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Expression and Purification of a Bispecific Antibody against CD16 and Hemagglutinin Neuraminidase (HN) in *E. Coli* for Cancer Immunotherapy

Mina Bahrololoumi Shapourabadi[1], Farzin Roohvand[1], Arash Arashkia[1], Nasir Mohajel[1], Shahriyar Abdoli[1], Zahra Shahosseini[1,2], Frank Momburg[3], Mostafa Jarahian[3], Mohsen Abolhassani[4], Kayhan Azadmanesh*[1]

Abstract

Background: Immunotherapy of cancer by bispecific antibodies (bsAb) is an attractive approach for retargeting immune effector cells including natural killer (NK) cells to the tumor if the proper expression and purification of the bsAb for such applications could be addressed. Herein, we describe *E. coli* expression of a recombinant bsAb (bsHN-CD16) recognizing NK-CD16 and hemagglutinin neuraminidase (HN) of Newcastle Disease Virus (NDV). This bsAb might be efficient for *ex vivo* stimulation of NK cells via coupling to HN on the surface of the NDV-infected tumor cells.

Methods: A bsAb-encoding pcDNA3.1 vector (anti-HN scFv-Fc-anti-CD16 scFv) was used as a template, and the scFv segments (after enzymatic digestion and cutting of the Fc part) were rejoined to construct the Fc-deprived bsAb (anti-HN scFv-anti-CD16 scFv; bsHN-CD16). The constructed bsHN-CD16 was inserted into the *Hin*dIII and *Bam*HI site of the T7 promoter-based pET28a plasmid. Following restriction analyses and DNA sequencing to confirm the cloning steps, bsHN-CD16 encoding pET28a was transformed into the *E. coli* (Rosetta DE3 strain), induced for protein expression by IPTG, and the protein was purified under native condition by Ni/NTA column using imidazole.

Results: Analyses by SDS-PAGE and Western Blotting using Rabbit anti-human whole IgG-HRP conjugate, confirmed the expression and purification of the bsAb with the expected full size of 55 kDa and yields around 8% of the total protein.

Conclusions: Results showed efficient production of the bsAb in *E. coli* for future large-scale purification.

Keywords: Bispecific Antibody, *Escherichia Coli*, Immunotherapy, Natural Killer Cell (NK Cell), Newcastle Disease Virus (NDV).

Introduction

Cancer with approximately 9.6 million (1 out 6) annual deaths, was one of the main causes of death worldwide in 2018 (1). Due to insufficiency of conventional cancer therapies, novel and promising treatment approaches such as immunotherapy are strongly sought.

Natural killer (NK) cells are non-B, non-T lymphocytes, and as a part of the innate immune system, they can recognize and kill virus-infected or transformed cells without prior sensitization (2). The activity of NK cells is regulated by a balance between inhibitory and activating receptors on the surface of the NK cells. There are different activating receptors on the surface of NK cells comprising: Natural killer receptors (NCRs), NKG2D, and CD16 (FcγRIII) (2, 3). It is shown that

1: Department of Molecular Virology, Pasteur Institute of Iran, Tehran, Iran.
2: Iran University of Medical Sciences, Tehran, Iran.
3: German Cancer Research Center (DKFZ), Heidelberg, Germany.
4: Immunology Department, Pasteur Institute of Iran.
***Corresponding author: Kayhan Azadmanesh; Tel: +98 21 641122511; E-mail: azadmanesh@pasteur.ac.ir.**
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decreased activity of peripheral blood lymphocyte specially NK cells is associated with higher cancer risk (4-6). Of note, the inhibitory effect of the tumor microenvironment might also decline the effector functioning of the NK cells by downregulation of activating receptors such as NCRs (7-9). Therefore, the development of therapeutic approaches to revive NK cells functioning is a major concern in the immunotherapy of cancer (10). Application of bispecific antibodies (bsAb) is one of the promising approaches to preserve the effector function of NK cells against tumor cells (11). Bispecific antibodies are protein engagers that bind to two distinct ligands at the same time and thereby activate the immune effector cell against the target cell (12). In this context, bsAbs which can effectively activate NK cells by binding to the EpCAM antigen (a tumor associated antigen) (13) or CD33 (14) on the surface of the tumor cell from one side and to the CD16 activating receptor on the surface of NK cells from the other side were reported. However, due to the immune suppressive condition of the tumor region, it might be needed to enhance the danger signals that are required to elicit a strong immune response similar to bacteria/virus infection (15). In this context, infection of the tumor cells by a safe, non-lytic virus with potent immuno-stimulatory effects, such as Newcastle Disease Virus (NDV) Ulster strain was reported (15). This strategy provided the required strong danger signals via the tumor cell surface-expressed, viral hemagglutinin-neuraminidase (HN) protein. Accordingly, bsAbs designed to attach the surface-displayed HN from one end and the CD3 or CD28 receptors on the T cells from the other end could efficiently activate these immune (T) cells against the tumor (16). This final report suggested the possibility of applying the same strategy to activate NK cells against tumor cells, too.

An important point for the success in the application of recombinant bsAbs is their efficient and appropriate expression in a proper host. To this end, the expression of several bsAbs in different expression systems, including *E. coli*, have been reported (13, 17-19). Indeed, *E. coli* expression system, due to the ease of manipulation, availability of a high number of vectors with various promoters and high yields, is the first choice in most of the recombinant protein expression applications (20-22). Also, there are plenty of reports on the application of this host for expression of bsAbs in proper structure and high yields (13, 17, 18, 20). In the present study, we successfully expressed a recombinant bsAb (bsHN-CD16) recognizing CD16 and NDV-HN in *E. coli* for the final application in cancer immunotherapy by activation and retargeting of NK cells towards NDV-infected tumor cells.

Materials and methods

Bacterial strains and Plasmids

E. coli DH5-alpha strain was used for cloning purposes. pET28a (Novagen, USA) was used as a bacterial expression vector, and *E. coli* strain Rosetta (DE3) was used as an expression host. Propagation of *E. coli* for protein expression was in 2x LB medium (Merck, Germany).

Cloning and construction of the recombinant bsAb-encoding plasmid

The primary bsAb-encoding construct (anti-HN scFv-Fc-anti-CD16 scFv) that had been subcloned in the pcDNA3.1(-) vector was engineered by Dr. Frank Momburg (German Cancer Research Center, DKFZ). The construct contained an scFv antibody derived from the hybridoma HN.B (23) (kindly provided by Dr. V. Schirrmacher, German Cancer Research Centre) and an scFv antibody derived from the hybridoma A9 (24) (kindly provided by Dr. Gerhard Moldenhauer, German Cancer Research Centre) linked by the Fc portion of human IgG1. This plasmid was used as a template, and the scFv segments (after enzymatic digestion and cutting of the Fc part) were rejoined to construct the Fc-deprived bsAb (antiHN scFv-anti-CD16 scFv; bsHN-CD16). The constructed bsHN-CD16 was inserted into the *Hin*dIII and *Bam*HI site of the T7 promoter-based pET28a plasmid. Colony-PCR, digestion analyses, and sequencing were performed to verify that the cassette is cloned correctly in the frame. All cloning and molecular procedures were based on conventional protocols (25).

Expression of the bsAb in E. coli

The recombinant plasmid was transformed into *E. coli* Rosetta DE3 competent cells. Transformed bacteria were grown in 800 ml 2x Luria Broth

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supplemented with 50 ug/ml kanamycin and 25ug/ml chloramphenicol. Induction of expression was done when OD_{600} reached 0.6 by the addition of 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) (Merck, Germany) at 37 °C in a shaker incubator. Three hours after induction, bacteria were harvested by centrifugation at 4000 x g, 15 minutes at 4 °C and the pellet stored at -70 °C after washing the pellet with PBS with the same centrifugation conditions.

Protein Purification

Protein purification was done under native condition by affinity chromatography using Ni/NTA column according to the manufacturer's protocol (The QIA expressionist ™. 2003). In brief, the bacterial pellet was thawed, and about 3 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) was added and gently shaken in the refrigerator for about 1 hour. Next, sonication was done to lyse the bacterial cell completely (240 W, 20 s sonication, 20 s rest on ice for 15 cycles). To remove the pellet, centrifugation was done with 10000g, 30 min at 4 °C. The supernatant was collected to be applied on the Ni/NTA column. About 1 ml of resin was added to the column, and after removing the alcohol, the resin was washed with 3 ml of the lysis buffer. Next, the lysate supernatant solution was mixed very gently (about 50 rpm) with the resin on a rotary shaker at 4 °C in a 15 ml falcon tube. The mixture was loaded into a column, and the flow-through was collected. The column was washed twice with 4 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) and then the protein was eluted 5 times with 0.5 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) which were collected in tubes to be analyzed by SDS-PAGE later. The purified protein under native condition was further passed through 30 kDa Amicon-filters to ensure removal of any protein bands below this size.

Protein characterization

The pellet from 1 ml of the medium before and after induction was characterized by SDS-PAGE and then by Western blotting using rabbit anti-human whole IgG HRP conjugate (Sigma, UK) using standard protocols (25). In brief, after separation by electrophoresis, protein bands were transferred to polyvinylidene difluoride membrane (PVDF) (Roche, Germany) in wet condition and blocked by 5% skim milk in PBS plus 0.1% Tween-20 (PBS-T) overnight at 4 °C. Blots were then incubated with anti-human whole IgG HRP conjugate with a concentration of 1:2000 in PBS-T for two hours. After washing steps with PBS-T, detection step was done by addition of 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich). Seven mg DAB in 10 ml PBS in the presence of 14 µl hydrogen peroxide was added to the blot, and the expected bands were observable after a few minutes.

Protein expression yield was measured by image analysis using the open source software, FiJi (26). Briefly, the image of the acrylamide electrophoresis gel was acquired by a commercial camera. The resulted image was converted to a 32-bit image, and the background was subtracted to reduce the noise level. Then the ratio of the area under the curve related to the protein of interest divided to the total area under the curve related to the bacterial crude lysate to obtain protein expression yield.

Results

Construction of the bsAb (Anti HN- Anti CD16)-encoding vector for expression in E. coli and restriction analyses

The primary bsAb (anti-HN scFv-Fc-anti-CD16 scFv) in pcDNA3.1(-) vector coding for a tetravalent bispecific antibody for secretory expression in mammalian cells was used as the template for developing bsAb coding sequence in bacterial vector. Since the Fc part in mammalians is post-translationally *N*-glycosylated and is not required for efficient production in bacteria, the Fc portion was deleted. To this end, first, the anti-HN part was separated by digesting the pcDNA3.1(-) plasmid containing the primary bispecific antibody by *Nhe*I at 37 °C. The intended band (about 940 bp) was extracted from the agarose gel and cloned into a new intact pcDNA3.1(-), which was digested by the same enzyme followed by 1 µg alkaline phosphatase, using T4 ligase. The positive clones with correct orientation were selected by colony-PCR using CMV-F and BGH-R universal primers, and digestion with *Eco*RI, where expected 960 and 5418 bp bands relating to the positive clones with correct orientation could be observed (Fig. 1). Since for developing a

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construct without Fc, it was necessary to separate the segment containing-CD16 scFv, the main bsAb-coding pcDNA3.1 plasmid was digested by *Hin*dIII and *Xba*I, and the 833 bp segment was extracted from the gel and cloned in a new pcDNA3.1 plasmid backbone. This construct was confirmed by digestion with *Pst*I restriction enzyme where the 4061 bp and 1840 bp segments could be seen on the gel (Fig. 2). Subsequently, to generate the pre-final construct (bsHN-CD16 in pcDNA3.1), the anti-HN coding plasmid was digested with *Nhe*I, while the anti-CD16 coding sequence was digested by *Xba*I restriction enzyme. Since *Nhe*I and *Xba*I create compatible cut sites, the anti-HN scFv coding sequence was inserted beside the anti-CD16 coding sequence inside the pcDNA3.1(-) by T4 ligase, after the alkaline phosphatase treatment for ten minutes. Colony-PCR and digestion with *Eco*RI confirmed the accuracy of cloning and the correct orientation (Fig. 3). Finally, the bacterial expression vector, pET28a, and the pre-final construct were digested by *Nco*I and *Hin*dIII. The cut with *Nco*I removed the ER leader sequence of the original construct. The insert (antiHN-antiCD16) was extracted from the gel and ligated to the digested vector according to the standard protocol by T4 ligase (25). The final construct was confirmed by colony-PCR, digestion by *Bst*EII restriction enzyme to generate expected 4676, 1357, and 809 bp segments, and finally sequencing (Fig. 4).

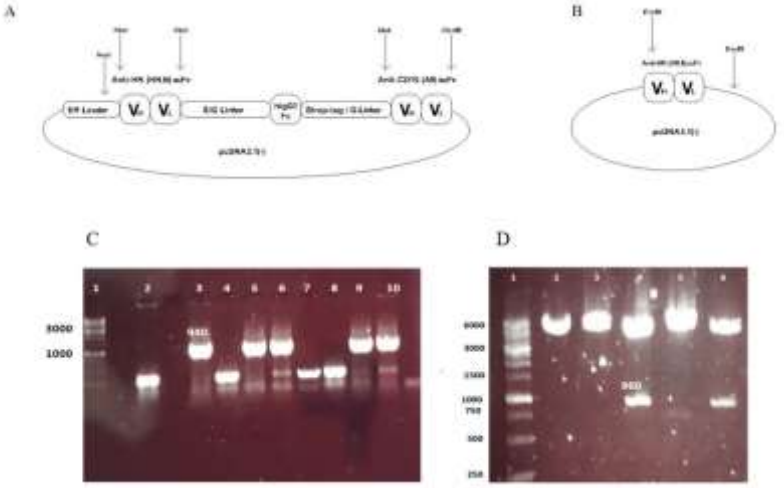

Fig. 1. Confirmation of the positive colons of pcDNA3.1(-) encoding anti-HN scFv. A. Schematic diagram of the primary (template) bsAb (antiHN scFv-Fc-anti-CD16 scFv) in pCDNA3.1 for expression in mammalian cells and the restriction cut sites used for cloning in this study. B. Anti-HN scFv in pcDNA3.1 generated in this step using *Nhe*I. C. Colony-PCR confirmation of the clones by CMV-F and BGL-R universal primer pairs. The band about 1000 bp was the desired band in the clones 3,5,6,9 and 10. D. Restriction Analysis: All the positive colons were digested with *Eco*RI to confirm the correct orientation by the production of a 960 bp fragment (lanes 4 and 6).

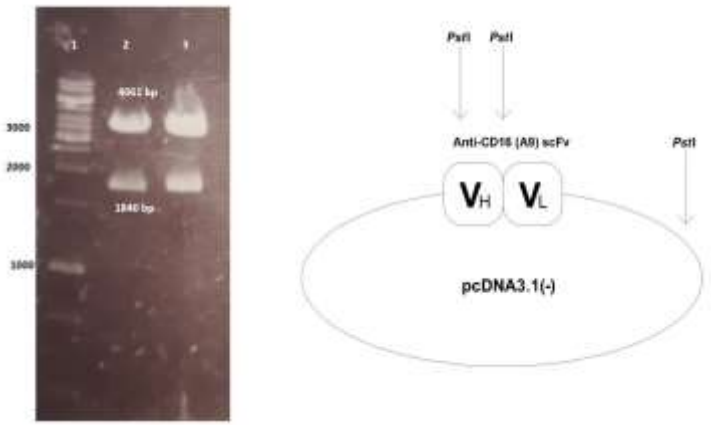

Fig. 2. Anti-CD16 scFv coding sequence in pcDNA3.1. The 833 bp sequence encoding CD16 scFv was separated from the primary mammalian template by *Hin*dIII and *Xba*I restriction enzymes and cloned into a new pcDNA3.1 backbone by T4 ligase. The positive clones were confirmed by digesting the anti-CD16 coding plasmid with *Pst*I restriction enzyme to produce 4061 and 1840 bp bands. A 206 bp segment was also produced, which cannot be seen on the gel. Results for two examined colonies are provided.

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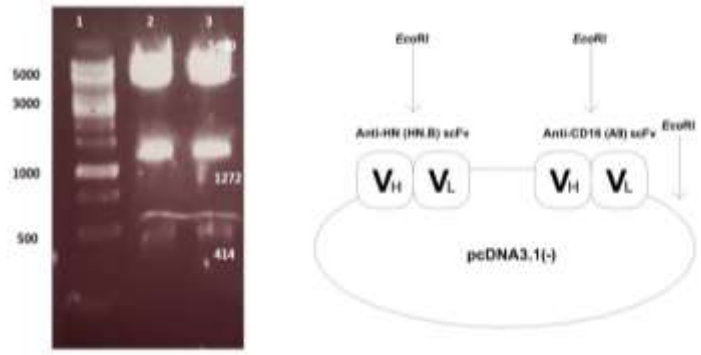

Fig. 3. Schematic diagram of the analysis and confirmation of the pre-final construct encoding anti-HN-antiCD16 sequence in pcDNA3.1 using *Eco*RI. Three expected 5400, 1272 and 414 bp segments were observed on the gel. Results for two examined colonies are provided.

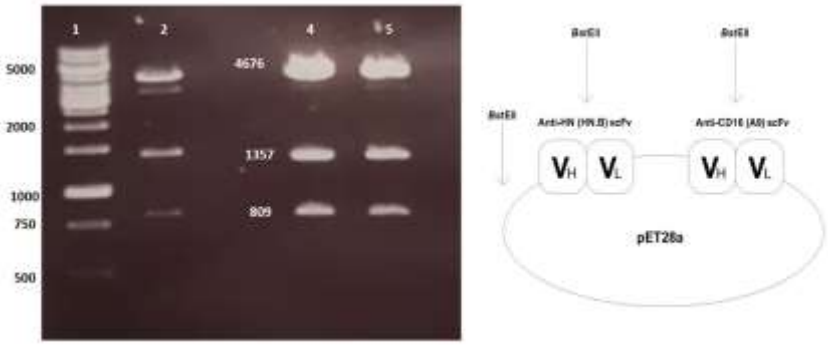

Fig. 4. Conformation of the final construct (pET28a encoding aHN-aCD16, bsAb minus Fc) in three clones by digestion using *Bst*EII restriction enzyme. The desired 4676, 1357 and 809 bp bands were observable. Clone number 5 was selected for the expression experiments.

Expression, purification, and characterization of the E. coli-derived bsHN-CD16 Ab

As shown in Fig. 5A, induction of the *E. coli* expression hosts harboring the pET28a-encoding bsAb by IPTG indicated the expected 55 kDa protein band corresponding to the bsHN-CD16. Western blotting by rabbit anti-human whole IgG HRP conjugate confirmed the nature of the expressed antibody (Fig. 5B). Image analysis of the SDS-PAGE protein bands (Fig. 5A) by Fiji software (26) indicated a yield of 8% of total bacterial protein, that showed more than 90% purity after purification with Ni-NTA method under native condition. This purity was further enhanced by passage through 30 kDa Amicon filters (Fig. 6).

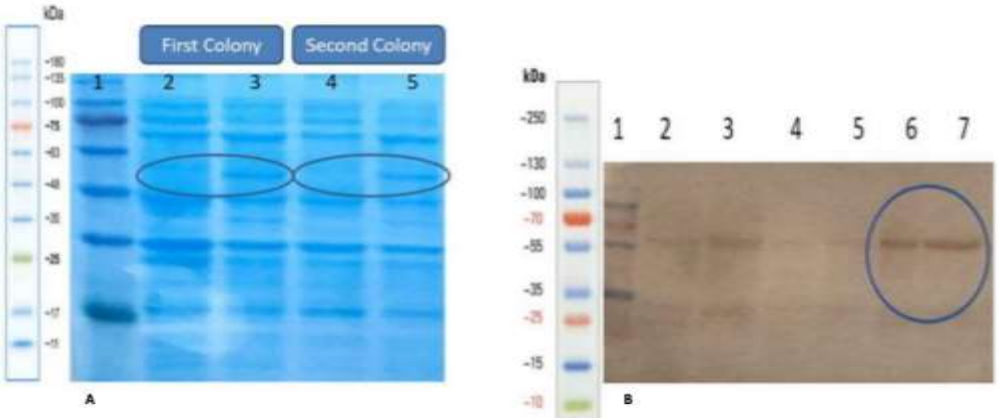

Fig. 5. A. Expression of bsAb minus Fc (bsHN-CD16) in *E. coli*, Rosetta (DE3). Induction for protein expression was done by 1 mM IPTG at OD 0.6. A. SDS-PAGE of the crude extracts on 12% acrylamide gel. The 55 kDa band showed expression of the desired protein. Lanes 2 and 4 were the bacterial lysate before induction and the lanes 3 and 5 were after induction (circled regions). B. Western blot on the crude lysate using rabbit anti-human whole IgG HRP conjugate. Lanes 4 and 5 were before induction, while lanes 6 and 7 were after induction (Lanes 2 and 3 were colonies with low expression levels).

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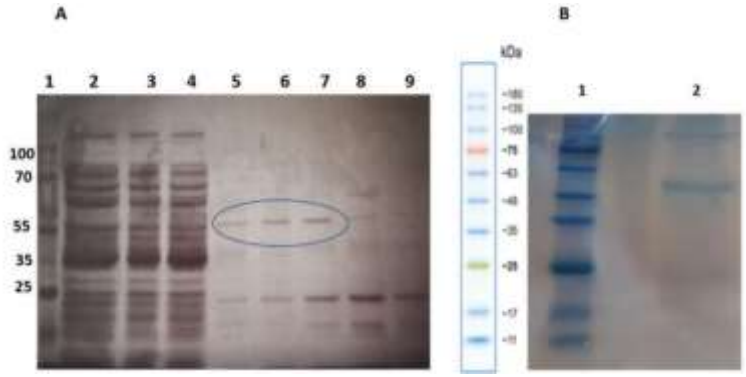

Fig. 6. The SDS-PAGE analysis of the purified bsAb protein on Ni/NTA column. A. Purification was done on Ni/NTA column chromatography system under native condition. Lane1: Ladder, Lane2: The supernatant (crude extract), Lane3: wash1, Lane4: wash2, Lane5-9: Elution1-5. B. Final purified protein was produced by passing the first three elution fractions (Fig. 6A) from the 30 kDa Amicon filter.

Discussion

In this study for the first time, to our best of knowledge, we have successfully cloned, expressed and purified a recombinant bispecific antibody (bsHN-CD16) in *E. coli* strain Rosetta (DE3) with acceptable yields for potential large-scale plans. This immune engager is supposed to bind to the viral HN protein displayed on the surface of the NDV infected tumor cells from one side and to the CD16 activating receptor on the surface of the NK cells from the other side. Since *E. coli* expression system, due to fast, cost-effective and efficient yield of protein production, is the first choice for protein production (20, 21), we decided to produce the bsAb in this bacterial expression system.

As shown in figures 1 to 3, the challenging step in the construction of the *E. coli*-expression vector for this bsAb was deletion of the Fc region. In fact, Fc fragment is a highly glycosylated structure in Abs and considering the unavailability of the glycosylation system in *E. coli* cells, presence of the Fc in the target constructs might result in the inhibition or reduction of the protein-expression yields in *E. coli* (22). Also, the CD16 on the NK cell might act as the receptor for the Fc part of the antibodies (FcγRIII) (27). Thus, deletion of the Fc part from the construct might ensure that binding of the bsAb to the CD16, occurs only via the CD16 scFv part. Consistent with our approach, there are other studies addressing the construction of an NK engager without Fc part for retargeting NK cells toward the tumor cells (13, 14, 17, 28).

Several other studies reported the expression of bispecific antibodies with the ability of engaging CD16 on the surface of the NK cells in *E. coli* system, including: bsCD16-CD133 (against colorectal cancer cell) (18), bsCD16-CD33 (against HL-60 cell line, a human leukaemia cell line) (14) or bsCD16-EpCAM (against HT-29 cell line, colorectal cell line) (29). Of note, most of these prior mentioned studies reported the use of BL2 strain of *E. coli* as an expression host. However, since the CD16 scFv in our engager (as well as in the prior reports) is a human-derived sequence, using Rosetta (DE3) strain in our study, as a BL21 derivative designed to enhance the expression of eukaryotic proteins, might be a better choice. The Rosetta (DE3) strain supplies tRNA genes for AGG, AGA, AUA, CUA, CCC, GGA (codons rarely used in *E. coli*) on a Col-E1 compatible chloramphenicol-resistant plasmid (30) and might decrease the need for codon optimization.

Image analysis by Fiji software (27) determined the yield of our bsAb expression, which was about 8% of the total bacterial proteins. Although this yield was acceptable, there are reported strategies to further improve the expression yields of bispecific antibodies in *E. coli* expression systems (28). Accordingly, in one study, incorporation of a segment of human muscle aldolase (hma) in the bsAb was reported to improve the expression yields by 20-fold which resulted in enhanced purity from 10% to 80% (31). In another study, the inclusion of the 218s linker (GSTSGSGKPGSGEGSTKG) improved proteolytic stability and reduced the degree of protein aggregation (28). Recently, by optimizing the cultivating media and induction

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condition (M9 minimal medium and 24 h shaking at 16 °C after induction by 1 mM IPTG), expression levels for a bsAb in *E. coli* BL-21 strain were improved to an acceptable level of 0.6 mg/200 ml (19). Therefore, it might be possible to further improve the expression level of our bsAb with similar strategies.

Due to the presence of 6xHis-tag (C-terminal poly-histidine tag with a free carboxyl group) in our bsAb, the purification of bsAb was performed by Ni/NTA column and under native condition. Although the yield of purification under native condition is less than denaturing condition, to avoid solubilizing and refolding steps, and to retain the natural structure of the bsAb, we decided to choose native purification protocol. As shown in Fig. 6A, several unwanted small sized bacterial proteins were present in our natively purified bsAb preparations which were removed by passing through a 30 kDa Amicon filter (Fig. 6B). Our approach is in contrast to several prior studies that had purified the *E. coli*-derived bsAbs under denaturing condition(13, 17, 31). In fact, despite an acceptable purity of 80 to 90% after denaturing condition of protein purification, returning the natural structure and biological activity to the produced antibodies would probably require a complex refolding processes (13, 17, 31).

Altogether, in the present study, to our best of knowledge, we provided the first report on successful cloning and expression of a bispecific antibody (bsHN-CD16) for recognition of the CD16 and NDV-HN in the Rosetta (DE3) strain of *E. coli* expression system. This bsAb is designed to bind to CD16 activating receptor on NK cells from one side and to the viral protein HN on the surface of the NDV-infected tumor cell from the other side. Further studies on this bsAb, including binding and cytotoxicity assays, may demonstrate the potential of this bsAb for application in cancer immunotherapy.

There is no conflict of interest.

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