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Nanobody production can be simplified by direct secretion from *Escherichia coli*

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ABSTRACT

It is well known that *camelids* (camels and llamas) have fully functional antibodies with only a heavy chain consisting of a single variable domain and two constant domains. This single variable domain is called a "nanobody" and many nanobodies are synthesized in the cytosol of *Escherichia coli,* however, most of the nanobodies become inclusion bodies without tags to enhance their solubility. We generated a vector system to enable the secretary expression of nanobodies in *Escherichia coli*. In this system, several NBs were secreted into the culture supernatant. Since the vector contained 6xHis tag and AviTAG, biotinylation (even fluorescent-labeled) of AviTAG was achieved during cell culture, and purification of the supernatant was a step by immobilized metal ion adsorption chromatography. The procedure described in this study is believed to be as simple as regular plasmid minipreps. Therefore, many laboratories can use this method.

1. Introduction

It is well known that *camelids* (camels and llamas) have fully functional antibodies with only a heavy chain consisting of a single variable domain and two constant domains [1]. This single variable domain was well expressed in the cytosol of *Escherichia coli* (*E. coli*) [2], and this structure is called "nanobody (NB)" [3]. NBs are expressed in the cytosol of *E. coli* but usually become inclusion bodies. Although the addition of various tags improves solubility [4,5], the purification process usually takes time to remove large amounts of endogenous proteins and the tags.

NBs have been used as a substitute for conventional antibodies for cell and molecular biology and as a candidate for future therapeutics. Furthermore, they are used in particular for super-resolution imaging because of their smaller size [6,7]. Thus, a simple soluble secretion expression system is desired. In this study, we described a new system to enable secretion of NBs *in E. coli*.

2. Materials and methods

2.1. Construction of pMAK461, pMAK462, pMAK463, and pMAK464

A parental vector "pMal-T-Avi-His/BirA" was kindly gifted by Dr. Tonia Rex *via* Addgene (Addgene plasmid # 102962; http://n2t.net/addgene:102962). This vector encodes the sequences of maltose binding protein (MBP), multi-cloning site (MCS), and AviTag to express designated recombinant protein fused to MBP at the C-terminus and AviTAG at the N-terminus, and also encodes the sequence of *birA* (Biotin ligase) driven by T7 promoter to biotinylate AviTAG. In the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) and biotin, *E. coli* generate MBP/biotinylated fusion protein. This vector was digested by *Mfe*I (NEB Japan, Tokyo, Japan) and *Eco*RI (NEB Japan) to remove the sequences of MBP, AviTAG, and 6 × His tag with two *Bam*HI sites (backbone A) (Fig. 1A). All polymerase chain reactions (PCR) in this study were carried out using PrimeSTAR® HS DNA Polymerase (TAKARA BIO, Otsu, Japan) according to manufacturer's instruction. pMAL-p5x (NEB Japan) was used for a PCR using two primers: pMAK461F1; 5′- TTTTCACGAG*CAATTG*ACCAACAAGGACC (The italic part; *Mfe*I recognition site) and pMAK461R1; 5′- GCACCTGGGATCCG ATTTTGGCGAGAGCCGAGG (fragment A) (Fig. 1B). pTP1183 was kindly gifted by Dr. Dirk Görlich *via* Addgene (Addgene plasmid # 104163; http://n2t.net/addgene:104163) [5]. This vector encodes the sequence of nanobody to recognize rabbit IgG-Fc (hereafter referred as $NB_{\alpha Rb\text{-}IgG\text{-}Fc}$), and was used for a PCR using two primers: pMAK461F2; 5′-CCAAAATC*GGATCC*CAGGTGCAATTGGTAGAGTCTG (The italic part; *Bam*HI recognition site) and pMAK461R2; 5′- GTGCCATTCGATT TTCTGAGCTTCGAAGATGTCGTTCAGTCC*GATATC*TCCACCGGAGGAG ACTGTGAC (The underlined part; AviTAG, the italic part; *Eco*RV recognition site). This amplicon was further amplified by a PCR using two primers: pMAK461F2 and pMAK461R3; 5′- CCTTGTAATC*GAATTC*TT

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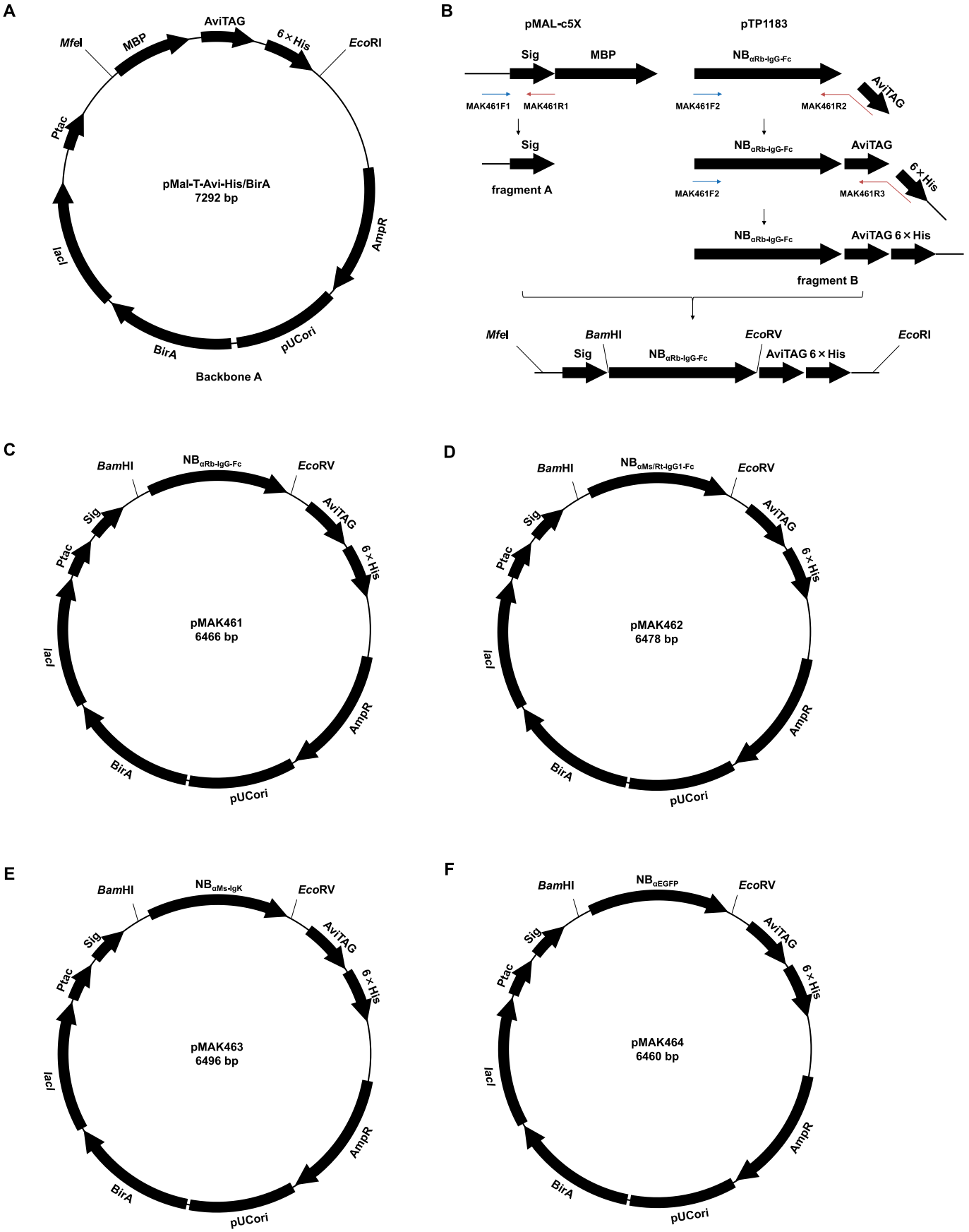

Fig. 1. (A) Map of pMAL-T-Avi-His/BirA. *Mef*I and *Eco*RI were used for cloning. (B) Preparation of insert to pMAL-T-Avi-His/BirA. (C) Map of pMAK461. (D) Map of pMAK462. (E) Map of pMAK463. (F) Map of pMAK464. MBP; Maltose Binding Protein, Sig; Signal peptide of MBP, Ptac; *tac* promoter, *lacI*; *lac* repressor, BirA; *E. coli* biotin protein ligase, pUCori; pUC origin of replication, AviTAG; AviTAG for specific biotinylation by BirA, 6 × His; 6 × histidine tag, and AmpR; ampicillin resistant marker.

AGTGGTGATGATGGTGATGGGAGCCGTGCCATTCGATTTTCTGAGC TTC (The italic part: *Eco*RI recognition site, the underlined part; 6 × His tag) (fragment B) (Fig. 1B). The fragments A and B were subjected to an overlap and extension PCR using two primers: pMAK461F1 and pMAK461R3. The resulted amplicon was fused to the backbone A using InFusion cloning kit (TAKARA BIO) according to manufacturer's instruction, and the resulted plasmid was pMAK461 (Fig. 1C).

pTP1112 and pTP1174, also gifted by Dr. Dirk Görlich *via* Addgene (Addgene plasmid # 104158; http://n2t.net/addgene:104158, and Addgene plasmid # 104162; http://n2t.net/addgene:104162), encode the sequence of nanobody to recognize mouse/rat IgG1-Fc and mouse kappa chain, respectively (hereafter referred as $NB_{\alpha Ms/Rt\text{-}IgG1\text{-}Fc}$ and $NB_{\alpha Ms\text{-}IgK}$). These vectors were used for PCRs using two primers: pMAK462F; 5′-CGCCAAAATC*GGATCC*CAGGTGCAATTGGTGGAG (The italic part; *Bam*HI recognition site) and pMAK462R; 5′- GTCGTTCAGT CC*GATATC*TCCACCGGAGGAGACTGTGACC (The italic part; *Eco*RV recognition site). Each amplicon was fused to pMAK461 digested by *Bam*HI (NEB Japan) and *Eco*RV (NEB Japan) using InFusion cloning kit (TAKARA BIO). The resulted plasmids were pMAK462 and pMAK463 (Fig. 1D–E).

pGEX6P1-GFP-nanobody, which encodes the nanobody recognizes enhanced green fluorescent protein (EGFP), was kindly gifted by Dr. Kazuhisa Nakayama directly (also abailable from Addgene plasmid # 61838; http://www.addgene.org/61838). This vector was used for PCR using two primers: pMAK464F; 5′-CGCCAAAATC*GGATCC*CAGGTGCA ATTGGTAGAGTCTG (The italic part; *Bam*HI recognition site) and pMAK464R; 5′- GTCGTTCAGTCC*GATATC*TCCACCGGAGGAGACTGT GAC (The italic part; *Eco*RV recognition site). The amplicon was fused to pMAK461 digested by *Bam*HI and *Eco*RV using InFusion cloning kit (TAKARA BIO). The resulted plasmids were pMAK464 (Fig. 1F).

2.2. Transformation of pMAK461, pMAK462, pMAK463, and pMAK464, and induction of NBs

These plasmids were individually transformed to SHuffle T7 Express competent *E. coli* (NEB Japan). The transformed cells were individually seeded onto a LB/Agar plate containing 100 μg/mL ampicillin. A single colony from each plate was picked up, and was transferred to 5 mL LB containing 100 μg/mL ampicillin. The cells were grown at 37 °C for 16 h.

The culture (150 μL) was diluted with 15 mL LB containing 100 μg/mL ampicillin. After 3 h of growth at 37 °C, 10 μL of 40 mg/mL IPTG (Nacalai Tesque, Kyoto, Japan) and 300 μL of 2.5 mM biotin (Fujifilm, Osaka, Japan) dissolved in 10 mM Bicine, pH 8.3 (Dojindo, Kumamoto, Japan) or 15 μL of 50 mM biotin dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Japan, Tokyo, Japan) were added to the culture to induce the protein expression. For fluorescent labeling, 50 mM biotin-4-fluorescein (AAT Bioquest, CA, USA) dissolved in DMSO was used instead of 2.5 mM biotin. The induction was carried out for 4 h at 30 °C and for 16 h at 25 °C. After the induction, the culture was centrifuged at 2330 *g* and 4 °C for 10 m. The supernatant was collected for further analysis.

2.3. Coomassie brilliant blue (CBB) stain and Western blot (WB) for recombinant NBs

The culture supernatants (5 μl) were electrophoresed on several sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). One gel was used for CBB stains with Bullet CBB Stain One (Nacalai Tesque) according to manufacturer's instruction. The other gels were used to transfer the recombinant NBs to PVDF membranes (Immobilon Western, Nihon Millipore, Japan) for WB. Four PVDF membranes were exposed to a horse radish peroxidase (HRP) conjugated polyclonal rabbit IgG (Bioss Antibodies Inc, Woburn, Massachusetts, USA), a HRP-conjugated polyclonal mouse IgG (Bioss Antibodies Inc), a HRP-conjugated polyclonal rat IgG (Bioss Antibodies Inc), or a HRP-conjugated streptavidin (SA) (Cell Signaling Technology Japan, Japan). One PVDF membrane was exposed to a polyclonal rabbit anti 6 × His TAG antibody (Medical and Biological Laboratories, Nagoya, Japan) and a HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology Japan). All membranes were visualized with a SuperSignal West Pico kit (Thermo Fisher Scientific, Japan) according to manufacturer's instructions.

2.4. Purification of recombinant NBs

To perform immobilized metal ion adsorption chromatography (IMAC), 250 μL of PureCube Ni-NTA Mag Beads (Cube Biotech, Monheim Germany) was poured into a 1.5 mL tube with a magnetic stand. The preserving solution was decanted, and then the beads were soaked with 1.5 mL of 20 mM Tris-HCl (pH 8.0)/500 mM NaCl/20 mM imidazole (hereafter referred as His-W/B buffer). The tube was gently rotated at room temperature for 5 m, and then it was set on the magnetic stand and the supernatant was decanted. The culture supernatant (15 mL) was mixed with 25 μL of 5 N NaOH and 1.7 mL of 5 M NaCl, and then the beads were added in a 50 mL tube. The tube was gently rotated at 4 °C for 16 h, and then it was set on a larger magnetic stand. The supernatant was decanted, 5 mL the His-W/B buffer was added, and then the tube was rotated at 4 °C for 5 m. After repeating this washing step, the supernatant was decanted, and then 1 mL 20 mM Tris-HCl (pH 8.0)/500 mM NaCl/500 mM imidazole (hereafter referred as His-E buffer) was added. After rotating the tube at room temperature for 5 m, the tube was set on the larger magnetic stand, and then the recombinant NB was eluted. The protein concentrations of eluted NBs were estimated by a densitometry.

3. Results

3.1. Construction of pMAK461, pAMK462, pMAK463, and pMAK464

We were able to successfully assemble these vectors (Fig. 1). All of the vital parts, such as the coding sequences for signal peptide of MBP, AviTAG, 6 × His tag, and NBs were verified by DNA sequencing (data not shown).

3.2. CBB stain and WBs for recombinant NBs

The expected size of $NB_{\alpha Rb\text{-}IgG\text{-}Fc}$ (pMAK461), $NB_{\alpha Ms/Rt\text{-}IgG1\text{-}Fc}$ (pMAK462), $NB_{\alpha Ms\text{-}IgK}$ (pMAK463), and $NB_{\alpha EGFP}$ (pMAK464) are 16.1 kDa, 16.4 kDa, 17.1 kDa, and 16.1 kDa, respectively. The CBB stain revealed that positive bands were observed in the sizes which was corresponding to the expected size of NBs (Fig. 2A). WB using a polyclonal rabbit anti 6 × His Tag followed by a HRP-conjugated anti rabbit IgG revealed that these bands were positive for 6 × His TAG (Fig. 2B). Also, WB using a HRP-conjugated SA revealed that these bands were positive for SA (Fig. 2C). WB using a HRP-conjugated polyclonal rabbit IgG revealed that only $NB_{\alpha Rb\text{-}IgG\text{-}Fc}$ was positive (Fig. 2D). WB using a HRP-conjugated polyclonal mouse IgG revealed that $NB_{\alpha Ms/Rt\text{-}IgG1\text{-}Fc}$ and $NB_{\alpha Ms\text{-}IgK}$ were positive (Fig. 2E). WB using a HRP-conjugated polyclonal rat IgG revealed that only $NB_{\alpha Ms\text{-}IgK}$ was positive (Fig. 2F). No positive stains were observed using these antibodies for $NB_{\alpha EGFP}$ as expected (Fig. 2D–F).

3.3. Purification of recombinant NBs

After being alkalified the culture supernatants with appropriate amounts of NaOH and increasing the concentration of NaCl, nickel magnetic beads saturated with the washing/binding buffer were directly mixed. After binding the recombinant NBs, the beads were washed with the washing/binding buffer, and then the recombinant NBs were eluted with the elution buffer. As shown in Fig. 3, each NB was

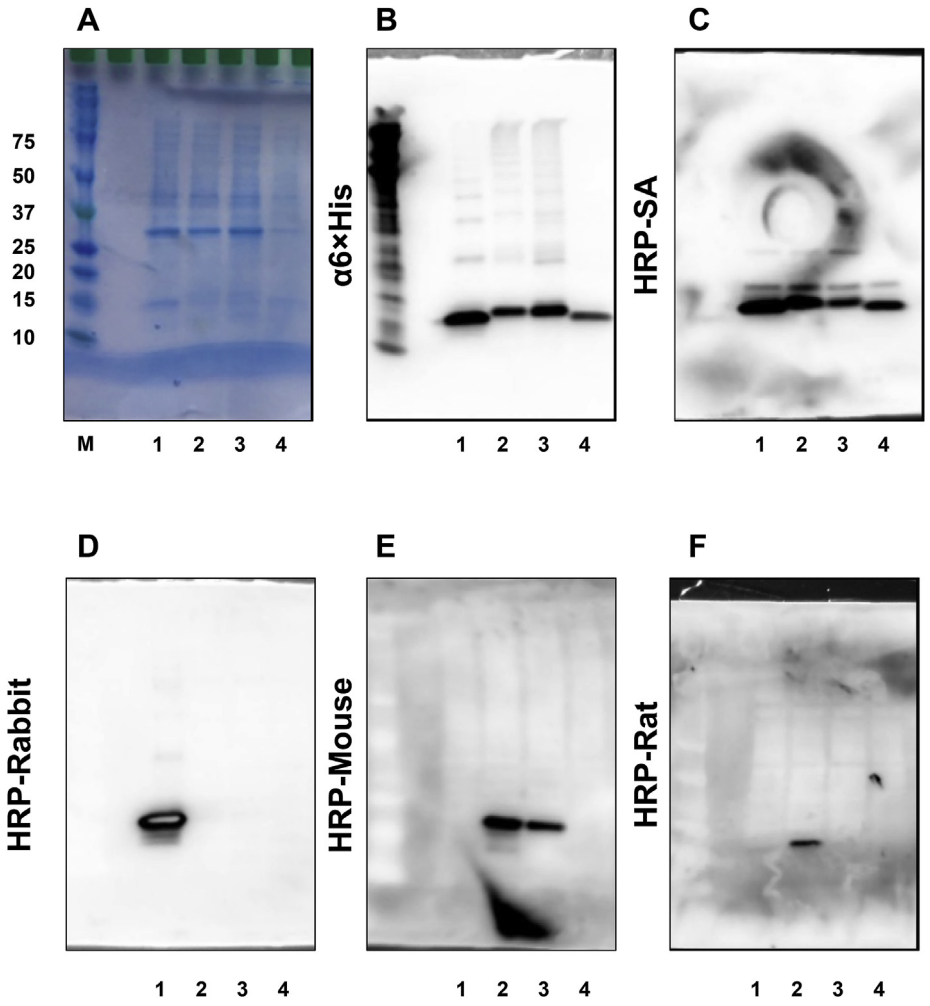

Fig. 2. (A) CBB stain. M; molecular marker. Left numbers were molecular weights (kDa). 1; $NB_{\alpha Rb\text{-}IgG\text{-}Fc}$ (pMAK461), 2; $NB_{\alpha Ms/Rt\text{-}IgG1\text{-}Fc}$ (pMAK462), 3; $NB_{\alpha Ms\text{-}IgK}$2; (pMAK463), 4; $NB_{\alpha EGFP}$ (pMAK464). (B) WB of 6 × His tag. (C) WB of SA. (D) WB of rabbit IgG. (E) WB of mouse IgG. (F) WB of rat IgG.

eluted as a single band. The concentration of each NB was assessed by comparing bands of serial concentrations of bovine serum albumin with densitometry. The range of concentrations of NBs was approximately between 25 and 200 μg/mL. Since the volume of culture supernatant was 15 mL and the volume of the elution was 1 mL, the yield will be around 1.67–13.3 mg if 1 L media are used although the conditions were not optimized yet.

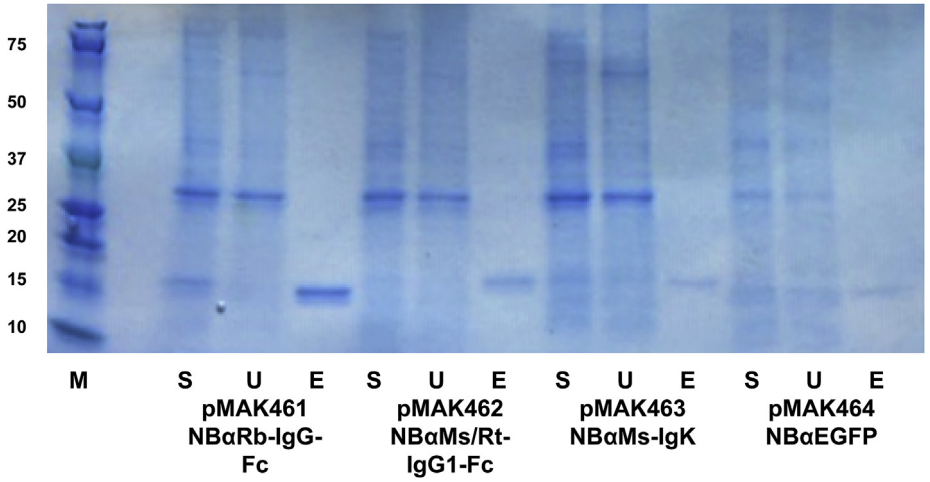

Fig. 3. CBB stain during the purification of NBs. M; molecular marker. Left numbers were molecular weights (kDa). S; the supernatants, U; unbound fractions, and E; the elution fractions.

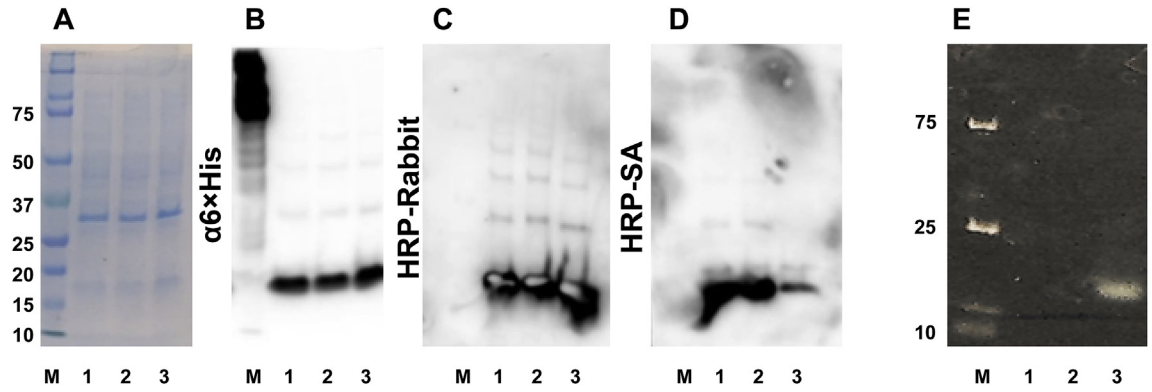

Fig. 4. (A) CBB stain of $NB_{\alpha Rb\text{-}IgG\text{-}Fc}$ (pMAK461) biotinylated by different biotin stock solutions. (1) 2.5 mM biotin dissolved in 10 mM Bicine pH 8.3, (2) 50 mM biotin dissolved in DMSO, and (3) 50 mM biotin-fluorescein dissolved in DMSO. M; molecular marker. Left numbers were molecular weights (kDa). (B) WB of 6 × His tag. (C) WB of rabbit. (D) WB of SA. (E) UV detection.

3.4. Biotinylation of recombinant NBs in E. coli

The AviTAG fused to the C-terminus of the NBs was efficiently biotinylated by *birA* (Fig. 4). Usually, such biotinylation was carried out by supplementation of biotin dissolved in Bicine. However, its solubility is not high, so a relatively large amount of biotin-containing solution must be added to the culture. If DMSO is used instead of Bicine, biotin can be dissolved at a higher concentration. Biotin dissolved in DMSO was successfully used in this system. In addition, fluorescently labeled biotin such as biotin-4-fluorescein is usually more difficult to dissolve in Bicine, however, DMSO is useful to dissolve it, which can be also used in this system.

4. Discussion

Several types of cells have been used to synthesize NBs. Among them, *E. coli* is often used because of its cost-effectiveness. An appropriate disulfide bond is believed to be necessary for almost all NBs to maintain a stable conformation. Periplasm of *E. coli* is maintained in the oxidized state and its cytoplasm is maintained in the reduced state. Thus, although NBs should be expressed in the periplasm, periplasm in *E. coli* is spatially restricted and production of NBs is limited. NEB sells SHffle *E. coli*, which allows disulfide bonding in the cytoplasm. Although many types of NB were highly expressed in the cytoplasm, most of them were shown as inclusion bodies. Several tags such as MBP or glutathione S-transferase (GST) are used to enhance solubility [4,5], thus, NB is usually expressed with one of these tags to enhance solubility, and these tags are used for affinity purification [4,5]. However, these tags are much larger than NBs, and removal of them are necessary in some cases.

We recently obtained information on detergents released from Sanyo Chemical (Kyoto, Japan) (The original paper was only written in a Japanese journal; KAGAKU to SEIBUTSU 52.No.3,2014, pp 184–188, and well summarized in their web site in English, https://www.sanyo-chemical.co.jp/eng/topics/2014/02/development-of-high-efficient-new-production-process-for-proteins.html). They have developed detergents that act to make the extracellular membrane of *E. coli* more permeable without reducing cell viability. Thus, the detergents can be used to continuously release periplasmic proteins towards the outside of the cell. It was thought that limited spatial problems in periplasmic expression of NBs could be avoided with detergent since NBs can be released continuously into the culture supernatant. To test this, we made pMAK461-464 and transformed them into SHuffle T7 Express competent *E. coli*. It was not expected that NBs would be observed in the supernatant without detergent after induction, but a number of NBs were observed.

A number of methods have been developed to purify recombinant proteins. Among them, His tag affinity purification is one of the most widely used [8]. A small His tag (usually 6 histidines) can be fused to either the N- terminus or C-terminus of the target protein. The His tag is captured by a commercially available resin bound nickel or cobalt ion, and the His tag fusion protein is easily eluted from the resin with imidazole. Since it usually does not interfere with the function of the target protein, removal of the His tag is not required. It is well known that Ni-NTA resin (to a lesser extent Co-NTA resin) retains some of the endogenous proteins extracted from *E. coli* lysates. Among them, ArnA (74.3 kDa) and SlyD (20.9 kDa) are the major contaminants that make His tag affinity purification difficult with *E. coli* lysates. Impurities are generally eliminated using a secondary chromatographic technique, or by expressing the recombinant protein in a *slyA* and *arnA* modified *E. coli* strain [9]. Affinity purification with the secondary tags is usually more expensive and inefficient compared to His tag purification. In our method, secreted NBs with a 6xHis tag at the C-terminus were easily purified by one-step IMAC using Ni-NTA.

Biotin stock solutions are usually prepared with 2.5–5 mM in 10 mM Bicine, pH 8.3, due to their low solubility. In this study, biotin or biotin-4-fluorescein were dissolved in DMSO and the concentration was adjusted to 50 mM. The NBs were uniformly biotinylated by using these stock solutions. These stock solutions can be stored as liquids at −20 °C and were proven to function as conventional stock solutions. Furthermore, direct labeling of NBs using biotin bound fluorophores would be beneficial to bio-imaging analyses.

The procedure described in this study is believed to be as simple as regular plasmid minipreps. Therefore, many laboratories can use this method. The vectors pMAK461-464 will be available from Riken Bio resource center (http://www.brc.riken.jp/lab/dna/) and Addgene (https://www.addgene.org/).

Authorships

T. I. planned this study. T.I. and K.H. performed the experiments. T.I. and K.U. analyzed the data and wrote the paper.

CRediT authorship contribution statement

Takayuki Iwaki: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Kimiko Hara:** Validation, Investigation. **Kazuo Umemura:** Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

We have no conflicts of interest to be declared. Also, we have no

commercial relationships with Addgene or Riken.

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