GENERATION OF MCHERRYBODY: AN ANTI-TRANSFERRIN RECEPTOR ANTIBODY VARIABLE FRAGMENT LINKED BY THE MCHERRY FLUORESCENT PROTEIN

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Abstract

A facile generation of a recombinant antibody fragment with intrinsic fluorescent properties of the monomeric mCherry fluorescent protein is described. The so-called mCherryBody was designed based on the structure model of the variable fragment of anti-transferrin receptor antibody LUCA31 and the X-ray crystallographic structure of the mCherry protein. mCherryBody was constructed to retain optimal spatial geometry between the C- and N-termini of the antibody light-chain (V_L) and heavy-chain (V_H) by mimicking the domains interface pairing in antibody Fab fragments and incorporation of the mCherry fluorescent protein as a bridging scaffold. The gene encoding the chimeric protein was cloned into the pJExpress414 expression vector, expressed and secreted into the periplasm of *Escherichia coli* NiCo21(DE3) for assembly and disulphide bond formation. Based on its amino acid sequence, mCherryBody was predicted to have a molecular weight of 51.46 kDa. The modular assembly used in the generation of mCherryBody may permit the interchange of binding sites and of fluorescent proteins to create robust panels of coloured antibody fragments. Thus, the mCherryBody platform facilitates rapid generation of colored single-chain variable fragment (scFv) chimeras that could be used for screening of antibodies against cell surface markers or receptors.

Key words: antibody variable fragment, fluorescent protein, molecular design, mCherry, transferrin receptor

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Introduction

Fluorescently labeled antibodies are indispensable tools in almost every field of biomedical diagnosis and research, allowing the detection of antibodies, antigens and virtually any antigenic protein in cells and tissues. The fluorescence detection can be performed by staining with either direct (primary) or indirect (secondary) antibodies. Antibodies or antibody fragments could be labeled with the fluorophores or fluorescent proteins by chemical conjugation with particular amino acid residues on antibody molecule.

With the advance of recombinant DNA technology fluorescent proteins could also be fused to various heterologous proteins to produce stable fusion proteins that retain their original biological activity while at the same time retaining the fluorescent properties of the fluorescent protein. The major challenge in the generation of such fusion proteins may reside in the different folding requirements for antibodies and fluorescent proteins. Fluorescent proteins are known to fold correctly under the reducing conditions found in the cytoplasm of *Aequorea* and other species in which they have been recombinantly expressed (Feilmeier *et al*., 2000). On the other hand, antibodies, either full-length IgGs or antibody fragments, contain disulfide bonds, so that they require an oxidizing environment for their correct folding. The formation of disulfide bridge is enabled in the bacterial periplasm or in the endoplasmic reticulum (ER) lumen of eukaryotic cells.

Nevertheless, fusion proteins composed of single chain variable antibody fragments (scFv) and green fluorescent protein (GFP) have been purified under native conditions from the bacterial periplasm (Casey *et al*., 2000), from the bacterial cytoplasm (Schwalbach *et al*., 2000; Morino *et al*., 2001) or expressed as bacterial cytosolic inclusion bodies (Oelschlaeger *et al*., 2002).

To date many antibody structures have been elucidated by X-ray crystallography and the distance between the C-terminus on the variable heavy-chain (V_H) and the N-terminus on the variable light-chain (V_L) has been determined to be approximately 34 – 35 Å (Arndt *et al*., 1998). The common strategy to recombinantly assemble the V_H and V_L domains of the antibody variable fragments is by incorporation of a flexible linker of 20 – 30 amino acids between the two termini to generate the scFv's. However, assembly of a scFv fragment with such long flexible linker often resulted in aggregation, reduced efficacy or affinity and decreased stability relative to the corresponding Fab fragment or whole IgG. (Worn and Plücktun, 2001).

Fluorescent proteins have in common a very rigid β-barrel structure that can withstand fusions to either N- or C-termini (Hink *et al*., 2000) and extensive permutations to two of the exposed loops while retaining the optical fluorescent properties (Pavoor *et al*., 2009). Additionally, our examination on 3D structures of various fluorescent proteins revealed that the distance between the N- and C-termini is approximately between 20 – 30 Å. One of such fluorescent protein is mCherry that derives from red fluorescent protein isolated from *Discosoma sp.*, commonly known as mushroom anemone (Shaner *et al*., 2004), and could be expressed in the periplasm of *E. coli* (Aronson *et al*., 2001).

In this paper, we demonstrate the molecular design, design of expression vector, assembly and expression of a V_L-mCherry-V_H-His6-tag molecule, designated mCherryBody, where the monomeric fluorescent protein mCherry is inserted as a rigid linker between the V_L and V_H domains of an anti-transferrin receptor antibody LUCA31. Transferrin receptor levels are elevated in various types of cancer cells, including breast cancer, cervical cancer and prostate cancer cells, and correlate with the aggressive or proliferative ability of tumor cells. Therefore, transferrin receptor is a potential target for drug delivery in the therapy of malignant cell growth (Qian *et al*., 2002).

Materials and Methods

Molecular design and visualisation. The crystal structure of the fluorescent protein mCherry was downloaded from Protein Database (PBD entry: 2H5Q; Shu *et al*., 2006). The structure models of the V_H and V_L domains of the anti-TfR antibody variable fragment were predicted using Swiss-Model Workspace (Arnold *et al*., 2006). The variable domain of anti-TfR antibody was constructed based on the crystal structure of the V_H domain of the monoclonal antibody 3B5H10, which recognizes the conformation of polyglutamine in the protein huntingtin (PDB entry: 3S96; Peters-Libeu *et al*., 2012) and the V_L domain of the anti-meningococcal subtype P1.4 PorA antibody (PDB entry: 2BRR; Oomen *et al*., 2005). The sequence identity between the V_H domain of anti-TfR antibody and V_H domain of 3S96 is 88.1%, whereas the V_L domain of anti-TfR antibody shares an overall 84.1% sequence identity with 2BRR. Further modelling was performed using Swiss-PdbViewer software version 4.1 (Guex and Peitsch, 1997). The structure model of mCherryBody was viewed using UCSF-Chimera software version 1.10 (Pettersen *et al*., 2004).

Construction of the gene encoding the protein mCherryBody. The amino acid sequence of the variable heavy and light chain domains of the scFv antibody fragment was deduced from the amino acid sequence of the anti-TfR monoclonal antibody LUCA31. The amino acid sequence of mCherry was obtained from PDB Bank (PDB entry: 2H5Q) or UniProtKB (ID: Q5S3G8). Between the C-terminus of the V_L domain and the N-terminus of mCherry a peptide linker $(Gly_3Ser)_2$ was inserted, and between the C-terminus of mCherry and the N-terminus of the V_H domain, a peptide linker $Gly_3Thr–Gly_3Ser$ was inserted to covalently link the domains in the sequence V_L-mCherry-V_H. At the N-terminus of the fusion protein, a sequence encoding the signal peptide *pel*B was added for localization of the protein into bacterial periplasm during expression, whereas at the C-terminus a sequence encoding six histidines (His6), which facilitate protein purification, was incorporated.

The constructed amino acid sequence for mCherryBody was used as blueprint to design the synthetic gene encoding mCherryBody by using the software Gene Designer and DNA2.0 algorithms (http://www.dna20.com). The synthetic gene was designed to use the codon preference for *E. coli* as the host for gene expression (http://www.kazusa.or.jp/codon/). The gene encoding mCherryBody was synthesized by DNA2.0 (Menlo Park, CA), and cloned into the expression vector pJExpress 414 (T7 Promoter, Amp^R, pUC origin). The expression vector harboring the gene encoding mCherryBody was designated pJE-mCherryBody.

Expression of mCherryBody in *E. Coli.* To express the chimeric protein mCherryBody, *E. coli* NiCo21(DE3) (Novagen) cells were transformed with the pJE-mCherryBody plasmid and plated onto LB agar supplemented with ampicillin (100 μg/mL final concentration). The cells were allowed to grow at 25ºC for 18 h and the following day, a fresh colony was inoculated into 3 mL of LB media (with antibiotics) and grown at 25ºC (with shaking at 250 rpm) for 16 h. Afterward, 100 mL of pre-warmed LB media, prepared in 500 mL Erlenmeyer flasks (with antibiotics), was inoculated with 1 mL of the overnight culture and grown at 25ºC (with shaking at 250 rpm) until the optical density at 600 nm had reached 0.5, then isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was added to the culture to final concentration of 0.2 mM and the cells were grown at 25ºC for an additional 16 h with shaking at 250 rpm.

Isolation of mCherryBody from *E. coli* periplasm. Bacterial cell pellet from 100 mL culture was resuspended in 10 mL of periplasmic buffer (50 mM PBS (pH 7.5), 300 mM NaCl, and 10 mM imidazole) and supplemented to a final concentration of 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Afterward, lysozyme was added to a final concentration of 0.5 mg/mL. The cell suspension was incubated at room temperature for 1 h (Kusharyoto *et al*., 2002), followed by centrifugation at 6000 rpm for 10 min at 4ºC. The supernatant was collected to give the periplasmic fraction and stored at 4ºC.

Purification of mCherryBody by IMAC.. The recombinant protein was purified using 1 mL of TALON™ matrix (Clontech) packed in a PD-10 column (GE Healthcare) (Kusharyoto *et al*., 2002). First, the resin in the column was equilibrated with 5 column volumes of equilibration buffer (50 mM PBS (pH 7.5), 300 mM NaCl, and 10 mM imidazole). Afterward, 10 mL of *E. coli* periplasmic fraction was loaded onto the column and the column was washed with 5 column volumes of washing buffer (50 mM PBS (pH 7.5), 300 mM NaCl, and 20 mM imidazole). The protein was eluted with 5 column volumes of elution buffer (50 mM PBS (pH 7.5), 300 mM NaCl, and 300 mM imidazole) and collected in 1 mL aliquots. Expression and purification of mCherryBody were verified by SDS-PAGE and Western blot using HisDetector™ Ni-HRP Conjugate Western Blot Kit (KPL) according to manufacturer's protocol.

Results

Molecular Design. The structure model of the anti-TfR variable antibody fragment LUCA-31 reveals that the distance between the Ser114 residue at the C-terminus on the variable light chain (V_L) and the Asp1 residue at the N-terminus on the variable heavy chain (V_H) is approximately 34.8 Å. Based on the X-ray crystallographic structure of the protein mCherry (PDB entry: 2H5Q), the residues Met5 at the N-terminus and Thr223 at the C-terminus of mCherry were identified to have a similar distance between them of approximately 30.8 Å without intersecting the rest of the protein. Direct fusion of the V_L to the N-terminus and V_H to the C-terminus of mCherry would have resulted in a linkage 7 to 9 Å shorter than the optimal spacing revealed by X-ray crystallographic structures of different antibody fragments (data not shown). This was overcome by modelling the N- and C-termini to include a flexible $(Gly_3Ser)_2$ linker between V_L and the N-terminus and a $Gly_4Thr\text{-}Gly_3Ser$ linker between V_H and the C-terminus of mCherry (Fig. 1). With the addition of these amino acid linkers to both ends between the variable antibody domains and mCherry, the closest distance between both protein domains can readily be extended to approximately 13 Å apart in the same orientation. On the other hand, the shortest distance between the two flexible linkers was approximately 22 Å. The resulted model could accommodate the variable

domains of the antibody with a molecular geometry approach that determined from the X-ray studies.

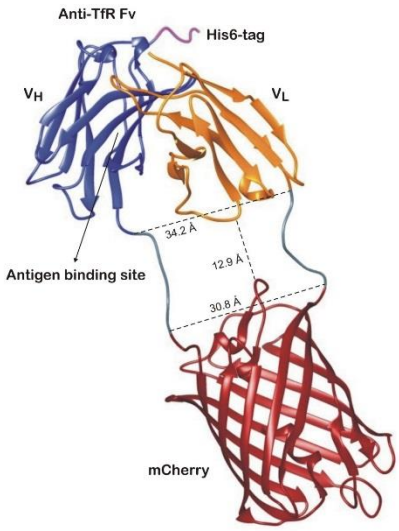

Figure 1: Ribbon representation of a 3D-structure model of mCherryBody with His6-tag (in violet) at the C-terminus. The V_H-chain of anti-TfR variable fragment (anti-TfR Fv) is shown blue, mCherry in red, V_L-chain in orange, and both linkers are shown in grey. All distances are indicated in angstrom (Å). The structure model is visualized using the program UCSF-Chimera

The predicted structure shows that the fluorescent protein mCherry would not interfere with the anti-TfR-Fv binding site which is fully available to contact the target ligand. This precise spatial geometry would permit the linkage of mCherry or any of its derivatives between the antibody V_H/V_L or V_L/V_H chains resulting in functional scFv fluorophore fusions.

Design of the Synthetic Gene. The gene encoding the chimeric protein V_L−mCherry−V_H was synthesized and cloned into the pJExpress414 expression vector. The synthetic gene was designed to include the *pel*B signal sequence to direct the expression of the protein into the periplasm of *E. coli*. As shown in the schematic representation of the synthetic gene (Fig. 2), the gene encoding the V_L domain of the antibody fragment is flanked by the restriction sites *Nco*I and *Bam*HI, while the V_H domain is flanked by *Kpn*I and *Xho*I, respectively. Using these restriction sites, genes encoding V_L and V_H domain of any kind of antibody variable fragments could be inserted into the plasmid without any changes in their DNA sequences, thus enabling generation of a fusion between any antibody variable fragments and mCherry as fluorophore. Different orientation of the variable fragment in the fusion protein, for instance V_H−mCherry−V_L, could also be chosen.

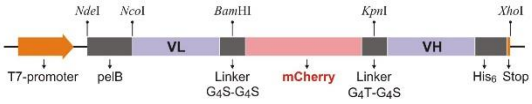

Figure 2: Schematic presentation of the expression cassette pJE-mCherryBody which has a T7 promoter to initiate transcription using T7 RNA polymerase, pelB sequence to translocate newly synthesized molecule into the *E. coli* periplasm. VL is the gene encoding the variable light chain of anti-TfR antibody LUCA31, mCherry is the gene of the fluorescent protein and VH is the gene for the variable heavy chain of anti-TfR antibody LUCA31. A sequence encoding His6-tag for purification by IMAC is added before stop-codon.

The restriction sites *Bam*HI and *Kpn*I could be used to insert a gene encoding any fluorescent protein other than mCherry. However, identification of appropriate amino acid residues at the N- and C-termini of the fluorescent protein should be carried out in such, that the N- and C- termini should protrude from the same plane with spacing of 20-30 Å apart and without intersecting the rest of the protein. In case that any other signal sequence will be used to translocate the protein expression into the *E. coli* periplasm, substitution of the *Nco*I site might be necessary, thus, the V_L or V_H domain could be inserted between the restriction site of choice and *Bam*HI.

Expression and Purification of mCherryBody. The expression of the recombinant protein mCherryBody took place in *E.coli* NiCo21(DE3) strain after induction with IPTG. Interestingly, during expression of mCherryBody, the bacterial cells turned their color milky white to cherry red, which might indicated that the protein was generated in *E. coli* cells and the fluorescent properties of mCherry was not compromised (Fig. 3 A). Further examination under microscope confirmed that the cells exhibited the characteristic red color (Fig. 3 B). Unfortunately, we were not able to observe and determine, whether the protein was in fact expressed in the periplasmic space of *E. coli*, although a signal peptide for localization into the periplasm was included in the design of the expression vector.

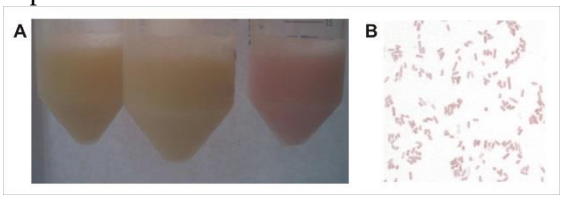

Figure 3: Expression of mCherryBody in *E. coli* NiCo21(DE3) cultivated at 25ºC. (A) Bacterial cells, which do not harbour the expression vector, cells before induction and red colored cells after induction with IPTG (from left to right). (B) Examination of the cells under microscope after induction with IPTG

The mCherrybody protein was recovered from the periplasmic extract and purified *via* IMAC on Co^{2+}-bound TALON™ matrix. Protein expression and purification processes were monitored by SDS–PAGE and Western blot analysis. Upon verification by SDS-PAGE, mCherryBody appeared as a single band at around 52 kDa with relatively high purity, which corresponded to the calculated molecular weight of mCherryBody with additional C-terminal His6-tag (Fig. 4). On SDS-PAGE gel, another protein band was also detected, corresponding to the host histidin- rich protein SlyD-CBD commonly co-purified by IMAC (Robichon *et al*., 2011). Using the bicinchoninic acid assay, the concentration of the recovered mCherryBody protein was determined to be approximately 0.8 mg/L *E.coli* culture (data not shown), which is rather low compared to the expression of other fusion proteins composed of a scFv fragment and a fluorescent protein (Morino *et al*., 2001; Oelschlaeger *et al*., 2002; Pavoor *et al*., 2009).

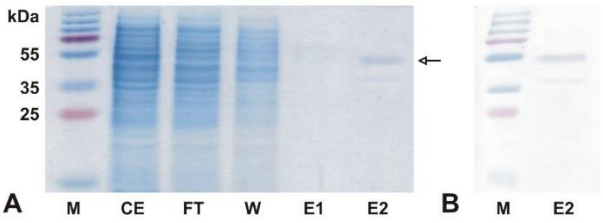

Figure 4: Verification of expression and purification of mCherryBody. (A) SDS-PAGE analysis of mCherryBody isolated from the periplasmic space of *E. coli* NiCo21(DE3). Lane M: protein marker; lane CE: periplasmic extract of *E. coli* NiCo21(DE3); lane FT: flow through of column containing Talon™ resin; lane W: fraction from washing steps; lane E1–E2: fractions from elution steps of purified mCherryBody. (B) Western blot analysis of the purified mCherryBody from elution fraction E2.

Discussion

Antibody molecules have been tagged with various fluorophores for their use as highly sensitive tools in imaging and for cell sorting. Commonly, antibodies are chemically conjugated *in vitro* to the fluorophores, such as FITC, CY-3, and CY-5. Such chemical conjugation is dependent on the availability of particular amino acid side chains in the antibody molecule. However, inappropriate conjugation in or in close proximity of the binding site can reduce the binding efficacy of the parental antibody. Besides, it is difficult to control the number of labels incorporated into each antibody molecule during the preparation that limits the utility of these reagents in quantitative analysis.

With the application of recombinant DNA technology, scFvs have been engineered to incorporate a free cysteine at the C-terminus for specific conjugation to other molecules or have been fused directly to fluorescent proteins for prokaryotic (Schwalbach *et al*., 2000; Morino *et al*., 2001, Kusharyoto *et al*., 2015) or eukaryotic expression (Peipp *et al*., 2004). However, conventional scFv's are not ideally suited for these applications. The major drawback of scFv fragments is that they are not as stable compared to whole antibody or Fab fragment (Worn and Pluckthun, 2001). Moreover, scFv fragments tend to aggregate or to form a mixture of multimers which may alter their binding kinetics (Holliger *et al*., 1993).

Despite these issues the concept of having a genetically encoded affinity label is very appealing, since it provides a simple platform for creating uniform diagnostic reagents. An alternative approach to circumvent the problem has been developed by modifying a fluorescent protein to exhibit two extended loops that can form recognition motifs while retaining the fluorophore properties (Pavoor *et al*., 2009). Antibody variable fragments may represent the best recognition motif, since their binding site is composed of V_H and V_L domains with a total of six loops with an extremely high combinatorially diverse CDRH3 and CDRL3 from natural and synthetic repertoires. Thus, antibody variable fragments could provide a vast range of high affinity binding sites (Lerner, 2006).

An attempt to combine the best features of both the antibody binding sites and the properties of encoded fluorophores in a single molecule has been made previously by inserting an enhanced green fluorescent protein (EGFP) between the V_H and V_L domains to spatially orientate the V_H/V_L interfaces to mimic the pairing observed in Fab structures (Yang *et al*., 1996). The resulted molecule did not bind to the target antigen, which might be due to fact that EGFP could exist as a dimer and its assembly could sterically hinder V_H/V_L domain pairing. Such obstacle could be overcome by using a fluorescent protein with β-barrel structures, which could be predominantly expressed as monomeric protein exemplified by mCherry (Campbell *et al*., 2002).

The constructed expression vector encoding mCherryBody in our study is based on the pJE-series as shown in Fig. 2. Although the use of the T7 promoter and *E. coli* NiCo21(DE3) host might enable overexpression of the recombinant protein, it is possible that the majority of the recombinant protein retained within the cytoplasmic space of *E coli* despite having a *pel*B leader sequence to direct the polypeptide into the bacterial periplasm. The recombinant mCherryBody itself need to be expressed in the periplasmic space since the formation of intramolecular disulfide bonds within the V_H and V_L domains requires an oxidizing environment provided by the bacterial periplasm. In such case, the amino acid sequence of the antibody variable fragment will limit the expression level of the recombinant protein, since mCherry could be expressed predominantly in the periplasm of *E. coli* (Aronson *et al*., 2011). Previously, we had been shown that the antibody scFv fragment of LUCA31 could be expressed only in limited amount in the periplasm of *E. coli* (Andriani *et al*., 2012). Therefore, the rather low expression yield of mCherryBody might be due to the difficulties in the expression of the variable fragment of LUCA31, which could not be compensated by the fusion with mCherry.

The protein mCherryBody could be expressed and purified from the bacterial periplasm in a sufficient quantity and purity, and the cells producing mCherrybody exhibited the observable intrinsic red color of mCherry, which may indicated that the fusion with antibody variable fragment of LUCA31 did not compromise the fluorescent properties of mCherry. However, the function and binding properties of mCherryBody still need to be verified by additional experiments using its target, transferrin receptor, or cells expressing it. The binding properties of mCherryBody depend on the binding affinity of the variable fragment of LUCA31, which, as monoclonal antibody, is able to bind to transferrin receptor

expressed on the surface of different cancer cell lines (Mather *et al*., 2009). Nevertheless, our platform could be applied to existing antibody and fluorescent protein repertoires to create next generation of diagnostic and cell sorting molecules. It may also be used to establish libraries of V_H and V_L domains that may readily be accessed using non-isotopic screening as an alternative to other display selection technologies.

Conclusion

The assembly of the CherryBody by incorporating the fluorophore as a bridge introduces the following properties: firstly, with an integrated fluorophore, a single reagent has been created that enables reduction in production time and cost. Secondly, the antibody binding site and fluorophore are stoichiometric, and thus the signal generated would be directly proportional to the amount of antibody bound. Thus, the mCherryBody platform could facilitate rapid generation of colored single-chain variable fragment (scFv) chimeras that could be used for screening of antibodies against cell surface markers or receptors.

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