Research paper

Blocking monocyte transmigration in in vitro system by a human antibody scFv anti-CD99. Efficient large scale purification from periplasmic inclusion bodies in E. coli expression system

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Article info

Article history:
Received 24 January 2014
Received in revised form 24 April 2014
Accepted 25 April 2014
Available online xxxx

Keywords:
Single chain variable fragment (scFv)
Purification
Aggregates
Periplasmic inclusion bodies
Blocking monocyte transmigration

Abstract

Migration of leukocytes into site of inflammation involves several steps mediated by various families of adhesion molecules. CD99 play a significant role in transendothelial migration (TEM) of leukocytes. Inhibition of TEM by specific monoclonal antibody (mAb) can provide a potent therapeutic approach to treating inflammatory conditions. However, the therapeutic utilization of whole IgG can lead to an inappropriate activation of Fc receptor-expressing cells, inducing serious adverse side effects due to cytokine release. In this regard, specific recombinant antibody in single chain variable fragments (scFvs) originated by phage library may offer a solution by affecting TEM function in a safe clinical context. However, this consideration requires large scale production of functional scFv antibodies and the absence of toxic reagents utilized for solubilization and refolding step of inclusion bodies that may discourage industrial application of these antibody fragments. In order to apply the scFv anti-CD99 named C7A in a clinical setting, we herein describe an efficient and large scale production of the antibody fragments expressed in E. coli as periplasmic insoluble protein avoiding gel filtration chromatography approach, and laborious refolding step pre- and post-purification. Using differential salt elution which is a simple, reproducible and effective procedure we are able to separate scFv in monomer format from aggregates. The purified scFv antibody C7A exhibits inhibitory activity comparable to an antagonistic conventional mAb, thus providing an excellent agent for blocking CD99 signaling. This protocol can be useful for the successful purification of other monomeric scFvs which are expressed as periplasmic inclusion bodies in bacterial systems.

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1. Introduction

CD99 is a relatively unique molecule unrelated to any other molecule in the human genome except the closely-related paralog CD99-like 2 (CD99L2), which may have arisen from a
common ancestral gene (Suh et al., 2003). The gene encoding CD99 is in the pseudoautosomal region of the human X chromosome (Smith et al., 1993). In mice, the region of the genome syntenic to the pseudoautosomal region of the human X chromosome is on chromosome 4 (Park et al., 2005) where the gene encoding for mouse CD99 determinant is located. CD99 is a type I transmembrane exclusively O-linked glycoprotein that in humans has an apparent molecular weight of 32 kDa on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Almost half the apparent molecular weight is due to carbohydrate modification (Schenkel et al., 2002). CD99 is expressed along the cell borders between endothelial cells. It is also expressed diffusely on the surfaces of most circulating blood cells, although the degree of expression varies considerably between subtypes of leukocytes and expression is lower than on endothelial cells. CD99 is also expressed on various tumors including Ewing’s sarcoma and primitive neuroectodermal tumor (Scotlandi et al., 1996). CD99 can act as an adhesion molecule, and I. cells transfected with CD99 aggregate in a homophilic manner, with CD99 on once cell binding to CD99 on the adherent cell (Schenkel et al., 2002). Homophilic interaction between CD99 on adjacent endothelial cells may help stabilize endothelial borders, but CD99 is not a part of the adherent junction complex. CD99 does, however, play a significant role in TEM of leukocytes. Similar to the regulation of TEM by platelet/endothelial cell adhesion molecule 1 (PECAM-1), homophilic interaction between CD99 at the endothelial cell border and CD99 on monocytes (Schenkel et al., 2002) and neutrophils (Lou et al., 2007) is required for transmigration. However, CD99 regulates a later step in transmigration than PECAM-1. Leukocytes in which PECAM-1 has been blocked by mAb can still be prevented from transmigrating if anti-CD99 mAb hec2 is added after the anti-PECAM-1 block has been removed. Conversely, if CD99 interaction is first blocked, leukocytes can no longer be inhibited from transmigrating by anti-PECAM-1 antibody when the anti-CD99 block is removed (Schenkel et al., 2002). In support of this, confocal images of leukocytes blocked in the act of transmigration by anti-CD99 show their leading edge under the endothelial cytoplasm, their cell body lodged at the border between endothelial cells, and the trailing uropod on the apical surface (Schenkel et al., 2002; Lou et al., 2007). As long as the block continues, they migrate along the junctions over the surface of the endothelium in this manner, unable to finish transmigration. Blocking antibodies against mouse CD99 inhibit inflammation in several animal models. Migration of T lymphocytes into skin (Bixel et al., 2004) and neutrophils and monocytes into the peritoneal cavity (Dufour et al., 2008) is blocked by interfering with CD99 function. Hence, the inhibition of TEM mediate by specific human mAb represents a potent and safe immunotherapeutic approach to treating inflammatory condition.

Monoclonal antibody therapy is the fastest growing sector of pharmaceutical biotechnology and a number of antibody-based biopharmaceuticals have been approved for different human pathologies including several inflammatory and immune diseases (Kotsovilis and Andreakos, 2014). Several factors determine the efficacy of these products, including target specificity, effector functions and xenogeneic origin of monoclonal immunoglobulin that alter the pharmacokinetic profile of the antibody, leading to severe toxicity and preventing repeat dosing (Mirick et al., 2004) responses to monoclonal antibodies. Today, with the advances of recombinant genetic strategies rodent antibodies have been genetically modified in chimeric and humanized version significantly reducing their immunogeneity (Presta, 2005). However, the main pitfalls of the use of conventional mAbs (usually IgG) is not only related to their immunogenicity but also to other biochemical and biological aspects such as slow rates of clearance that may affect the physiology of normal tissues and inappropriate activation of Fc receptor expressing cells that can lead to massive cytokine release which could be associated to severe side effects. In this context, the ability to produce fully human antibody fragments has represented a significant breakthrough in the field of antibody engineering.

Antibody fragments in particular in scFv format with their small size (27–32 kDa), display better tissue penetration compared to full length monoclonal antibodies without any significant toxicity. ScFvs represent the smallest stable antibody fragments still capable of specifically binding an antigen. They are structured as a single polypeptide chain incorporating a heavy chain variable (VH) and a light chain variable (VL) region of an antibody, linked by a flexible linker. The extremely versatile format of scFvs can be tailored by genetic engineering to improve affinity and stability (Todorovska et al., 2001). They can be modified in their size, pharmacokinetics, immunogenicity, specificity, valency and effector functions (Hudson and Souriau, 2003). Moreover, scFvs can be easily expressed and produced in E. coli in large quantity (Kripityanov and Little, 1999). However, the expression of heterologous proteins in E. coli often encounters the formation of inclusion bodies (IBs), which are insoluble and non-functional protein aggregates. For the successful production of antibody fragments from inclusion bodies, refolding step is required for solubilization and functional recovery of the protein (Gautam et al., 2012). However, these procedures represent complex biochemical approaches thus discouraging industrial production. Therefore a simple and effective method is required for biological and medical utilization of scFv antibodies.

In this context, herein we describe an efficient and simple procedure for large scale production of scFvs in the E. coli system from periplasmic inclusion bodies. Furthermore, related methodologies to obtain monomeric soluble biologically active scFv are in detail described. ScFvs were purified with a His<sub>6</sub>-tag using immobilized metal affinity and anion chromatography avoiding gel filtration chromatography approach, and laborious refolding step pre- and post-purification phase. Biological assays show that the anti-CD99 scFv C7A subjected to this procedure is fully active for specific binding and blocking activity of TEM.

2. Material and methods

2.1. Cloning

scFv C7A isolated from the ETH-2 human scFv displayed phage library (Viti et al., 2000) by bio-panning approach and affinity maturing as previously described (Neri et al., 1996). scFv anti-CD99 was cloned into a pET22b(+) vector which includes pelB sequence (scFv C7A) or pET45b(+) vector which does not include pelB sequence (scFv C7A NO pelB) (Novagen, Merck KGaA, Darmstadt, Germany) by amplifying the sequence from pDN332 including the D3SD3–FLAG–His<sub>6</sub>-tag at the C-terminus.

Please cite this article as: Moricoli, D., et al., Blocking monocyte transmigration in in vitro system by a human antibody scFv anti-CD99. Efficient large scale purification from... J. Immunol. Methods (2014), http://dx.doi.org/10.1016/j.jim.2014.04.012
For cloning in the pET22b (+) the scFv sequence was amplified using the primers NcoI Fw 5'-CCAGCCGCCCATGGCGAGGTG-3' and EcoRI Rev 5'-ACAACTTCAAGCCTTAAGTGGATG-3'. For cloning in the pET45b (+) the scFv sequence was amplified using the primers NcoI Fw 5'-CCAGCCGCCCATGGCGAGGTG-3' and PstI Rev 5'-ACACTTCTGAGCTTAAGTGGATG-3'. Amplicons were digested together with pET22b (+) or pET45b (+) vector, with NcoI and EcoRI enzymes or NcoI and PstI (New England Biolabs, Ipswich, MA, USA) at 37 °C for 3 h. The digested products were purified and ligated together with T4 DNA ligase (Promega, Madison, WI, USA) at 4 °C overnight. The ligation mix was transformed into E. coli strain BL21 (DE3) (F′ ompT hsdSb(rB−λdcm−) gal dcm (DE3)) for protein expression. Positive clones were screened for correct insertion by colony polymerase chain reaction and sequencing.

2.2. Expression

E. coli BL21 (DE3) starter culture grown to an O.D.₆₀₀ of 2.0 in a shaking incubator set at 37 °C and 200 rpm was inoculated for large scale production into 20 l Bioreactor (Biostat C, Sartorius). The fermentation phase was carried out inoculated for large scale production into 20 l Bioreactor 2.0 in a shaking incubator set at 37 °C and 200 rpm was filtered using 0.45 μm sterilizing filter (Merck Millipore) and centrifuged at 8000 rpm for 60 min at 4 °C. Finally the supernatant was filtered using 0.22 μm sterilizing filter (Millipore).

2.3. Cell lysis and solubilization of inclusion bodies

Collected cells were suspended in 7 l lysis buffer containing: 20 mM Imidazole, 0.5 M NaCl and 20 mM phosphate buffer pH 7.5, disrupted using a homogenizer (GEA Niro Soavi) at 680 bar and centrifuged at 8000 rpm for 60 min at 4 °C. The pellet was resuspended in 7 l of solubilization buffer containing: 8 M Urea, 20 mM Imidazole, 0.5 M NaCl and 20 mM phosphate buffer pH 7.5 and incubated for 16 h under agitation at 21 °C and centrifuged at 8000 rpm for 60 min at 4 °C. Finally the supernatant was filtered using 0.45 μm sterilizing filter (Merck Millipore).

2.4. Purification

Purification was performed on an AKTA explorer 100 (GE-Healthcare) and BPG 100/500 column (GE-Healthcare). All packed chromatography columns were cleaned and deproteinized by flowing 1 M NaOH through the column at 40 ml/min for 2 h and washed with water for injection (WFI) until the pH of the column effluent was < pH 8.0. The first step consisted of an IMAC chelating sepharose fast flow (GE Healthcare) a Ni-chelating column charged with 0.1 M NiSO₄. The column was equilibrated with a buffer containing: 8 M Urea, 20 mM Imidazole, 0.5 M NaCl and 20 mM phosphate buffer pH 7.5 (buffer A). After sample loading and a washing step with equilibration buffer, a linear gradient from 8 M to 0 M of Urea equivalent to three column volumes (CV) was used to elute Urea. The gradient was applied by using buffer A and buffer A without Urea. The column was washed with 70 mM Imidazole in 0.5 M NaCl and 20 mM phosphate buffer pH 7.5 (buffer A). After sample loading and a washing step with equilibration buffer, a linear gradient from 8 M to 0 M of Urea equivalent to three column volumes (CV) was used to elute Urea. The gradient was applied by using buffer A and buffer A without Urea. The column was washed with 70 mM Imidazole in 0.5 M NaCl and 20 mM phosphate buffer pH 7.5. scFv anti-CD99 was eluted in a single peak with 250 mM Imidazole in 0.5 M NaCl and 20 mM phosphate buffer pH 7.5. A further DEAE Sepharse fast flow (GE Healthcare) column using differential salt elution was used to separate monomer scFv from scFv anti-CD99 aggregates. The column was equilibrated with 50 mM NaCl in 1.5 mM EDTA, 10% (v/v) glycerol and 20 mM phosphate buffer pH 7.5. The IMAC eluate pool was diluted tenfold with 1.5 mM EDTA 10% (v/v) glycerol, 20 mM phosphate buffer pH 7.5, and applied to the column. After washing with equilibration buffer, bound scFv monomer was eluted with 0.25 M NaCl in 1.5 mM EDTA, 10% (v/v) glycerol and 20 mM phosphate buffer pH 7.5 and collected in a single peak based on 280 nm absorbance. scFv aggregates were eluted with 0.5 M NaCl in 1.5 mM EDTA, 10% (v/v) glycerol, 20 mM phosphate buffer pH 7.5, then collected in a single peak based on 280 nm absorbance. Finally the monomer scFv C7A eluted with 0.25 M NaCl was filtered using a Chromasorb 50 ml device (Merck Millipore) for endotoxin reduction and then filtered using a 0.22 μm sterilizing filter (Merck Millipore).

2.5. Protein concentration

Total protein concentration was determined using the Bio-Rad protein assay, a method based on the Bradford assay (Zor and Selinger, 1996), using BSA as a standard. Color development and absorbance values were measured using a Spectophotometer Shimadzu UV-1601 and compared to standard curves.

2.6. SDS-PAGE and Western blotting analysis

SDS-PAGE under reduced conditions was carried out according to Laemmli (1970). The samples were run on 12% gels at 100 V for 90 min using BioRad mini protein system (BioRad Laboratories). The resolved protein samples were visualized by staining with Comassie brilliant blue. Western blotting (WB) analysis samples were separated by SDS-PAGE at 12% (w/v) and transferred overnight at +4 °C on nitrocellulose membranes (GE Healthcare) (Towbin et al., 1979) Membranes were saturated with Tris buffer saline (TBS) containing 5% (w/v) non-fat dry milk for 1 h at room temperature and then incubated for 3 h at +4 °C with an anti-polyhystidine monoclonal antibody (AbD Serotec, Oxford, UK) 1/1000 diluted in TBS containing Tween 0.05% (w/v) (TTBS) with 5% (w/v) non-fat dry milk. After incubation with goat anti-mouse IgG-HRP conjugated (BioRad) for 1 h at room temperature the immunoreactive bands were revealed by the ECL detection system (GE Healthcare). Images derive from SDS-PAGE and WB collected and analyzed by a Chemi Doc XRS (BioRad).

2.7. Gel filtration chromatography

Size exclusion chromatography (SEC) was used to evaluate the structure of the scFvs. SEC was performed with AKTA Explorer 100 using a Hi-load Superdex 75 16/60 (GE Healthcare) column equilibrated with PBS (phosphate buffer saline) pH 7.5, at a flow rate of 1 ml/min. 2 ml of purified recombinant antibodies was applied to the column. scFvs were detected by its absorbance at 280 nm. The molecular weights were estimated based on standard curve calibrating the system with proteins of known molecular weight: Albumin 67,000 Da, Ovalbumin 45,000 Da, Carbonic Anhydrase 30,000 Da, Trypsin Inhibitor 20,100 Da and Thiamine 3337.27 Da. The void volume (Vo) was determined using Blue Dextran 2000. The log molecular mass of each standard was plotted against the fraction of the stationary
gel volume available for diffusion (Kav) to generate a calibration curve. The proportion of monomer to the total was determined by Unicorn software (GE Healthcare) by integration of the relative area under the chromatographic profile corresponding to each peak.

2.8. Endotoxin content

Bacterial endotoxin levels were determined with the Limulus amebocyte lysate (LAL) kit (PBI International) in the purified scFv C7A preparation following the instruction manual.

2.9. Production of the recombinant human CD99 antigen fragment (rCD99) for enzyme-linked immunosorbent assay (ELISA)

_E. coli_ TG1 cells transformed with recombinant plasmids pQE30a–CD99/His (Gellini et al., 2012) were grown in a fermentation process at 37 °C in 5 l of vegetable peptone broth (VPB): vegetable peptone 18 g/l, yeast extract 3 g/l, potassium phosphate dibasic 2.5 g/l, sodium chloride 5 g/l, dextrose 2.5 g/l containing 100 µg/ml ampicillin, until the optical density (OD) at 600 nm was 0.6. Induction of the rCD99 fragment was obtained by adding 1 mM IPTG. Induced cultures were then incubated for a further 3 h at 37 °C. Finally, the cells were harvested by centrifugation at 5000 rpm for 30 min at 4 °C. For protein purification, the pellets were resuspended in 50 ml/l of a buffer containing 20 mM phosphate buffer pH 7.5, 1.5 mM EDTA, and homogenized at 680 bar. The cell lysis solutions were centrifuged at 8000 rpm for 60 min at 4 °C and the supernatant filtered through 0.45 µm filter (Merck Millipore). Purification was performed by using AKTA explorer chromatography system (GE-Helthcare). The first step consisted of an IMAC Chelating Sepharose fast flow (GE-Helthcare) a Ni-chelating column charged with 0.1 M NiSO4. The column was washed with 50 mM Imidazole in 500 mM NaCl and 20 mM phosphate buffer pH 7.5. After sample loading and a washing step with equilibration buffer, the column was washed with 50 mM Imidazole in 500 mM NaCl and 20 mM phosphate buffer pH 7.5. rCD99 fragment was eluted in a single peak with 250 mM Imidazole in 500 mM NaCl and 20 mM phosphate buffer pH 7.5. The IMAC eluate pool was collected in a single peak based on 280 nm absorbance. The purified protein was filtered through a 0.22 µm membrane filter (Merck Millipore) and collected at −80 °C.

2.10. ELISA assay

2HB 96-well plates (M-Medical) were coated with 100 µl/well of rCD99 antigen or BSA 5 µg/ml diluted in carbonate buffer pH 9.6 (100 µl/well) and kept at 37 °C for 16–18 h. After five washes in PBS containing Tween-20 0.05% v/v (TPBS), the plate was blocked with bovine serum albumin (BSA) 1% (w/v) in PBS (PBSS) (150 µl/well) and kept for 1 h at 37 °C. After washing in TPBS scFvs were diluted in PBSS. They were tested (100 µl/well) at different concentrations and the plate was incubated for 90 min at 37 °C. Thereafter the plate was incubated with a freshly prepared mixture containing an anti-scFv polyclonal antibody (Diatheva) pre-diluted 1/1000 (v/v) in PBSS (100 µl/well) and kept for 1 h at 37 °C. The immunoreactive signals were highlighted after further addition of a goat anti-rabbit HRP-conjugated antibody (BioRad) 1/1000 (v/v) diluted in PBSS (100 µl/well) with ABTS (Roche Diagnostic) as substrate. The absorbance values were obtained reading at 405 nm with a microplate reader 45 min after the addition of the staining solution (BioRad).

2.11. Cell culture

All procedures were approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine. Peripheral blood mononuclear cells (PBMC) were collected and isolated from blood of healthy human volunteers, diluted to 4 × 10⁶ PBMC/ml in Medium 199 plus 0.1% human serum albumin as described by Muller and Weigl (1992). Human umbilical vein endothelial cells were cultured from umbilical cords obtained from healthy women after normal delivery.

2.12. Flow cytometry

Flow cytometry was performed on a FACSscan flow cytometer (Becton-Dickinson, NJ, All USA). PBMC staining and washing steps were conducted in PBS without divalent cations plus 1% heat-inactivated fetal bovine serum (FBS). Cells were lifted with trypsin-EDTA and incubated in the presence of 10 µg/ml of scFv C7A. Finally cells were incubated with mouse anti-His6 monoclonal antibody (Serotec) pre-diluted 1/1000 (v/v) and with a goat anti-mouse FITC conjugated antibody (Sigma Aldrich) diluted 1/200 (v/v).

2.13. Transendothelial migration (TEM) assay

The monolayer of human umbilical vein endothelial cells grown on hydrated collagen gels made in 96-well culture dishes were used within three days of achieving confluence. As soon as the PBMC were isolated, aliquots were mixed with an equal volume of control antibody (mAb hec1, recognizes VE-cadherin but does not block TEM), anti-CD99 mAb hec2 (Schenkel et al., 2002; Lou et al., 2007), or scFv C7A also in an equal volume of control antibody (mAb hec1, recognizes VE-cadherin but does not block TEM), anti-CD99 mAb hec2. The monolayer was washed twice with warm Medium 199 and then 100 µl of PBMC suspension was added to each well of replicate monolayers of endothelial cells. The co-cultures were returned to the CO2 incubator for 60 min. Then the monolayers were washed sequentially with 100 µl EGTA in PBS then warm PBS with calcium and magnesium. They were then fixed in 2.5% glutaraldehyde overnight then stained and evaluated for transmigration as described by Muller et al. (1993). Briefly, after staining the fixed monolayers with modified Wright–Giemsa stain, co-cultures were removed from the individual wells, mounted on slides and examined by Nomarski optics at 600×. Monocytes in focus above the level of the endothelial cell were counted as adherent but not...
transmigrated. Monocytes in focus below the level of the endothelial cell (i.e. in the collagen gel) were counted as transmigrated. The percent transmigration was calculated as the number of transmigrated cells divided by the sum of the transmigrated plus adherent cells.

3. Results

3.1. Expression of scFv C7A

Sequencing of the expression construct revealed that the scFv anti-CD99 C7A is in VH-linker-VL format with the 15 amino acid (Gly4Ser) 3 linker residues. Protein expression was induced in E. coli BL21 (DE3) strain transformed with pET22b-scFv C7A construct. It was induced at 10 O.D. by adding 1 mM IPTG for 3 h at 37 °C. In this condition the yield of scFv was approximately 35 mg/l. Fig. 1a shows SDS-PAGE and WB of the soluble and insoluble quantity of scFv obtained after cell lysis and solubilization of inclusion bodies. The highest percentage of scFv C7A (85%) is included in the insoluble fraction (Fig. 1b). Therefore it was decided to purify scFv starting from periplasmic inclusion bodies.

3.2. Purification and biophysical analysis of scFv C7A

After the solubilization of the inclusion bodies by 8 M Urea buffer, the supernatant was loaded onto an IMAC nickel column which binds scFv C7A via the histidine tag. In this step the column was equilibrated in the presence of 8 M Urea. Subsequently a linear gradient (3CV) in column Urea elimination was used (from 8 M Urea to 0 M). A washing step by 70 mM Imidazole was used for removing the residual high and low molecular weight contaminant host protein in the scFv sample. Elution by 250 mM Imidazole was revealed scFv 95% purity as estimated by SDS-PAGE analysis loading 5 μg/lane (Fig. 1c). The IMAC-eluate was diluted 1/10 and...
loaded onto a DEAE-Sepharose column; this step allowed the separation of aggregates scFv C7A from monomer. The scFv monomer was eluted at 0.25 M NaCl while aggregates were eluted at 0.5 M NaCl (Fig. 2a). SDS-PAGE analysis of the 0.25 M NaCl eluate showed the presence of highly pure band of 33 kDa which corresponded to scFv C7A and confirmed by WB analysis with 99% purity estimated on loading 10 μg/lane. Peak eluted at 0.5 M NaCl also showed the presence of pure band scFv C7A (Fig. 2b) but gel filtration analysis clearly showed that scFv anti-C7A eluted at 0.25 M. NaCl consisted of a monomer forms (Fig. 2c) while the scFv eluted at 0.5 M NaCl consisted of aggregate forms (Fig. 2d). Table 1 summarizes the yield, the percentage of purity and the percentage of total scFv C7A monomer in the purification steps. The monomeric scFv at 0.25 M NaCl was tested for endotoxin contamination before and after Chromasorb device endotoxin removal. The endotoxin derived from bacterial cell wall has a number of biological effects on mammalian cells and it would interfere with bioactivity assays or to be inappropriate for therapeutic conditions for human use. The LAL assay indicated a lipopolysaccharide (LPS) concentration of about 1000 EU/ml (1 EU/μg) after anionic chromatography step. The chromasorb device, reduced endotoxin content on the scFv to 1 EU/ml (0.001 EU/μg) which is well within a range that can be used for clinical use and for cell migration assays.

### 3.3. Biological characterization of scFv C7A

The binding properties of the purified antibody were tested in ELISA assays, where it manifested a very strong binding to purified extracellular fragment rCD99 (Fig. 3a). In contrast, no binding to rCD99 was observed with an irrelevant scFv subjected to the same procedure. No binding was detected either when the plates were coated with an unrelated protein, e.g., BSA. The binding occurred in an ELISA plate as well as in the Western blotting denaturating gel system indicating that

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**Fig. 2.** (a) Final purification step using DEAE Sepharose. Chromatogram shows elution in two peaks at 0.25 M and 0.5 M NaCl. (b) The protein profile of the two peaks was analyzed by SDS-PAGE. Low molecular weight marker (lane M); eluate 0.25 M NaCl (5 μg) (lane 1); eluate 0.5 M NaCl (2.5 μg) (lane 2) eluate 0.25 M NaCl (10 μg) (lane 1). This is confirmed by WB analysis with anti-His-tag antibody. Eluate 0.25 M NaCl (5 μg) (lane 1); eluate 0.5 M NaCl (2.5 μg) (lane 2) eluate 0.25 M NaCl (10 μg) (lane 1). (c) Gel filtration of scFv C7A monomer eluted with 0.25 M NaCl, the presence of aggregates is <1%. The peak integration was calculated with Unicorn software (GE Healthcare). (d) Gel filtration of scFv anti-C7A aggregates eluted with 0.5 M NaCl that were eluted in proximity of the void volume (Vo).

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the scFv recognized its target site in rCD99 both as a folded sequence and as a linear epitope. We also estimate the affinity of scFv C7A for the rCD99 protein. The determination of antibody affinity was performed by equilibrium saturation analysis using a competition ELISA assay according to Rath et al. (1988) with minor modifications. The average scFv $K_d$ was about $2.4 \times 10^{-8}$ M (data not shown). Flow cytometry studies showed that scFv C7A binds the external domain of CD99 cell adhesion molecule expressed on human monocyte cell lines (Fig. 3b). Staining of CD99 by mAb hec2 on freshly isolated monocyte resulted in similar staining profiles (data not shown). To further evaluate the biological activity of the scFv C7A, we performed a transendothelial migration monocyte blocking assay, utilized for previous antibody anti-CD99 blocking studies (Schenkel et al., 2002; Lou et al., 2007). The results show that scFv C7A blocked monocyte transmigration in a concentration-dependent manner. A concentration of 20 μg/ml scFv blocked transmigration by >70% (Fig. 3c). Interestingly, the monovalent scFv had about the same ability to block transmigration as of bivalent mAb hec2 at the same concentration. The result of the stability study in ELISA assay (Fig. 3d) shows that scFv C7A antibody at the reference value of 20 μg/ml retains the ability to react with the rCD99 protein.

### Table 1

<table>
<thead>
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<th>Purification step</th>
<th>Yield (mg)</th>
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<th>% monomer by SEC</th>
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![Fig. 3](image_url)

**Fig. 3.** (a) Specific binding of scFv C7A (from 40 to 0.15 μg/ml) to rCD99 (5 μg/ml) analyzed by ELISA, no reactivity was determined to BSA. As negative control was used a scFv anti-CEACAM1 to rCD99. Lower binding of scFv C7A NO pelB aggregates compared with scFv C7A monomer was also shown (b) The binding profile by flow cytometry of one representative experiment of three performed of scFv C7A (10 μg/ml) samples to monocyte cells. (c) TEM analysis: negative control (NC) monocyte migration (70%) in the presence of non-blocking anti-VE-cadherin; hec2 mAb positive control (20 μg/ml) confirms the ability of anti-CD99 to block transmigration. The different concentrations of scFv C7A (from 1 to 20 μg/ml) shows the antibody fragment ability to blocking monocyte transmigration. At a concentration of 20 μg/ml of scFv C7A monovalent, %TEM was 21%. Values are mean ± SEM of three experiments performed with six replicates in each experiment. P values vs. control for hec2 = 0.0003, for scFv 2 μg/ml = 0.008, for scFv 5 μg/ml = 0.0011, for scFv 20 μg/ml = 0.0006. (d) Relative binding level (%) to rCD99 (5 μg/ml) of scFv C7A (20 μg/ml) at time 0 and after a storage periods for 3, 6, 9, 12 months at −80 °C, −20 °C and +4 °C determined by ELISA.

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after one year when stored at −80 °C and −20 °C compared to the value at time zero. In contrast a strong decrease of binding was observed when scFv C7A was stored at +4 °C.

3.4. Expression, purification and characterization of scFv C7A NO pelB

The expression and purification were performed in the same conditions of scFv C7A. The expression of scFv C7A NO pelB construct seems to behave in the same way of scFv C7A, with a percentage of scFv cytoplasmic inclusion bodies around 81% (Fig. 4a–b). The first purification step by IMAC chromatography revealed a similar peak profile compared to scFv C7A (Fig. 4c); SDS-PAGE and WB confirm this result. The substantial difference was appreciated in the final purification step DEAE chromatography; in fact, Fig. 4d shows that no peak elution was obtained at 0.25 M NaCl, while peak eluted at 0.5 M NaCl showed the presence of pure and apparently correct molecular weight of scFv C7A NO pelB (~32 kDa) as show in SDS-PAGE analysis and confirmed by WB (Fig. 4d). Gel filtration analysis demonstrated that scFv C7A NO pelB eluted at 0.5 M NaCl consisted mainly of aggregates (Fig. 4e) assuming that scFv from cytoplasmic inclusion bodies remains in an unfolding aggregation state, while scFv derived from periplasmic inclusion bodies has the ability of correctly folding through this simple and fast refolding method. Finally scFv C7A NO pelB purified was tested in ELISA assay; the test revealed that also aggregated forms had some activity, although considerably lower than scFv C7A in monomeric form (Fig. 3a).

4. Discussion

Migration of monocytes from the bloodstream across vascular endothelium is required for entry into sites of inflammation. Transendothelial migration of monocytes initially involves tethering of cells to the endothelium, followed by loose rolling along the vascular surface, firm adhesion to the
endothelium and diapedesis between the tightly apposing endothelial cells (Sullivan and Muller, 2014). Diapedesis by monocytes occurs through interaction between PECAM-1 on both the monocyte and the endothelial cells, followed by similar homophilic adhesion via CD99. After penetration of the endothelial basement membrane, monocytes migrate through the extracellular matrix of the tissues where they may migrate to sites of inflammation (Muller, 2014). Several publications show clearly that the blocking antibodies against CD99 inhibit inflammation in vitro and in vivo models (Bixel et al., 2010; Dufour et al., 2008). These findings indicate that the transmembrane CD99 protein represents a valid pharmacological target for the treatment of inflammatory process.

mAbs are an increasingly important class of therapeutic agents. Intact antibodies are bivalent specific target reagent with antigen binding site located on the two Fab tips and recruitment of effector functions mediated by the stem Fc domain. This last domain recruits cytotoxic effector functions through complement as well as by direct binding to Fc receptors on leukocytes. However there is a range of therapeutic applications in which the Fc-mediated effects are not required and are even detrimental. For example with antibodies that work by reducing the ability of inflammatory cells to pass into inflamed tissue, an inappropriate activation of Fc receptor-expressing cells can lead to massive cytokine release and associated toxic effects (Holliger and Hudson, 2005). Added to this, monoclonal antibody producing technology is very laborious and time consuming (Frenzel et al., 2013). These limitations of traditional techniques have led several research groups to investigate the use of the E. coli system in producing scFv antibodies (Huang et al., 2012).

Recombinant DNA technologies have made possible the modification of an antibody into smaller binding fragments such as scFv, in which the antigen binding sites can be retained. A bacterial expression system offers an economical alternative with the potential for high product yields, short fermentation durations and ease of scalability (Meyer and Schmidhalter, 2012). To date, the bacterial expression system is most often applied for the production of scFv compared to the various expression strategies available (Nelson, 2010; Reichert, 2012). For medical applications scFvs are needed in large amounts, and the ability to produce high yields in E. coli has gained considerable interest.

There are a number of different strategies used to express the recombinant antibody fragments in E. coli (Katsuda et al., 2012). Soluble periplasmic and cytoplasmic expressions have been extensively employed in the small scale laboratory production of various soluble scFv antibodies, but scalability issues involved with these processes make them difficult to implement in an industrial setting. (Wang et al., 2013) In fact, due to the intrinsic high growth rate of E. coli, high cell density cultures (HCD) are currently used for the industrial production of antibody fragments (Spadiut et al., 2014). Normally, over-expression in HCD of scFvs in the cytoplasm of E. coli leads to the accumulation of IBs (Kim et al., 2011). For that reason, IBs must be re-natured in vitro to improve the correct folding of functional antibody by means of appropriate rearrangement of the disulfide bonds. HCD fermentation and high throughput purification of the recombinant protein from inclusion bodies of E. coli are the two major bottlenecks for the cost effective production of therapeutic proteins (Panda, 2003).

Highly efficient refolding methods are required to provide enough active scFv for therapeutic or diagnostic use.

To date, a plethora of refolding and purification systems from cytoplasmic insoluble scFv has been reported, i.e., gel filtration chromatography, elaborate dialysis, or use reagent as ionic detergents (Bu et al., 2013; Ortega et al., 2013; Ong et al., 2012; Liu et al., 2006), unfortunately many of these biochemical processes are not suitable as a source of therapeutic antibodies for future industrial processes. Furthermore many of this published methods lacking a biophysical characterization in gel filtration chromatography to verify the correct conformational state of the scFv (Kou et al., 2007; Robert et al., 2006). In addition as demonstrated in this work for scFvs and by Garcia-Fruitos et al. (2005) for enzymes and proteins, the IBs’ aggregation state may have a biological activity and therefore may have been led to think that the refolding methods utilized are effective, whereas with many probabilities it may be a mixture of monomers–dimers and aggregates of scFv inapplicable to therapeutic human use.

To date, all four monovalent scFvs (ESBA105, Efungumab, Pexelizumab and Aurograb) in clinical development and expressed in E. coli, were produced as cytoplasmic inclusion bodies. The refolding methods used for these scFv are in all cases elaborate and time consuming: for example Pexelizumab and ESBA105 purification methods include into a production process a long dialysis step for scFv refolding and gel filtration chromatography for eliminating the aggregates (Evans et al., 2002; Der Maur et al., 2009; Ottiger et al., 2009). Furthermore monomer scFv Efungumab final purified product contained 10.5% of dimer (Matthews et al., 2003; Burme and Wechner, 2009) and for monomer scFv Aurograb approximately 18% of the molecule exists as a homodimer (Burme and Matthews, 2008) indicating that purification processes for these scFvs are unable to be fully efficient in the refolding of scFv in monomeric state.

The study of purification and characterization of scFv C7A by the presence or absence of pelB leader sequence in the construct, has allowed the understanding of interesting aspects: unlike cytoplasmic inclusion bodies, a significant part of the periplasmic protein aggregates has correctly formed disulfide bridges. This periplasmic space is identified to contain protein such as chaperones and disulfide isomerases which assist the proper folding of antibody fragment. This conclusion also was derived from previous observation that the redox reshuffling of disulfide bridges in scFv isolated from periplasmic inclusion bodies did not increase the yield of soluble and functional product after refolding (Kipriyanov et al., 1994). The driving force behind periplasmic aggregation might be hydrophobic interactions between the molecules of folding intermediates (Dill, 1990). In this case, high protein concentrations derive from HCD cultures of the secreted antibody fragment in the periplasmic space would favor the formation of insoluble aggregates over correct folding (Bowden and Georgiou, 1990; Kieh labeling et al., 1991).

To date, few cases reported purification of antibody fragments from periplasmic insoluble material (Casalvilla et al., 1999; Smallshaw et al., 1999; Moosmayer et al., 1995; Kipriyanov et al., 1995, 1996). Furthermore, no gel filtration characterization was used to verify the correct folding of purified scFv (Casalvilla et al., 1999; Smallshaw et al., 1999; Moosmayer et al., 1995). In addition, Kipriyanov et al. (1995, 1996) uses a laboratory scale method for scFv production consisting of refolding by slow dialysis and final gel filtration to...
separate the various forms of purified scFv. Until now, there was no paper that describes a large scale method for expression and purification and characterization of monomeric antibody fragments from periplasmic inclusion bodies suitable for clinical development.

In the present study, a human anti-CD99 scFv C7A was constructed and expressed in E. coli BL21 (DE3) as periplasmic inclusion bodies. A convenient procedure of two steps on-column purification and refolding was provided. Large scale production at 15 l scale of scFv C7A with a total yield of 500 mg (~35 mg/l) render the process optimal for a high production yield of drug substances. For antibody purification and refolding procedure neither use of dialysis step or additives inappropriate for human use were required. The denatured inclusion body samples were loaded directly on column. In the first chromatographic step an affinity His-tag allows the elimination on-column of Urea by the application a linear gradient step. At the same time a washing step by 70 mMimidazole buffer was able to eliminate most bacterial contaminants and to obtain a scFv at 95% purity. The second chromatographic step of an anion exchange chromatography separated the aggregates from monomeric active form and avoids the use of gel filtration chromatography in the final step. The principle is simple but very effective. The use of differential salt elution has allowed us to discriminate the monomeric forms of the scFv that have less negative charges compared to aggregated forms of scFvs with more negative charges and therefore a higher binding capacity towards the resin positively charged DEAE resin. The eluted monomeric scFv C7A shows a 99% purity by SDS-PAGE and gel filtration analysis. The endotoxin content was 1 EU/ml (0.001 EU/µg) by using the chromasorb salt tolerant device before final sterilization step, making the antibody suitable for cellular assays and for clinical application. The refolded scFv showed correct structure by analytical gel filtration and high binding affinity towards its cognate antigen CD99 in ELISA and flow-cytometry studies. Activity assay showed that the scFv C7A is able to block monocyte transendothelial migration with an activity level comparable to anti-CD99 mAb hec2 which was obtained from the eukaryotic expression system. Furthermore, our results show that biological specificities are unchanged for at least 12 months when the purified scFv C7A is stored on −20 °C and −80 °C.

In conclusion, by using the purification procedure herein reported and described biologically active scFv C7A were obtained. The materials and methods utilized allowed a large scale production of a safe antibody which is appropriate for industrial requirements and clinical application. This work would be important to help industrial development of inclusion bodies expressed scFv biopharmaceuticals, redirecting the scFv industrial large scale production from cytoplasmic to scFv periplasmic inclusion bodies’ expression. We have tested the production protocol with other two different scFvs starting from periplasmic inclusion bodies with similar results (data not shown). The method here proposed, may be also applicable to other scFvs in monomeric conformation.

References


