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Site-selective functionalization of nanobodies using intein-mediated protein ligation for innovative bioconjugation

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Running head

Bioconjugation by Intein-mediated protein ligation

Abstract

An expression strategy is presented in order to produce nanobodies modified with a clickable alkyne function at their C-terminus via the Intein-mediated Protein Ligation (IPL) technique. The protocol focuses on the cytoplasmic expression and extraction of a nanobody-intein-chitin binding domain

(CBD) fusion protein in *E. coli* SHuffle® T7 cells, in the commonly used Luria-Bertani (LB) medium. The combination of these factors results in a high yield and nearly complete alkynation of the nanobody at its C-terminus via IPL. The resulting alkynated nanobodies retain excellent binding capacity towards the nanobody targeted antigen. The presented protocol benefits from time- and cost-effectiveness and allows a feasible up-scaling of functionalized (here alkynated) nanobodies. The production of high quantities of site-specifically modified nanobodies paves the way to i) novel biosurface applications that demand for homogeneously oriented nanobodies having their active site fully accessible for target (e.g. biomarker) binding, and ii) innovative applications such as localized drug delivery and image guided surgery by covalent 'click' chemistry coupling of these alkynated nanobodies to a multitude of azide-containing counterparts as there are drug containing polymers and contrast labeling agents.

Key words

Protein functionalization, intein, nanobody, click chemistry

1 Introduction

When considering the coupling of a protein of interest (POI) to e.g. labeling moieties or material surfaces, the effectiveness of the resulting construct is strongly dependent on the applied conjugation methodology. Ideally, the conjugated protein should maintain its function (e.g. catalytic activity for enzymes, antigen binding potential for antibodies) making targeted, or site-specific, modification of proteins desirable. In addition, for most applications the covalent coupling of the protein to the surface or other functional compounds is required.

Proteins are composed of various amino acids and thus generally contain multiple reactive functionalities (e.g. carboxylic acids, amines, thiols, alcohols) [1]. The possibility of possessing multiple copies of these functional groups and/or their presence in the binding pockets, excludes many conventional chemical conjugation techniques (e.g. amide bond formation via carbodiimide chemistry) since such methods do not allow the targeting of a specific region (i.e. a particular amino acid within the protein of interest) without altering the protein's conformation.

To circumvent this issue, bioorthogonal handles (i.e. not occurring naturally within a particular protein) can be introduced to subsequently allow the selective coupling of the protein to a complementary functionality. In this respect so-called click reactions have gained a lot of interest as such reactions are very efficient, highly specific and can be performed under the mild reaction conditions associated with protein modifications. The Cu-catalyzed Huisgen 1,3-dipolar cycloaddition (or Cu-catalyzed azide-alkyne cycloaddition, CuAAC) is a well-known example of such efficient coupling methodologies [2]. In this reaction an azide and alkyne react to form a stable 1,2,3-triazole ring (*see* **note 1**). The modification of the protein at a single site would thus enable the site-specific coupling of the POI to molecules and surfaces decorated with the complementary chemical functionality.

In the current chapter the intein-mediated protein ligation methodology is introduced as a method to selectively introduce a particular chemical handle at the C-terminus of the POI [3, 4]. Inteins (also called protein introns) are self-cleavable peptide sequences ending in an N-terminal cysteine [5]. This cysteine facilitates an N-S shift at the C-terminus of the POI thereby forming a thioester linkage (Fig. 1). The

formed thioester is prone to nucleophilic attack and can be cleaved via the addition of thiol-containing compounds (e.g. dithiothreitol, DTT; sodium 2-mercaptoethane sulfonate, MESNA) in what is called native chemical ligation [6, 7].

By fusing the POI with an intein sequence (*see* **note 2**), the N-S shift can be exploited to make the POI susceptible to functionalized thiols. Within this protocol, the focus lies on a cysteine derivative containing an alkyne functionality (in short cysteine-alkyne linkers, Fig. 2A), which would enable the subsequent conjugation to various azide-functionalized compounds/surfaces [8–10]. These include azide-functionalized biotin for application in streptavidin-based assays (Fig. 2B), fluorescent markers for imaging applications (Fig. 2C), azide-pending polymers for controlled drug delivery applications (Fig. 2D) or azide-functionalized surfaces for biosensor development (Fig. 2E). The choice for introducing an alkyne-functionality at the protein C-terminus rather than an azide is explained in **note 3**.

In the current protocol the intein-mediated protein ligation technique is applied as a straightforward method to introduce an alkyne functionality at the C-terminus of a nanobody. Nanobodies are single domain antigen-binding proteins derived from camelid antibodies (or heavy chain antibodies) [11, 12]. Nanobodies exhibit similar binding capacities and affinities towards their antigen than conventional immunoglobulins, but are much smaller, more thermostable and resistant to pH changes [13]. These properties, together with the fact that nanobodies are encoded by a single gene, make these proteins an interesting alternative for conventional antibodies in biosensing and targeted drug delivery applications [12, 14]. IPL is particularly interesting for functionalizing nanobodies as the C-terminus is located at the opposite site of the binding paratope, which contains the N-terminus [15]. Conjugating nanobodies at the C-terminus via the IPL methodology thus guarantees the target binding potential of the nanobody remaining unaffected.

The presented protocol describes the cloning of a nanobody in a commercially-available plasmid, the production and purification of a nanobody-intein fusion construct out of the cytoplasm of fermented *E. coli* cells, the generation of alkynated nanobodies and the conjugation of azidified substrates to the

alkyne-functionalized nanobody by CuAAC. Also methods are provided to evaluate the success of the conjugation reaction.

2 Materials

Unless stated otherwise, all reagents mentioned in this protocol were purchased from Sigma-Aldrich and used as received. Aqueous solutions and buffers are prepared using Milli-Q water (ρ = 18.2 MΩ).

2.1 Molecular cloning of the nanobodies

1. Plasmids containing nanobody sequences are needed. In our studies we have used pHEN6 plasmids containing coding for nanobodies followed by His_6 coding for purification purposes [16]. Dilute each template plasmid to 100 pg/mL in sterile water
2. Forward primer (5'-GGTGGTCATATGCAGGTGCAGCTGCAGGAGTCT-3' , 10 µM concentration in sterile water).
3. Reverse primer (5'-GGTGGTCTCGAGTGAGGAGACGGTGACCTGGGTCCC-3', 10 µM concentration in sterile water)
4. Thermocycling device
5. 5 units/µL Taq polymerase (e.g. Dream Taq from Thermo Fisher Scientific)
6. Taq polymerase buffer (e.g. Dream Tag buffer from Thermo Fisher Scientific)
7. 10 mM dNTPs
8. 10 units/µL restriction enzymes NdeI, XhoI, (e.g. from Thermo Fisher Scientific).
9. QIAquick Gel Extraction Kit (Thermo Fisher Scientific)
10. 1U/µL T4 DNA Ligase
11. 10x T4 DNA ligase buffer
12. Double-digested (NdeI, XhoI) pMXB10 plasmid (New England Biolabs, *see* Fig. 3)

2.2 Transformation and culture

1. Sterile Plates, flasks, glass tubes, pipette tips
2. Glycerol stock *E.coli* BL21(DE3) at -80°C (e.g. from Thermo Fisher Scientific)

3. Luria-Bertani (LB) growth medium containing, per L water, 10g tryptone, 5g yeast extract and 10g NaCl, adjusted to pH7.0 with NaOH and autoclaved
4. 200 µg/mL plasmid from section 3.1 (*see* Fig. 3)
5. 100 mg/mL Ampicillin stock solution
6. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution in Milli-Q
7. Heating block or water bath (42 °C)
8. Incubator (37 °C)
9. LB^{AMP} (LB culture medium containing 100 µg/mL Ampicillin)
10. LB^{AMP} agar plates (LB medium containing 100 µg/mL Ampicillin and 1.5 w/v % agar)

2.3 Protein extraction

1. Solid Phase Extraction (SPE) Cartridges
2. Eppendorf vials
3. Falcon tubes
4. Micropipettes and tips
5. Bacterial Protein Extraction Reagent (B-PER, Thermo Fisher Scientific) containing 1U/mL DNaseI

2.4 Protein purification and IPL-mediated alkynation

1. Solid Phase Extraction Cartridges
2. Eppendorf vials
3. Falcon tubes
4. Micropipettes and tips
5. 50% (w/v) chitin slurry in 20% (v/v) ethanol (New England Biolabs)
6. 3 M stock solution of dithiothreitol (DTT) in Milli-Q
7. 3 M stock solution of sodium 2-mercaptoethane sulfonate (MESNA) in Milli-Q
8. 1 M stock solution of Tris(2-carboxyethyl)phosphine (TCEP) in Milli-Q
9. 1 M stock solution cysteine-alkyne linker in Milli-Q
10. Column buffer (CB) consisting of 20 mM HEPES-NaOH pH 8.5, 0.5 M NaCl and 1 mM EDTA

2.5 CuAAC click reaction

1. Alkyne-functionalized nanobody prepared in section 3.4
2. Azide functionalized binding partner such as those depicted in Fig. 3 (e.g. N-(3-azidopropyl)biotinamide from TCI).
3. CuAAC buffer: 1 mM Tris-(2-carboxyethyl)phosphine (TCEP), 0.1 mM Tris-(benzyltriazolylmethyl)amine (TBTA) and 1 mM $CuSO_4$ in PBS buffer pH 7.4.

2.6 Assessment of the click reaction efficiency to azidified biotin by western blotting

1. 15% SDS-PAGE gel
2. Amersham Hybond™-LFP PVDF membrane (GE Healthcare and Life Sciences)
3. 5% (w/v) bovine serum albumin (BSA)
4. Tris-buffered saline (50 mM Tris, 150 mM NaCl) containing 0.1 % Tween 20 (TBST)
5. Streptavidin-alkaline phosphatase conjugate (2 mg/mL, e.g. from Thermo Fisher Scientific)
6. Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolul phosphate (NBT/BCIP) ready-to-use solution (e.g. from Sigma-Aldrich)

2.7 Electrospray ionization Fourier transform mass spectrometry

1. HPLC set-up (Dionex 3000) equipped with auto-injector and coupled to the mass spectrometer (Orbitrap Velos Pro from Thermo Fisher Scientific)
2. Dionex Acclaim PolarAdvantage II C18 reverse-phase 2.0x10 mm guard column (particle diameter 5µm, porosity 0.0120 µm)
3. Solvent A: 0.1% (v/v) formic acid in Milli-Q water
4. Solvent B: HPLC grade acetonitrile

3 Methods

3.1 Molecular cloning of the nanobodies in the pMXB10 plasmid

This section describes the cloning of the nanobody (from a template plasmid) into the pMXB10 plasmid. As such the nanobody is cloned, via a linker peptide, in frame with an intein and a chitin binding domain (CBD) (*see* Fig. 3)

1. Thaw all reagents on ice.
2. Prepare a master mix containing 36.5 µL Milli-Q water, 5 µL Taq polymerase buffer, 5 µL dNTPs, 1 µL forward and 1 µL reverse primer, 1 µL nanobody-coding template plasmid and 0.25 µL Taq polymerase
3. Place the 96 well in a thermocycler, using the following temperature program:
    - 5 min at 95°C
    - 30 cycles of subsequently 30 s at 95°C, 30 s at 55°C and 45 s at 72°C
    - A final elongation step of 10 min at 72°C
4. Digest the gene fragment using the appropriate restriction enzyme by mixing the reagents in the following ratios: 6.5 µL of Milli-Q water, 2 µL of 10x restriction buffer, 0.5 µL NdeI, 1 µL XhoI (10 units μL^{-1}), 10 µL PCR product.
5. Incubate this solution for 2 hours at 37°C and purify using the QIAquick gel Extraction kit.
6. Ligate the digested gene fragments into the pMXB10 expression vector by preparing a reaction mixture containing 100 ng double-digested and purified plasmid, 50 ng double-digested PCR-fragment (1-3 molar excess relative to the plasmid), 1 µl T4 DNA ligase and 1 µL 10x T4 DNA Ligase buffer and sterile water for a total volume of 10 µL (*see* **note 4**).
7. The solution is incubated at room temperature for 10 minutes followed by incubation at 4°C until applied in transformation, which should take place on the same day.

3.2 Transformation and culture

1. Take a vial of glycerol stock *E. coli* BL21 (DE3) and the plasmid solution (Fig. 3) and thaw on ice for 5 min.

2. Pipette 1 µL of the plasmid solution to the cells and do not mix (200 ng of plasmid per 50 µL of cell suspension).
3. Incubate the cells on ice for 30 min.
4. Give a heat shock by quickly transferring the cell vial to a heat block or water bath at 42°C for 30 sec.
5. Put the vial containing the cells immediately back on ice for 2 min.
6. Suspend the cells in 1 mL LB (without Amp) and incubate at 37°C for 1 hour while shaking.
7. Spread the cells in 2 LB^{Amp} plates (100 µg/mL, 1.5 % Agar) at a ratio of 1:9.
8. Incubate the plates overnight at 37 °C (*see* **note 5**).
9. Select a single colony and inoculate 3 mL of LB^{Amp} (100 µg mL^{-1}) in a glass tube (*see* **note 6**).
10. Incubate the tube overnight at 37 °C.
11. Inoculate 300 mL liquid LB^{Amp} with the 3 mL of pre-culture (=1:100).
12. Incubate at 37 °C with shaking until an optical density (OD) of 0.5-0.6 is obtained (approx. 2.5-3 hours).
13. Add IPTG to a final concentration of 1 mM to induce expression.
14. Incubate for 3 hours at 37 °C.
15. Harvest the cells via centrifugation (5000 g for 10 min at room temperature) (*see* **note 7**).

3.3 Protein extraction

1. Resuspend the cell pellet from 300 mL bacterial culture in 6 mL B-PER supplemented with DNAseI by pipetting up and down.
2. Incubate for 15 min at room temperature.
3. Centrifuge at 15000 g, 30 min, 4 °C.
4. Collect the supernatant for purification.

3.4 Protein purification and IPL-mediated alkynation

1. Pre-wet the filter frits of 3 empty SPE columns with distilled water.
2. Add the appropriate amount of chitin slurry to each column (2.5 mL per column considering the 300 mL of growth medium) and let equilibrate by gravity.

3. Wash each column with 10 column volumes of Milli-Q water and subsequently 10 column volumes of column buffer (CB).
4. Divide the cell lysate in 3 portions and load each portion on a chitin column
5. Wash each column with 20-30 column volumes of CB.
6. Quickly flush (< 5 min) the columns with 1.5-3 mL of the freshly prepared cleavage buffers (*see* **note 8**):
   a. Column 1: column buffer (negative control)
   b. Column 2: 30 mM DTT in CB
   c. Column 3: 30 mM cysteine-alkyne, 30 mM MESNA and 1 mM TCEP in CB
7. Close all SPE columns by putting a stopper on the column exit.
8. Add 1 column volume of the selected cleavage buffer to each column, mix gently and incubate overnight at 4 °C.
9. Elute the columns with 1.5 column volumes of column buffer while collecting the samples in falcon tubes.
10. The collected samples are finally purified via dialysis against PBS buffer (pH 7.4) with at least 3 buffer changes at 2 hour intervals.

3.5 CuAAC click reaction with alkynated nanobody

1. Mix 10 µM purified alkyne-functionalized nanobody from section 3.4 with 0.2 mM azide-functionalized substrate (*see* Fig. 2 and **note 9**) in CuAAC buffer. As an easy quantifiable substrate we here use azide-biotin.
2. Shake the solution for 2 hours at room temperature

3.6 Assessment of the click reaction efficiency

1. Perform electrophoresis on the nanobody-biotin conjugate using a 15% SDS-PAGE gel.
2. Transfer the proteins to an Amersham Hybond^{TM}-LFP PVDF membrane.
3. Block the blot with a 5% BSA solution for 1.5 hours.
4. Incubate the membrane for 1 hour in a sufficient amount of TBST containing 2 µg/mL streptavidin-alkaline phosphatase conjugate.

5. The proteins are finally visualized by adding 1 mL of the ready-to-use NBCP/BCP solution (approx. 10 minutes development) (*see* **note 10**).

3.7 Electrospray ionization Fourier transform mass spectrometry

1. Trap and further desalt 30 µL aliquots of the dialyzed or desalted nanobodies and nanobody-conjugate solutions (20 µM) for 5 min on a C18 reverse-phase guard column at a flow rate of 0.5 mL min^{-1} using 0.1 % (v/v) formic acid in Milli-Q (solvent A) as mobile phase.
2. Elute the sample in 50% (v/v) acetonitrile in solvent A at a flow rate of 0.1 mL min^{-1} into an electrospray ionization (ESI) source with sheath gas (8 arbitrary flow rate units). The source voltage and capillary temperature were +4 kV and 275°C, respectively.
3. Collect mass spectra in full scan mode in the mass range of 110-2000 Thomson at a resolution of 3×10^4 full width at half maximum with automatic gain control set to 1×10^6 ions in 100 ms and without microscan averaging.
4. Average the scans recorded in the 4-7 min timeframe.
5. Deconvolute the average spectrum using Promass software for Xcalibur v2.8 (Novatia LLC) for molecular weight determination.
6. Compare the deconvoluted masses to the corresponding theoretical values derived from CLC Main Workbench 6 software (*see* Fig. 5 and **note 11**).

4 Notes

1. The CuAAC reaction discussed in this chapter requires the presence of Cu(I) ions to catalyze the conjugation reactions. Since Cu^+ ions are known to complex to the formed 1,2,3-triazole ring and possibly to the protein, this may exclude the application of the CuAAC reaction for certain applications. For this reason, an alternative pathway has been proposed which relies on highly strained alkynes (e.g. cyclooctyne) [17, 18]. Due to the ring strain in this functional group, a strain promoted azide-alkyne click reaction (SPAAC) can be performed in absence of a metal catalyst, thereby further broadening the scope of the azide-alkyne click reaction.

2. The presented protocol lists the optimized conditions for one particular nanobody construct characterized by a LEY spacer between the nanobody and the intein domain. Especially the tyrosine adjacent to the intein domain has been reported to facilitate the thiol-initiated cleavage of the protein-of-interest from the intein. Other constructs exhibiting EFLEY or His_6 spacers have also been produced via the presented protocol. Translation of the IPL methodology to other proteins and/or substrates will probably require some optimization in terms of product yield and modification efficiency.
3. Cysteine-alkyne is preferred for the native chemical ligation over cysteine-azide based on the inherent reactivity of organic azides [19]. Azides are considered manageable when i) the number of nitrogen atoms (N_N) in the compound does not exceed the number of carbon atoms (N_C) and ii) the equation $(N_C + N_O)/ N_N \geq 3$ is fulfilled, with N_O the number of oxygen atoms in the molecule. Considering a cysteine analogue carrying an azide functionality, the latter equation is not met unless very long spacers are introduced. Therefore, from a safety point of view, the azide functionality is generally introduced on the surface of a solid support or on the compound with the highest molecular weight, e.g. biotin, 5-carboxy-fluoresceine (5-FAM) and poly(ethyl-2-oxazoline).
4. It is advised to verify that the insert was ligated correctly into the vector via sequencing prior to applying the recombinant plasmid in the transformation of *E. coli*.
5. If it is not possible to immediately continue with the following step of the presented protocol (i.e. the preculture of a single colony), it is advised to store the plates at 4°C to prevent the overgrowth of the bacteria.
6. The yield of the fusion protein can be further increased by applying the *E. coli* Shuffle® T7 strain and the nutrient-rich EnPresso® medium (supplemented with 100 ng /mL ampicillin) [8]. For EnPresso® the manufacturer's specifications were followed by dissolving the tablets in Milli-Q water, then 1/2000 of reagent A and bacterial preculture were added for growth at 30 °C overnight. The next day, 0.5 mM IPTG, reagent A (1/2000) and the booster tablet were added, followed by protein expression for 24 hours. As can be seen in Fig. 4, yields in excess of 40 mg mL^{-1} could be reached by combining the Shuffle® T7 strain with the EnPresso®

growth medium. However, it should be noted this increase in yield comes at a cost since EnPresso® is more expensive and the procedure is more time-consuming (48 hours) than when using LB medium (3 hours).

7. In case the harvested cells cannot be used in the subsequent protein extraction step, the cells should be stored at -20°C in order to prevent protein degradation taking place prior to the protein extraction step.
8. It is advised to flush the column with cleavage buffer, to make sure the entire chitin column experiences the same cleavage conditions. If this step is omitted, adding cleavage buffer to the column will efficiently cleave off the nanobodies from the chitin beads near the top, while nanobodies near the column outlet are unaffected by the added thiols. Losses of protein during this step are limited since the intein-mediated cleavage does not take place instantaneously. However, the flushing step should not take more than 5 minutes.
9. Next to azido-biotin, the alkynated nanobodies have been successfully conjugated to 5-carboxy-fluorescein-azide (5-FAM azide) and azidified poly(ethyl-2-oxazoline) (pEtOx-N_3). pEtOx-N_3 (M_n 5 and 10 kg /mol) was kindly provided by prof. Richard Hoogenboom (Ghent University). For these azides, slightly modified conditions were applied during the click reactions as shown in table 1. In the case of 5-FAM, reaction efficiencies were higher when using sodium ascorbate as the reducing agent (5 mM) and Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) as copper ligand (0.1 mM). These reagents were also beneficial for the conjugation to pEtOx, but only 10 equivalents of the azidified polymer were added to the reaction mixture since polymers reduce the solution's viscosity.
10. Although the methodology described in section 3.6 enables the visualization of successful click reactions with azido-biotin as reagent, similar results can be obtained in a more straightforward manner when using alternative azide-pending compounds. E.g. when azide-functionalized 5-carboxy fluorescein (5-FAM, Fig. 2) is used in click reactions, proteins that were successfully coupled to 5-FAM will fluoresce under direct UV illumination. In addition, coupling the nanobodies to high molecular weight azides (e.g. polymers carrying an azide end-group) will

result in an apparent change in molecular weight compared to unmodified protein when the gel is stained (e.g. with the ready-to-use instant blue stain from Expedeon).

11. Although the above-mentioned evaluation of the click efficiency via SDS-PAGE is straightforward for high molecular weight compounds, this approach provides only qualitative information. Mass spectrometry was shown to be a semi-quantitative method to determine the degree of alkynation of the nanobodies (Fig. 5). In addition, this technique clearly indicates that only a single alkyne functionality is introduced in the POI via IPL.

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Figure captions

Fig. 1: Scheme showing the IPL mechanism. The protein of interest is C-terminally ligated to an intein-chitin binding domain (intein-CBD). The expressed fusion protein is purified via affinity chromatography on a chitin column and subsequently cleaved by addition of thiol-based nucleophiles. When cysteine-alkyne linkers are applied, on column cleavage of the POI occurs simultaneous to protein functionalization yielding alkynated proteins.

Fig. 2: Chemical structure of the **(A)** cysteine-alkyne, **(B)** azido-biotin, **(C)** 5-FAM-azide, **(D)** PEtOx-N_3 polymers and **(E)** azide-functionalized surfaces to be coupled to the alkynated nanobodies by means of CuAAC 'click' chemistry.

Fig. 3: Scheme for cloning and expression of the selected nanobody variant. The IPL-mediated production strategy of unmodified and alkynated nanobodies is shown with corresponding yields displayed at the bottom. Reproduced with permission from [10].

Fig. 4: Expression yield of the alkynated nanobody (NbVCAM1-LEY-alkyne) in the SHuffle® T7 *E. coli* strain using different media: Luria-Bertani (LB), terrific broth (TB) and EnPresso®. All cultures were grown in triplicate and the reported values correspond to the averages with their standard deviations. Reproduced with permission from [10].

Fig. 5: ESI-FTMS spectra of desalted and buffer-exchanged NbVCAM1-LEY-alkyne (using amicon concentrator with N_2-aerated PBS buffer) showing quasi only mono-alkynated NbVCAM1-LEY-alkyne. The representative structure of the corresponding nanobody species is displayed next to the corresponding mass peaks. Reproduced with permission from [10].

Table captions

Table 1. Overview of CuAAC conditions applied for the bioconjugation of alkynated nanobodies with various azide-containing reaction partners.

Table 1

Reagents	Binding partner			
	Surface bound azides[8]	Azido-biotin	5-FAM-azide	pEtOx-N_3
Alkynated nanobody	0.5 µM[1]	10 µM	10 µM	100 µM
Azide-functionalized compound	-	200 µM	200 µM	1000 µM
Catalyst	0.5 mM $Cu(CH_3CN)_4^+PF_6^-$	1 mM $CuSO_4$	1 mM $CuSO_4$	1 mM $CuSO_4$
Reducing agent	0.65mM sodium ascorbate	1 mM TCEP	5 mM sodium ascorbate	5 mM sodium ascorbate
Ligand	0.5 mM TBTA	0.1 mM TBTA	0.1 mM THPTA	0.1 mM THPTA
Reaction time (h)	16	2	1.5	0.75
Reaction Yield (%)	N.A.[2]	40	50-60	80

[1] Reaction performed in 95-5 mixture of acetate buffer (pH 4) and DMSO

[2] Successful deposition was observed, but this cannot be expressed in terms of yield.

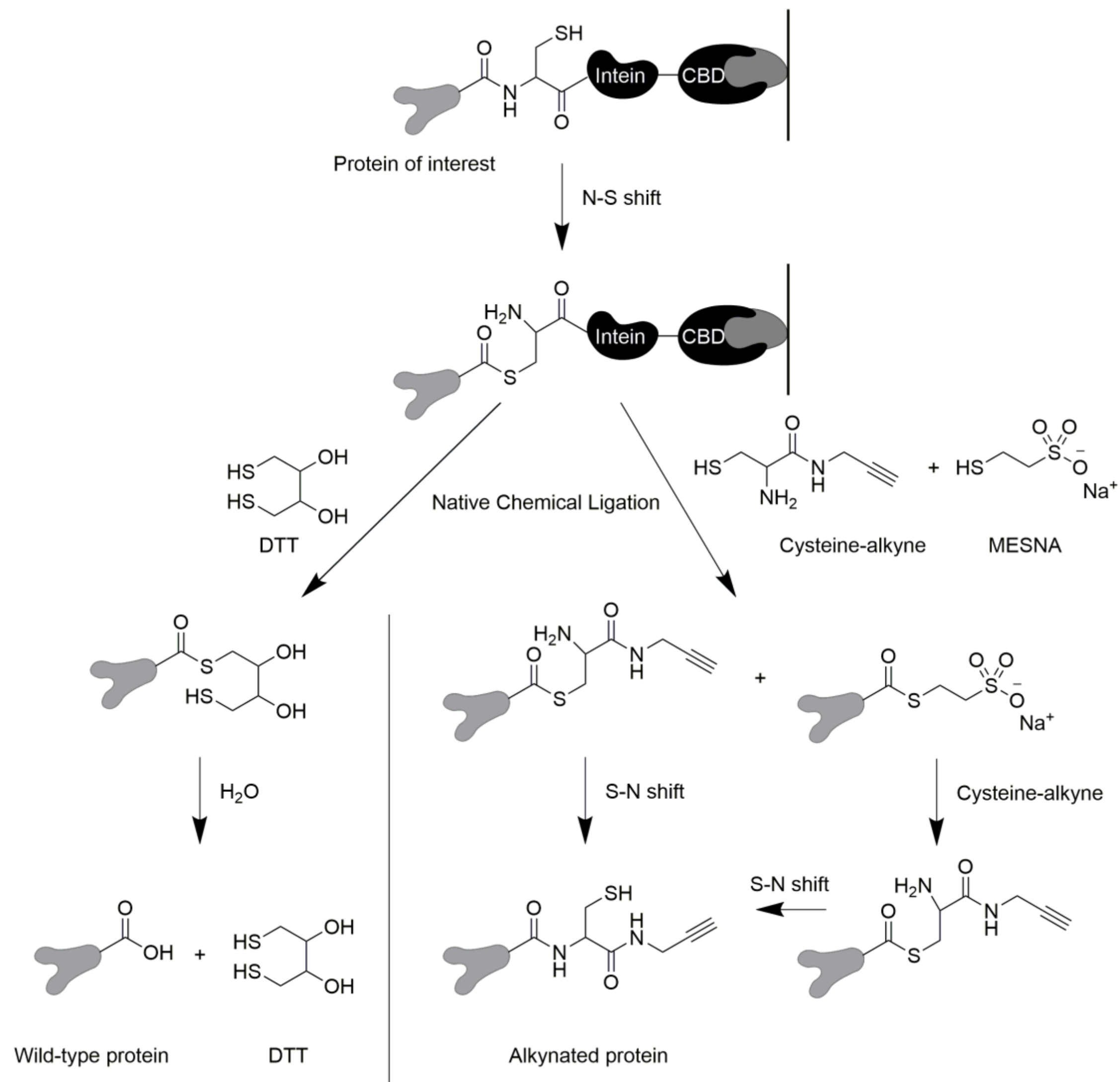
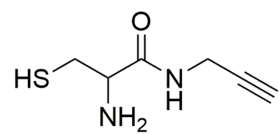


FIGURE 1



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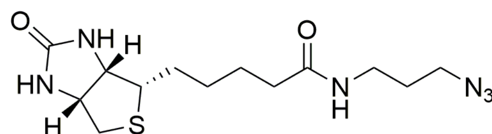

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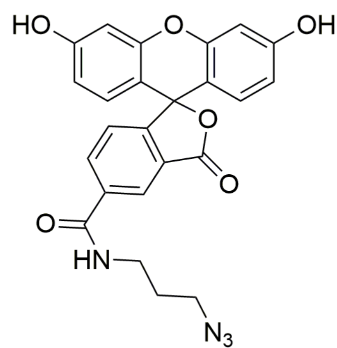

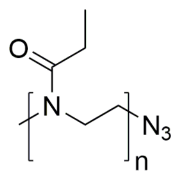

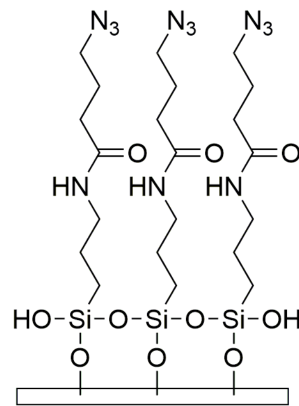

C

D

E

FIGURE 2

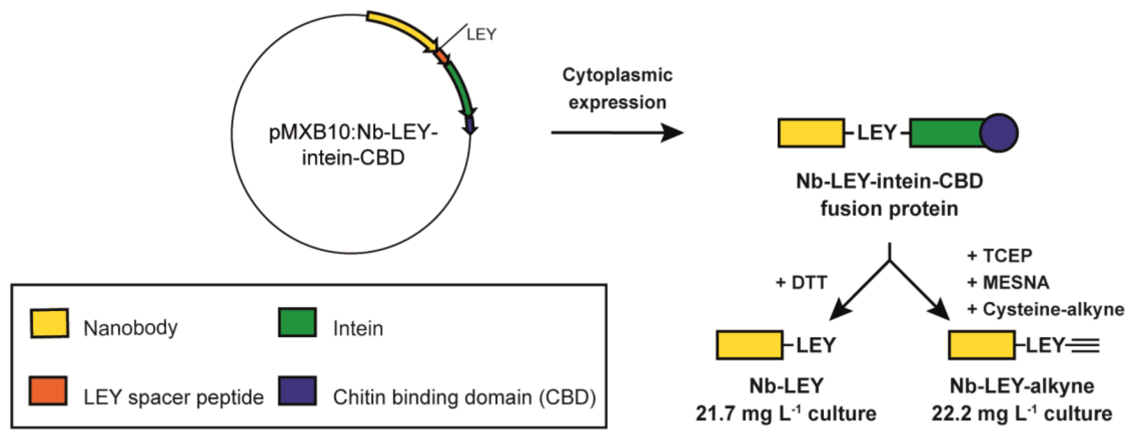

FIGURE 3

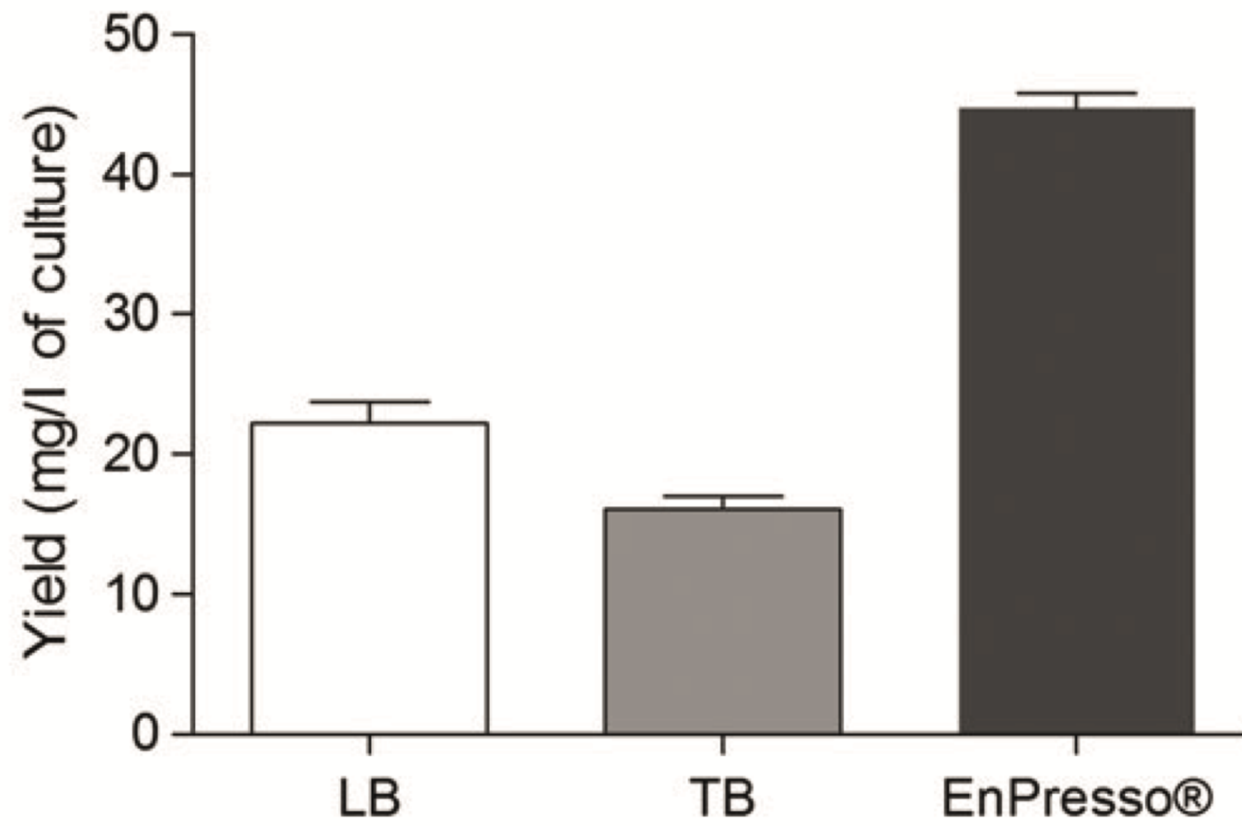

FIGURE 4

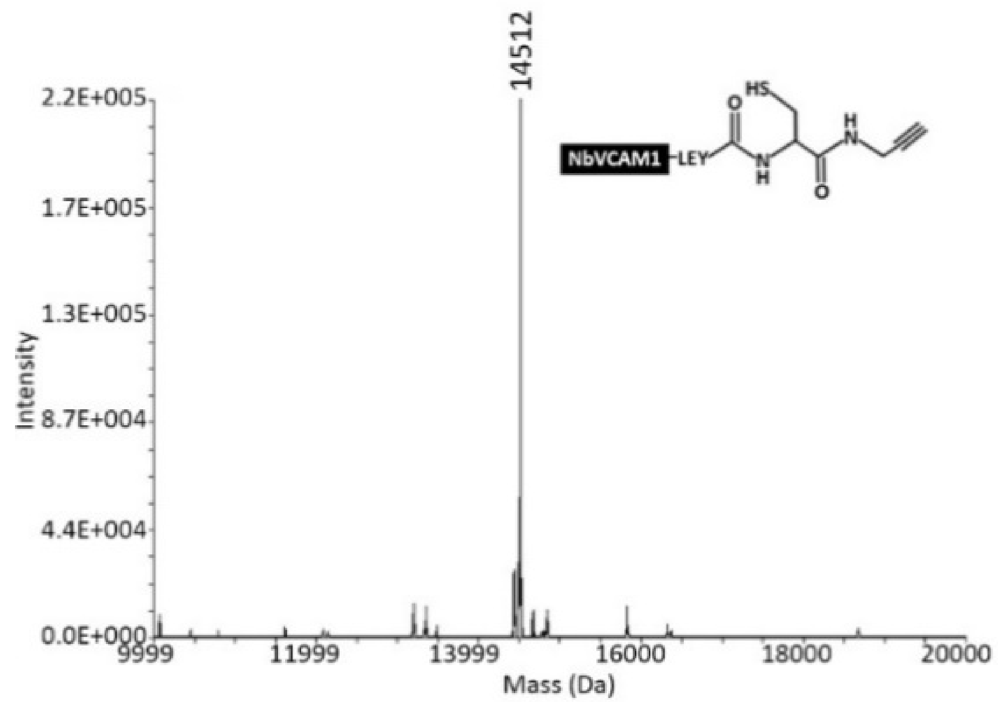

FIGURE 5