2014•2015
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Modification of nanobodies with 'click' functionalities using the amber suppression technique

Promotor :
Prof. dr. Wanda GUEDENS
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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Samenvatting

Introductie

Nanobodies (Nbs) zijn zeer geschikt als receptor molecule voor biosensoren vanwege hun hoge specificiteit en klein formaat. De huidige immobilisatie technieken leiden tot een willekeurige orientatie van de Nbs. Dit vermindert de sensitiviteit en reproduceerbaarheid van de biosensensoren wat een nieuwe methode voor de covalente en georiënteerde immobilisatie van Nbs noodzakelijk maakt. 'Klik'-chemie is een groep reacties die vlot verlopen onder milde reactie condities. Dit maakt deze reacties ideaal voor gebruik met eiwitten

Niet-natuurlijke amino zuren (nnAZ) maken het mogelijk om nieuwe functionele groepen zoals 'klik'-groepen te introduceren in *E. Coli.* nnAZ kunnen in *E. Coli* geïntroduceerd worden door middel van het hercoderen van een van de drie stop codons met behulp van een orthogonaal tRNA synthetase (RS)/ tRNA paar. Het amber codon (TAG) is het meest gebruikte codon voor suppressie. Het van Methanosarcina Mazei afkomstige pyrrolysine (pyl)RS/$tRNA^{pyl}$ is een natuurlijk voorkomend paar dat codeert voor het amber codon. Twee mutaties in het pylRS (RS^{FA}) maken het aminoacyleren van verscheidene lysine derivaten efficiënter. Het is onlangs aangetoond dat de sequentie rond het amber codon, amber suppressie efficiëntie beïnvloed.

Het doel van deze studie was het toevoegen van propargyl-lysine (PrK) in Nbs door middel van amber suppressie. De hypothese was dat het aanpassen van de sequentie rond het amber codon en het gebruik van het gemuteerde pylRS, amber suppressie efficientie zou verbeteren in Nbs tegen VCAM-1 en LOX-1 onder expressie gebracht in E. Coli.

Methoden

Nbs tegen VCAM-1 en LOX-1 inclusief amber codon en aangepaste sequentie daarrond zijn tezamen met RS^{FA} onder expressie gebracht in *E. Coli*. Gebruikte *E. Coli* varianten waren WK6, SHuffle, en JX33. Amber suppressie werd onderzocht met Western Blot en Ni-NTA opzuivering.

Resultaten

Nbs tegen LOX-1 konden niet worden gedetecteerd in de verscheiden *E. Coli* soorten. Nbs tegen VCAM-1 waren aanwezig maar er was geen amber suppressie. Endogene suppressie was zichtbaar in Nbs tegen VCAM-1

Discussie en conclusie

Amber suppressie met PrK was niet succesvol met behulp van pylRS^{FA}. Cyclooctyne-lysine is eerder met succes ingevoegd in eiwitten met behulp van pylRS^{FA} en kan mogelijk als alternatieve 'klik'-groep dienen in Nbs. PrK is succesvol toegevoed aan fluorescente eiwitten met behulp van pylRS onder expressie van een andere vector. Deze vector dient getest te worden voor suppressie in de Nbs die zijn gebruikt in deze studie.

Abstract

Introduction

Nanobodies (Nbs) are excellent receptor molecules in bio-sensing applications due to their high specificity and small size. Current immobilisation techniques cause random orientation of Nbs on the surface decreasing sensitivity and reproducibility of biosensors. A method is required that covalently immobilizes Nbs in an oriented manner on a surface. 'Click'-chemistry is a set of reactions that proceed rapidly under mild reaction condition making them ideal for use with proteins.

Unnatural amino acids (uAAs) allow introduction of new functional groups into *E. Coli*. uAAs can be incorporated by recoding one of three stop codon with an orthogonal tRNA synthetase (RS)/tRNA pair. The amber codon (TAG) is most used for suppression in *E. Coli*. The *Methanosarcina Mazei* pyrrolysine (pyl)RS/tRNApyl pair is a naturally occurring pair encoding the amber codon. Introducing two mutations into pylRS (RS^{FA}) improved aminoacylation efficiency of various lysine derivatives. It was recently shown that the sequence context around the amber codon influences amber suppression efficiency.

The aim of this study was to incorporate propargyl-lysine (PrK) into Nbs using amber suppression. We hypothesized that adapting the sequence context and using RS^{FA} would improve amber suppression efficiency in Nbs against VCAM-1 and LOX-1 expressed in *E. Coli*.

Methods

Nbs against VCAM-1 and LOX-1 containing the amber codon and adapted sequence context were co-expressed with RS^{FA} in *E. Coli*. *E. Coli* strains used were WK6, SHuffle, and JX33. Amber suppression was investigated with Western Blot and Ni-NTA purification.

Results

Nbs against LOX-1 could not be created in multiple *E. Coli* strains. Nbs against VCAM-1 were created but amber suppression was not successful. Endogenous suppression was observed in Nbs against VCAM-1.

Discussion and Conclusion

Amber suppression with PrK was not successful using $pylRS^{FA}$. Cyclooctyne-lysine has previously been incorporated into proteins using $pylRS^{FA}$ and could be used as alternative 'click'-group in Nbs. In another study PrK has been incorporated into fluorescent proteins using pylRS expressed in a different vector. This vector should be tested for suppression of Nbs used in this study. Successful suppression might be achieved in BL21 and Top10 cells.

List of abbreviations

amino acid	AA
amino acyl tRNA synthetase	aaRS
Antibody	Ab
Bacterial protein extraction reagent	B-PER
copper (I) catalyzed azide alkyne cycloaddition	CuAAC
Escherichia coli	*E. Coli*
Green fluorescent protein	*GFP*
Heavy chain antibodies	HcAbs
Heavy chain	H-chain
Hypervariable complementart determining region	CDR
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Leptin type oxidized low density lipoprotein receptor 1	LOX-1
light chan	L-chain
Methanocaldococcus jannaschi	Mj
Methanosarcina Barkeri	*M. Barkeri*
Methanosarcina Mazei	*M. Mazei*
Nickel-Nitrilotriacetic acid	Ni-NTA
nitro bleu tetrazolium chloride - 5-Bromo-4-chloro-3-indolyl phosphate	NBT-BCIP
N^{ε}-(o-azidobenzyloxycarbonyl)-L-lysine	AzLys
optical density	OD
oxidized low density lipoprotein	oxLDL
p-azido-L-phenylalanine	AzPhe
polymerase chain reaction	PCR
Polyvinylideenfluoride	PVDF
Propargyl-Lysine	PrK
pyrrolysyl-tRNA synthethase	pylRS
release factor 1	RF-1
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Strain-promoted azide alkyne cycloaddition	SPAAC
TBS	tris buffered saline
tough broth	TB
Tris buffered saline - tween 20	TBST
Tris EDTA sucroe buffer	TES
Tris(2-carboxyethyl)phosphine	TCEP
unnatural amino acids	uAAs
Vascular cell adhesion molecule 1	VCAM-1
Yellow fluorescent protein	YFP

1. Introduction

1.1 Biosensors

Biosensors are a powerful tool for the analysis of biomolecular interactions in clinical, biochemical and environmental analysis. In a medical setting biosensors offer the potential to provide cheap, accurate, real-time, information in a first line or home setting. One such biosensor is the glucose sensor that accurately measures blood glucose levels in diabetic patients (1, 2). These properties could be useful in the management of growing health concerns such as atherosclerosis and metabolic syndrome (3). A typical biosensor consists of a biorecognition platform, a transducer and an instrument interface, that translates the reaction between the analyte and the biorecognition layer into a measurable signal (2). Many biosensors involve protein-protein or protein-ligand interactions. These include antibodies (Abs) or antibody-derivatives (e.g. nanobodies), enzymes, or receptors (Fig 1.1A) (1, 4).

Vascular cell adhesion molecule 1 (VCAM-1) and leptin type oxidized low density lipoprotein receptor 1 (LOX-1) are two proteins that have been reported as potential biomarkers for the diagnosis of atherosclerosis (5, 6). VCAM-1 is a membrane protein present on endothelial cells and is responsible for monocyte recruitment in relation to inflammation. In inflammatory processes such as unstable atherosclerotic plaques, VCAM-1 is expressed on the endothelium (6). LOX-1 is a scavenger receptor involved in the uptake of oxidized low-density lipoproteins (oxLDL). Uptake of oxLDL is correlated to unstable atherosclerotic plaque formation (5). Detection of soluble VCAM-1 and LOX-1 could aid in the diagnosis of atherosclerosis (5, 6).

1.2 Nanobodies are sensitive and specific receptor molecules

Nanobodies (Nbs) are the smallest independent antigen-binding domain. They are derived from heavy chain antibodies (HcAbs) found in species of *Camelidae*. Whereas classical Abs are composed of two heavy chains (H-chain) and two light chains (L-chain), HcAbs contain only the H-chains. The H-chain of HcAbs is composed of three instead of four globular domains. The CH2-CH3 domains are highly homologous with classical Abs. The CH1 domain is missing in HcAbs reducing the antigen-binding fragment to one variable domain; the VHH domain (Fig 1.1 B). The VHH domain is adapted compared to the VH domain from classical Abs, to become a functional antigen binding site (7). Both the VH and VHH domain consist of four framework regions that surround three ,hypervariable, complementarity

determining regions (CDR). The Nb is the single VHH entity with a size of 15 kDA, compared to 150 kDa for antibodies, 2.5 nm in diameter and 4 nm in height (Fig. 1.1B) (7, 8). In the VHH domain CDR-1 and CDR-3 are more extended than in the VH domain. A disulfide bond between CDR-1 and CDR-3 serves to shape the loops in which CDR-1 and CDR-3 are located. This is thought to assist in antigen-binding through reducing the entropic gap between the bound and unbound state of the Ab and antigen.

In a biosensor context the Nb's small size allows immobilization of a larger number of receptor molecules, improving sensitivity and allowing miniaturization of the sensor (4, 9). Nbs offer long shelf-life, high melting points (67-78 ºC), stability in basic and acidic environment, and resistance against proteases. This allows Nbs to survive in harsh conditions such as the stomach and gut. The VH domain, in classical Abs, contains hydrophobic amino acids (AAs) to bind the L-chain. In the VHH domain these hydrophobic AAs have been replaced by hydrophilic AAs, which increases the solubility of Nbs compared to other antibody fragments in physiological conditions (7, 8).

1.3 A method for covalent orient immobilisation of proteins is required

The sensitivity and specificity of a biosensor is largely dependent on the biorecognition layer. Vital to this is the correct immobilization of the receptor molecule so that the recognition site is maximally exposed to the target without affecting its activity. This is especially of importance when miniaturizing the sensor to the micro- or nanoscale, since reducing the surface size limits the amount of receptors that can be placed onto the sensor (10). To produce a reusable biosensor with a long shelf-life, covalent, thus stable, coupling is necessary. Therefore methods are required for efficient, oriented, and covalent immobilization of proteins, without affecting their structure or function (11, 12).

Covalent immobilization of proteins, without affecting protein structure and function, requires an immobilization strategy that can be carried out under mild reaction condition. These include; low temperature, neutral pH, and normal pressure, to prevent denaturation of proteins. Furthermore, the end product should be free of contaminating side products to prevent interference with the sensor or toxicity to living cells. 'Click'-chemistry, defined by Sharpless et al. in 2001, provides a set of reactions that can fulfil these criteria. 'Click'-chemistry must be high yielding and proceed rapidly under mild reaction conditions (13).

Additionally, it should produce a minimal amount of by-products, proceed in water or under solvent free conditions and be insensitive to water and oxygen (13-15). Most 'click'-groups are bio-orthogonal, i.e. not present in nature. Thus, no unwanted side reactions will occur within the protein or with other biomolecules, offering the potential of oriented immobilization (15).

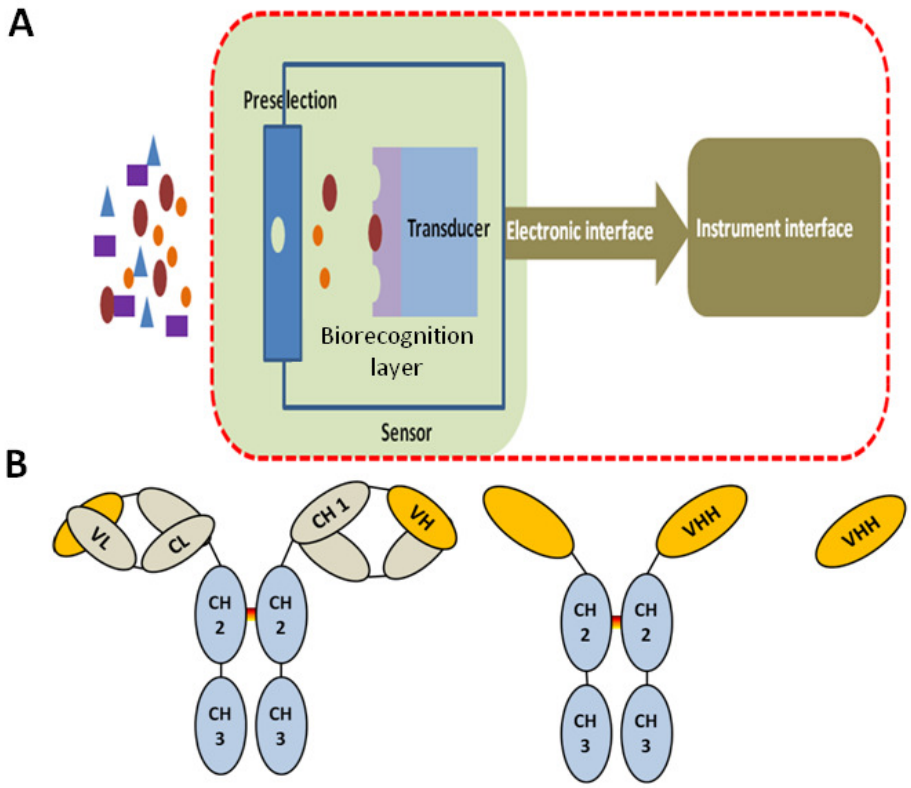

Figure 1.1 A) Schematic setup of a biosensor showing the biorecognition layer on the transducer and the signal being transferred via electronic interface to provide a visual signal on the instrument interface. **B)** Classical Ab (left) containing both H-chain and L-chain, HcAB (centre) consisting of only H-chain and lacking CH 1 domain, and Nb (right) being the single domain antigen-binding entity derived from HcAb

1.4 Click Chemistry

There are a variety of click-reactions that can be used for protein modification, including the well know Huisgen copper (I) catalyzed azide alkyne cycloaddition (CuAAC), Strain-promoted azide alkyne cycloaddition (SPAAC), Staudinger ligation, thiol-ene additions, and Diels-Alder reactions.

1.4.1 Azide alkyne cycloaddition reactions

Azide alkyne cycloaddition in its classic form has limited applicability in biological systems due to the requirement of high temperatures and a final mixture of 1, 4- and 1, 5-triazoles. The use of Cu (I) as a catalyst accelerates the reaction, allowing it to proceed at room temperature and resulting in the formation of only 1, 4-triazoles (Figure 1.2A). However, copper is cytotoxic to both bacterial and mammalian cells, which makes the reaction unsuitable in combination with living cells or tissue (15). An alternative reaction has been proposed by Bertozzi et al. making use of ring strained alkynes to accelerate the reaction (Fig. 1.2B) (16). The ring strain caused by bond angle deformation greatly reduces the activation barrier making the reaction proceed without the use of copper as catalyst. Using SPAAC cyclooctyne was successfully bound to azidosugars in Jurkat cells after 1h incubation at room temperature (16), demonstrating its possibility for the use in living systems.

1.4.2 Staudinger Ligation

An alternative reaction that makes use of azides is the Staudinger reaction. In Staudinger reactions an azide reacts with a phosphine resulting in the formation of an aza-ylide. However in water the aza-ylide hydrolizes to form a primary amine and a phosphine oxide (10). Bertozzi et al. developed an alternative Staudinger reaction, the Staudinger ligation, with an esther strategically placed on one of the phospine's aryl substituents resulting in the formation of a stable amide bond (Fig. 1.2C). Staudinger ligation takes place at room temperature in water and at neutral pH. However, phosphine reagents oxidize in air reducing their shelf life and the reaction suffers flow sluggish kinetics, making it less suitable to track cell processes (14).

1.4.3 Thiol-ene reaction

Another highly efficient reaction is that between thiols and reactive carbon-carbon double bonds (Fig. 1.2D). This reaction occurs via activation with radicals or light. Activation by light offers the advantage of surface patterning with photolithography. A major drawback of the use of thiols is that, being present in cysteine, they are not bioorthogonal This makes the reaction less suitable for oriented immobilization of proteins (10).

1.4.4 Diels-Alder cycloaddition

Diels-Alder reaction occurs between a diene and a dienophile to form a six membered unsaturated ring structure (Fig. 1.2E). Diels-Alder reactions are fast and efficient in water and are chemoselective. Diels-Alder has been used for peptide modification and linking between proteins and carbohydrates and for the labelling of DNA and RNA fragments with fluorescent markers (17).

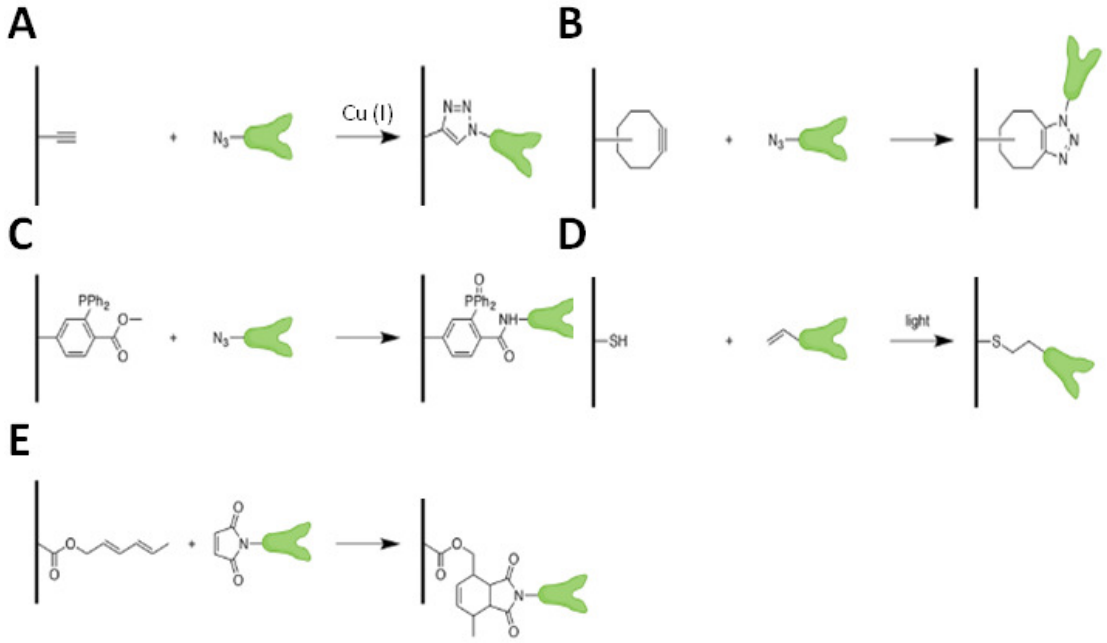

Figure 1.2 A) CuAAC **B)** SPAAC **C)** Staudinger ligation **D)** Thiol-ene **E)** Diels alder (10)

1.5 Non-covalent Oriented immobilisation of proteins

An oriented immobilization of proteins requires a site-specific modification to incorporate the binding site. Several site-specific, non-covalent, strategies exist that make use of complementary affinity reactions between biomolecules. This includes the His-tag, FLAG-tag, and peptide epitope tags, or the use of biotinylated proteins. However, these strategies do not offer the same stability as covalent bonds and often make use of bulky binding groups.

1.6 Unnatural amino acids allow incorporation of 'click'-groups in proteins

Alternatively a method has been developed to incorporate unnatural AAs (uAAs) into proteins. This offers the possibility of site-specifically incorporating click-groups for covalent binding into the protein via the protein translational machinery (10, 12). Most species are

limited to 20 natural amino acids for the production of proteins which are encoded by 61 triplet codons. The three remaining codons, TAG (amber), TAA (ochre) and TGA (opal), are stop codons that terminate translation (18). The encoding of a non-natural AA requires;

1) A codon that does not encode for any of the endogenous AAs.
2) A functional aminoacyl tRNA synthetase (aaRS) / tRNA pair that acts independently from the endogenous aminoacylation machinery of the cell. The suppressor tRNA should not be aminoacylated by endogenous aaRS and the introduced aaRS should not aminoacylate endogenous tRNA. Also the introduced aaRS must only charge the cognate tRNA with the uAA and not any endogenous AAs.
3) Efficient transportation of the uAA into the cell or its synthesis in the cell. The uAA must be non-toxic and stable to the cell's metabolic enzymes (19, 20).

Since the three stop codons do not encode for a tRNA, these can be reprogrammed to encode for the uAA. In *E. Coli* the most used codon for the introduction of uAAs is the amber codon as this is the least present stop codon (only 7% of *E. Coli* genes end in TAG) reducing the effect of stop codon suppression on endogenous protein translation to a minimum. Work is also being done on using 4 – base decoding pairs for the introduction of uAAs. This greatly expands the amount of codons available for introduction of novel AAs, allowing introduction of multiple uAAs into the translational machinery (19).

1.7 Natural aaRS/tRNA pairs exist that are orthogonal in *E. Coli*

To obtain orthogonal aaRS/tRNA pairs, it is possible to evolve endogenous aaRS/tRNA for the binding of uAAs. However, this has led to misaminoacylation of native tRNAs by the evolved aaRS. Also the creation of synthetic tRNA has not led to efficient aminoacylation and protein formation. Studies have identified several aaRS/tRNA pairs from other species that are orthogonal in *E. Coli*. The tyrosyl-tRNA synthetase and cognate tRNA from the *Methanocaldococcus jannaschi* (*Mj*TyrRS/*Mj*tRNA^{tyr}) was the first to be discovered. It has been used to encode *p-azido-L-phenylalanine* (AzPhe) in *E. Coli*. AzPhe containing proteins were site-specifically modified with phosphine via Staudinger ligation and were selectively labelled with alkyne-fluorescent probes using Huisgen [3+2] cycloaddition (19, 21, 22).

Pyrrolysine is a lysine derivative containing a methyl-pyrroline moiety on it (Fig. 1.3A) that is found in *Methanosarcina Barkeri* and *Methanosarcina mazei (M mazei).* Pyrrolysine is directly esterified to its specific tRNA (tRNA^{Pyl}) by pyrrolysyl-tRNA synthethase (PylRS).

$tRNA^{Pyl}$ contains the CUA anticodon with its complementary UAG codon and can be readily used for amber suppression. Pyrrolysine has been translationaly incorporated into *E. Coli* using PylRS/$tRNA^{Pyl}$ from both *M. Mazei* and *M. Barkeri* (23).

1.8 Mutations in PylRS offers improved amber suppression and allows incorporation of additional lysine-derivatives

Yanagisawa et al. (2008) have further developed the *M. Mazei* PylRS/$tRNA^{Pyl}$ to function for different lysine and pyrrolysine derivatives. It was found that the presence of the N^{ϵ}-carbonyl group was essential for esterification by PylRS. Applying two mutations to the PylRS improved aminoacylation to $tRNA^{Pyl}$. The mutation Y384F enhanced *in vitro* aminoacylation and *in vivo* amber suppression and the mutation Y306A expanded the AA binding pocket, allowing the esterification of ring structured N^{ϵ}-carbonyl lysine derivatives as well as improving amber suppression. Combining mutation Y384F and Y306A led to successful encoding of N^{ϵ}-(o-azidobenzyloxycarbonyl)-L-lysine (AzLys) (Fig. 1.3B) in *E. Coli* and the production of a milligram quantity of protein, containing AzLys at the amber site, which could be site-specifically modified with a fluorescent probe (23).

1.9 The sequence context around the amber codon affects suppression efficiency

A limiting factor for amber suppression efficiency is release factor 1 (RF-1) which terminates translation in response to TAG. The TAG-codon is competitively recognized by RF-1 and the amber suppressor tRNA, resulting in protein expression levels around 20% compared to proteins without the amber codon. Recently it has been demonstrated that the 6 nucleotides upstream and the six nucleotides downstream of the amber codon influence amber suppression and the resulting protein translation. Pott et al. (2014) created a library with sequence NNK NNK TAG NNK NNK with N being any of the four bases (A, T, G, C) and K being either G or T. The N positions were characterized by a low GC content (22,5% for $tRNA^{Pyl}$/PylRS) and high presence of A (77.5% for $tRNA^{Pyl}$/PylRS). It was shown that optimizing the surrounding sequence context increased yield by 70-100% compared to sense codon translation. Furthermore, this increase was independent of the used non-natural AA and observed in multiple proteins, demonstrating that sequence context is of importance for optimization of amber suppression (24).

1.10 Aim and hypothesis

The aim of this study was to incorporate Propargyl-Lysine (PrK, Fig. 1.3 C) into Nbs against VCAM-1 and Nbs against LOX-1 through amber suppression. It was hypothesized that adapting the sequence context around the amber codon and adding mutation Y384F and Y306A in PylRS (RSFA) would improve amber suppression efficiency in Nbs against VCAM-1 and LOX-1 expressed in *E. Coli*.

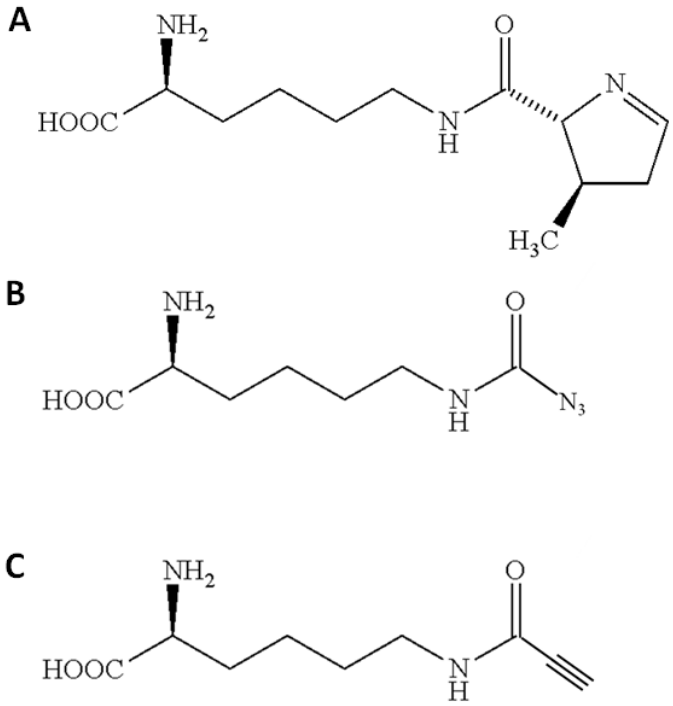

Figure 1.3 Structure of **A)** Pyrrolysine, **B)** AzLys **C)** PrK

2. Methods and materials

2.1 Materials

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich. Primers for PCR reactions were purchased from Integrated DNA technologies, PCR reagents were obtained from Thermo Scientific. Culture media ingredients were purchased from BD. PCR was performed in the MJ Mini^{TM} Personal Thermal Cycler (Biorad). Agarose gel electrophoreses was done in Mini-Sub® Cell GT Cell (Bio-Rad). Sodium sodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot were done in the Mini-PROTEAN® Tetra cell (Bio-Rad). PowerPac^{TM} Basic (Bio-Rad) was used as power supply for Western Blot and gel electrophoreses. Bacterial colony size was estimated by measuring the optical density (OD) using Ultrospec 10 cell density meter (Amersham Biosciences)

2.2 Nanobodies

Nbs targeting VCAM-1 and LOX-1 were kindly provided by Professor S. Muyldermans from the Vrije Universiteit Brussel. A 6-His tag was present at the C-terminus for purification purposes. For each Nb three genetic variations were used (Table 2.1, Fig 2.1). For amber suppression the TAG-codon was incorporated at the C-terminus, before the His-tag (VCAM-1_TAG/LOX-1_TAG). To investigate the effect of sequence context on amber suppression efficiency (VCAM-1_Pott/LOX-1_Pott), 2 additional codons were included at each side of the amber codon. Finally a Wild type Nb (VCAM-1_WT/LOX-1_WT) containing only the Nb-gene and His-tag was used as negative control.

Table 2.1 List of genetic variations for Nbs against VCAM-1 and LOX-1

Nbs against VCAM-1	**Nbs against LOX-1**
VCAM-1_TAG	LOX-1_TAG
VCAM-1_Pott	LOX-1_Pott
VCAM-1_WT	LOX-1_WT

Nbs were ligated into the pBAD- and pMES4-vectors for cytoplasmic and periplasmic protein expression respectively (supplemental figure S1 and S2). Transcription of the pBAD vector is under control of the Arabad promoter. pMES4 transcription is regulated by the lac-operon.

pBAD and pMES4 possessed the ampR gene, responsible for ampicillin resistance, as selection marker. In the pBAD vector the nanobody construct, and the His-tag was incorporated between the restriction sites NcoI and XhoI. In the pMES4 vector the restriction sites used were Eco9II and BamHI and they confined only the His-tag and the amber-suppression construct. Additionally, the pMES4 vector contained a pelB leader sequence to direct the Nbs to the periplasm. PylRS and $tRNA^{pyl}$ were ligated into the pEVOL system (supplemental figure S3). pEVOL contains the *CmR* gene responsible for chloramphenicol resistance as a selection marker (25).

2.3 *E. Coli*

Top10F' cells were used for cloning and amplifying of plasmids. For periplasmic expression WK6 cells were used. For cytoplasmic expression JX33 and SHuffle strains were used. In the JX33 strain, RF-1 is knocked out to increase amber suppression efficiency (26). SHuffle cells were used because this strain was reported to be capable of improved disulfide bonding in the cytoplasm (27).

Chemical transformation

Either 4 µl of ligation mixture or 100-200 ng of plasmid DNA was added to 50 µl of chemically competent cells. In the case of co-transformation of both the pEVOL vector and the pBAD or pMES4 vector, 100-200 ng of DNA of each was added. For chemical transformation the bacteria and DNA were left to incubate on ice for 45 minutes. Cells were then subjected to a heat shock of 42 ºC for 1 minute and put back on ice for a further 5 minutes. Subsequently 800 µl of preheated (37 ºC) recovery medium (2-YT medium, 1% glucose) was added and cells were incubated for 60 minutes at 37 ºC while shaking at a speed of 220 rpm. After incubation cells were transferred to 2-YT agarose plates containing 100 µg/ml ampicillin, and/or 50 µg/ml chloramphenicol.

Electrical transformation

Either 1 µl of ligation mixture or 100-200 ng of plasmid DNA was added to 50 µl electrocomptent cells. For electroporation, cells received a 1.8 kV electronic pulse for approximately 5.5 ms using the micropulser (Bio-Rad). 800 µl of preheated (37 ºC) recovery medium was added and cells were incubated at 37 ºC for 45 minutes. After incubation cells were transferred to 2-YT agarose plates containing 100µg/ml ampicillin, and/or 50 µg/ml chloramphenicol. Agarose plates were incubated overnight at 37 ºC

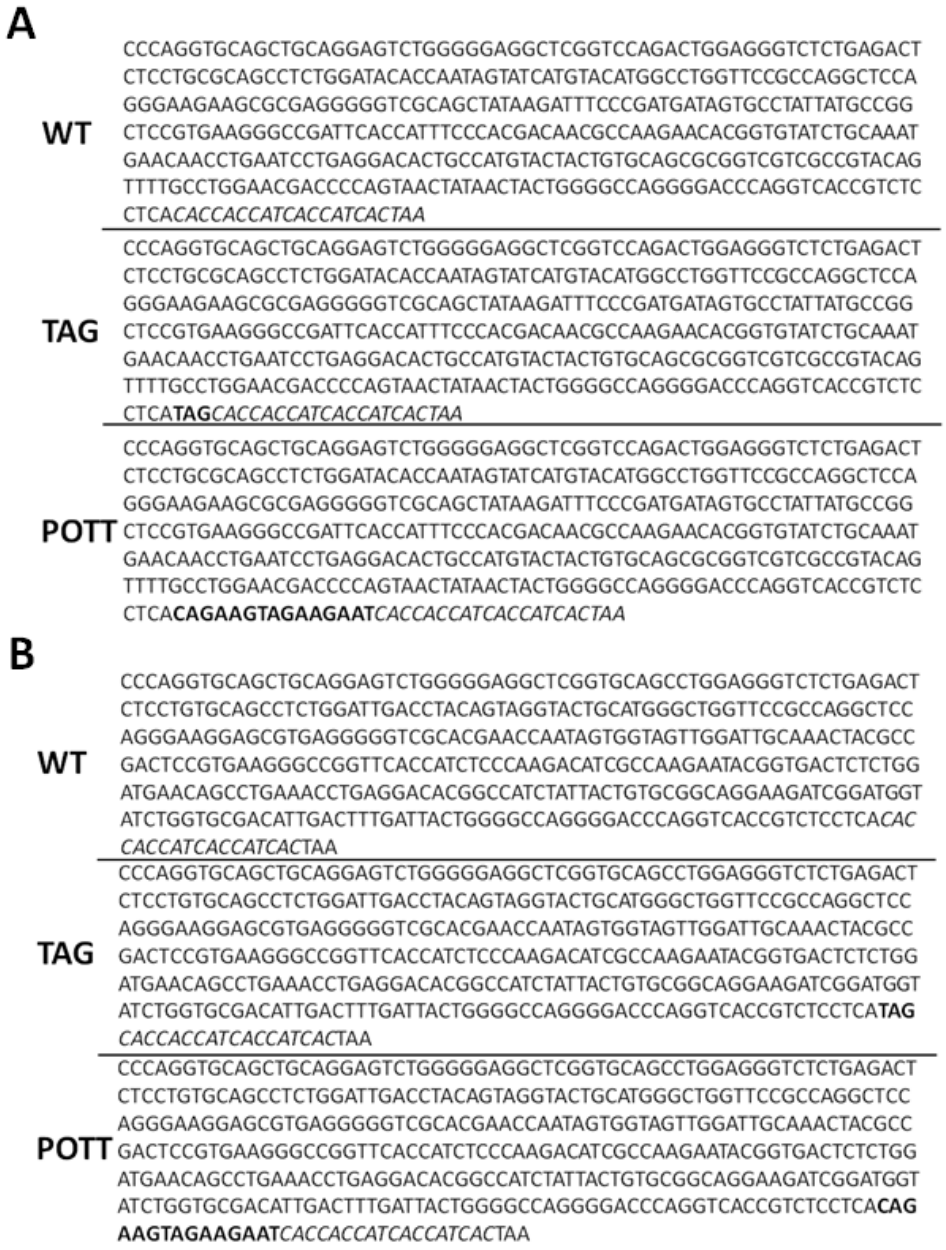

Figure 2.1 Sequences for **A)** VCAM-1 Nb and **B)** LOX-1 Nb. Mutations compared to wild type are written in bold and His-tag is marked in italic. TAG represents Nb with amber codon included prior to His-tag, POTT is sequence with amber codon and adapted sequence context round the amber codon.

2.4 PCR

PCR was performed following the Phire Hot start II DNA Polymerase protocol (table 2.2). 30 denaturation extension cycles were run for each sample. The amplified DNA was purified using QIA quick purification kit (Qiagen) according to protocol with the exception that the sample was washed three times rather than once.

Restriction was performed on PCR product and Vector using FastDigest restriction enzymes and FastDigest green buffer (table 2.3). Restriction enzymes for pMES4 vector and PCR product were ECO 9II and BamHI and for the pBAD vector and PCR product were NcoI and XhoI. Restriction analysis was performed at 37 ºC for 20-60 minutes. Digestion products were loaded on a 0.8% agarose gel and gel electrophoreses was performed at 120V. Purification was performed using QIA quick gel extraction kit (Qiagen). Vector and PCR product were ligated at 22ºC for 20-40 minutes using T4 DNA ligase and T4 10x buffer (table 2.4).

Table 2.2 Phire Hot start II PCR protocol

Reaction mixture		Running Protocol			
	Volume (µl)	Cycle step	Temp. (ºC)	Time (s)	Cycles
DNA template	0.5-1.0 (100-200 ng)	Initial denaturation	98	30	1
Phire hot start DNA polymerase	1.0	Denaturation	98	5	30
5x Phire reaction buffer	10.0	Extension	72	10-15/ kb	
dNTP	1.0 (200µM each nucleotide)	Final extension	72	1 min	1
Forward Primer	0.25				
Reverse Primer	0.25				
MilliQ water	36.5-37.0				

Table 2.3 Restriction analysis reaction mixture

	PCR product	Vector (plasmid DNA)
DNA (µl)	20.0	30.0
Enzyme (µl/enzyme)	2.0	2.0
Buffer (µl)	4.0	4.0
MilliQ water (µl)	32.0	30.0

Table 2.4 Ligation reaction mixture

	Volume (µl)
Vector	1.0
PCR product	1.0
T4 DNA ligase	0.25
T4 10x buffer	2.0
MilliQ water	15.75

2.5 Protein expression and extraction

Periplasmic expression

WK6 cells were cultured in flasks in either 2YT-medium or TB-medium containing 2mM PrK (SiChem), 100µg/ml ampicillin, and 50 µg/ml chloramphenicol, at 37 ºC and 220 rpm. At an OD_{600} of 0.6-0.9 OD_{600} Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM final concentration) was added to induce protein expression. Cells were cultured overnight at 28 ºC until an OD_{600} of 20-30 was reached. Culturing without PrK was done as negative control

Periplasmic protein extraction was performed by centrifuging cultures at 8000g for 8 minutes. Cells were then resuspended in 2 ml Tris EDTA sucrose buffer (TES) and incubated for one hour at room temperature on a shaker. After first incubation 4 ml of 1:4 diluted TES buffer was added and cells were incubated on ice for another two hours while shaking. Finally $MgCl_2$ was added to a final concentration of approximately 15 mM and the cells were centrifuged for 30 minutes at 8000g and 4 ºC and the supernatant containing the periplasmic extract was collected.

Cytoplasmic expression

JX33 or SHuffle cells were cultured in shake flasks in either 2YT-medium or TB-medium containing 2mM PrK, 100 µg/ml ampicillin, and 50 µg/ml chloramphenicol, in an incubator at 37 ºC and 220 rpm. At an OD_{600} of 0.6-0.9 arabinose was added to a concentration of 1mM to induce protein expression. Culturing without PrK was done as control

Cytoplasmic extraction was carried out with bacterial protein extraction reagent (B-PER) according to manufacturer's instructions. Cultures were centrifuged at 5000 g for 10 minutes. Subsequently the cells were resuspended in 4 ml B-PER per mg of cells and incubated for 15 minutes. Finally, the solution was centrifuged at 15000 g for 30 minutes at 4 ºC and the supernatant containing the cytoplasmic extract was collected.

Nickel-Nitrilotriacetic acid (Ni-NTA) purification

Ni-NTA agarose beads were obtained from QIAGEN. 2 ml cleared lysate was added to 0.5 ml of beads and mixed on a rotary shaker at 4 ºC for 1 hour and 220 rpm. The mixture was loaded onto a column and the flow through was collected for SDS-PAGE analysis. Samples were washed twice with either 2.5 ml PBS for periplasmic extracts or 2.5 ml 10mM imidazole wash buffer for cytoplasmic extracts. Both wash fractions were collected for SDS-PAGE

analysis. 0.5 ml 500 mM imidazole elution buffer was added four times and each elution fraction was collected separately for further analysis.

2.6 SDS-PAGE

PageRuler^{TM} Protein Ladder (Thermo scientific) was used as protein weight reference for SDS-PAGE and Western Blot. For SDS-PAGE an SDS-gel was cast consisting of a 15% separating gel and a 4% stacking gel (table 2.5). Gel electrophoresis was done at 120V for approximately two hours. Prior to loading proteins were denatured by boiling 10µl sample in 10µl 2x sample buffer for five minutes.

Table 2.5 SDS-gel composition

Component	Stacking	Separating
	4%	15%
Polyacrylamide 30%	0.75 ml	5 ml
Tris-HCl 1,5M pH=8,8		3.75 ml
Tris HCl 0,5M pH=6.8	2.5 ml	
SDS 10%	50 µl	100 µl
Water	1.5 ml	1.1 ml
APS	30 µl	100 µl
TEMED	10 µl	15 µl

2.7 Western Blot

Western Blots were performed using either the His-tag or the click group for detection. Proteins were transferred from the SDS-gel to a PVDF membrane via overnight electrotransfer at 20V in Toubin buffer.

A ‘click’-reaction was executed with the Nb and biotin azide prior to SDS-PAGE. For this, 200 µl Nb was incubated at room temperature for two hours with 21.8 µl click mixture (table 2.6). A 5% bovine serum albumin (BSA, Amresco) in Tris buffered saline (TBS)-tween

solution was used for blocking and straptavidine-alkaline-phosphatase was used for the detection of biotinylated moleculed. The membrane was developed in nitro bleu tetrazolium chloride - 5-Bromo-4-chloro-3-indolyl phosphate (NBT-BCIP).

For detection using the His-tag a 5% skimmed milk solution (Merck) in TBS-tween was used as blocking reagent. Mouse-anti-His was used as primary antibody and Goat-anti-mouse-alkaline-phosphatase was used as secondary antibody. NBT-BCIP was used to develop the membrane.

Table 2.6 'Click'-reaction mixture	
	Volume (µl)
Biotin-azide (20mM)	2
TCEP (50 mM)	4
TBTA (1,7 mM)	11.8
$CuSO_4$ (50 mM)	4

3. Results

3.1 The 'Click'-group could not be detected in Nbs against VCAM-1 from WK-6 cells

WK6_VCAM-1_Pott_RSFA and WK6_LOX-1_Pott_RSFA were cultured in the 2YT medium in the presence (PrK+) or absence (PrK-) of PrK. To detect successful amber suppression Western Blot analysis was performed on the periplasmic extract directed at the 'click'-group. Although a signal was observed around 20 kDa, on the Western Blot, this level did not correspond to the normal 15 kDa value for the Nbs (fig 3.1 B, D). The signal was not only observed in the sample with 'click'-group and 'click'-reaction but also in the negative controls, i.e. PrK- and/or no click reaction was performed. The SDS-PAGE gel that was run as a control for the Western Blot did show bands at 15 kDa in the case of VCAM-1 (fig 3.1 A) but not LOX-1(fig 3.1 C). No 20 kDa band was observed in the SDS-analysis of either VCAM-1 or LOX-1. Together, these results suggest that Nbs against VCAM-1 but not LOX-1 were produced in WK6 and that PrK was not incorporated into the Nb.

The absence of the 'click'-group can be explained by termination at the amber codon or endogenous suppression. Ni-NTA purification was performed on VCAM-1 to detect the presence of the His-tag which would indicate endogenous suppression. SDS-analysis of the Ni-NTA purification did not show band in the elution fractions of either PrK+ or PrK- indicating the absence of the His-tag (fig 3.2).

3.2 Nbs against VCAM-1 from SHuffle cells were no longer present after Ni-NTA purification

In order to demonstrate amber suppression in the cytoplasm, VCAM-1_Pott_RSFA was expressed in SHuffle cells. Cells were cultured in 2YT-medium in the presence or absence of PrK (PrK+, PrK-). VCAM-1 WT Nbs from WK6 cells were used as reference. Ni-NTA purification was performed on the cytoplasmic extract from the SHuffle cells to isolate the Nbs from the cytoplasmic extract through the His-tag. SDS-PAGE on the reference Nb and the Ni-NTA fractions was performed. A clear band was visible at 15kDa in the VCAM-1 WT lane but not in any of the other lanes (fig 3.3). Bands were still visible in the elution fractions around 25 kDa and 40 kDa. This could indicate that the His-tag was present in heavier proteins, possibly indicating the presence of aggregates.

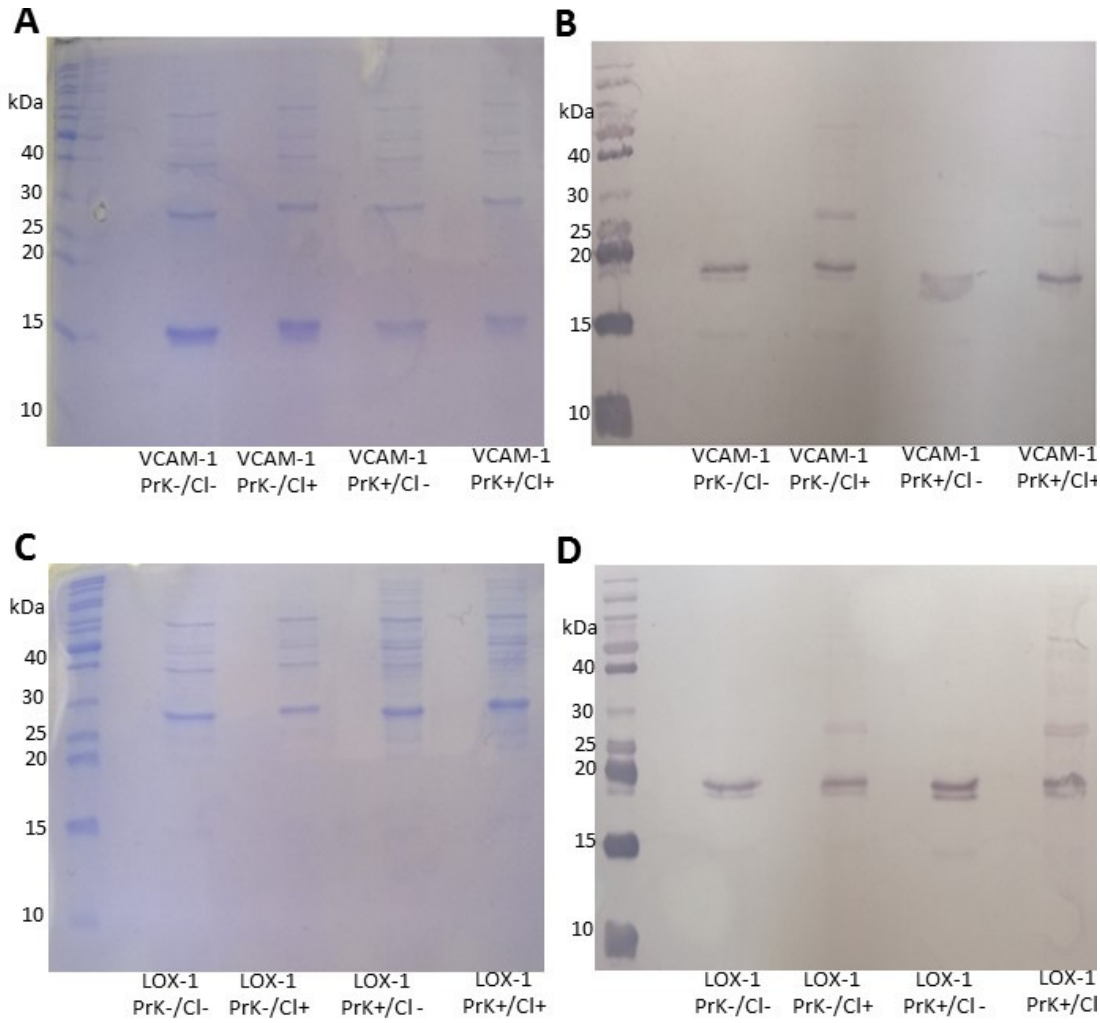

Figure 3.1 Nb expression in WK6 cells did not result in successful amber suppression. **A)** SDS gel analysis periplasmic expressions from WK6_VCAM_Pott_FA, **B)** Western Blot analysis of WK6_VCAM_Pott_FA directed at PrK, **C)** SDS gel analysis periplasmic expressions from WK6_VCAM_Pott_FA, **D)** Western Blot analysis of WK6_VCAM_Pott_FA directed at PrK. PrK- and PrK+ indicate culture in the absence or presence of PrK respectively, Cl + and Cl - indicate if a click reaction was performed (Cl +) or not (Cl -).

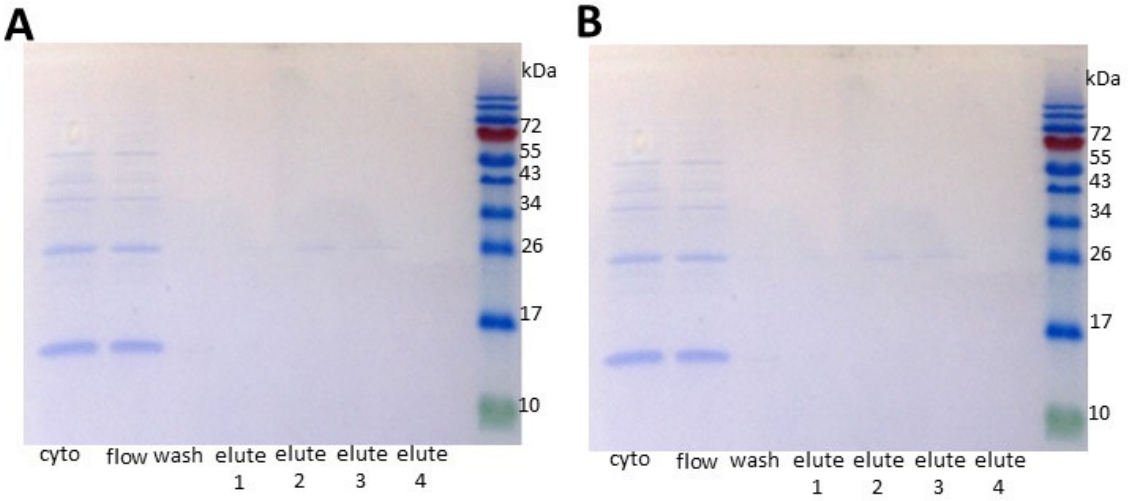

Figure 3.2 SDS-gel analysis of Ni-NTA purification indicates absence of His-tag on VCAM-1 Nbs obtained from WK6 cells. **A)** Ni-NTA purification fractions of VCAM-1 from WK6 cells with PrK present during cell culture, **B)** Ni-NTA purification fractions of VCAM-1 from WK6 cells with PrK not present during cell culture. cyto is protein extract prior to Ni-NTA purification, flow and wash are fractions collected after loading and washing, elute 1-4 are the fractions collected from subsequent elution steps.

3.3 Western Blot showed bands that could indicate the presence of the His-tag on Nbs against VCAM-1 from JX33 cells

RF-1 is knocked out in the JX33 strain. RF-1 is responsible for translation termination in response to the amber codon. Therefore, amber suppression should be more efficient in this *E. coli* strain. VCAM-1_Pott_RSFA was expressed in JX33 cells, which were cultured in the presence of absence of PrK (PrK+/PrK-). A Western Blot targeting the His-tag was performed. Bands were visible below 15 kDa in the cytoplasmic extract of VCAM-1 of both conditions with and without PrK (Fig. 3.4 B). Although this is too low to be the Nb, the SDS-PAGE gel shows lanes that are not entirely straight. This makes it difficult to distinguish the exact weight of the protein and it is possible that the bands are actually at 15 kDa (Fig. 3.4 A). Bands were visible at the same level in the SDS-analysis, and in both the Western Blot and SDS-analysis the bands were smeared rather than distinct. However, since the bands are present in PrK+ and PrK-, this would suggest that there is at least partial endogenous suppression if the bands are indeed representing the Nb.

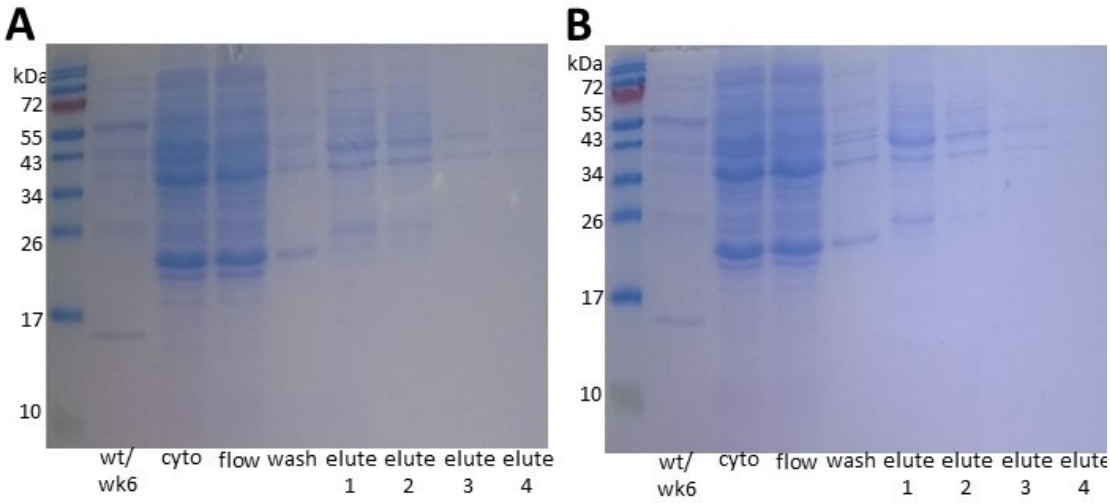

Figure 3.3 SDS-gel analysis of Ni-NTA purification indicates absence of His-tag on VCAM-1 Nbs obtained from SHuffle cells. **A)** Ni-NTA purification fractions of VCAM-1 from SHuffle and reference Nbs from WK6 cells, **B)** Ni-NTA purification fractions of VCAM-1 from Shuffle cells with PrK not present during cell culture and reference Nbs from WK6 cells. WT/WK6 are reference Nbs, cyto is protein extract prior to Ni-NTA purification, flow and wash are fractions collected after loading and washing, elute 1-4 are the fractions collected from subsequent elution steps.

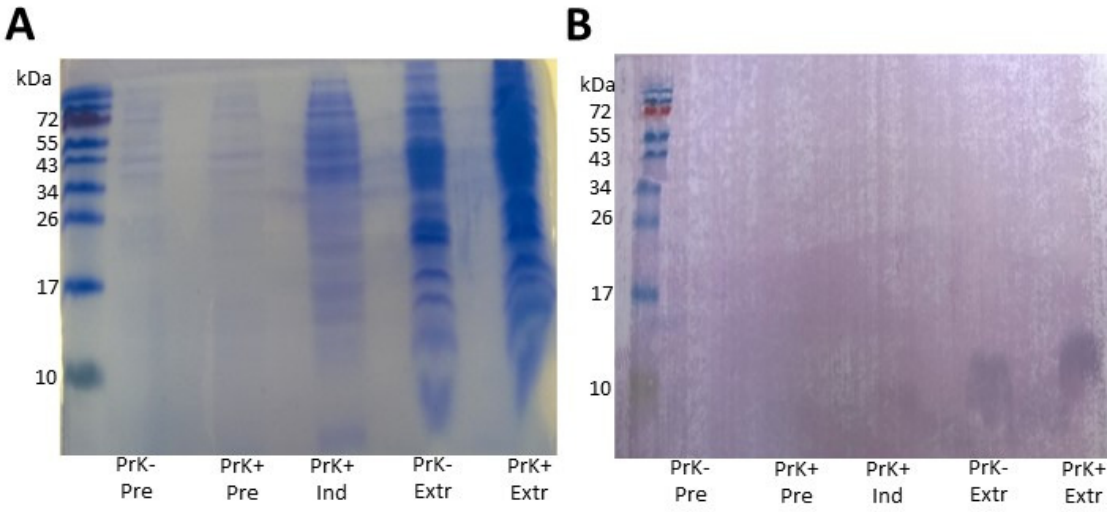

Figure 3.4 A) SDS-gel analysis of JX33_VCAM_FA, Pre is culture sample prior to induction with arabinose, Ind is culture sample at the end of the cell culture process and Ext is cytoplasmic extract. PrK + and PrK – indicate culturing in the presence (+) or absence (-) of PrK in the culture medium **B)** Western Blot of JX33_VCAM_FA directed at His-tag.

3.4 Ni-NTA purification did not provide bands at 15 kDa in the elution fraction of LOX-1 expressed in JX33 cultured in TB medium

It is possible that co-transformation of pEVOL and pBAD/pMES causes a metabolic strain on the *E. Coli* when cultured in 2-YT medium which could explain the absence of Nbs against LOX-1. Since Nbs against LOX-1 were not present, expression experiments were repeated using TB medium rather than 2-YT. LOX-1_Pott, LOX-1_TAG, and LOX-1_WT in combination with RSWT of RSFA were expressed in JX33 cells rather than only investigation LOX-1_Pott_RSFA. Culturing was done in presence (PrK+) or absence (PrK-) of PrK. Ni-NTA purification was performed on the cytoplasmic extract to isolate the Nb with a His-tag. The band between 10-17 kDa was observed in the flow through of the Ni-NTA purification (fig 3.5 B) but was not observed in the elution fractions (fig 3.5 C and D). However, bands were still visible in the elution fraction around 43 kDa and in the second elution fraction additional bands were observed around 26 kDa (fig 3.5 C). Despite using β-mercapthoethanol as reducing agent for SDS-analysis, it is possible that these bands are dimers of the actual Nb.

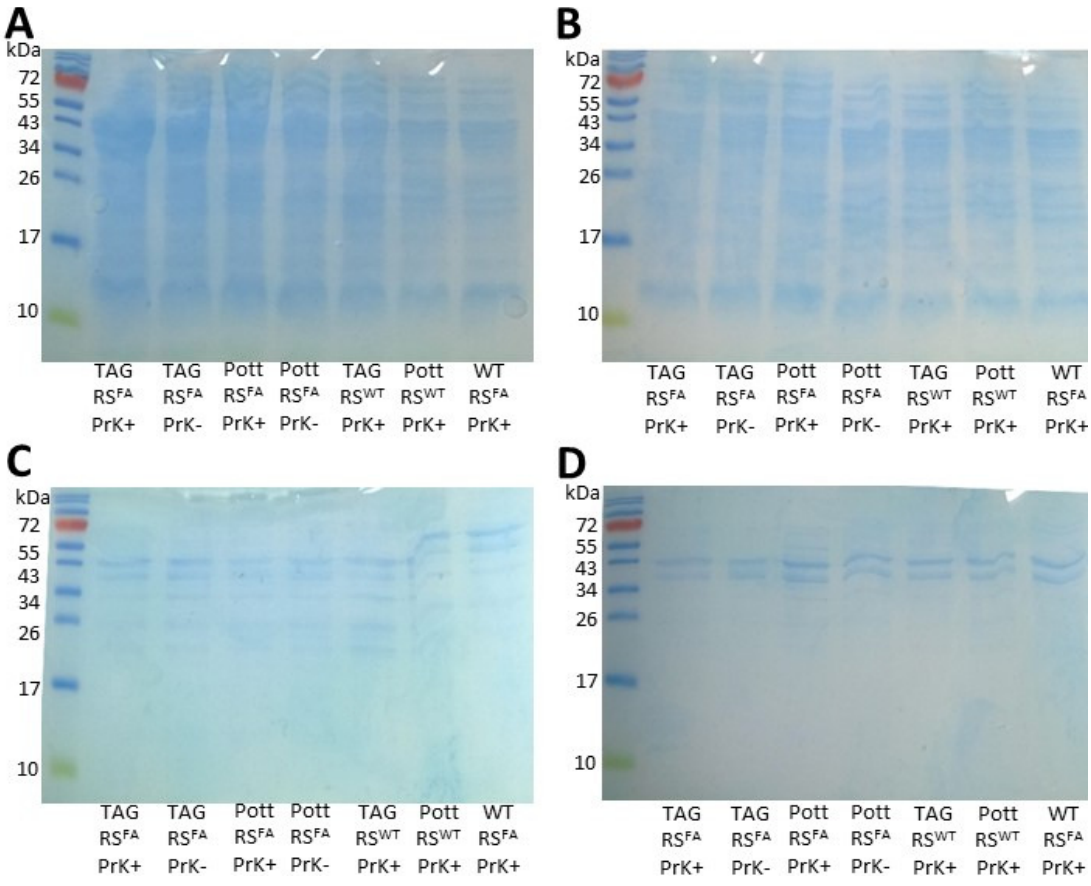

Figure 3.5 SDS-gel analysis of Ni-NTA purification of LOX-1 Nbs from JX33 cells cultured in TB medium **A)** cytoplasm, **B)** flow through, **C)** and **D)** elution fractions. Cells were cultured in the presence (PrK+) or absence (PrK-) of PrK. TAG, Pott and WT represent Nb sequence

4. Discussion

4.1 Overview of results

The goal of the study was to demonstrate that adapting the sequence context around the amber codon, and using PylRS^{FA} rather than wild-type PylRS would improve amber suppression efficiency in the LOX-1 and VCAM-1 Nbs. No clear evidence was found for the successful incorporation of PrK into Nbs via amber suppression. Several conditions were checked. First, the, theoretically, most optimal sequence for amber suppression was used. I.e. the adapted sequence context, as proposed by Pott et al., was tested as well as the mutated pylRS^{FA}. Both Nbs against LOX-1 and VCAM-1 were investigated using three different *E. Coli strains,* WK6, SHuffle, and JX33 cells cultured in 2YT-medium. Nbs against LOX-1 could not be created. Therefore, the other sequences for Nbs against LOX-1 were investigated, i.e. WT and TAG, as well as wild-type pylRS. These sequences were tested in JX33 in TB rather than in 2YT medium to provide a richer growth medium.

4.1.1 Absence of LOX-1 could be attributed to inclusion bodies

The 15 kDa band representing LOX-1 Nbs was not detected on SDS-gel analysis in all conditions. The absence of LOX-1 Nbs could be explained by the formation of inclusion bodies. Inclusion bodies can occur due to incorrect disulfide bond formation or insolubility of the protein (28). However, one of the major strengths of Nbs reported is their good solubility compared to other Ab fragments due to the absence of the hydrophobic region that is present on the VH domain of classic Abs (7, 8, 29). In WK6 cells Nbs were expressed in the periplasm, where in *E. Coli* cystein oxidation takes place to form disulfide bonds. This ensures correct folding making the presence of inclusion bodies less likely in WK6 (28).

4.1.2 Multimers of Nbs against LOX-1 from JX33 cells could be present despite the use of β-mercaptho-ethanol

SDS-gel analysis of Ni-NTA purification of Nbs against LOX-1 from JX33 cells, cultured in TB medium, did provide bands at 26 and 43 kDa in the elute. This could indicate the occurrence of dimers and trimers in the SDS-gel analysis. β-mercaptho-ethanol was added as a reducing agent to break down disulfide bonds in order to prevent the occurrence of multimers on SDS-gel, making it less likely that the observed bands were in fact aggregates of the Nb. Several studies have reported proteins that resisted the dissociation by SDS and β-mercaptho-ethanol (30, 31). Moreover, one study indicated the possibility that SDS caused

dimerization of β-amyloid, although no mechanism or conclusive evidence was given for this theory (31). Therefore it remains possible that the bands visible on the SDS-gel are in fact multimers of Nbs against LOX-1. Identifying the His-tag using Western Blot could confirm if this is indeed the case. SDS-gel analysis of the periplasmic extract of LOX-1 Nbs from WK6 cells did show bands around 26 kDa. Although Western Blot was performed to identify the 'click'-group, the presence of the His-tag was not investigated in this instance. It is possible that these bands are due to dimerization, but could have been caused by native proteins present in the periplasm. The presence of the His-tag in the absence of the 'click'-group would indicate a high degree of endogenous suppression whereas the absence of His-tag and 'click'-group indicates translation of termination. RF-1 is normally responsible for terminating the translation at the amber codon (26). It has been reported that RF-1 knockout increases endogenous suppression due to decreased termination efficiency at the amber codon (32, 33).

4.1.3 Nbs against VCAM-1 were observed on SDS but PrK was not incorporated

Although the 15 kDa band was not detected for Nbs against LOX-1 from WK6 cells, it was observed in the SDS-gel analysis of VCAM-1. This suggests that Nbs against VCAM-1 could be produced successfully. The Western Blot to identify the click-group on Nbs against LOX-1 and VCAM-1 from WK6 cells did show bands around 20 kDa. The exact nature of these bands could not be identified. No corresponding band was visible on the SDS-gel analysis of either VCAM-1 or LOX-1. Also, the signal was observed in conditions in which PrK was not present and in which no 'click'-reaction had been performed. Therefore, it is not possible that these signals correspond to the presence of the 'click'-group and most likely represent an artefact. Both blots were done simultaneously which could explain why the artefact is visible on all samples at the same level. These results suggest that amber suppression with PrK was not successful in WK6 cells. The fact that the Nbs could not be isolated with Ni-NTA purification further supports the conclusion that amber suppression was not successful and the amount endogenous suppression was low. Else this would have resulted in the presence of the His-tag. To confirm the production of Nbs against VCAM-1, as suggested by the SDS-analysis, Mass spectrometry of the protein sample could be performed, since this method allows identification of different proteins (34).

4.1.4 Results on Nbs against VCAM-1 from JX33 are inconclusive but could indicate the endogenous suppression

Analysis of Nbs against VCAM-1 from JX33 cells is inconclusive, yet suggests that there was no amber suppression. A Western Blot to identify the His-tag showed faint bands in the cytoplasmic extract from VCAM-1 in the presence and absence of PrK. These bands were below the 15 kDa range and were weak and smeared. Therefore it is not likely that these bands represent the Nb with the His-tag. However, the corresponding SDS-analysis showed that the lanes did not run straight and lanes were observed corresponding to the bands on the Western Blot. This made it more difficult to estimate the correct weight of the protein and the possibility that it is the Nb cannot be entirely excluded. Repeating the experiment can provide information on the exact weight of the bands. In the case that it is the Nb, the fact that the band was on both the sample with and without PrK suggests that the presence of the His-tag was caused by endogenous expression.

4.1.5 Bands observed on SDS analysis could indicate multimers of Nbs against VCAM-1 from SHuffle cells

The elution fractions from Ni-NTA purification from Shuffle cells expressing Nbs against VCAM-1 showed no band at the 15 kDa level. In line with the expression experiment from LOX-1 in JX33, bands were observed in the elution fractions at higher levels. In both the PrK+ and PrK- condition were observed at the same level in the elution fraction. If the bands indeed represent multimers of the Nb this would indicate that the His-tag was present in both conditions, meaning that there is a high degree of endogenous suppression but no amber suppression. The presence of multimers still needs to be confirmed by performing Western Blot targeting the His-tag. Although the presence of β-mercaptho-ethanol makes the occurrence of multimers less likely it cannot be excluded given other reports stating that multimers can occur in SDS-analysis despite the presence of β-mercaptho-ethanol (30, 31).

4.2 Future outlook

4.2.1 Detection of the His-tag using Western Blot to confirm endogenous suppression

So far, conditions tested did not show amber suppression in any of the conditions tested. The possibility that multimers can form in SDS-PAGE analysis despite the presence of β-mercaptho-ethanol could point to the presence of the His-tag in Nbs, in both the conditions were expression was induced in the presence and in the absence of PrK. If this is indeed the

case, it would indicate that endogenous suppression occurred. To confirm this, a Western Blot directed at the His-tag should be performed.

4.2.2 Investigating the presence of inclusion bodies

The presence of inclusion bodies should be investigated, especially for LOX-1 Nbs. Several protocols exist to solubilize and extract inclusion bodies, using Guanidine·HCl (8 M) and dithiothreitol (DTT) as solubilisation agents (35). Commercial alternatives for extraction of inclusion bodies are also available (36, 37).

4.2.3 Testing of other sequences and the use of TB medium should be performed

So far only amber suppression of LOX-1 in JX33 cells has been investigated in TB medium. Further analysis of Nbs against LOX-1 in WK6 or SHuffle cells is necessary, since the 15 kDa band was observed for VCAM-1 Nbs but not LOX-1 Nbs. Only VCAM_Pott_FA has been tested because this was expected to give the most efficient amber suppression. As amber suppression has not yet been achieved VCAM_TAG and RS^{wt} should be examined for successful suppression with these genetic variations.

4.2.4 Investigating a different vector for expression of pylRS/$tRNA^{pyl}$

Recently, a study on the crystal structure of pylRS revealed that PrK could be esterified in the active site of pylRS *in vitro (38)*. Kaya *et al.* could incorporate up to three PrK AAs into yellow fluorescent protein (YFP) in the BL21 *E. Coli* strain (39). Kaya *et al.* used the pET-Duet1 vector rather than the pEVOL vector, which was used in this study, for expression of pylRS/$tRNA^{pyl}$. Although pEVOL was reported to have better amber suppression efficiency with tyrRS than pET, no comparative study was found for amber suppression efficiency of pylRS with pEVOL compared to pET. Repeating experiments using pET-Duet1 as vector for pylRS/$tRNA^{pyl}$ might prove successful in this study.

4.2.5 Using Top10 and BL21 cells can be used for incorporation of uAA

The pEVOL vector was originally designed with the tyrosyl-RS/$tRNA^{tyr}$ rather than pylRS/$tRNA^{pyl}$. The use of pEVOL for $pylRS^{FA}$/$tRNA^{pyl}$ was reported to successfully incorporate a cyclooctyne-lysine in green fluorescent protein (GFP) using Top10 *E. Coli*, demonstrating the possibility to use pEVOL for pylRS/$tRNA^{pyl}$ (40). Since Top10 cells were used for successful amber suppression of GFP it is worthwhile to investigate expression of our Nbs in Top10 cells. Kaya et al. incorporated YFP in BL21 cells (39). Although SHuffle

cells are derived from BL21 cells, expression experiments have not yet been performed using BL21 in this study (27). Since BL21 cells were successful in incorporating PrK in YFP, repeating expression experiments in BL21 is recommended. In this study SHuffle cells were chosen above BL21 to minimize the occurrence of inclusion bodies. In SHuffle cells the oxidative environment in the cytoplasm and the presence of disulfide bond isomerise, should ensure correct disulfide bonds in these cells (27). Since BL21 cells do not possess disulfide bond isomerise the possibility of inclusion body formation should be taken into account.

4.2.6 Use of cyclooctyne-lysine as alternative 'click'-AA

Since the ultimate goal of this study is the oriented immobilization of Nb for improved sensor sensitivity, the use of alternative 'click'-amino acids should be considered. Since cyclooctyne-lysine was successfully incorporated using pylRS^{FA}/tRNA^{pyl}, expressed through pEVOL, this provides an interesting starting point (40). The use of cyclooctyne-lysine offers the additional advantage over PrK that SPAAC excludes the need for copper, making it suitable for living cells and *in vivo* testing as well.

5. Conclusions

Nbs against VCAM-1 but not LOX-1 could be created in WK6 and SHuffle cells. We have not shown successful incorporation of PrK using amber suppression using VCAM-1_POTT and pylRS^{FA}. Experiments will need to be repeated with VCAM-1-TAG and pylRS^{WT}. The use of a different vector for pylRS and using different 'click'-amino acids could prove successful. Since amber suppression has not been successful in this study, no conclusions can be made on the effect of sequence context on amber suppression efficiency.

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7. Supplemental

Contents

Supplemental table 1 Stock and buffer solutions	
1X PBS	140 mM PBS
	3 mM KCl
	10 mM Na2 PO4
	10 mM KH2 PO4
	pH 7.4
Toubin blotting buffer	25 mM TRIS
	192 mM glycine
	20% methanol
1X SDS-PAGE running buffer	25 mM TRIS
	200 mM glycine
	3.5 mM SDS
2X sample buffer	0.5 M TRIS-HCL pH 6.8
	4.4% SDS
	10 mg/ml bromophenol blue
	20 % v/v glycerol
	10 mM β-Mercapthoethanol
TBST	12.5 mM TRIS
	150 mM NaCl
	0.05% Tween 20
TES	30 mM TRIS
	1 mM EDTA
	0.05 M ethanolamine
Imidazole 1M stock solution (filtered)	3.4 g Imidazol
	50 ml Water

Supplemental table 2 Culture media	
2YT medium	16 g Bacto-tryptone
	10 g bacto yeast extract
	5 g NaCl
	Adjust volume to 1000 ml with MiliQ water
TB Medium	900 ml A + 100 ml B
A	13.33 g bacto-tryptone
	26.67 g bacto yeat extract
	4.44 ml 100% glycerol
	Adjust volume to 1000 ml with MiliQ water
B	2.31 g KH_2PO_4
	12.54 g K_2HPO_4
	Adjust volume to 100 ml with MiliQ water

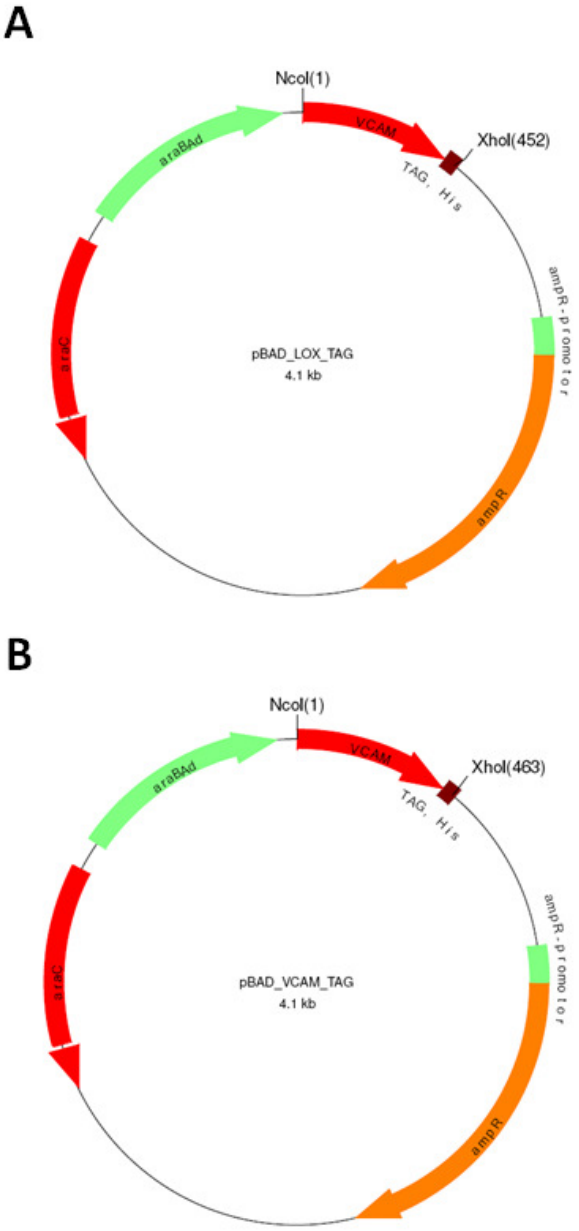

Figure S.1 Graphic representation of plasmids from vectors pBAD with **A)** LOX-1_TAG and **B)** VCAM-1_TAG

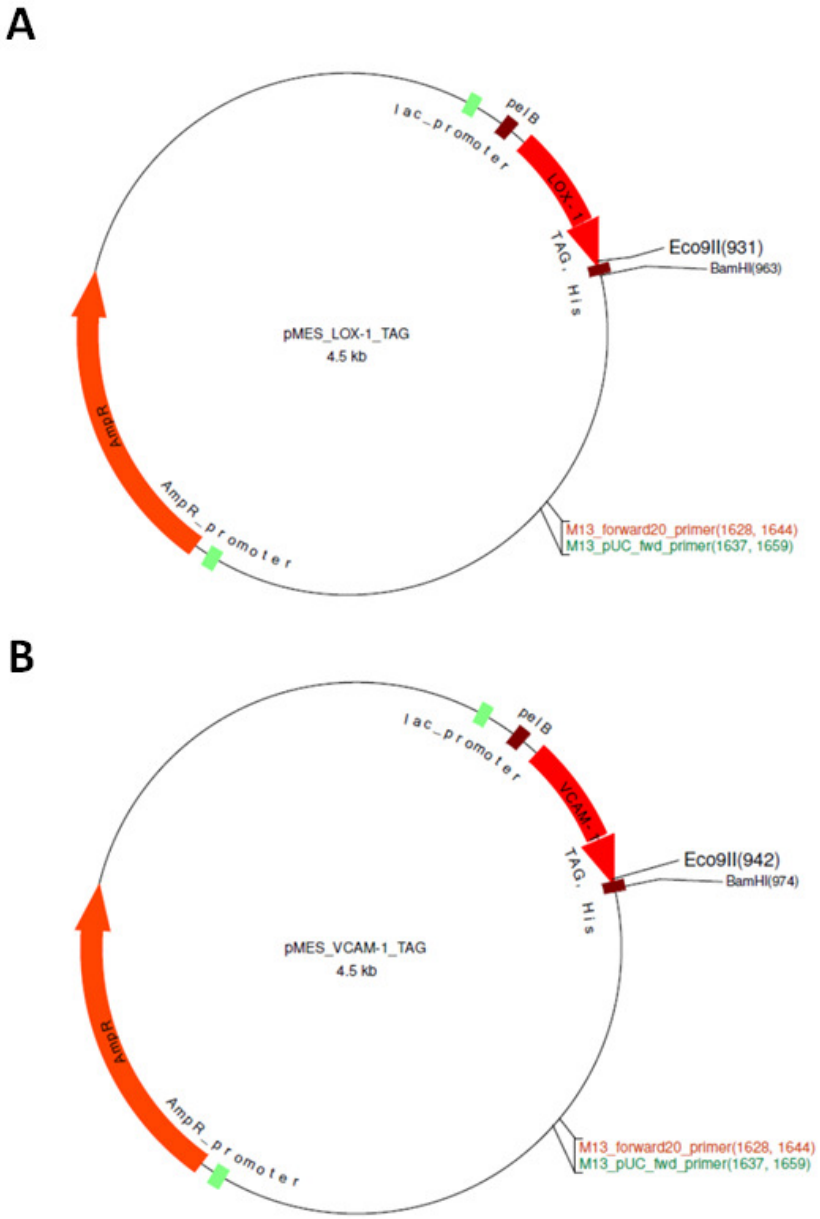

Figure S.2 Graphic representation of plasmids from vectors pMES4 **A)** wih LOX-1_TAG and **B)** VCAM-1_TAG

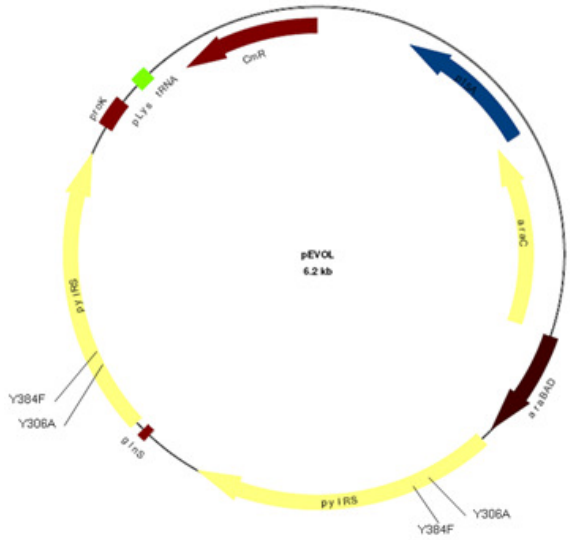

Figure S.3 Graphic representation of plasmid from pEVOL with pylRS^{FA} and tRNA^{pyl}

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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Modification of nanobodies with 'click' functionalities using the amber suppression technique

Richting: **master in de biomedische wetenschappen-bio-elektronica en nanotechnologie**
Jaar: **2015**

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Voor akkoord,

Huizing, Lennart

Datum: **9/06/2015**