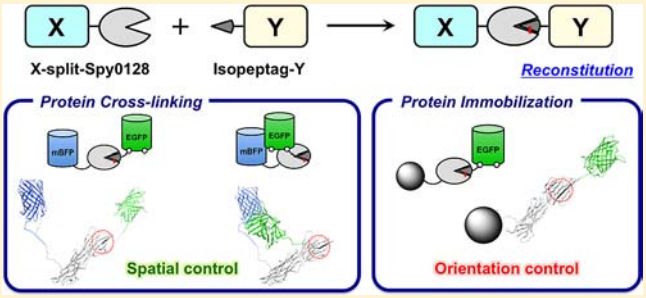
Supporting Information

ABSTRACT: Site-specific cross-linking techniques between proteins and additional functional groups have become increasingly important for expanding the utility of proteins in biochemistry and biotechnology. In order to explore powerful techniques for practical bioconjugation applications, we have validated a technique mediated by a unique property of *Streptcoccus pyogenes* pilin subunit Spy0128, an autocatalytic intramolecular isopeptide formation in Spy0128. Recently, it has been revealed that Spy0128 can be split into two fragments (split-Spy0128 (residues 18−299 of Spy0128) and isopeptag (residues 293−308 of Spy0128)) that were capable of forming an intermolecular covalent complex. We focused on this unique reconstitution property and first studied the bioconjugation of blue and green fluorescent proteins, enabling the direct monitoring of cross-linking reactions by Förster resonance energy transfer (FRET). A fluorescence lifetime study shows that spatial control of two proteins on the Spy0128 scaffold is possible when one protein is fused to the N-terminus of split-Spy0128 and another one is tethered at the N- or C-terminus of the isopeptag. Furthermore, we demonstrated site-specific protein immobilization mediated by the reconstitution of split-Spy0128 and isopeptag. In this case, a split-Spy0128 mutant with a free N-terminal Cys residue was first immobilized onto beads chemically modified with a maleimide group through a Michael addition process. Then, an isopeptagged protein was successfully immobilized onto the split-Spy0128-immobilized beads. These results suggest that Spy0128 is a potent proteinaceous scaffold available for bioconjugation both in solution and at a solid surface.



INTRODUCTION

Site-specific cross-linking techniques between proteins and additional functional groups (e.g., fluorophores, proteins, ligands, and insoluble carriers) have become increasingly important for expanding the utility of proteins in biochemistry and biotechnology.[1−3] In order to achieve the labeling of proteins *in vitro* and *in vivo* without disturbing their native functions, it is desirable to develop methods for site-specific modification of proteins. In particular, direct covalent cross-linking methods between proteins and proteins (peptides) have important applications in creating novel artificial proteins. Some protein coupling techniques include enzymatic methods (e.g., transglutaminase (TGase),[4−6] sortase[7,8]) and chemical ligation utilizing an intein system,[9,10] however, also have a few drawbacks. TGases can catalyze the cross-linking reaction of specific Gln- and Lys-containing peptides fused to a target protein. However, mammalian TGase has low inherent stability and bacterial TGase intrinsically shows low substrate specificity especially for primary amino groups; thus, the reaction conditions must be optimized to prevent target proteins from nonspecific modification. Although Sortases strictly recognize the LPXTG motif and conjugate it with an N-terminal oligoglycine, yields of the ligation are routinely low due to the reverse hydrolytic reaction. An intein system allows N-terminal cysteine derivatives to be conjugated to C-terminal thioesters of intein fusion proteins. This system has been proven to be useful, but it is known that the intein domains are potentially unstable and show low activity depending on the fusion protein.[9,10] In this study, we explored an efficient bioconjugation tool for cross-linking between biomolecules including peptides and proteins with high specificity.

We focused on a backbone pilin protein Spy0128 from an M1 strain of *Streptcoccus pyogenes*.[11−13] Spy0128 has a two-domain, irregular all-β structure. It has high thermal stability, mechanical stability, and protease resistance. The stabilities are as a result of two intramolecular isopeptide bonds within a subunit. One isopeptide bond is formed between Lys^{36} and Asn^{168} in the N domain and the other is between Lys^{179} and Asn^{303} in the C domain. It was revealed that $Glu^{117, 258}$ near to $Lys^{36, 179}$ and $Asn^{168, 303}$ is involved in the important formation of the isopeptide bonds.[13] Recently, it has been demonstrated that Spy0128 can be split into two fragments (split-Spy0128

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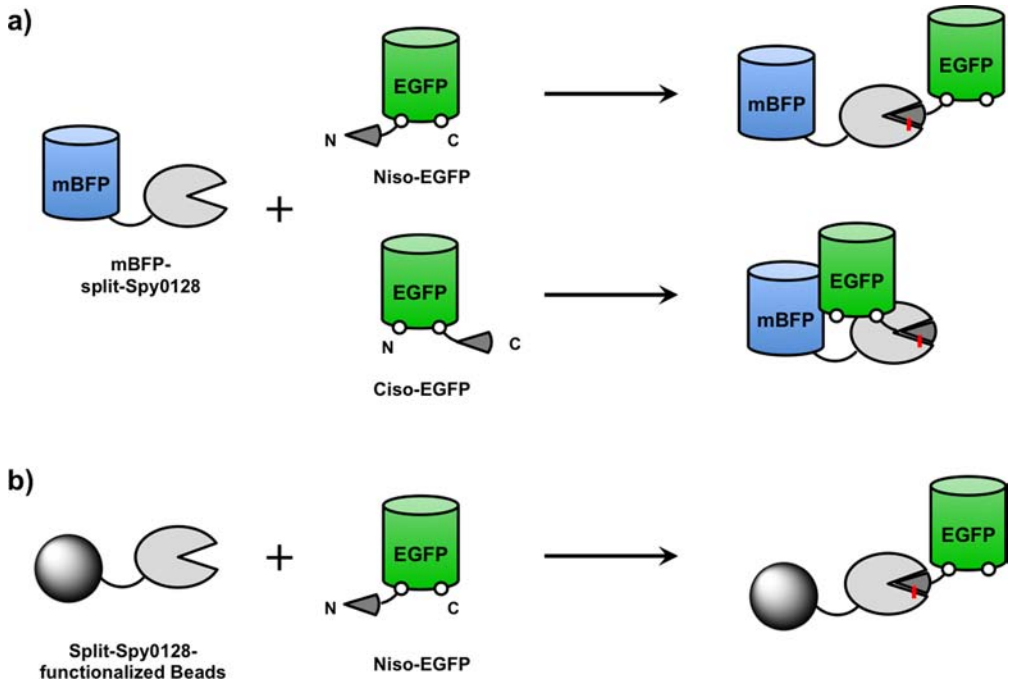

Figure 1. (a) Cross-linking between mBFP-split Spy0128 and isopeptag-EGFPs. The EGFPs, of which an isopeptag was fused to the N- or C-terminus, were abbreviated as Niso-EGFP and Ciso-EGFP. (b) Immobilization of Niso-EGFP onto split-Spy0128-immobilized beads.

(residues 18−299 of Spy0128) and isopeptag (residues 293−308 of Spy0128)) that were capable of forming an intermolecular covalent complex which was useful for irreversible peptide targeting.[14] The reconstitution of Spy0128 was a highly efficient reaction and independent of most reaction conditions such as temperature (4−37 °C), pH (6−8), salts, and detergents.

Herein, we explored the potential of split Spy0128 as a bioconjugate tool for site-specific cross-linking between proteins and proteins (peptides). Little method has been reported which can be used to connect two or more biocomponents via side-chain cross-linking of proteins without the use of a post-translational modification enzyme. The technique is expected to prove useful for the development of artificial proteins like branched proteins and *in vivo* multifunctional covalent protein complexes which have been fabricated *in vitro* until now.[15−17]

In this work, we attempted the heteroconjugation of biomolecules mediated by reconstitution of split-Spy0128 and isopeptag to utilize the split Spy0128 as a bioconjugate tool. We first present the bioconjugation of blue and green fluorescent proteins (monomeric blue fluorescent protein (mBFP), enhanced green fluorescent protein (EGFP)), enabling the direct monitoring of the cross-linking reactions by Förster resonance energy transfer (FRET) (Figure 1a). We then prepared three recombinant proteins, mBFP fused to the N-terminus of split-Spy0128 (designated as mBFP-split-Spy0128), and isopeptag tethered at the N- or C-terminus of the EGFP (each protein is designated as Niso- or Ciso-EGFP) and investigated how the insertion site of isopeptag fused to EGFP affected the reactivity and FRET efficiency. In addition, we discuss whether spatial control of the two proteins on the Spy0128 scaffold is possible by a fluorescence lifetime study. Furthermore, we demonstrated site-specific protein immobilization mediated by the reconstitution of split-Spy0128 and isopeptag. In this case, a split-Spy0128 mutant with a free N-terminal Cys residue was first immobilized onto beads chemically modified with a maleimide group through a Michael addition process. Then, an isopeptagged protein was successfully immobilized onto the split-Spy0128-immobilized beads (Figure 1b).

EXPERIMENTAL PROCEDURES

Materials. UV−vis spectroscopy was performed on a UV/vis spectrophotometer V-560 (Jasco). Fluorescence spectroscopy was performed on a PerkinElmer LS55 fluorescence spectrometer (PerkinElmer, Inc.). Circular dichroism (CD) spectroscopy was performed using a JASCO J-725G spectropolarimeter (Jasco). Mass spectra were recorded with a Bruker Autoflex-III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-ToF MS) utilizing α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. Fluorescence microscopy was carried out using an Olympus IX70 fluorescence microscope (Olympus). Genes encoding monomeric blue fluorescent protein (mBFP) and split-Spy0128 were synthesized by Integrated DNA Technologies, Inc. DNA primers were synthesized by Genenet Co., Ltd. Ligases and restriction enzymes were purchased from TOYOBO and Takara. Amino-PEGA resin was purchased from Merck. Immobilized TCEP disulfide reducing gel was purchased from Thermo Fisher Scientific. All other chemicals were obtained from commercial suppliers unless otherwise indicated.

Genetic Manipulation. Amino acid sequences of the N- and C-terminal regions of recombinant proteins prepared in this study are shown in Table 1. Primer nucleotide sequences used for polymerase chain reaction (PCR) are shown in Supporting Information Table S1 and Table S2. All inverse polymerase chain reactions (iPCR) were performed using a KOD Plus Mutagenesis Kit (TOYOBO). Isopeptag (TDKDMTITFTNKKDAE) was genetically attached to the N- or C-terminus of enhanced green fluorescent protein (EGFP) via a GGGS linker. The resultant N- or C-terminal

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Table 1. Recombinant EGFPs and Split-Spy0128s Prepared in This Study

protein	amino acid sequence of N- and C-terminal regions
wild-type EGFP	MVSKG-----DELYK**HHHHHH**
Niso-EGFP	M**TDKDMTITFTNKKDAEGGGS**VSKG-----DELYK**HHHHHH**
Ciso-EGFP	**MHHHHHH**MVSKG-----DELYK**GGGSTDKDMTITFTNKKDAE**
split-Spy0128	**MHHHHHHGS**ATTVHG-----DMTI
mBFP-split-Spy0128	**MHHHHHH**MSELI--(mBFP)--GS--ATTVHG−(Spy0128 (18−299))−DMTI
Cys-split-Spy0128	**MCGSHHHHHHGS**ATTVHG-----DMTI
split-Spy0128 (E117A)	**MHHHHHHGS**ATTVHG--(E117A)--DMTI
Cys-split-Spy0128 (E117A)	**MCGSHHHHHHGS**ATTVHG--(E117A)--DMTI

isopeptagged EGFP were designated as Niso-EGFP and Ciso-EGFP. A DNA fragment encoding wild-type EGFP was amplified by PCR using pET32 b562-EGFP as the template DNA.[18] After PCR amplification with primer **1** and primer **2** containing *Nde*I and *Eco*RI restriction sites, the gene was cloned into a bacterial expression plasmid vector, pET22b (+)- (Novagen), by digestion with the restriction enzymes (pET22 wild-type EGFP). The amino acid sequence of wild-type EGFP is shown in the Supporting Information. To yield pET22 Niso-EGFP, isopeptag was inserted into the N-terminus of wild-type EGFP twice. To attach the isopeptag(−8) (FTNKKDAE) and GGGS linker with wild-type EGFP, iPCR was conducted using pET22 wild-type EGFP as the template with primer **3** and primer **4** (pET22 Niso(−8)-EGFP). Next, to obtain pET22 Niso-EGFP, iPCR was conducted using pET22 Niso(−8)-EGFP as the template with primer **5** and primer **6**. In a similar fashion, to attach the GGGS linker and the isopeptag(−8) with the N-terminal 6 × His tagged EGFP, iPCR was conducted using pET22 N-terminal 6 × His tagged EGFP as the template with primer **7** and primer **8** (pET22 Ciso(−8)-EGFP). Next, to obtain pET22 Ciso-EGFP, iPCR was conducted using pET22 Ciso(−8)-EGFP as the template with primer **9** and primer **10**.[19]

Glutathione S-Transferase (GST)-split-Spy0128 (18−299) gene on pUC minusMCS (Medical & Biological Laboratories Co., Ltd.) was digested with *Nde*I and *Eco*RI, and placed into the pET22b (+) vector. To obtain pET22 split-Spy0128, iPCR was conducted using pET22 GST-split-Spy0128 as the template with primer **11** and primer **12**.

A DNA fragment encoding mBFP was amplified by PCR with primer **13** and primer **14**, while a DNA fragment encoding pET22 split-Spy0128 was amplified by iPCR using pET22 GST-split-Spy0128 as the template DNA with primer **15** and primer **16**. To obtain pET22 mBFP-split-Spy0128, the two fragments were ligated using an In-Fusion Dry-Down PCR Cloning Kit. The amino acid sequence of mBFP-split-Spy0128 is shown in the Supporting Information. To obtain pET22 Cys-split-Spy0128, iPCR was conducted using pET22 split-Spy0128 as the template with primer **17** and primer **18**. E117A mutations of split-Spy0128 and Cys-split-Spy0128 were introduced to pET22 split-Spy0128 and pET22 Cys-split-Spy0128 by iPCR with primer **19** and primer **20** (pET22 split-Spy0128 (E117A), pET22 Cys-split-Spy0128 (E117A)).

Expression and Purification of the Recombinant Proteins. Recombinant plasmids were transformed into *E. coli* BL21 (Takara Bio Inc.), and transformants were grown in Luria−Bertani (LB) medium supplemented with 100 μg mL^{-1} ampicillin at 37 °C in a shaker at 200 rpm. After reaching an OD_{600} of ∼0.6, protein expression was induced with 0.5 mM isopropyl thiogalactosidase (IPTG) and the culture was incubated overnight at 25 °C. Cells were harvested by centrifugation, resuspended in TBS buffer (25 mM Tris-HCl, 137 mM NaCl, 2.68 mM KCl), and lysed by sonication on ice. The cell debris was pelleted by centrifugation and the supernatants were prepared for protein purification. The supernatant was purified using Ni−NTA columns (GE Healthcare). Finally, the aqueous solution was desalted using a PD-10 column with 10 mM Tris-HCl buffer (pH 8.0) as the eluent. mBFP-split-Spy0128 was further purified by anion exchange chromatography using a Hi TrapQ HP column (GE Healthcare) with a NaCl gradient (0−1 M) in 10 mM phosphate buffer (pH 7.0). The aqueous solution was desalted as presented above.

The EGFPs were quantified using an extinction coefficient of 55 000 M^{-1} cm^{-1} at 488 nm.[20] The mBFP-split-Spy0128 was quantified using an extinction coefficient of 52 000 M^{-1} cm^{-1} at 400 nm.[21] The other proteins were quantified using the Bicinchonic acid assay (BCA assay) and bovine serum albumin as the standard.

Conjugation between Split-Spy0128-fusion Protein and Isopeptagged Protein. A total of 10 μM split-Spy0128 proteins and 10−30 μM isopeptagged EGFPs (Niso-EGFP, Ciso-EGFP) were combined in reaction buffer (50 mM Tris-HCl (pH 8)). The reaction mixtures were incubated at 25 °C for ∼24 h in the dark. The reaction products were analyzed by SDS-PAGE. To follow the time course of the protein conjugation reaction, a small aliquot of the reaction mixture was mixed with a standard sample buffer solution for SDS-PAGE analysis and subjected to heat-treatment at 95 °C for 15 min to terminate the reconstitution reaction. The SDS-PAGE gel was stained by Coomassie brilliant blue (CBB) staining. The band intensities were quantified using a *CS analyzer 2.0* (Atto Corporation).

FRET Analysis. A total of 10 μM mBFP-split-Spy0128 and 10 μM isopeptagged EGFPs (Niso-EGFP, Ciso-EGFP) were combined in reaction buffer (50 mM Tris-HCl (pH 8)). The reaction mixtures were incubated at 25 °C for ∼24 h in the dark to follow the FRET. The time course of fluorescent spectral change was measured in the range between 400 and 600 nm at an excitation wavelength of 365 nm. The temperature was maintained at 25 °C.

Fluorescence Lifetime Study. Fluorescence lifetimes were measured by a single-photon counting system using a dye (PBD, Exciton, Inc., 360 nm), a Pulsed Nitrogen/Dye Laser (USHO KEC-100), and a steakscope (Hamamatsu Photonics, C4334) equipped with a polychromator at 293 K.[22]

MALDI-ToF MS Characterization. The reaction mixtures were desalted and concentrated using a ZipTip (C18 type, Millipore), and washed with a 0.1% TFA solution. The protein adsorbed on the tip was eluted with aqueous CH_3CN solution

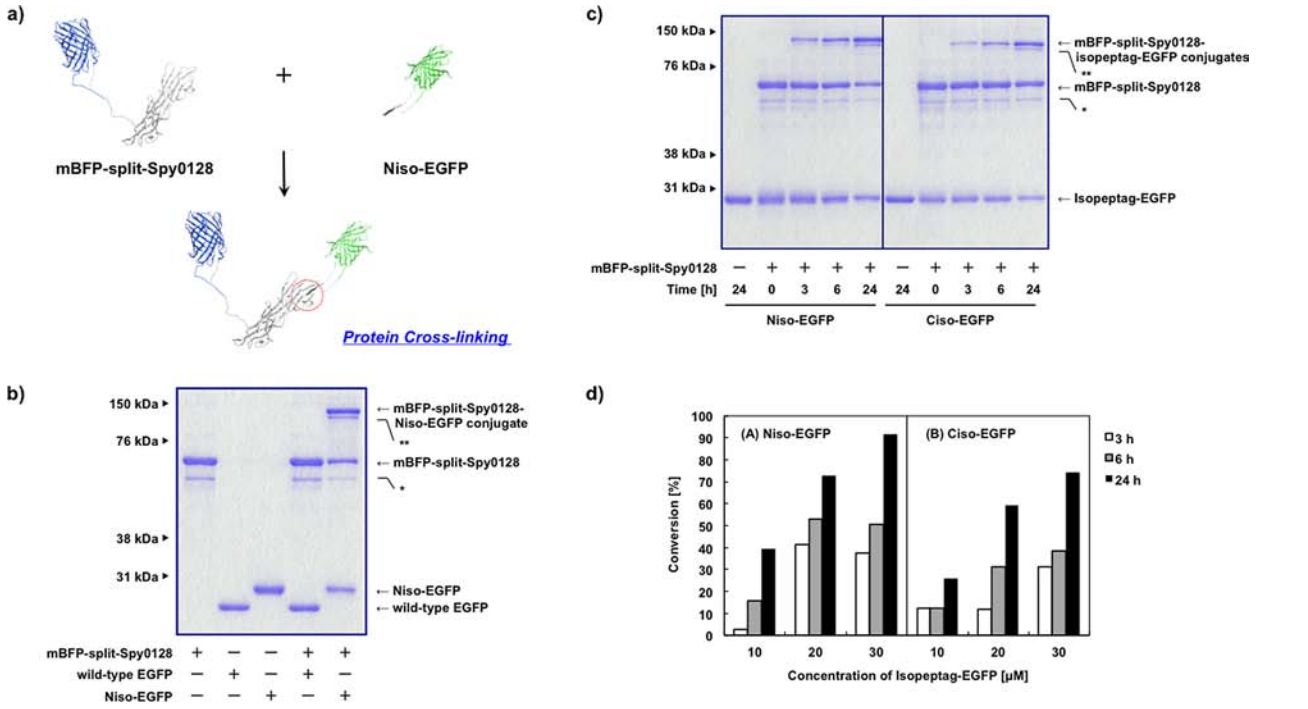

Figure 2. (a) Schematic illustration of conjugation between mBFP-split-Spy0128 and Niso-EGFP. (b) SDS-PAGE analysis of site-specific conjugation between mBFP-split-Spy0128 and Niso-EGFP. The reaction mixtures were incubated at 25 °C for 24 h in the dark. (c) Influence of the insertion site of isopeptag fused to EGFP. (b,c) A total of 10 μM mBFP-split-Spy0128 and 10 μM isopeptagged EGFPs (Niso-EGFP, Ciso-EGFP) were combined in a reaction buffer (50 mM Tris-HCl, pH 8). Degradation products of mBFP-split-Spy0128 and the conjugate are marked with * and **. (d) Efficacy of split-Spy0128-mediated conjugation between two proteins at different concentrations of (A) Niso-EGFP and (B) Ciso-EGFP with mBFP-split-Spy0128 (10 μM). The ligation efficiency was monitored by SDS-PAGE and the protein band intensities were quantified using a *CS analyzer 2.0*.

(0.1% TFA solution/CH_3CN = 50:50) containing CHCA (10 mg/mL), and the solution was directly subjected to MALDI-ToF MS analysis.

CD Spectroscopy. The proteins were prepared at a concentration of 0.1 mg/mL in 10 mM sodium phosphate buffer (pH 7.0). Wavelength scans between 200 and 250 nm in a cell with a 0.1 cm path length at a scan speed of 20 nm/min at 25 °C were completed. The spectra were accumulated five times with a bandwidth of 1.0 nm.

Preparation of Maleimide-Functionalized PEGA Resin (1). Amino-PEGA resin methanolic suspension (10 μmol amine sites) was washed with dichloromethane (DCM) (5 mL × 5) and *N*,*N*-dimethylformamide (DMF) (5 mL × 5) and drained. *N*-(6-Maleimidocaproyloxy) succinimide (EMCS) (30 μmol), HBTU (30 μmol), HOBt (30 μmol), and *N*,*N*-diisopropylethylamine (DIEA) (60 μmol) were dissolved in DMF (1 mL), and then the mixture was added to the washed resin. The mixture was stirred for 3 h, drained, and washed with DMF (5 mL × 5), DCM (5 mL × 5), and methanol (5 mL × 5) to obtain maleimide-functionalized PEGA resin **1**. The resin was subjected to the Kaiser test.[23] The resin gave a negative Kaiser test result and was stored swollen in methanol.

Immobilization of Cys-split-Spy0128 onto Maleimide-Functionalized PEGA Resin (2). In subsequent experiments, coupling regents were dissolved in the minimum amount of solvents (0.1−0.5 mL) in accordance with the number of moles of reactive sites. The maleimide-PEGA resin (200 nmol maleimide sites) was washed with deionized water (0.5 mL × 5) and 10 mM phosphate buffer (pH 7.0) (0.5 mL × 5) and then drained. The Cys-split-Spy0128 (6 nmol in 10 mM phosphate buffer (pH 7.0)) subjected to a reduction treatment with immobilized TCEP disulfide reducing gel was added to the maleimide-PEGA resin (200 nmol maleimide sites) and incubated for 3 h at room temperature. The reaction solution was drained, and then the resin was washed with 10 mM phosphate buffer (pH 7.0) (0.5 mL × 5). The immobilized Cys-split-Spy0128 on the maleimide-PEGA resin (200 nmol maleimide sites) was roughly equivalent to 1.7 nmol (calculated from the BCA assay of the supernatant of the reaction solution). 2-Mercaptoethanol (20 μmol in 10 mM phosphate buffer (pH 7.0)) was added to the Cys-split-Spy0128-immobilized PEGA resin and incubated for 1 h to block any unreacted maleimide groups. The blocking solution was drained and washed with 50 mM Tris-HCl (pH 8) (0.5 mL × 5). To confirm the immobilization of Cys-split-Spy0128 on the maleimide-functionalized PEGA resin, the resin was incubated with Alexa488 labeled anti-His tag antibody (sufficient to cover the beads, 1 μg/mL in PBS, Medical & Biological Laboratories Co., Ltd.) for 1 h at 37 °C. The solution was drained, washed with PBS (0.5 mL × 5), and the beads were examined by fluorescence microscopy.

Immobilization of Isopeptag-EGFP onto Cys-split-Spy0128-Immobilized Beads (3). Cys-split-Spy0128-immobilized beads were washed with 50 mM Tris-HCl (pH 8) and drained. Isopeptag-EGFP (10 nmol, 50 mM Tris-HCl (pH 8)) was added to Cys-split-Spy0128-immobilized beads (0.4 nmol Cys-split-Spy0128), and incubated for 12 h at room temperature. The reaction solution was drained, and then the beads were washed with 50 mM Tris-HCl (pH 8) (0.5 mL × 5) and examined by fluorescence microscopy.

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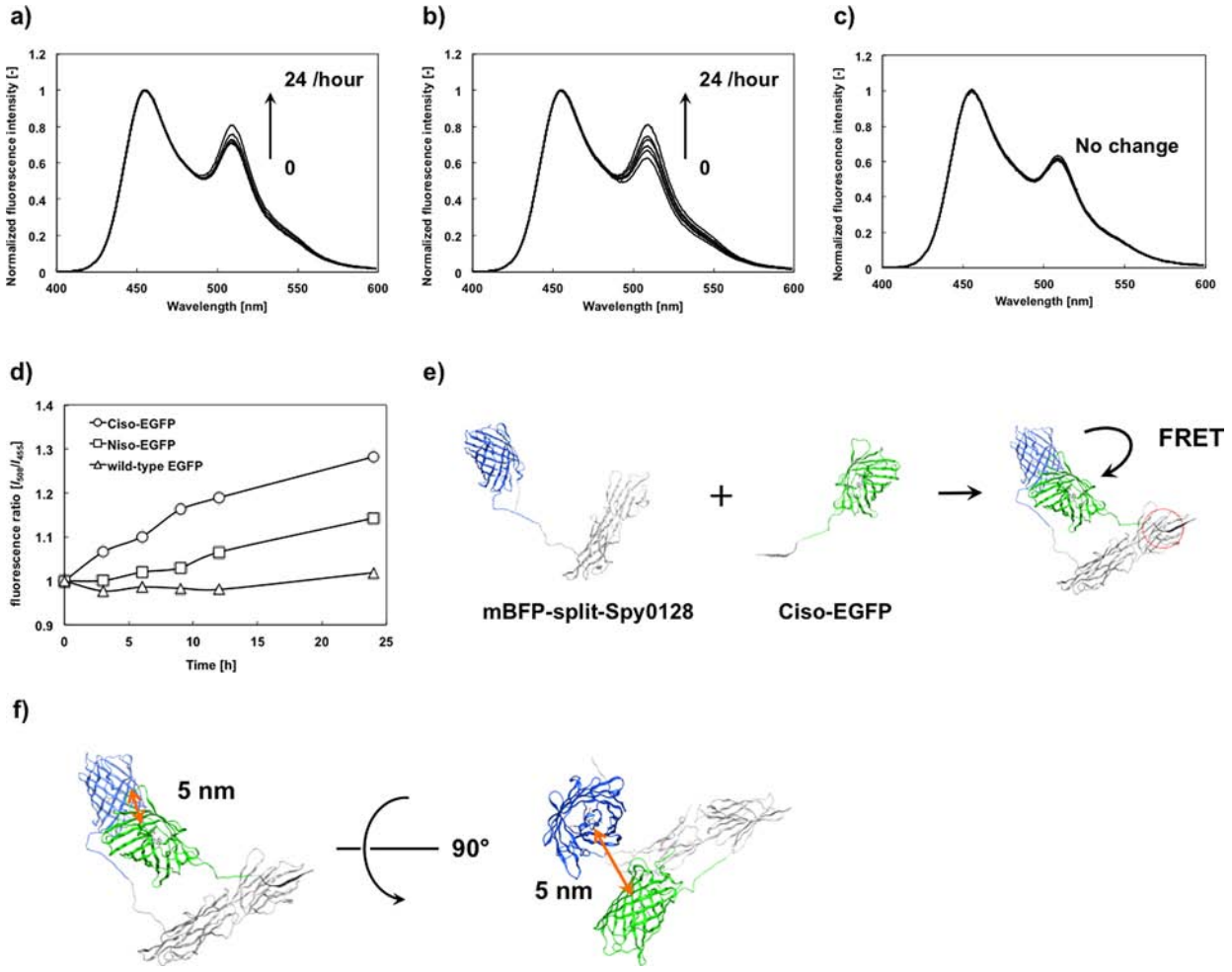

Figure 3. (a–c) Time-dependent emission spectra of mBFP-split-Spy0128-isopeptag-EGFPs, obtained by incubation with mBFP-split-Spy0128 and isopeptag-EGFPs (a, Niso-EGFP; b, Ciso-EGFP; c, wild-type EGFP). (d) Time profile of the emission intensity ratio (I_{508}/I_{455}) with different isopeptag-EGFPs. (e) Schematic illustration of conjugation between mBFP-split-Spy0128 and Ciso-EGFP. (f) Modeling of mBFP-split-Spy0128-Ciso-EGFP based on the fluorescence lifetime study.

RESULTS AND DISCUSSION

Conjugation between Split-Spy0128-Fusion Protein and Isopeptagged Protein. To study the ability of split Spy0128 to be used as a bioconjugate tool, we first conducted the cross-linking of blue and green fluorescent proteins (mBFP and EGFP) mediated by the reconstitution of split-Spy0128 and isopeptag, enabling the direct monitoring of cross-linking reactions by Förster resonance energy transfer (FRET). When mBFP was conjugated with EGFP within the range of 3–10 nm, FRET may take place whereby the energy exciting mBFP (the donor) transfers to the EGFP (the acceptor). We prepared three recombinant proteins. mBFP was fused to the N-terminus of split-Spy0128 because the C-terminus of split-Spy0128 takes part in the reconstitution reaction. Isopeptag was tethered at the N- or C-terminus of the EGFP. They are abbreviated as mBFP-split-Spy0128, Niso-EGFP, and Ciso-EGFP. mBFP-split-Spy0128 was incubated with Niso-EGFP at 25 °C for ~24 h in the dark (Figure 2a). SDS-PAGE analysis showed that the reaction mixture containing mBFP-split-Spy0128 and Niso-EGFP gave the desirable protein band, while there were no significant changes in the bands of control samples; this suggested that the isopeptag was site-specifically conjugated to the split-Spy0128 (Figure 2b). In a similar fashion, the ligation reaction with Ciso-EGFP was carried out to confirm the dependence of the insertion site of the isopeptag. Ciso-EGFP was also conjugated with mBFP-split-Spy0128; however, the yield of the mBFP-split-Spy0128-Ciso-EGFP conjugate was slightly lower than that of the mBFP-split-Spy0128-Niso-EGFP conjugate (Figure 2c). This is thought to be due to steric hindrance between EGFP and the N domain of Spy0128. As shown in Figure 2d, the ligation efficiency was improved by the addition of an excessive amount of the reaction partner (isopeptag-EGFPs). The cross-linking reaction between mBFP-split-Spy0128 and Niso-EGFPs was almost completed (>90%) in approximately 24 h at 30 μM Niso-EGFP. To identify whether the new band obtained by SDS-PAGE analysis represents mBFP-split-Spy0128-isopeptag-EGFP conjugates, the reaction products were investigated by MALDI-ToF MS analysis. The results revealed that mBFP-split-Spy0128-isopeptag-EGFP conjugates (with Niso-EGFP; found *ca.* 88 kDa, calcd 88 127, with Ciso-EGFP; found *ca.* 88 kDa, calcd 88 127 Da) were composed of one molecule of mBFP-split-Spy0128 (found: *ca.* 58 kDa, calcd: 58 301 Da) and one molecule of isopeptag-EGFP (Niso-EGFP; found *ca.* 30 kDa, calcd 29 844 Da, Ciso-EGFP; found *ca.* 30 kDa, calcd 29 844 Da) (Figure S1). Judging from the mass numbers of mBFP-split-Spy0128 and Ciso-EGFP, a Met residue was removed presumably by methionine aminopeptidase specifically liberat-

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ing the N-terminal Met residue from the proteins.[24] Therefore, this method is a site-specific and efficient protein cross-linking technology.

Subsequently, the direct monitoring of cross-linking reactions between the two different fluorescent proteins was conducted with FRET analysis. When mBFP-split-Spy0128 was incubated with isopeptag-EGFP in equal amounts, the fluorescence of EGFP relative to mBFP increased gradually in a time-dependent manner (Figure 3a−c). This indicates that the FRET between mBFP and EGFP occurred by cross-linking. Figure 3d shows the time profile of the emission intensity ratio (I_{508}/I_{455}) with different isopeptag-EGFPs (Niso-EGFP (square), Ciso-EGFP (circle), and wild-type EGFP (triangle)) at an excitation wavelength of 365 nm. The FRET efficiency of Ciso-EGFP was higher than that of Niso-EGFP despite the high cross-linking efficiency of Niso-EGFP. The FRET efficiency between two fluorophores depends heavily on their distance. This result suggested that each of the resultant adducts was different in the distance between mBFP and EGFP due to the difference of the insertion site of isopeptag, and that Ciso-EGFP was located on the same side as mBFP, while Niso-EGFP was not (Figures 2a, 3e).

In order to examine the results in more detail, the fluorescence lifetimes of mBFP-split-Spy0128-isopeptag-EGFP conjugates were measured. The fluorescence decay profile of mBFP-split-Spy0128 was first fitted by the following equation: $I(t) = A_1 \exp(-t/\tau_1)$ [A_1, amplitude; τ_1, fluorescence lifetime] and determined τ_1 for mBFP-split-Spy0128 (2.7 ns). We then analyzed the reaction mixture containing mBFP-split-Spy0128 and mBFP-split-Spy0128-Ciso-EGFP by the following equation: $I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$ [A_1 and A_2, amplitude; τ_1 (2.7 ns) and τ_2, fluorescence lifetime].[25] The obtained fluorescence decay profile was well fitted to the above biexponential equation ($\chi^2 = 1.2$); as a result, we obtained the fluorescence lifetime of mBFP-split-Spy0128-Ciso-EGFP (τ_2 = 0.71 ns) and their amplitudes (A_1 = 0.76, A_2 = 0.24). The values of the amplitude corresponded approximately to the conversion of the mBFP-split-Spy0128-Ciso-EGFP conjugate (26%, Figure 2d), indicating the adequacy of analysis was justified. In general, when FRET can occur, a shortened fluorescence lifetime of the donor molecule is observed due to the presence of an acceptor molecule. The observed fluorescence lifetime of the mBFP-split-Spy0128-Ciso-EGFP conjugate (τ_2: 0.71 ns) decreased by 2 ns compared to that of mBFP-split-Spy0128 (τ_1: 2.7 ns). This may be ascribed to the intramolecular energy transfer from mBFP to EGFP in the mBFP-split-Spy0128-Ciso-EGFP conjugate. The fluorescence lifetime of the mBFP-split-Spy0128-Niso-EGFP conjugate was not accurately obtained, implying that the mBFP was spatially far away from the EGFP in the mBFP-split-Spy0128-Niso-EGFP conjugate, and the intramolecular energy transfer from mBFP to EGFP could not occur.

The rate constant for fluorescence energy transfer (k_{ET}) of mBFP-split-Spy0128-Ciso-EGFP conjugate was estimated by the equation $k_{ET} = 1/\tau_2 - 1/\tau_1$. The value of k_{ET} was estimated to be 1.0×10^9 s^{-1}. k_{ET} is also defined by the equation $k_{ET} = (1/\tau_1)(R_0/R)^6$, where R_0 is the characteristic transfer distance, and is defined by the equation $R_0 = 9.7 \times 10^2 (J \kappa^2 n^{-4} \Phi_D)^{1/6}$ [nm].[25] J is the overlap integral of the emission spectrum of the donor and the absorption spectrum of the acceptor. Spectral overlap between mBFP-split-Spy0128 emission and EGFP absorption is shown in SI Figure S2. From the result, we calculated the J value. Assuming that the orientation of a mutual acceptor and donor is random, a value of 2/3 was used for κ^2, a relative orientation factor between the interacting dipoles. n is the refractive index of the medium and Φ_D is the quantum yield of mBFP.[21] We can estimate R_0 (5.8 nm) with the values. Consequently, R can be calculated to be 4.9 nm. When we roughly simulated the molecular modeling of the mBFP-split-Spy0128-Ciso-EGFP conjugate, the distance between two interchromophores of the fluorescent proteins was around 5.0 nm (Figure 3f), and thus, it supported the validity of the obtained values.[26]

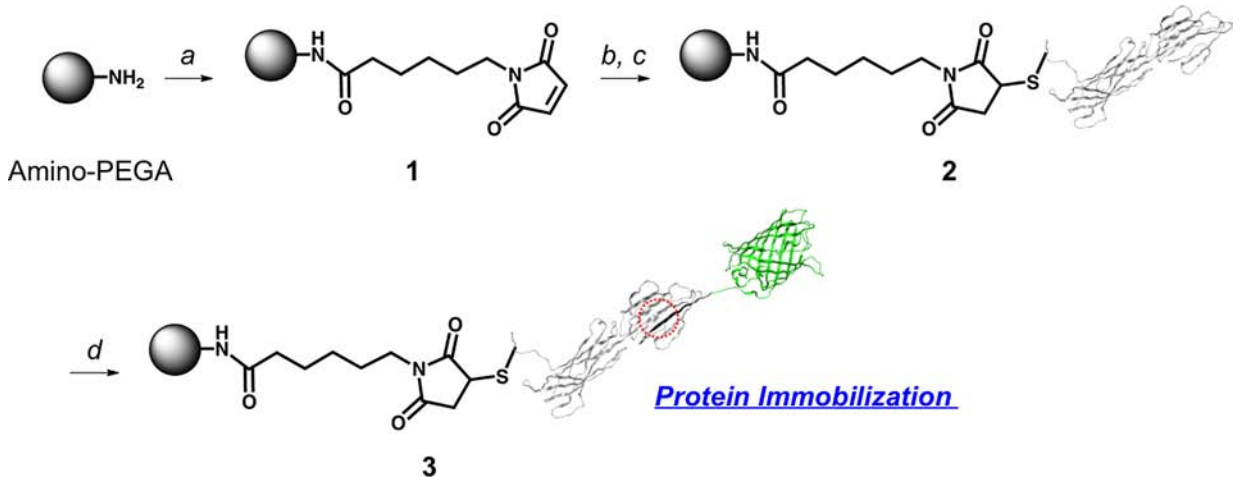
These results demonstrated that the split-Spy0128-mediated method is capable of not only cross-linking between two proteins, but also providing spatial control of them on the Spy0128 scaffold. This reconstitution reaction forms a covalent amide bond between the Lys and Asn side chains. In fact, the split-Spy0128-fusion protein was conjugated with isopeptag fused to the N- and/or C-terminus of the target proteins. Therefore, split Spy0128 could be a potent scaffold for the development of an artificial protein assembly using bioconjugation.

Site-specific bioconjugation techniques with post-translational modifying enzymes (e.g., TGase, sortase) have been explored.[3] Short peptide-tag-selective protein-labeling technologies with the enzymes have been employed to minimize the influence of labeling on the functionality of target proteins. Meanwhile, the relatively large size of split-Spy0128 may influence the expression and intrinsic properties of target proteins. Even so, split-Spy0128 has some advantages over other methods. First, split-Spy0128 protein is stable and is easy to express in *E. coli*. The specificity of reconstitution reaction is higher than that of microbial TGase.[4] The yield of sortase-mediated peptide ligation is not always high owing to the reversible reaction, whereas split-Spy0128-mediated ligation proceeds sufficiently because it irreversibly forms an isopeptide bond.[8] Since incorporation of two split Spy0128 fragments into target proteins is feasibly conducted by standard genetic manipulation, the present technique is applicable to the development of artificial proteins *in vivo*, which is still difficult with TGase and sortase.[4−8]

Immobilization of Isopeptagged Proteins onto Split-Spy0128-Immobilized Beads. Finally, we verified the applicability of the present technology for protein immobilization onto beads, which is important for the development of technologies such as protein arrays and nanofabrication.[27−29]

Split-Spy0128 and isopeptagged recombinant proteins are inactive individually; however, when the two proteins encounter each other, the reconstitution reaction can occur. In our approach, split-Spy0128 is first immobilized onto beads, then isopeptagged proteins are immobilized onto the split-Spy0128-immobilized beads by a reconstitution reaction. The spontaneous covalent immobilization of proteins is achieved by preparation of isopeptag (only 16 residues) fused to recombinant proteins. The key to our approach is the oriented immobilization of split-Spy0128. Since the C domain of split-Spy0128 takes part in the reconstitution reaction, it is better to immobilize at the N terminus to achieve efficient immobilization. Therefore, we focused on introducing a unique Cys residue to the target protein because the Cys residue can be selectively alkylated with maleimide.[30] Importantly, Spy0128 contains no Cys residues. Thus, a single thiol group can be engineered into split-Spy0128 by site-directed mutagenesis. First, a split-Spy0128 mutant with a free N-terminal Cys residue (Cys-split-Spy0128) was prepared (Table 1). The cross-linking

Scheme 1. Schematic Representation of Immobilization of Cys-split-Spy0128 and Isopeptag-EGFP onto Beads[a]



[a](a) EMCD, HBTU, HOBt, DMF, 3 h; (b,c) Cys-split-Spy0128, sodium phosphate buffer (pH 7.0), 3 h; 2-mercaptoethanol, sodium phosphate buffer (pH 7.0), 1 h; (d) isopeptag-EGFP, Tris-HCl buffer (pH 8.0), 12 h.

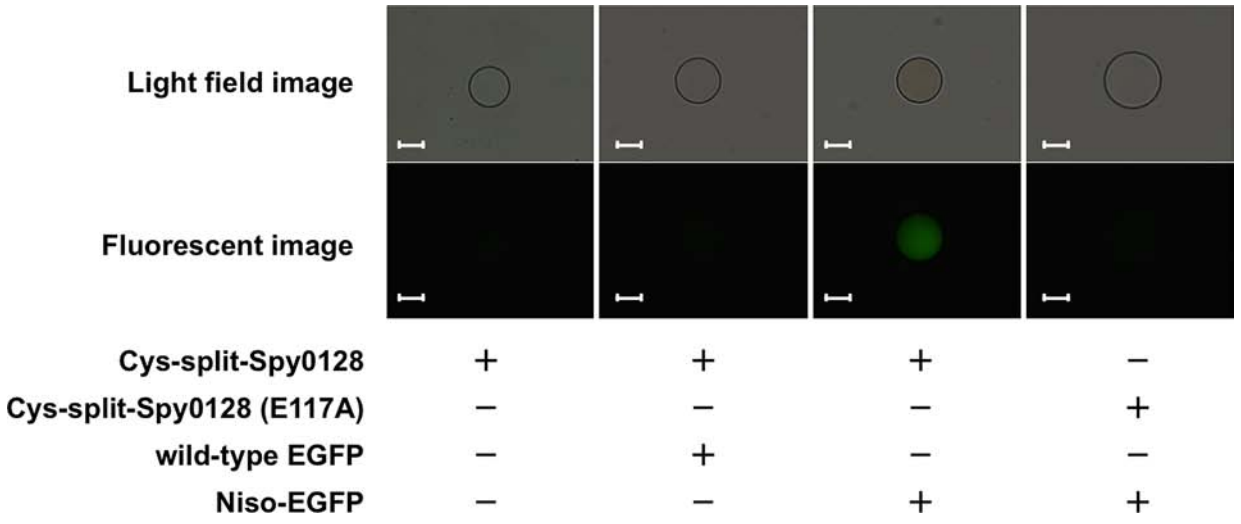


Figure 4. Microscope image of split-Spy0128-immobilized beads treated with isopeptag-EGFP. (Top row) Light field image. (Bottom row) Fluorescent image with a band path (460−490 nm) filter. The scale bars are 50 μm.

reaction between Cys-split-Spy0128 and isopeptagged recombinant proteins in a homogeneous system was tested. As was the case with split-Spy0128, Cys-split-Spy0128 was conjugated with isopeptagged proteins (SI Figure S3a). However, an undesired band corresponding to a Cys-split-Spy0128 dimer was observed (SI Figure S3a, lane 1). Split-Spy0128 contains one isopeptide in the N domain. In a previous report, it was discussed that the isopeptide was formed either intermolecularly or intramolecularly.[14] In order to examine the cause directly, we prepared a Cys-split-Spy0128(E117A) control, lacking Glu as the catalytic site in the N domain, and analyzed it by SDS-PAGE analysis (SI Figure S3b). The dimer of Cys-split-Spy0128 (E117A) was not detected, indicating that the side reaction proceeded in the N domain of split-Spy0128. Intriguingly, no conjugate was observed for Cys-split-Spy0128 (E117A) (SI Figure S3, lane 5), implying that the lack of the isopeptide bond in the N domain causes the structural alteration of split-Spy0128 and leads to no reaction between Cys-split-Spy0128 and isopeptag-EGFP. The secondary structures of the proteins were evaluated by CD spectroscopy (SI Figure S4).[31] It was revealed that the proper folding of the N domain is necessary for the reconstitution reaction. These results are consistent with the observation that the domain−domain interactions within Spy0128 might be required for the reactivity of isopeptide bond formation.[32]

Next, Cys-split-Spy0128 was first immobilized onto beads chemically modified with the maleimide group through a Michael addition process. The immobilization of Cys-split-Spy0128 was assessed using fluorescein-conjugated anti-His_6 antibody (data not shown). In solution, we observed the higher conjugation yield with Niso-EGFP than with Ciso-EGFP (Figure 2). Therefore, we employed Niso-EGFP to avoid steric hindrance in split-Spy0128-mediated protein immobilization. The Cys-split-Spy0128-immobilized beads **2** were then incubated with Niso-EGFP in 50 mM Tris-HCl buffer (pH 8) for 12 h (Scheme 1). The resultant beads were washed with reaction buffer and examined by fluorescence microscopy. Figure 4 shows a fluorescence image of EGFP-immobilized beads. Control experiments with Cys-split-Spy0128(E117A) and wild-type EGFP provided only the background signal, while bead **3** exhibited a clear fluorescent signal. This indicated

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that cross-linking between split-Spy0128 and isopeptagged proteins on the beads were achieved. These results suggest that Spy0128 is a potent proteinaceous scaffold available for bioconjugation both in solution and at a solid surface. Using maleimide derivatives containing various functional molecules (e.g., synthetic polymers, fluorophores), this strategy can be applied to wide-ranging bioconjugation applications.

CONCLUSIONS

In summary, we demonstrated that split Spy0128 is a potent bioconjugate tool for biochemistry and biotechnology. We succeeded in controlling the distance between two cross-linked proteins mediated by the reconstitution of split-Spy0128 and isopeptag. Genetic incorporation of a Cys residue into split-Spy0128 enabled the immobilization of the Cys-split-Spy0128 on the maleimide-functionalized resin, and then the isopeptag-EGFP was immobilized on the split-Spy0128-immobilized beads. These results suggest that Spy0128 is a potent proteinaceous scaffold available for bioconjugation both in solution and at a solid surface. The method presented here should be generally applicable to combinations involving different recombinant proteins and functional groups containing maleimide. Recent reports on a new split protein (second immunoglobulin-like collagen adhesive domain (CnaB2)), which can cross-link between two proteins in minutes, may be useful for increasing the reconstitution reactivity.[33]

ASSOCIATED CONTENT

Supporting Information

Protein sequences of wild-type EGFP and mBFP-split-Spy0128, primer lists for the isopeptag-EGFPs and the split-Spy0128 proteins, MALDI-Tof MS analysis of mBFP-split-Spy0128-isopeptag-EGFP conjugates, spectroscopic characterization and SDS-PAGE analysis of the recombinant proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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