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# Cytoplasmic versus periplasmic expression of site-specifically and bioorthogonally functionalized nanobodies using expressed protein ligation

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## Abstract

Site-specific functionalization of nanobodies after introducing bioorthogonal groups offers the possibility to biofunctionalize surfaces with a uniformly oriented layer of nanobodies. In this paper, expressed protein ligation (EPL) was used for site-specific alkylation of the model nanobody NbBcII10. In contrast to EPL constructs, which are typically expressed in the cytoplasm, nanobodies are expressed in the periplasm where its oxidizing environment ensures a correct folding and disulfide bond formation. Different pathways were explored to express the EPL constructs in the periplasm but simultaneously, the effect of cytoplasmic expression on the functionality of NbBcII10 was also evaluated. By using *Escherichia coli* SHuffle<sup>®</sup>T7 cells, it was demonstrated that expression of the EPL complex in the cytoplasm was readily established and that site-specifically mono-alkynated nanobodies can be produced with the same binding properties as the non-modified NbBcII10 expressed in the periplasm. In conclusion, this paper shows that periplasmic expression of the EPL complex is quite challenging, but cytoplasmic expression has proven to be a valuable alternative.

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**Keywords:** Nanobodies; Expressed protein ligation; Periplasmic expression and extraction; Click chemistry; CuAAC

**Abbreviations:** EPL, expressed protein ligation; CBD, chitin binding domain; Nb, nanobody; SPR, surface plasmon resonance; BcII, *Bacillus cereus*  $\beta$ -lactamase; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

# 1 Introduction

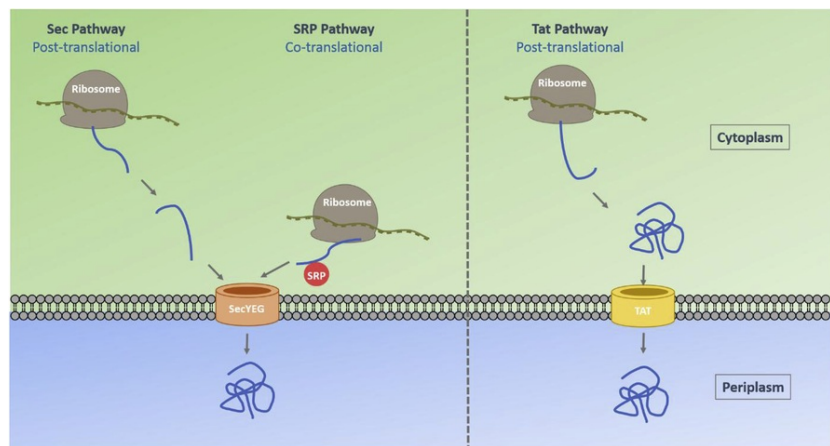
The oriented and covalent coupling of proteins to functionalized surfaces is a crucial step in the production of bioactive surfaces such as found in biosensors [1]. Nowadays, immobilization is mostly based on physical adsorption, resulting in a weak, non-covalent and non-oriented coupling of these proteins [2–4]. To strengthen the coupling, covalent attachments involving endogenous reactive groups (from lysines) are often used (e.g. EDC/NHS couplings) [5,6]. However, these couplings still result in a non-oriented immobilization because the endogenous groups occur at multiple locations spread over the protein's surface. Alternatively, a site-specifically introduced affinity tag (e.g. His<sub>6</sub>-tag) on the protein can be used to associate with a properly functionalized biosensor surface (e.g. Ni-NTA), but this interaction is weak and easily disrupted [7,8].

Therefore, the site-specific introduction in the protein of a single bioorthogonal group to react covalently with a complementary group on the biosensor surface, seems an attractive approach for a robust and directed protein immobilization. Such a site-specific functionalization and immobilization on a sensor surface will result in a higher specificity and selectivity of the envisaged antigen-protein interaction. In this paper, expressed protein ligation (EPL) was used for the introduction of an alkyne click functionality onto a single-domain antibody fragment, i.e. a nanobody (Nb), to develop site-specific functionalized probes for subsequent generation of oriented and covalently coupled protein layers onto a solid carrier.

EPL is a technique that involves the recombinant expression of a protein of interest fused with a mutated Mxe GyrA intein and a chitin binding domain (CBD). The mutant intein will facilitate an N,S-acyl shift in the peptide bond between the protein of interest and the intein, resulting in a thioester bond. Moreover, the CBD enables purification of the expressed protein on a chitin column. The protein of interest can subsequently be disconnected from the protein complex via a reaction between the newly formed thioester bond in the protein and a thiol group containing nucleophile [9,10]. Through careful design of so-called bifunctional linkers containing both a thiol group as nucleophile and a bioorthogonal group such as an alkyne, the protein can readily be site-specifically functionalized at its C-terminus [11,12].

Nbs, also known as single-domain antibody fragments (sdAbs) or VHHs, are the recombinant autonomous antigen-binding domains of heavy-chain-only antibodies that occur in species of the *Camelidae* family [13,14]. They are an interesting alternative for monoclonal antibodies in diagnostic applications due to their small size (~15 kDa) and their high specificity and affinity for their antigen [15]. Furthermore, they have a well-conserved structure, are relatively easy to express in *Escherichia coli* (*E. coli*), they are encoded by only one gene (which facilitates an easy genetic manipulation) and they remain soluble and stable at elevated temperatures [15–17].

In general, Nbs are expressed in the periplasm of *E. coli* [18,19]. The oxidizing environment of the periplasm facilitates protein folding and the periplasmic extracts enriched with the recombinant Nb facilitates subsequent purification. The periplasmic transport of the Nb is provoked by the N-terminal pelB leader sequence of the Sec pathway, which enables transport through the inner membrane of bacteria (Fig. 1). EPL-based constructs, however, are preferentially expressed in the cytoplasm [20–22] and previous reports already indicated that the pelB leader sequence is not practical for the Nb-intein-CBD fusion protein [23]. We therefore explored alternative strategies to produce Nb-intein-CBD fusion proteins.



**Fig. 1 Three periplasmic secretion pathways for proteins in *E. coli*.** The Sec pathway is a post-translational pathway in which the protein is fully secreted by ribosomes. Afterwards, the leader sequence enables correct folding of the protein by transporting it to the periplasm via the SecYEG translocon. The SRP pathway uses the same translocon as the Sec pathway but here SRP is binding to the leader sequence, and translocates the protein-ribosome complex to the SecYEG translocon during the

protein synthesis. The Tat pathway uses a different approach since it is transporting folded proteins from the cytoplasm to the periplasm using the Tat translocon.

alt-text: Fig. 1

Three pathways allowing periplasmic transport of recombinant proteins in *E. coli* are known: the Sec pathway, the signal recognition particle (SRP) pathway, and the twin-arginine translocation (Tat) pathway (Fig. 1). The Sec pathway is a post-translational pathway in which the protein is synthesized completely, i.e. with a leader sequence, and released from the ribosome after which it is directed to the Sec-translocase [24]. Common leader sequences used for this pathway are pelB, ompA, ompF and malE [25]. The efficacy of these four Sec leader signals was evaluated to produce the Nb-intein-CBD fusion in the periplasm. The second pathway for periplasmic transport is the SRP pathway. This pathway also targets the Sec-translocase but it is a co-translational pathway. SRP binds to the leader sequence after its translation from the ribosomes and the entire complex of SRP-ribosome-nascent protein is targeted to the Sec-translocase [24]. Commonly used leader sequences dedicated to the SRP pathway are DsbA and TolB [26]. These two leader sequences were also tested in this study on their ability to produce Nb-intein-CBD fusions in the bacterial periplasm. The third pathway, the Tat pathway is not interesting for Nb transport because this pathway transports folded proteins into the periplasm using Tat translocons [27].

In the present study, a nanobody against *Bacillus cereus*  $\beta$ -lactamase (BcII) [19,28,29], NbBcII10, is used as a model to evaluate various approaches for the periplasmic transport of the Nb-intein-CBD fusion protein. Furthermore, the requirement for periplasmic expression of Nbs alkynated by EPL was investigated by comparing the functionality of Nb-intein-CBD fusion protein expressed in the cytoplasm and native non-functionalized NbBcII10 expressed in the periplasm. To this end, the binding capacities of both formats were analyzed using ELISA and surface plasmon resonance (SPR).

## 2 Materials and methods

### 2.1 Materials

The primers were synthesized by Integrated DNA technologies (IDT). Materials for the PCR and molecular cloning were purchased from Thermo Scientific. Sanger sequencing was performed by LGC Genomics Germany. Growth media components were purchased from Becton Dickinson (BD) Biosciences. The BcII antigen was kindly provided by Prof. André Matagne (Université de Liège, Belgium). The pTXB1 vector, chitin resin, *E. coli* BL21(DE3) and SHuffle®T7 competent cells were purchased from New England Biolabs. Materials and chemicals for the SPR experiments were purchased from GE Healthcare. All other chemicals were purchased from Sigma unless stated otherwise.

### 2.2 Molecular cloning

The pTXB1 vector was used to express the Nb-intein-CBD fusion protein. The different leader sequences used for the different constructs are summarized in Table 1.

**Table 1** Overview of the DNA sequences of the different leader sequences used for the periplasmic expression of NbBcII10 [25,26].

alt-text: Table 1				
Construct	Leader	DNA sequence leader	Pathway	Accession no.
a	pelB	5´ ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC 3´	Sec	P11431 <sup>b</sup>
b	ompA	5´ ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC 3´	Sec	V00307 <sup>a</sup>
c	ompF	5´ ATG ATG AAG CGC AAT ATT CGT GCA GTG ATC GTC CCT GCT CTG TTA GTA GCA GGT ACT GCA AAC GCT 3´	Sec	J01655 <sup>a</sup>
d	malE	5´ ATG AAA ATA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC 3´	Sec	V00303 <sup>a</sup>
e	DsbA	5´ ATG AAA AAA ATT TGG CTG GCG CTG GCG GGC CTG GTG CTG GCG TTT AGC GCG AGC GCG 3´	SRP	P0AEG4 <sup>b</sup>
f	TolB	5´ ATG AAA CAG GCG CTG CGT GTG GCG TTT GGC TTT CTG ATT CTG TGG GCG AGC GTG CTG CAT GCG 3´	SRP	P0A855 <sup>b</sup>
g	None	None	N.A.	N.A.

<sup>a</sup> GenBank.  
<sup>b</sup> Uniprot.

The *NbBcII10* gene was amplified from the pHEN6:*pelB*-NbBcII10-His<sub>6</sub> vector [19] using different primers to generate different amplicons to clone in the pTXB1 vector. The *pelB*-NbBcII10 insert was made using the *pelB\_fw* forward primer (5'-GGTGGTCATATGATGAAATACCTATTGCCTACGGCAGCCG-3') and *pelB\_rv* reverse primer (5'-GGTGGTTGCTCTTCGCATGAGGAGACGGTGA-3'). The gene of the other leader sequences was not available, so these leader sequences were added to the Nb by two subsequent PCR reactions. The forward primers are summarized in Table 2, the reverse primer, *leader\_rv* (5'-GGTGGTTGCTCTTCCGCATGAGGAGACGGTGA-3') was the same for all the reactions.

**Table 2** Overview of the different forward primers used for the attachment of different leader sequences to NbBcII10 in order to direct the periplasmic expression of the Nb-intein-CBD fusion protein.

alt-text: Table 2	
Leader	Primer sequence
OmpA_1	5'-TGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCAGGTGCAGCTGGTGGAGTC-3'
OmpA_2	5'-GGTGGTCATATGATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTT-3'
OmpF_1	5'-GATCGTCCCTGCTCTGTTAGTAGCAGGTACTGCAAACGCTCAGGTGCAGCTGGTGGAGTC-3'
OmpF_2	5'-GGTGGTCATATGATGATGAAGCGCAATATTCGTGCAGTGATCGTCCCTGCTCTGTTAG-3'
malE_1	5'-CGCATTAACGACGATGATGTTTTCCGCCTCGGCTCTCGCCCAGGTGCAGCTGGTGGAGTC-3'
malE_2	5'-GGTGGTCATATGATGAAAAATAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGACGATGATGT-3'
DsbA_1	5'-CCTGGTGCTGGCGTTTAGCGCGAGCGCGCAGGTGCAGCTGGTGGAGTC-3'
DsbA_2	5'-GGTGGTCATATGATGAAAAAATTTGGCTGGCGCTGGCGGGCCTGGTGCTGGCGTTTAG-3'
TolB_1	5'-GGCTTTCTGATTCTGTGGGCGAGCGTGCTGCATGCGCAGGTGCAGCTGGTGGAGTC-3'
TolB_2	5'-GGTGGTCATATGATGAAACAGGCGCTGCGTGTGGCGTTTGGCTTTCTGATTCTGTGGGC-3'
None	5'-GGTGGTCATATGCAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTCGGTG-3'

As a positive control, a variant of construct (a) was made containing two stop codons (TAA-TGA) downstream of the *pelB*-NbBcII10 gene (construct (a')). This *pelB*-NbBcII10-TAA-TGA insert was made using the *pelB\_stop\_fw* primer (5'-GGTCACGGTCACGGTGGTCATATGATGAAATACCTATTGCCTACGGCAGCCGCTG-3') and the *pelB\_stop\_rv* primer (5'-GGTGGTTGCTCTTCCGCATCATTATG AGGAGACGGTGACCTGGG-3').

For cytoplasmic expression, no leader sequence is needed and the insert only consists of NbBcII10 (last primer of Table 2). In contrast to the other constructs, a different reverse primer (5'-GGTGGTTGCTCTTCCGCAGTACTCGAGTGAGGAGACGGTGACCTGGGT-3') was used to add three amino acids (LEY) at the C-terminus of the Nb.

All PCRs were performed using Phusion Hot Start II high-fidelity DNA polymerase for 30 s at 98 °C, followed by 30 cycles of 8 s at 98 °C and 15 s at 72 °C. The final elongation step was performed for 8 min at 72 °C resulting in the final inserts. Next, the Nb constructs and pTXB1 vector were digested using Fastdigest *Nde*I and *Sap*I restriction enzymes using a standard protocol as provided by the supplier, and purified using PCR cleanup or gel extraction kits. After purification, the vector and insert were ligated using T4 ligase according to manufacturer's protocol. The ligated constructs were subsequently transformed into chemically competent TOP10F' cells using the heat shock method, plated on Luria-Bertani (LB) agar, containing 100 µg/ml Ampicillin (LB<sup>amp</sup>) and incubated for 16 h at 37 °C. Afterwards, individual colonies were randomly selected, cultured and DNA was extracted to check the constructs by means of Sanger sequencing.

## 2.3 Expression and extraction of the Nb-intein-CBD fusion protein

### 2.3.1 Periplasmic expression in E. coli BL21(DE3)

Constructs (a)-(d) and (a') were transformed into chemically competent *E. coli* BL21(DE3) cells and cultured in 50 ml LB<sup>amp</sup> medium until an optical density OD<sub>600</sub> between 0.6 and 0.9 was reached. Next, expression for 16 h at 28 °C was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested by centrifugation and a periplasmic extraction was performed using an osmotic shock. To this end, the cell pellet was resuspended in Tris-EDTA-Sucrose (TES) solution and incubated on ice for 1 h while shaking. Next, an osmotic shock was achieved by adding a double amount of TES diluted 1:4 with Milli-Q water and the cell

suspension was shaken on ice during 2 h. Afterwards,  $\text{MgCl}_2$  was added to a final concentration of 10–15 mM. After centrifugation, the supernatant was collected for further use.

### 2.3.2 Periplasmic expression in *E. coli* Lemo21(DE3)

To increase the periplasmic extraction yield, a BL21(DE3) variant, *E. coli* Lemo21(DE3) was used. This strain is especially designed for the expression of poorly soluble proteins. The strain contains an additional plasmid, i.e. pLemo, that can produce T7 lysozyme, a natural inhibitor of T7 RNA polymerase. The expression of T7 lysozyme is under control of a rhamnose inducible promotor. By varying the concentration of rhamnose, and consequently the concentration of T7 lysozyme, the activity of T7 RNA polymerase can be modulated resulting in a controlled regulation of the target protein expression [30,31]. Constructs (a)–(f), the negative control (g) and positive control (a') were transformed into chemically competent Lemo21(DE3) cells and expression was performed in the same way as for normal BL21(DE3) except that only 400  $\mu\text{M}$  IPTG was used to induce the expression and 100  $\mu\text{g/ml}$  chloramphenicol was added to the medium to preserve the pLemo vector. In addition, to regulate the expression level of T7 lysozyme, varying concentrations of rhamnose (final concentration between 0 and 2000  $\mu\text{M}$ ) were tested. Periplasmic extraction was performed as described for BL21(DE3) or as described by Schlegel et al. [31].

### 2.3.3 Cytoplasmic expression in *E. coli* SHuffle®T7

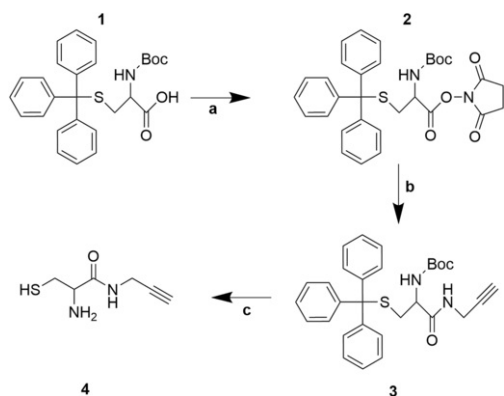
Construct (g) was transformed into chemically competent *E. Coli* SHuffle®T7 cells. These cells are derived from the *E. coli* K12 strain and are especially developed to facilitate disulfide formation in the cytoplasm. The *NbBcII10*-gene containing SHuffle®T7 cells were cultured in 300 ml  $\text{LB}^{\text{amp}}$  medium until an  $\text{OD}_{600}$  between 0.6 and 0.9 was reached. Expression was induced by adding IPTG to a final concentration of 1 mM during 3 h at 37 °C. Afterwards, the cells of a 300 ml culture were harvested using centrifugation and extracted by resuspending the cell pellet in 6 ml B-PER solution (Thermo Scientific), supplemented with 6 U DNaseI (Thermo Scientific), and incubated for 15 min at room temperature. After centrifugation for 30 min at 20 000*g* and 4 °C, the supernatant was collected for further use.

### 2.3.4 Periplasmic expression in *E. coli* WK6

Non-modified NbBcII10-His<sub>6</sub> (pHEN6 vector) was expressed in *E. coli* WK6 cells (5 mg/l culture) as described by Conrath et al. [19]. NbBcII10-HLC (pHEN25 vector) is expressed following the same protocol. This expressed protein contains at its C terminal end a spacer of 14 amino acids, a hexahistidine tag and a cysteine, which will cause a dimerization of the Nb [32].

## 2.4 Synthesis of the 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide bifunctional linker

2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide was synthesized (Fig. 2) by dissolving 2 mmol (0.9272 g) N-(tert-Butoxycarbonyl)-S-trityl-L-cysteine (**1**), 2.2 mmol (0.4217 g) of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 2.2 mmol (0.2532 g) N-hydroxysuccinimide (NHS) in 30 ml dichloromethane (DCM) and stirring for 16 h at room temperature. After extraction with water, the DCM was dried over  $\text{MgSO}_4$  and evaporated under reduced pressure. 1 mmol (0.5607 g) of the resulting white powder (**2**) was dissolved in 20 ml dry DCM together with 2 mmol (0.1372 ml) propargylamine and 2 mmol triethylamine (0.279 ml) and stirred continuously for 16 h under  $\text{N}_2$  atmosphere at room temperature. The resulting product (**3**) was purified using column chromatography over silica with ethylacetate/DCM (1/24) as eluent. The solvent was removed under reduced pressure. To remove the protective Boc and trityl groups, 100 mg of the dry purified product (**3**) was dissolved in 3 ml trifluoroacetic acid (TFA), 100  $\mu\text{l}$  water and 100  $\mu\text{l}$  triisopropylsilane. After 30 min of stirring at room temperature, the TFA was removed under reduced pressure and the product (**4**) was dissolved in 20 ml DCM. The mixture was extracted 5 times with water and the water phase was lyophilized.



**Fig. 2** Synthesis pathway of 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide (**4**). (a) EDC, NHS, DCM (b) propargylamine, triethylamine, DCM (c) TFA, water, triisopropylsilane.

## 2.5 Site-specific alkynylation of NbBcII10 by expressed protein ligation

After a cytoplasmic extraction, expressed in *E. coli* SHuffle®T7, the supernatant was slowly added to columns containing chitin beads that were equilibrated with running buffer (20 mM HEPES, 500 mM NaCl and 1 mM EDTA at pH 8.5). After extensive washing with running buffer, the columns were incubated overnight at 4 °C with cleavage buffer (CB; 20 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1 mM tris(2-carboxyethyl)phosphine (TCEP) at pH 8.5). While one column was incubated with CB supplemented with 30 mM 2-mercaptoethane sulfonate Na (MESNA), another column was incubated with CB supplemented with 30 mM MESNA and 1 mM of the bifunctional linker 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide. After overnight incubation, the columns were eluted using 1.5 column volumes of running buffer and immediately dialyzed against PBS using an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane having a molecular weight cut-off of 3 kDa (Merck Millipore).

## 2.6 SDS-PAGE and electrospray ionization mass spectrometry

All protein extracts were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were boiled in a 2~~×~~ or 5~~×~~ SDS sample buffer containing β-mercaptoethanol for 5 min and analyzed by 12% SDS-PAGE.

The cytoplasmic extracts were, after alkynylation using EPL and dialysis, analyzed by electrospray ionization mass spectrometry (ESI-MS) as described by Ta et al. [23].

## 2.7 Clickability of NbBcII10-LEY-alkyne using western blot

To test the clickability of the EPL-functionalized NbBcII10, a copper-catalyzed azide-alkyne cycloaddition (CuAAC) was performed in solution. In 200 µl phosphate buffered saline (pH 7.4), containing purified NbBcII10-LEY-alkyne, an excess of azide functionalized biotin derivative N-(3-azidopropyl)-5-(2-oxohexahydro-1H-thieno [3,4-d]imidazol-4-yl)pentanamide [11] (180 µM, dissolved in DMSO) was added together with 900 µM TCEP, 90 µM tris(benzyltriazolylmethyl)amine (TBTA, dissolved in DMSO) and 900 µM CuSO<sub>4</sub>. As a negative control non-alkynated NbBcII10 was used. After 2 h of shaking at 21 °C, the reaction products were, without any further purification, boiled with 2~~×~~ SDS sample buffer. Afterwards, gel electrophoreses was performed using a 12% SDS-PAGE gel with a molecular weight marker. Subsequently, proteins were transferred to a polyvinylidene fluoride (pvdf) membrane by diffusion overnight. The next day, the membrane was blocked in 5% Bovine Serum Albumin (BSA) solution in Tris Buffered Saline Tween (TBST: 25 mM Tris, 150 mM NaCl, 0,05% Tween-20, pH 8.0) for 2 h. To detect the presence of biotin, the membrane was incubated with streptavidin alkaline phosphatase (1/1000) in TBST for 1 h. Finally, visualization of the biotinylated sample was performed by soaking the membrane for a few seconds in a bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NTB) solution that will react with the alkaline phosphate on the protein resulting in a purple precipitate visible on the membrane.

## 2.8 Enzyme-linked immunosorbent assay

To test the capacity of alkynated NbBcII10 to bind to its cognate BcII antigen, a competitive enzyme-linked immunosorbent assay (ELISA) was performed. Non-modified NbBcII10-His<sub>6</sub> was used as a control. A 96-well microplate (Thermo scientific Nunc Maxisorp™) was coated with 200 µl of a 1 µg/ml BcII antigen solution in ELISA coating buffer (0.1 M NaHCO<sub>3</sub>, pH 8.2) and incubated overnight at 4 °C while shaking. Next, the microplate was rinsed 5 times using TBST buffer, incubated for 7 h with a 2% (w/v) skimmed milk powder in TBST solution at 4 °C while shaking and again washed 5 times using TBST buffer. The NbBcII10-His<sub>6</sub> was then added in a concentration range between 5 and 250 ng/ml to one part of the plate, a combination of NbBcII10-LEY-alkyne (concentration range 250–49 750 ng/ml) and NbBcII10-His<sub>6</sub> (fixed concentration of 250 ng/ml) was added to the other part. After overnight incubation at 4 °C while shaking, the plate was washed 5 times with TBST buffer. Next, the plate was incubated with a monoclonal mouse anti-histidine antibody (1 µg/ml) for 3 h at 21 °C, rinsed again 3 times with TBST and incubated with anti-mouse IgG-alkaline phosphate antibody (0.1 µg/ml) for 2 h at 21 °C. Finally, the plate was rinsed 3 times with TBST and 200 µl of a ready-to-use 2~~p~~-nitrophenylphosphate (pNPP) solution was added. After 6.5 min, the reaction was blocked with 50 µl NaOH solution (3 M) and absorbance was measured at 405 nM (OD<sub>405</sub>) using a FLUOStarOmega Reader (BMG Labtech).

## 2.9 Surface plasmon resonance analysis

Surface plasmon resonance (SPR) affinity measurements were performed using a Biacore T200 (GE Healthcare). A CM5 SPR chip was coated with 348 RU of BcII antigen (MW 24 962 Da) by means of an EDC/NHS coupling. A kinetic study was performed as described in the manufacturer's protocol using a 2-fold serial dilution (500–1.95 nM) of NbBcII10-His<sub>6</sub>, NbBcII10-HLC and four different batches of NbBcII10-LEY-alkyne. A flow rate of 30 µl/min in HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA; 0.005% Tween20; pH 7.4) was used, combined with an association phase of 120 s and a dissociation phase of 600 s. For regeneration after binding, 100 mM glycine-HCl (pH 2.5) was used. The data were fitted using a 1:1 binding stoichiometry with drift and RI2 kinetic model (Biacore T200 evaluation software).

## 3 Results and discussion

## 3.1 Periplasmic expression

Nbs, including NbBcII10, are generally expressed with a pelB leader signal sequence for secretion in the bacterial periplasm [19]. In 2009, Reulen et al. [33] reported the possibility to express a Nb-intein-CBD fusion protein in the periplasm using this N-terminal pelB leader sequence. However, this approach failed for a broad panel of antibody fusion partners and the periplasmic expression of antibody-intein fusions in prokaryotes seems to have its limitations [20]. Recent experiments further supported the difficulties to reproduce this strategy for other Nb-intein-CBD fusion proteins [23].

In our study the contribution of the leader signal and the corresponding targeted translocation pathway in the expression level of this fusion was primarily assessed. To this end, NbBcII10 preceded by the pelB (a and a'), ompA (b), ompF (c) or malE (d) signal sequences was cloned between the *Nde*I and *Sap*I restriction sites in the pTXB1 vector and transformed in the *E. coli* strain BL21(DE3), expressing T7 RNA polymerase [6,34,35]. The ability of these four constructs (a)-(d) to export the NbBcII10-intein-CBD fusion protein to the periplasm using the Sec pathway was assessed by monitoring the SDS-PAGE band pattern for the presence of a 43 kDa protein band in the different periplasmic fractions. The absence of any significant protein band corresponding to the NbBcII10-intein-CBD fusion protein, even after enrichment on chitin beads confirmed the previously reported practical problems to transport the Nb-intein-CBD fusion protein through the inner membrane of BL21(DE3) cells via the Sec pathway [23]. As a positive control, construct (a') was cloned in the pTXB1 vector in the same way as construct (a-d) but due to the two stop codons between the pelB-NbBcII10 gene and the intein gene, only pelB-NbBcII10 was expressed and transported to the periplasm. As shown in the [Supplementary Information Fig. S1](#) (right Lane 1), NbBcII10 was clearly present in the periplasmic extract indicating that the intein-CBD part prevents the transport of the NbBcII10-intein-CBD fusion protein to the periplasm.

Wagner et al. [30] noted that BL21(DE3) cells are sensitive to the toxic effect of overexpressing membrane proteins by the formation of inclusion bodies in the cytoplasm. Similarly to our Nb-intein-CBD fusion proteins, the same translocation pathways, Sec and SRP, are used by membrane proteins [36]. However, expression of NbBcII10-intein-CBD fusion proteins is difficult to control in BL21(DE3) cells as it is driven by the T7 RNA polymerase, both under control of IPTG, and poorly adjustable [37]. To solve these issues, Wagner et al. developed the Lemo21(DE3) cell line, derived from BL21(DE3), where the activity of the T7 RNA polymerase is precisely controlled using its natural inhibitor, T7 lysozyme. Following this approach, overexpression of the recombinant target protein can be avoided. The pLemo vector expresses T7 lysozyme, which is under control of a rhamnose inducible promotor, allowing full control of its expression by varying the rhamnose concentration.

For this experiment, seven different constructs were transformed into competent Lemo21(DE3) cells: the previously tested constructs (a)-(d) preceded by leader sequences targeting the Sec pathway, two additional constructs incorporating the SRP-pathway targeting signal sequences DsbA (e) and TolB (f) and finally one construct devoid of any signal sequence as negative control (g). Different rhamnose concentrations were tested during expression ranging from 0 to 2000  $\mu$ M and two extraction methods were evaluated (osmotic shock and snap-freezing/vortexing). Of note, in absence of rhamnose there is no functional difference between BL21(DE3) and Lemo21(DE3) cells. For all tested rhamnose concentrations and with both extraction methods no protein band corresponding to the NbBcII10-intein-CBD fusion protein (43 kDa) could be detected in the periplasmic fraction by SDS-PAGE ([Fig. S1](#)). Here again, we observe that the positive control (a') was expressed and successfully transported to the periplasm. Consequently in absence (corresponding to the BL21(DE3) system) or presence of rhamnose in Lemo21(DE3) cells, the periplasmic transport of the nanobody complex is blocked by the intein-CBD part.

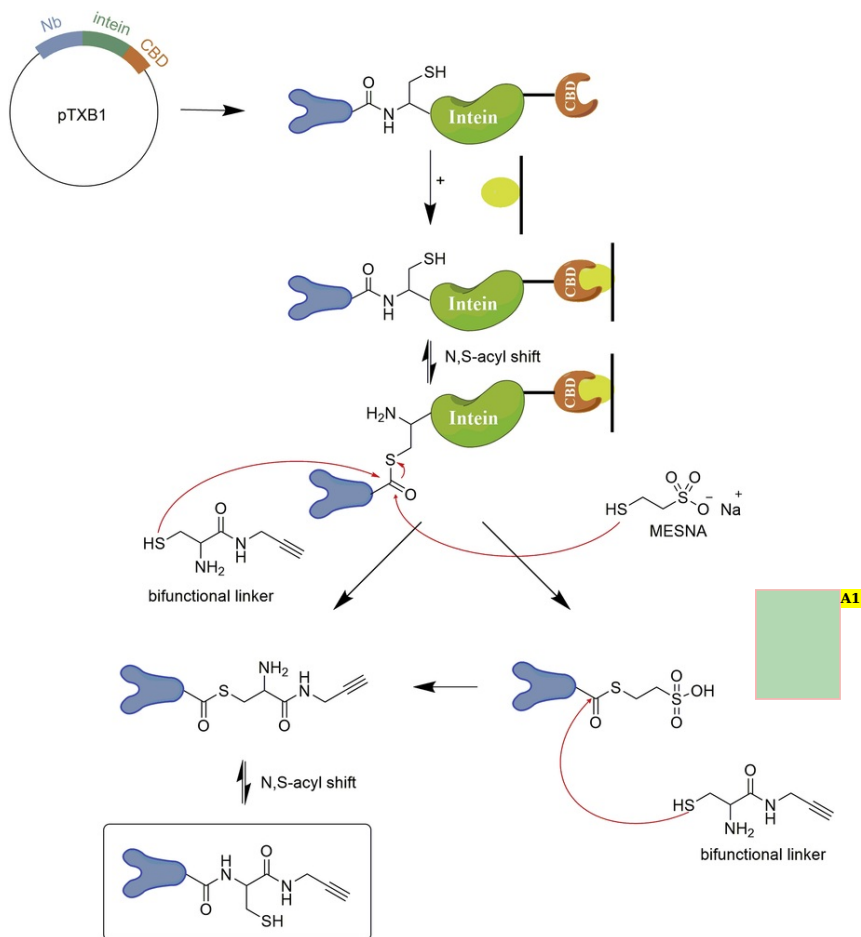
These results confirm the difficulties reported by Ta et al. [23] and Marshall et al. [20] regarding the periplasmic expression of antibody fusion proteins, and therefore raise the question about the need for periplasmic expression of Nbs.

## 3.2 Cytoplasmic expression

Periplasmic extraction might have advantages in terms of protein folding, but there are also a number of disadvantages such as a decreased yield due to saturation of the secretion systems, protein aggregation in the cytoplasm (inclusion bodies) as well as a more time consuming expression process [38].

To circumvent these drawbacks, an increasing amount of research has been performed on the cytoplasmic expression of Nbs [23,39–41]. Zarschler et al. [41], for example, reported a strategy for the cytoplasmic expression of Nbs using SHuffle®T7 cells, with proper intradomain disulfide bridge formation. Conversely, Ta et al. [23] reported no affinity loss after cytoplasmic extraction of NbVCAM1 using (Stop here left column on page 6 and start right column on page 6.) SHuffle®T7 cells. Consequently, we also transformed our pTXB1 construct (g) in SHuffle®T7 cells to evaluate cytoplasmic expression of our NbBcII10-intein-CBD fusion protein. To guarantee an optimal EPL efficiency, three amino acids, leucine (L), glutamine (E) and tyrosine (Y), were also added downstream of the NbBcII10 protein. Firstly, tyrosine is one of the preferred amino acids at the cleavage site for EPL [35] and secondly, the addition of a peptide spacer between the NbBcII10 and the intein enhances the independent folding of both proteins [23]. After expression in SHuffle®T7 cells, the Nb-fusion was extracted from the cytoplasm with B-PER solution containing DNaseI, followed by purification on a chitin column. EPL was then subsequently performed on this column ([Fig. 3](#)).





**Fig. 3 Scheme of the expressed protein ligation strategy.** For the site-specific alkylation of NbBcII10, the NbBcII10-intein-CBD fusion protein was expressed in SHuffle®T7 cells using the pTXB1 vector. After expression, the fusion protein was purified from the protein extract using a chitin column that binds the CBD present in the fusion protein. Next, the intein will facilitate an N,S-acyl shift of the amide bond between the NbBcII10 (C-terminally) and the intein. The bifunctional linker 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide reacts with the thioester bond resulting in the splicing of NbBcII10 from the column (*left*), and the alkylation of the NbBcII10. In order to increase the yield of the reaction, 2-mercaptoethane sulfonate Na (MESNA) was added at high concentration to facilitate splicing from the column by a nucleophilic attack followed by an exchange of MESNA for the bifunctional linker (*right*).

alt-text: Fig. 3

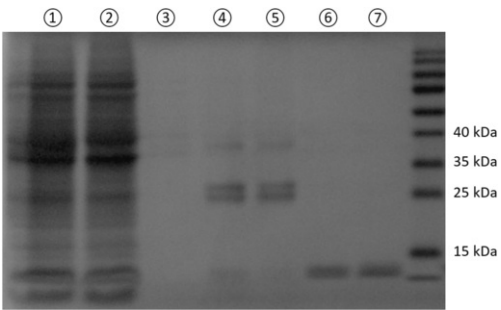
#### Annotations:

A1. This figure (Fig.3) needs to be placed underneath left and right column at the bottom of page 6.

### 3.3 Expressed protein ligation

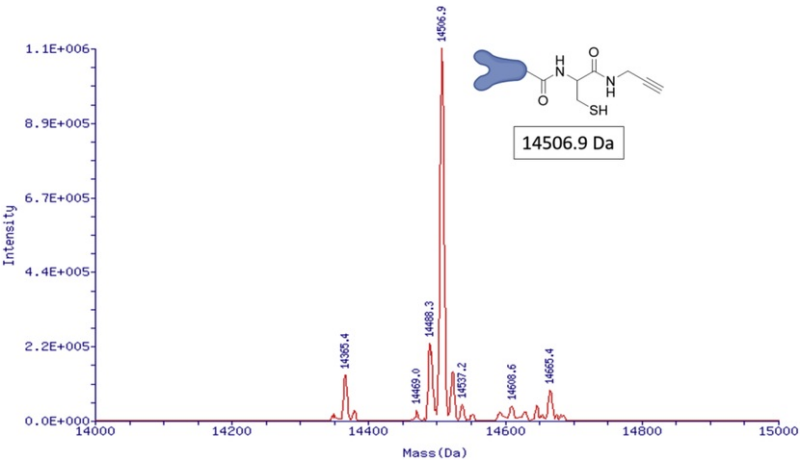
As illustrated in Fig. 3, the NbBcII10-intein-CBD fusion protein first binds to an affinity matrix composed of chitin resin. [This is the end of the right column on page 6 and start of the left column on page 7.] Subsequently, an N,S-acyl shift, facilitated by the intein, will alter the amide bond between the NbBcII10 and the intein into a less stable thioester bond. The thiol functionality of the bifunctional linker 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide will act as a nucleophile and will break the thioester bond, resulting in a 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide functionalized NbBcII10. A second N,S-acyl shift then turns the formed thioester bond back into a stable amide bond, leaving a C-terminal alkynated NbBcII10. To increase the yield of this process, 2-mercaptoethane sulfonate Na (MESNA) was added to the reaction with the bifunctional linker. MESNA will also react via the thioester bond between the

NbBcII10 and the intein, resulting in a thioester intermediate that is spliced from the column [35]. This MESNA intermediate can (This is the end of the left column on page 7 and import figure (Fig. 4) here. After Fig.4 the right column on page 7 starts. Underneath the left and right columns on page 7 figure (Fig. 5) is inserted.) afterwards undergo a nucleophilic attack by the 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide, thereby exchanging MESNA for the bifunctional linker. The characteristics of the final alkynated protein, NbBcII10-LEY-alkyne (yield of 3.5 mg/l culture), was evaluated on SDS-PAGE (Fig. 4) and by electrospray ionization mass spectrometry (ESI-MS) (Fig. 5). Both assays confirmed the theoretically calculated mass of 14 507 Da of the mono-alkynated NbBcII10-LEY.



**Fig. 4 SDS-page profile of the EPL process.** Lane ① represents the cytoplasmic extract, lane ② and ③ are respectively the first and the last washing step after binding of the Nb-intein-CBD fusion protein on the chitin column. Lane ④ and ⑤ are extraction samples of the chitin beads after the reaction with respectively MESNA and MESNA + bifunctional linker showing a mass of 28 kDa which corresponds to the molecular mass of the intein-CDB complex and a faint band of 43 kDa which corresponds to the mass of remaining total NbBcII10-intein-CBD fusion. Finally, lane ⑥ is the eluate of the column after incubation in cleavage buffer containing only MESNA resulting in non-alkynated, MESNA functionalized NbBcII10 and lane ⑦ is the eluate after incubation with cleavage buffer containing MESNA and bifunctional linker resulting in alkynated NbBcII10.

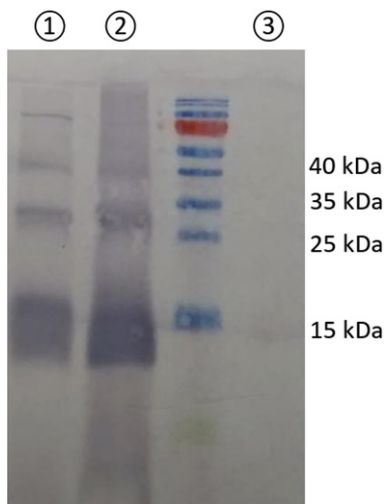
alt-text: Fig. 4



**Fig. 5 Electrospray ionization mass spectrometry (ESI-MS) spectrum of C-terminally, mono-alkynated NbBcII10-LEY.** The theoretical mass is 14 507 Da.

alt-text: Fig. 5

In order to test the ability of the alkynated NbBcII10 to click on an azide functional group, a click reaction was performed in solution with a biotin-azide derivative. The biotinylated Nb was subsequently visualized in Western Blot with a streptavidin alkaline phosphatase conjugate. As shown in Fig. 6, a color (Here ends the text in the right column on page 7 and insert figure (Fig. 6) here in the right column next to Fig 4. in the left column. After fig.6 the text in the left column on page 8 starts.) reaction appeared at 14.5 kDa in lane 1 and 2, confirming the presence of biotin on the Nb as a result of a successful click reaction. The negative control, lane 3, that contains non-alkynated NbBcII10 showed no reaction. Of note, due to the absence of reducing agent in the sample, dimers are also visible around 29 kDa.

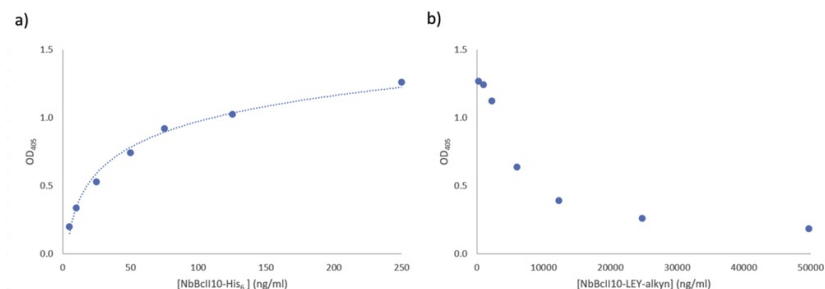


**Fig. 6 Western blot of the click-reaction product between NbBcII10-LEY-alkyne and a biotin-azide derivate.** Lanes ① and ② contain the reaction product (amount applied in lane ② is 10 times higher than lane ①), lane ③, the negative control, contains non-alkynated NbBcII10.

alt-text: Fig. 6

### 3.4 Functionality test of NbBcII10-LEY-alkyne

Finally the functionality of NbBcII10 after cytoplasmic extraction of the Nb-intein-CBD complex and after alkylation using EPL remains to be demonstrated. To this end, the affinity of NbBcII10-LEY-alkyne for its antigen BcII was evaluated in a competitive enzyme-linked immunosorbent assay (ELISA). First, the unmodified, native NbBcII10-His<sub>6</sub> at a concentration range between 0 and 250 ng/ml was used as a reference. The ELISA showed a clear logarithmic correlation between the OD<sub>405</sub> signal and the concentration of NbBcII10-His<sub>6</sub> (Fig. 7a). Next, in addition to 250 ng/ml NbBcII10-His<sub>6</sub>, a concentration range between 250 and 49 750 ng/ml NbBcII10-LEY-alkyne was added to BcII coated wells and the antigen captured NbBcII10-His<sub>6</sub> was detected. As shown in Fig. 7b, the OD<sub>405</sub> signal decreases with higher NbBcII10-LEY-alkyne concentrations indicating that NbBcII10-LEY-alkyne competes with NbBcII10-His<sub>6</sub> for binding the BcII antigen, thereby reducing the amount of detectable His<sub>6</sub>-tag.



**Fig. 7 Enzyme-linked immunosorbent assay (ELISA) results** (a) showing the binding of NbBcII10-His<sub>6</sub> to the BcII antigen and (b) competitive ELISA results showing the decrease in binding of NbBcII10-His<sub>6</sub> (250 µg/ml) after adding an increasing concentration of the NbBcII10-LEY-alkyne (250–49750 ng/ml).

alt-text: Fig. 7

In order to compare the affinity of NbBcII10-LEY-alkyne for BcII lactamase with that of NbBcII10-His<sub>6</sub>, an antigen binding study using SPR was performed. The association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rate constants between NbBcII10-LEY-alkyne and BcII were measured, from which the dissociation equilibrium constant ( $K_D$ ) can be calculated. Of note, due to the use of a cysteine-based bifunctional linker during the EPL process, i.e. 2-amino-3-mercapto-N-(prop-2-yn-1-yl)propanamide, a cysteine is incorporated at the C-terminus of the Nb as shown in Fig. 3. As a result, spontaneous dimerization of the Nbs is expected to occur, which could potentially influence the SPR experiment.

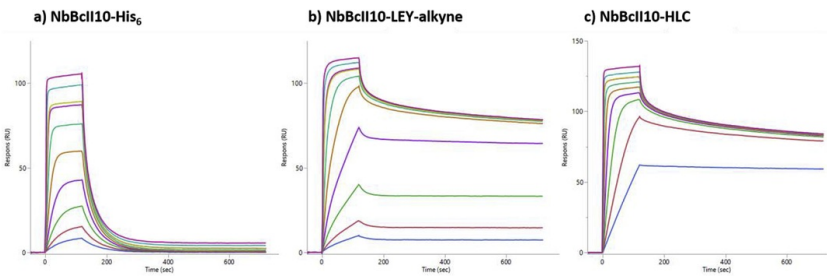
Indeed, under these circumstances a 1:1 binding model can no longer be assumed and therefore an additional control measurement was included. This control is the NbBcII10-HLC having an additional cysteine at its C-terminus and which is expressed in the periplasm and isolated in the same way as the unmodified NbBcII10-His<sub>6</sub> [32]. Table 3 reveals the improvement of the K<sub>D</sub> for the NbBcII10-LEY-alkyne recognition of BcII, compared with that of the non-modified NbBcII10-His<sub>6</sub>. This K<sub>D</sub> decrease (Here ends the text in the left column on page 8 and starts the right text in the right column on page 8. Underneath both columns on page 8 figure (Fig.7) is inserted.) is mainly caused by a 10-fold drop in the k<sub>off</sub>-value, as observed for the four independent batches of NbBcII10-LEY-alkyne. The variation in k<sub>on</sub>-values between NbBcII10-His<sub>6</sub> and the alkynated Nbs is marginal, whereas the k<sub>off</sub> values differ by nearly a factor 10. Note that a similar effect on the k<sub>off</sub> is observed for the dimerized NbBcII10-HLC.

**Table 3** Affinity constants (association rate constant k<sub>on</sub>, dissociation rate constant k<sub>off</sub>, and dissociation equilibrium constant K<sub>D</sub>) of the binding between NbBcII10 variants and BcII.

Nanobody	k <sub>on</sub> (1/M.s)	k <sub>off</sub> (1/s)	K <sub>D</sub> (M) <sup>a</sup>
NbBcII10-His <sub>6</sub>	1,24 × 10 <sup>6</sup>	2,23 × 10 <sup>-2</sup>	1,78 × 10 <sup>-8</sup>
NbBcII10-LEY-alkyne-1	9,67 × 10 <sup>5</sup>	1,35 × 10 <sup>-3</sup>	1,39 × 10 <sup>-9</sup>
NbBcII10-LEY-alkyne-2	4,44 × 10 <sup>5</sup>	1,26 × 10 <sup>-3</sup>	2,82 × 10 <sup>-9</sup>
NbBcII10-LEY-alkyne-3	5,16 × 10 <sup>5</sup>	1,32 × 10 <sup>-3</sup>	2,57 × 10 <sup>-9</sup>
NbBcII10-LEY-alkyne-4	3,70 × 10 <sup>6</sup>	1,70 × 10 <sup>-3</sup>	4,60 × 10 <sup>-10</sup>
NbBcII10-HLC	2,85 × 10 <sup>7</sup>	2,56 × 10 <sup>-3</sup>	8,99 × 10 <sup>-11</sup>

<sup>a</sup> K<sub>D</sub> = k<sub>off</sub>/k<sub>on</sub>.

When construing the sensorgram of NbBcII10-LEY-alkyne in Fig. 8b, however, it is clear that the dissociation phase of NbBcII10-LEY-alkyne deviates from that of the unmodified NbBcII10-His<sub>6</sub> (Fig. 8a). After an initial rapid drop in signal during the wash phase, the further release from the antigen apparently slows down and eventually stops to reach a (nearly) constant signal. Likewise, the sensorgram of NbBcII10-HLC (Fig. 8c) shows a very similar pattern. Clearly, this emphasizes the contribution of a fraction Nb proteins that are dimerized Nbs through their C-terminal cysteine residues within these samples. Of note, after every association-dissociation cycle, full regeneration of the surface was achieved with 100 mM glycine-HCl (pH 2.5).



**Fig. 8** SPR sensorgrams of the BcII antigen binding study of NbBcII10-His<sub>6</sub> (a), NbBcII10-LEY-alkyne (b) and NbBcII10-HLC (c). NbBcII10-LEY-alkyne and NbBcII10-HLC have a similar pattern with an improved k<sub>off</sub> in comparison with NbBcII10-His<sub>6</sub>.

alt-text: Fig. 8

In conclusion, neither the cytoplasmic extraction nor the site-specific alkylation of the NbBcII10-LEY-alkyne had any effect on the association rate constant relative to the native Nb. The dissociation rate constant, however, and consequently the dissociation equilibrium constant (K<sub>D</sub>) showed an improvement by almost a factor 10, most likely due to dimerization of the probe. This clearly demonstrates that the functionality of the antigen-recognizing moiety is not affected by the C-terminal functionalization.

Nonetheless, for diagnostic applications, where the Nb is immobilized to the surface rather than the antigen, this fractional dimerization should not raise any concerns.

To summarize, in this study, an alkyne was site-specifically introduced as a bioorthogonal group on a single-domain antigen binding fragment without compromising its functionality. Through a copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) [42] these probes can subsequently uniformly be immobilized in a oriented fashion on azide functionalized surfaces [43]. This strategy can also easily be extended to alternative click chemistries, using other bioorthogonal groups, such as the strain-promoted alkyne-azide cycloaddition (SPAAC) [44], Staudinger ligation [45], Diels-Alder cycloaddition [1] or the more recently developed strain-promoted inverse electron-demand Diels-Alder cycloaddition (SPIEDAC) [46] by simple adjustment of the bifunctional linker (Here ends the text in the right column on page 8 and above the text in the right column on page 9 Table 3 and Fig.8 are inserted. Then starts the text in the left column on page 9 i.e. Conclusions and further till the end of the text.).

## 4 Conclusions

In this study, it was proven that it is possible to site-specifically alkynated NbBcII10 using EPL and couple the alkynated Nb to an azide functionality. With respect to the periplasmic expression of the Nb-intein-CBD complex, several leader sequences were evaluated but none of them were successful. Nevertheless, by using SHuffle®T7 cells and appending a small peptide spacer (LEY) to the C-terminus of the Nb, fully functional Nb-LEY-alkyne was produced through cytoplasmic expression and alkylation by EPL. This demonstrates that Nb-intein-CBD fusion complexes, prepared for alkylation by EPL, do not necessarily need to be expressed in the periplasm to remain active and that post-translation alkylation using EPL has no influence on the binding activity of NbBcII10.

## Author contributions

B.B., W.G. and P.A. conceived and designed the experiments; B.B. performed the experiments in collaboration with R.H.; B.B, W.G. and P.A. analyzed the data; C.V., S.M. assisted on the affinity studies and, together with N.D., they contributed with their general NbBcII10 expression experience.

## Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pep.2017.02.009>.

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## Appendix A. Supplementary data

The following is the supplementary data related to this article:

## Supporting information

alt-text: Supporting information

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### Highlights

- In this study, it was proven that it is possible to site-specifically mono-alkynate NbBcII10 using EPL and couple the alkynated Nb to an azide functionality. (Nb= Nanobody; EPL = Expressed Protein Ligation).
  - With respect to the periplasmic expression of the Nb-intein-CBD complex, several leader sequences were evaluated but none of them were successful.
  - By using SHuffle®T7 cells and appending a small peptide spacer (LEY) to the C-terminus of the Nb, fully functional Nb-LEY-alkyne was produced through cytoplasmic expression and alkylation by EPL.
  - Nb-intein-CBD fusion complexes, prepared for alkylation by EPL, do not necessarily need to be expressed in the periplasm to remain active and t post-translation alkylation using EPL has no influence on the binding activity of NbBcII10. (The new highlights can be found in Q1 )
- 

## Queries and Answers

**Query:** Highlights should only consist of "125" characters per bullet point, including spaces. The highlights provided are too long; please edit them to meet the requirement.

**Answer: Highlights**

It was proven that it is possible to site-specifically mono-alkynate NbBcII10 using Expressed Protein Ligation.

The site-specific mono-alkynated NbBcII10 was successfully coupled to an azide functionality.

Several leader sequences were evaluated for the periplasmic transport of the Nb-intein-CBD complex but none of them were successful.

Fully functional Nb-LEY-alkyne was produced through cytoplasmic expression in SHuffle®T7 cells and alkylation by EPL.

Nb-intein-CBD fusion complexes, prepared for alkylation by EPL, do not necessarily need to be expressed in the periplasm.

Post-translational alkylation using EPL has no influence on the binding activity of NbBcII10.

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