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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 alkynation 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®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.

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_6-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

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

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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.

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* β-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

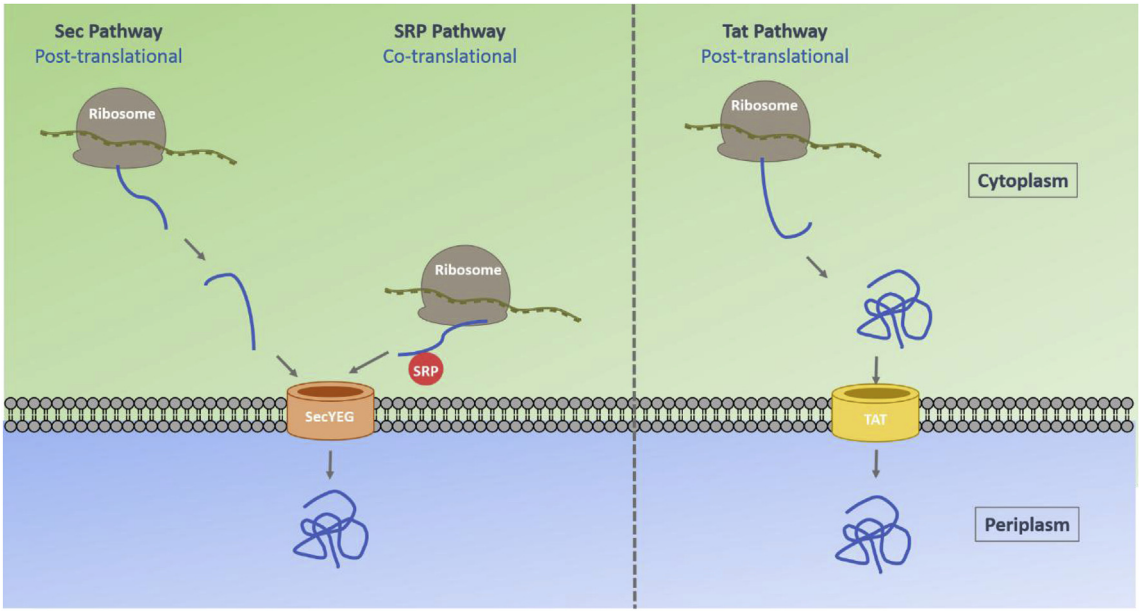

Fig. 1. Three periplasmic secretion pathways for proteins in *E. coli*. The Sec pathway is a post-translational pathway in which the protein is fully secreted by ribosomes. Afterwards, the leader sequence enables correct folding of the protein by transporting it to the periplasm via the SecYEG translocon. 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.