Introduction

The single-domain antibody (sdAb) is the smallest functional antibody fragment. It was originally derived from the variable domains of heavy-chain antibody (HCAb) found in either camel or shark. The HCAb is composed of the heavy chain (HC) only; it is devoid of both the light chain (LC) and the first constant domain (CH1) (Hamers-Casterman et al., 1993). Alternatively, sdAb can be engineered from the variable domains of either the heavy chains or the light chains of conventional antibodies (Famm et al., 2008). The variable domain of the HCAb (VHH) can bind to its corresponding antigen different from the conventional antibody that requires variable domains from both heavy chain and light chain for antigen binding. The stability of single-chain VHH in the absence of VL was proposed to be mediated by four amino acid substitutions in the framework-2 region (Muyldermans et al., 1994; Vu et al., 1997). A variety of sdAbs for tumor-associated antigens have been isolated (Roovers et al., 2007; Behar et al., 2009; Baral et al., 2011). Due to the nature of small size, sdAb is more suitable for genetic and molecular manipulation than a conventional antibody with VL and VH domain (Harmsen and De Haard 2008). In this study, we have fused an sdAb to a self-associating peptide to form multimers. Three different peptides were used in the strategy to produce multivalent and multimeric sdAbs targeting different epitopes on EGFR is more potent in the inhibition of tumour growth than that using bivalent, monospecific sdAb (Roovers et al., 2011). Although VHH fragment derived from HCAb is highly stable and soluble (Muyldermans 2001), due to its monovalent interaction to the antigen there is a disadvantage with fast dissociation rates in the non-equilibrium environment of the vasculature and tissues. To circumvent the problem the multimerization strategies were adopted to slow the dissociation rates as well as provide other benefits (Holliger and Hudson 2005). The strategies used for the multimerization include: domain-swapping phenomenon, linear fusion of genes, chemical linking, self-associating peptides, and heterodimerization modules (Deyev and Lebedenko 2008). In this study, we have fused an sdAb to a self-associating peptide to form multimers. Three different peptides were used in the strategy to produce multivalent recombinant multimers and the multimers were tested for their avidities and specificities against correspondent antigens.

Right-handed coiled coil (RHCC) is a c-terminal part fragment of the tetrabrachion protein complex derived from S-layer of the archaeabacterium Staphylothermus marinus (Peters et al., 1995; 1996) with an extreme thermal stability. The crystal structure reveals that RHCC folds into a parallel right-handed coiled-coil tetramer with four large internal cavities, which are capable of binding heavy metal ions (Stetefeld et al., 2000). RHCC has been utilized as a carrier for the anticancer drug cisplatin (Eriksson et al., 2009).

Cartilage oligomeric matrix protein (COMP) is an oligomeric glycoprotein found in all types of cartilage (Hedbom et al., 1995; 1996) with an extreme thermal stability. The crystal structure reveals that RHCC folds into a parallel right-handed coiled-coil tetramer with four large internal cavities, which are capable of binding heavy metal ions (Stetefeld et al., 2000). RHCC has been utilized as a carrier for the anticancer drug cisplatin (Eriksson et al., 2009).

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The C-terminus part of C4bp responsible for the polymerization of the molecule (Villoutreix et al., 1998). The C-terminal parts of both α- and β-chains are responsible for the polymerization of complement activation (Gigli et al., 1983). The C-terminal parts of both α- and β-chains are responsible for the polymerization of the molecule (Villoutreix et al., 1998). The amphipathic helices and two cysteine residues in the C-terminus of the C4bp α-chain (C4bpα) have been extensively studied for their roles in polymerization (Kask et al., 2002). The C-terminus part of C4bpα has been fused to various proteins and to single chain variable fragment (scfv) as an oligomerization domain (Oudin et al., 2000; Ogun et al., 2008).

In this study we report that sdAb derived from the variable domains of HCαb of Llama could be fused to three different multimeric peptides to create tetrameric, pentameric and heptameric molecules. These multimers were successfully expressed and correctly folded using prokaryotic expression systems. The avidities and specificities of these antibodies were assessed using biophysical and biological methods. The potential of the multimeric antibodies for further applications will be discussed.

Materials and methods

Construction of plasmids

The cDNA encoding sdAb EG2, which is specific for the extra-membrane domain of EGFR, was synthesized using published information (Bell et al., 2010) (patent WO2008/141449 A1), and inserted into pVT2 vector with the corresponding restriction enzymes BbsI and BamHI. The cDNA encoding coiled-coil domain of COMP (COMPcc) forms two internal hydrophobic pores capable of storage and delivery of vitamin D and other hydrophobic compounds (Guo et al., 2002). C4-binding protein (C4bp) is an abundant plasma glycoprotein that plays an important role in the classical pathway of complement activation (Gigli et al., 1979). Electron microscope images show that C4bp is a spider-like structure composed of seven α-chains and one β-chain (Dahlback et al., 1983). The C-terminal parts of both α- and β-chains are responsible for the polymerization of the molecule (Villoutreix et al., 1998). The amphipathic helices and two cysteine residues in the C-terminus of the C4bp α-chain (C4bpα) have been extensively studied for their roles in polymerization (Kask et al., 2002). The C-terminus part of C4bpα has been fused to various proteins and to single chain variable fragment (scfv) as an oligomerization domain (Oudin et al., 2000; Ogun et al., 2008).

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Expression and purification

The procedure for the expression and purification of EG2, EG2-RHCC, EG2-COMPcc and EG2-C4bpα were similar. BL21 cells with pET30a-EG2-RHCC, pET30a-EG2-COMPcc, pET30a-EG2-C4bpα and TG1 cells with pVT2-EG2 were cultured in Luria−Bertani (LB) medium containing corresponding antibiotics (kanamycin or ampicillin) at 37°C while shaking at 220 r.p.m. until OD600 reached 0.6. Then, isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM, and the BL21 cells were cultured overnight and the TG1 cells were cultured at 22°C for 22 h. The cells were harvested by centrifugation, and the pellets were resuspended in phosphate-buffered saline (PBS) with extra salt (400 mM/mI NaCl). Then, lysozyme was added for cell lysis at room temperature. After 30 min, the mixture was sonicated using a sonic dismembrator until it became clear, then centrifuged for 20 min at 12000 r.p.m. followed by another 20 min at 14000 r.p.m. The supernatant were filtered through a 0.22-μm filter and the sdAbs were purified by affinity chromatography on NiSO4-charged chelating sepharose Fast Flow gel (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer’s instructions. The elution of recombinant multimers and sdab EG2 was concentrated and further purified by Superdex™ 200 size-exclusion chromatography using the AKTA purifier 2000 system (GE healthcare Bio-Science AB).

For analysis of the multimeric state, the recombinant multimers and the sdAb EG2 under both reduced (+ DTT) and non-reduced (− DTT) conditions were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. In addition, native EG2-RHCC (− DTT, − SDS) was run on Native-PAGE (− DTT, − SDS) gels, and EG2-COMPcc and EG2-C4bpα under non-reduced (− DTT) conditions were run on 6% SDS-PAGE gels. All proteins in gels were stained by Coomassie brilliant blue R250.

Surface plasmon resonance

The binding specificities of EG2, EG2-RHCC, EG2-COMPcc and EG2-C4bpα to EGFR was measured using a BIACORE 3000 instrument (Biacore Inc., Uppsala, Sweden). Briefly, 1200RU EGFR (recombinant Human EGFR Fc chimera, 344-ER-050, R&D Systems, Minneapolis, MN, USA) were immobilized on channel 2 of CM5 chips (Biacore Inc.). Different concentrations of EG2, EG2-RHCC, EG2-COMPcc and EG2-C4bpα were injected into channels 1 and 2 at a flow rate of 30 μl/min to record the signals; 10 mM NaOH was used as a regenerating buffer. All steps were performed at 25°C, and signals were recorded as sensograms.
Flow cytometry analysis

A431 cells and 293T cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA). EG2, EG2-RHCC, EG2-COMPcc, EG2-C4bpα, and anti-tumor necrosis factor α (TNFα), anti-TNFα-RHCC, anti-TNFα-COMPcc and anti-TNFα-C4bpα (Lifei Wang and Xiaoli Liu et al., manuscript in preparation) were used as primary antibodies. 1 x 10⁶ cells were washed and resuspended in staining buffer (SB) containing PBS plus 0.5% bovine serum albumin plus 2 mM EDTA. The cells were incubated first with recombinant multimers or sdAbs on ice for 30 min and then with secondary antibodies, mouse anti-His IgG (Beijing Cowin Biotech Co. Ltd, Beijing, PR China). Subsequently, the cells were washed with 3 ml SB, and the pellets were resuspended in 100 μl of SB containing 5 μl of FITC-labeled goat anti-mouse IgG (Epigen Biotech). After incubation on ice for 30 min, the cells were washed and resuspended in 200 μl SB then analyzed by flow cytometry with a Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA, USA). All FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Results

Expression of recombinant multimers

The sdAb EG2 used in this experiment was derived from Ilama and was specific for the extracellular domain of EGFR. The molecular architecture of the sdAb EG2 is shown in Fig. 1A. The sdAbs were fused to the self-assembling peptides RHCC, COMPcc or C4bpα to make recombinant multimers EG2-RHCC, EG2-COMPcc or EG2-C4bpα, respectively. The molecular architectures of recombinant multimers are depicted in Fig. 1B. The molecular weight of EG2, EG2-RHCC, EG2-COMPcc and EG2-C4bpα calculated from the amino acid composition are 16.7 kDa, 94.5 kDa, 116.4 kDa and 162.6 kDa, respectively.

EG2-RHCC, EG2-COMPcc and EG2-C4bpα with C-terminal 6×His tags were expressed as soluble proteins in the cytoplasm of *Escherichia coli* strain BL21 and EG2 was expressed as soluble protein in the periplasmic cavity of *E. coli* strain TG1. The proteins were purified through two steps. First, the cell lysates were passed through the immobilized metal affinity chromatography columns and eluted by the addition of imidazole. Second, the eluted proteins were concentrated and passed through a Superdex™ 200 gel
filtration column. The size-exclusion chromatography profiles of recombinant multimers and the sdAb EG2 in PBS are showed in Fig. 2. The size-exclusion chromatography profiles of the molecular weight makers thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (44 kDa) in PBS are also showed in Fig. 2 and their elution positions are indicated in the figure. The elutions of all three recombinant multimers run out later than thyroglobulin (669 kDa) and ferritin (440 kDa), earlier than aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (44 kDa). The elution of the sdAb came out after ovalbumin (44 kDa). Furthermore, the order of elutions of the recombinant multimers was always found to be EG2-C4bp, EG2-COMPcc and EG2-RHCC under the same condition (column and buffer). All three recombinant multimers were eluted before aldolase (158 kDa) (Fig. 2), which is theoretically larger than EG2-COMPcc and EG2-RHCC, possibly due to less compacted structures of the multimers containing relatively long linker (Fig. 1B). Nevertheless, the results of size-exclusion chromatography suggest that all three recombinant multimers exist neither as monomer nor as irregular aggregates.

The multimeric states and the molecular weights of EG2-RHCC, EG2-COMPcc and EG2-C4bp were also investigated by SDS-PAGE (Fig. 3). In 12% SDS-PAGE gels, EG2-RHCC appeared as a band at ~25 kDa under both reduced and non-reduced conditions (Fig. 3A, left) and migrated ~95 kDa under native condition in native gels (Fig. 3B, left), suggesting that EG2-RHCC may be a multimer relied on hydrophobic interactions without inter-chain disulfide bonds. In contrast, the bands of EG2-COMPcc and EG2-C4bp appeared at ~25 kDa under reduced condition, while migrated above 116 kDa under non-reduced conditions in 12% SDS-PAGE gels (Fig. 3A, middle and right), indicating that the maintain of the multimeric states of EG2-COMPcc and EG2-C4bp is relied on the inter-chain disulfide bonds. In 6% SDS-PAGE gels, EG2-COMPcc and EG2-C4bp appeared as bands of ~130 and 170 kDa under non-reduced conditions (Fig. 3B, middle and right), which were slightly larger than those predicted from the amino acid composition. The results of SDS-PAGE consisted with the fact that COMPcc and C4bp possess inter-chain disulfide bonds while RHCC do not. In 15% SDS-PAGE gels, EG2 migrated <18.4 kDa under both reduced and non-reduced conditions (Fig. 3C).

Based on the results of size-exclusion chromatography and SDS-PAGE together with the native structures of the self-associating peptides used here, it is concluded that recombinant multimers EG2-RHCC, EG2-COMPcc and EG2-C4bp formed tetrameric, pentameric and heptameric molecules, respectively.

The specific bindings of recombinant multimers to their antigen EGFR

The abilities of antigen recognition for recombinant multimers were tested by surface plasmon resonance (SPR) (Fig. 4). Specific recognitions between immobilized EGFR and the recombinant multimers could be clearly seen from the curves in the sensorgrams, in which the signals [response units (RU)] increased as the concentration of the recombinant proteins increased (Fig. 4A–C). From the results we could
also infer slower dissociation rates of the recombinant multimers (Fig. 4A–C) compared with that of the monomeric form of sdAbs EG2 (Fig. 4D), which consists with the previous report that multivalent constructs of EG2 showed slower $k_{\text{off}}$ when interacted with an immobilized protein (Bell et al., 2010). In each round, when dissociation was ended, the curve did not return to the baseline and 10 mM NaOH was used as the regenerating buffer. Theoretically, the binding ratios between divalent EGFR (recombinant EGFR Fc chimera) and tetrameric EG2-RHCC, pentameric EG2-COMPcc and heptameric EG2-C4bp $\alpha$ are $2:4$, $2:5$ and $2:7$, respectively. None of these results strictly fit the interaction model of the BIAevaluation software 4.1 and the precise quantitative analysis of EGFR-recombinant antibodies was not feasible.

Next, the abilities of the recombinant multimers to specifically recognize EGFR expressed on the cell surface were analyzed. The cell line A431 expressing EGFR on its surface was used as a target cell and anti-TNF$\alpha$-RHCC, anti-TNF$\alpha$-COMPcc anti-TNF$\alpha$-C4bp $\alpha$ antibody multimers were served as negative controls for binding. As shown in the Fig. 5, the A431 cells were stained by EG2-RHCC, EG2-COMPcc and EG2-C4bp $\alpha$ very well. As expected, there was no staining with anti-TNF$\alpha$-RHCC, anti-TNF$\alpha$-COMPcc and anti-TNF$\alpha$-C4bp $\alpha$ multimers (Fig. 5A). Similarly, there was no binding seen when EGFR negative 293T cells were stained with multimers EG2-RHCC, EG2-COMPcc and EG2-C4bp $\alpha$ (Fig. 5B) and EG2 monomer (Fig. 5D). The results indicate that the recombinant multimers specifically recognized the EGFR expressed on A431 cells. When A431 cells were incubated with a monomeric form of EG2 sdAb, there was no staining, the same as the cells stained with anti-TNF$\alpha$ negative control (Fig. 5C). This results possibly due to the fast $k_{\text{off}}$ when EG2 sdAb interacted with EGFR (Fig. 4D). The overall results of FACS showed that all three recombinant multimers improved the affinity or avidity of sdAb EG2 for EGFR antigen.

**Discussion**

Our study has shown that the sdAb EG2 can be fused with three different self-associating peptides to form recombinant tetramers, pentamers and heptamers. In addition, all recombinant multimers retain their antigen-specific recognition and improve the affinity or avidity of the sdAb. The success of fusing one sdAb with three different self-associating peptides means that one can choose different self-associating peptides to obtain suitable number of multimeric state and desired properties attaching to a particular self-associating peptide, besides the general advantages brought by multimerization such as slower dissociation rate and greater tumor localization (Carter 2006). Recent publications described the binding ability of COMPcc and RHCC to biologically relevant molecules such as vitamin A and the anti-cancer drug cisplatin. The authors proposed that these binding properties could be utilized to develop a targeted system to deliver drugs directly...
to cancer cells, thereby circumventing the toxic effects on healthy cells (McFarlane et al., 2009). Our results and other related work (Zhu et al., 2010) represent an important step toward this goal. The recombinant antibodies will act as precision-guided munitions, in which the sdAbs is responsible for targeting while the oligomerization domain plays a role in carrying cytotoxic compound. Our results with C4bpα was among the highest number of valency for an antibody (Deyev and Lebedenko 2008), providing a choice for making recombinant multimer with the highest avidity possible.

We expressed recombinant multimers directly in the cytoplasm of E.coli stain BL21 using ordinary LB medium and relatively short time period compared with previous works (Stone et al., 2007; Bell et al., 2010). In our experiments, all three self-associating peptides, regardless of the source of derivation, human or archaeabacterium, could organize into the correct conformation in a soluble form in the cytoplasm and could be purified as soluble proteins. In previous studies, the production of tetramer RHCC was accomplished through manipulation of the refolding process (Stetefeld et al., 2000), and the formation of the pentamer complex of COMPcc was achieved using the glutathione redox system (Efimov et al., 1996). The highly solubility of the particular fold of EG2 could be further exploited to work on an expression and purification system for a protein of interest with sdAb EG2 as an expression tag.

A potential application of multimeric antibody is to construct bi-specific or multi-specific constructs for various applications. For example, incorporation of an anti-CD3 antibody along with a tumor-specific antibody would be used as a therapeutic for antigen-directed T-cell therapy.

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**References**


Single-domain antibody multimers with self-associating peptides


