Camel Single-domain Antibodies as Modular Building Units in Bispecific and Bivalent Antibody Constructs*

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Single-domain antibodies against various antigens are isolated from the unique heavy-chain antibodies of immunized camels and llamas. These minimal sized binders are very robust and bind the antigen with high affinity in a monomeric state. We evaluated the feasibility to produce soluble, functional bispecific and bivalent antibodies in Escherichia coli with camel single-domain antibody fragments as building blocks. Two single-domain antibody fragments were tethered by the structural upper hinge of a natural antibody to generate bispecific molecules. This linker was chosen for its protease resistance in serum and its natural flexibility to reorient the upstream and downstream located domains. The expression levels, ease of purification, and the solubility of the recombinant proteins were comparable with those of the constituent monomers. The individual moieties fully retain the binding capacity and the binding characteristics within the recombinant bispecific constructs. The easy generation steps and the biophysical properties of these bispecific and bivalent constructs based on camel single-domain antibody fragments makes them particularly attractive for use in therapeutic or diagnostic programs.

Bispecific antibody constructs have a great potential in therapeutic and diagnostic applications where cross-linking of unrelated antigens is required. For example, the immune activation or the cellular targeting of cytotoxic T-cells to mediate killing of tumor cells often benefit from the availability of bispecific antigen binders. Originally, bispecific antibodies were generated by fusing two carefully selected hybridomas (1). The disadvantages of this approach are the low yield of functional bispecific protein due to the frequent association between the noncognate antibody chains and the presence of the Fc part that might lead to illegitimate targeting to cells containing Fc receptors. The latter inconvenience is easily avoided by building smaller bispecific antigen-binding fragments obtained by tethering scFv fragments (2). The scFv fragments, formed by genetic engineering and expressed in Escherichia coli, contain only the VH and VL domains joined by a synthetic linker of about 15 amino acids. Multiple strategies were employed to connect different scFvs, e.g. chemical cross-linking with C-terminal cysteine residues (3), adding naturally associating helices from a four-helix bundle (4), adding leucine zippers (5), adding a CH3 domain with either a knob or hole at the interacting surfaces (6), or by connecting CH1 and CL domains to the respective scFv fragments (7). However the stability of these innovative bispecific constructs remains questionable. Bridging scFvs with a third polypeptide linker to form a bispecific unit has met with limited success, since such constructs contain three linkers known to be susceptible to proteolysis and leading to aggregation. An alternative was provided by the coexpression of two hybrid scFvs made by the VH of a first antibody and the VL gene of a second antibody with a shortened linker of 0–5 amino acids and vice versa (8). Two polypeptide chains are then forced to dimerize, since the shortened linker does not allow pairing of the domains within one polypeptide chain. The heterodimers of these two polypeptide chains carry two antigen-binding sites in the same molecule. Unfortunately, also homodimers that are nonfunctional might be formed, and therefore, the functional expression of these so-called diabodies often remains low. Moreover, the aberrant angle between the VH-VL pairs may be detrimental to antigen binding (9).

It is clear that the functional bispecific antibody constructs are in need of minimal size, absence of linker peptides prone to aggregation or susceptible to proteolysis, high expression yields, high solubility, and high stability. Theoretically, the usage of the single-domain antigen-binding sites derived from camel heavy-chain antibodies as the modular unit could generate such constructs (10). It has been demonstrated that serum of Camelidae (camels and llamas) contains an important fraction of functional, heavy-chain immunoglobulins that are naturally devoid of light chains (11). These heavy-chain antibodies recognize the antigen by one single domain (referred to as VHH) that can be cloned from the blood lymphocytes of an immunized dromedary (12). The VHHs are easily produced as recombinant proteins in bacteria and yeast, appear to be quite soluble and robust, and do not have the tendency to aggregate. The affinity of the isolated, single-domain antibody fragments is in the same range as that of scFv fragments. In addition, the VHHs seem to be directed toward epitopes that are not immunogenic for conventional antibodies, such as the active site of enzymes, resulting in potent enzyme inhibitors (13).

In this study, we constructed several bispecific (and bivalent) antibodies by genetically linking two distinct VHHs with the natural hinge of llama heavy-chain antibodies and report on their performance.

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The abbreviations used are: Fc, effector function fragment; cAb, camel single-domain antibody fragment; Fv, variable fragment of antibodies; VHH, variable domain of heavy chain of camel heavy-chain antibodies; NAG, N-acetyl-N,-acetylated-chitotriose; scFv, single chain variable fragment; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Nmc-A, nonmetal carbapenemase of class A; ELISA, enzyme-linked immunosorbent assay.
Cloning of Bispecific Antibodies—By standard DNA cloning techniques we assembled an expression vector (pKC6) to generate the bispecific proteins VHH1-LH-VHH2, tagged by six histidines. “LH” stands for “long hinge” and is the structural upper hinge of the llama IgG2a (14). The N-terminal antibody fragment is cloned into the expression vector using the restriction enzymes NcoI and BstEII. The C-terminal fragment is inserted using PstI and NotI. The amino acid sequences of linker and the three VHHS used in this study are shown in Fig. 1. The expression of protein is under control of the lac promoter.

Purification of the Bispecific or Bivalent Antibodies—The bacterial cells (WK6 E. coli) containing the proper plasmids were grown, and expression of the recombinant proteins was induced (1 mM isopropyl-

SDS-PAGE and Western Blot—Samples were analyzed by an SDS-PAGE (12% acrylamide) stained by Coomassie Brilliant Blue or in Western blot using an anti-histidine monoclonal antibody (Serotec) and an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NET) substrate (Sigma).

Biotinylation of Antigen—Nmc-A (nonmetallo carbapenemase of class A) and lysozyme were biotinylated by incubating the antigen with a 5× molar excess of biotin-X-N-hydroxysuccinimide (Calbiochem) on ice followed by extensive dialysis against PBS.

ELISA—For ELISA experiments, the antigens (1 μg/ml in PBS) were coated overnight at 4 °C in a 96-well plate (Nunc Maxisorb). After blocking, bispecific proteins were detected and with an anti-histidine monoclonal antibody (Serotec) and an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) with p-nitrophenyl phosphate substrate (Sigma). The signal was measured at 405 nm.

For the sandwich ELISA, bispecific constructs were applied to antigen-coated wells; after washing we added biotinylated Nmc-A or lysozyme.Captured biotinylated protein was detected by extravidin alkaline phosphatase conjugate (Sigma) and substrate, as described above.

Gel Filtration—Binding of the bispecific antibodies to the different antigens in solution was tested on an analytical gel filtration column (Superdex HR 75–10/30, Amersham Pharmacia Biotech) used at a flow rate of 0.3 ml of PBS/min. Bispecific antibodies (150 μg/200 μl) were preincubated for 1 h with equimolar amounts of their antigen before loading.

Biosensor Experiments—A biosensor (IAsys, Fisons) was used to measure the antigen affinity, the biospecificity, and to map the epitope.

Affinity Measurements—Lysozyme and Nmc-A were immobilized (13) with a coupling efficiency around 1000 arc seconds. After equilibration of the cuvette with PBS, different concentrations of the bivalent or bispecific proteins (final concentration 10–70 nM) were directly added to the sample cuvette (each concentration was measured in duplicate). In the IAsys biosensor, the sample is continuously stirred, and binding is continuously followed. Association and dissociation rate constants were determined with FASTFIT software. Kd was calculated as koff/kon.

Bispecificity Measurements—On a lysozyme cuvette, bispecific protein was added; after washing away nonbound protein, either α-amylase or Nmc-A was added, and additional binding was measured.

Epitope Mapping—Binding of the bispecific constructs, harboring the lysozyme-specific fragment, into the active site of lysozyme was illustrated in a displacement assay. The lysozyme cuvette was saturated with 1 mM NAG, to block all accessible active sites of the coupled lysozyme. Bispecific protein was added, and binding was monitored.

Amylase Inhibition Assay—The enzymatic activity of α-amylase was determined as described previously (13).

Stability Assay—The stability of the bispecific protein in PBS (85 μg/ml) or in mouse plasma (20 μg/ml) was evaluated with the biosensor by measuring the residual antigen binding activity after different incubation periods at 37 °C. Aliquots were also analyzed by SDS-PAGE after incubation.

RESULTS

We started from camel single-domain antibodies (cAb) currently investigated in our laboratory to illustrate their potential as modular building blocks in bispecific constructs. cAbLys3 binds to, and is a competitive inhibitor of, hen egg white lysozyme with a Kd of 50 nM. Its crystal structure in complex with antigen and the third antigen binding loop mimicking the natural substrate of lysozyme was described previously (16, 17). The inhibitor cAbAMD9 binds to porcine pancreatic α-amylase with a Kd of 4 nM (13). The cAbLA01 binds the Nmc-A with nanomolar affinity; this fragment was isolated from a phage library (12).

Cloning and Expression of Bispecific and Bivalent Constructs

The following five bispecific and bivalent constructs were assembled: cAbLys3-cAbAMD9, cAbAMD9-cAbLys3, cAbLA01-cAbLys3, cAbLys3-cAbLA01, and cAbLys3-cAbLys3. The gene segments encoding the bispecific constructs were cloned into an expression vector pKC6 (Fig. 1) between a PelB leader signal and a C-terminal hexahistidine tail for periplasmic transport and for purification of the recombinant protein, respectively. After screening for the presence of recombinant protein in the periplasm and for the best expressing clones, larger scale productions (up to 5 liters) of the bispecific proteins were performed.

The recombinant proteins were extracted from the periplasm and purified by immobilized metal affinity chromatography and gel filtration. The elution profile of the gel filtration indicates a molecular weight of 37,000 for the recombinant proteins, whereas 15,000 is found for a VHH domain (Fig. 2A). The SDS-PAGE of the recombinant protein confirmed its apparent molecular weight and indicated a purity above 90% as evaluated by Coomassie staining (Fig. 2B).

Our recombinant protein constructs have a good solubility behavior and have no tendency to aggregate even at 3–4 mg/
Bispecific Antibodies

Fig. 2. A, size exclusion chromatography profile of a single-domain antibody fragment (red) and a bispecific antibody (black). The elution volumes of molecular weight standards for this column are given at the top; B, SDS-PAGE from a single-domain antibody (cAbLys3, lane 2) and the bispecific protein (cAbLys3-cAbβLA01, lane 3), the apparent molecular masses of the size markers (lane 1) are 182 kDa, 115 kDa, 84 kDa, 62 kDa (pink), 51 kDa, 38 kDa, 26 kDa, 20 kDa, 15 kDa, and 9 kDa (from top to bottom).

ml. The purified recombinant proteins were obtained in yields in the range of 0.4–0.8 mg/liter culture in shake flasks. This is similar to what is obtained for the single-domain antibody fragments (12). It shows that the larger size and the presence of the llama IgG2a upper hinge linking the individual fragments does not hamper secretion and accumulation of soluble recombinant proteins.

Functionality and Bispecificity of the Constructs

The purified recombinant proteins were tested for specific antigen recognition in a solid phase ELISA on lysozyme, Nmc-A, or α-amylase. It was concluded that the original antigen specificity of the VHH was retained, independent of its position in the construct.

The bispecific proteins should bind both antigens simultaneously, and this was tested in ELISA, by gel filtration and by biosensor measurements.

ELISA—A sandwich ELISA was performed to demonstrate the simultaneous binding of the two fragments present in the bispecific proteins cAbβLA01-cAbLys3 and cAbLys3-cAbβLA01. Biotinylated lysozyme was added after binding of the bispecific proteins on immobilized Nmc-A. Good signals were obtained when the biotinylated lysozyme captured by the bispecific protein was detected with extravidin alkaline phosphatase conjugate (data not shown). Background signals were observed whenever an irrelevant protein was coated in the wells of the microtiter plates.

Biosensor Measurements—cAbβLA01-cAbLys3 or cAbLys3-cAbβLA01 were loaded to a cuvette containing immobilized lysozyme. After removal of the unbound material, the addition of Nmc-A resulted in a further signal increase (Fig. 3). The binding of Nmc-A is specific, since no signal increase was observed in the absence of the bispecific construct (Fig. 3, curves a–f).

The generality of the bispecificity of our constructs was confirmed with cAbAMD9-cAbLys3 or cAbLys3-cAbAMD9, and α-amylase as second antigen. Superposition of the different sensograms revealed that the position of each antibody fragment in the bispecific protein does not affect the binding capacity of its constituents.

Affinity and Epitope Mapping of the Bispecific Antibodies

The affinity and kinetic rate constants of each entity in the bispecific protein were compared with those of the single-domain format to evaluate the possible mutual interference between the two subunits. In addition, the binding epitope of the cAbLys3 and cAbAMD9 in the bispecific protein was mapped and compared with the epitope recognized by the original mo-
**Bispecific Antibodies**

| Protein          | Cuvette coupled with | $k_{on}$ | $k_{off}$ | $K_D$ |
|------------------|----------------------|----------|-----------|-------|
| cAbLys3          | Lysozyme             | 52       | 0.0030    | 57    |
| cAbLys3-cAbβLA01 | Lysozyme             | 50       | 0.0027    | 54    |
| cAbβLA01-cAbLys3 | Lysozyme             | 12       | 0.0026    | 215   |
| cAbLys3-cAbLys3 | Lysozyme             | 60       | 0.0007    | 11    |
| cAbLys3-cAbβLA01 | β-Lactamase          | 19       | 0.0005    | 26    |
| cAbβLA01-cAbLys3 | β-Lactamase          | 27       | 0.0006    | 22    |
| cAbβLA01         | β-Lactamase          | 62       | 0.0016    | 26    |

**TABLE I**

Kinetic rate constants, $k_{on}$ (10$^3$ M$^{-1}$ s$^{-1}$) and $k_{off}$ (s$^{-1}$), and equilibrium dissociation constant $K_D$ of bispecific, bivalent, and their single-domain antibody fragments as determined with IAsys biosensor.

Stable bispecific proteins (37 °C, plasma) are required for therapeutic or in vivo diagnostic applications (2, 18).

**Stability at 37 °C**—The residual binding capacity and bispecificity of cAbAMD9-cAbLys3 after 1.5- to 72-h incubations at 37 °C was tested by biosensor measurements. Samples collected at different time points were analyzed by following the binding on a sample cuvette coupled with lysozyme, and thereafter α-amylase was added, and its capturing was evaluated. The binding ability of the bispecific construct remained maximal (100%) for up to 28 h of incubation at 37 °C. A 2% decrease in binding activity was observed between 28 and 44 h. Prolonged incubation reduced the signal gradually to 75% by 76 h (Fig. 5). In parallel, samples were analyzed by SDS-PAGE to verify the occurrence of proteolytic. For samples stored over 30 h at 37 °C, we noticed the appearance of a minor band with a slight increase in mobility. This small shift in mobility can be attributed to the loss of the histidine tail from the bispecific protein, as demonstrated by Western blot experiments using an anti-histidine monoclonal antibody (data not shown).

**Stability in Mouse Plasma**—cAbAMD9-cAbLys3 was added to heparinized mouse plasma and incubated at 37 °C for increasing time periods up to 44 h. Biosensor measurements with a sample cuvette coupled with lysozyme proved that the binding capacity, and bispecificity of the bispecific construct remained unchanged under these conditions. Longer incubation times were not tried, because VHH monomers and dimers have an in vivo half-life of less than 2 h. Despite this fast clearance, they managed to target specifically the tumor tissue.

**Bivalent Constructs of cAbs**

Cloning two identical VHH genes in tandem in our expression vector pKC6 generates a bivalent recombinant protein that should possess a higher functional affinity (avidity). The cloning was performed with the cAbLys3 VHH gene.

**ELISA**—In an ELISA with coated lysozyme the bivalent protein and its single-domain fragment showed a similar binding pattern. Starting from a concentration of 10 μg/ml (which corresponds to 0.5 μM bivalent protein and 1 μM single-domain antibody) followed by serial dilutions, it is noted that the half-maximal signal in both cases is reached at about the same mol binding sites per well. The binding was specific, since the signal was reduced to background levels when 1 mM lysozyme was added or in absence of coated lysozyme.

**Biosensor**—In conditions of high antigen density to allow multivalent binding, the $k_{on}$ of the bivalent construct is in the same range as cAbLys3 alone. In contrast, the $k_{off}$ decreases by a factor of 4–5, leading to an avidity enhancement by a factor of 5 (Table I).

**DISCUSSION**

Bispecific antibodies carry great expectations in tumor diagnosis and therapy (19–21). The first clinical attempts in human tumor patients used bispecific antibodies from murine origin produced by hybrid hybridomas (1). The production and purification of these are extremely cost- and labor-intensive, and their use might induce a strong immune response against the mouse immunoglobulin. Moreover, their large size and presence of the Fc might impair a good tumor targeting (22). Smaller constructs, such as the bispecific scFv fragments.

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2. V. Cortez-Retamozo, M. Lauwereys, H. G. Gholamreza, M. Gobert, K. Conrath, S. Muyldermans, P. De Baetselier, and H. Revets, submitted for publication.
obtained by recombinant DNA technology, might circumvent the above shortcomings and might soon replace the hybrid hybridomas (2). Unfortunately, the bispecific constructs based on scFv also face some problems. The variable and unpredictable expression yields observed for different scFvs hamper their widespread use. Moreover, the linker connecting the VH and VL in the scFv (and often that between different scFvs as well) provokes aggregation of the recombinant material. Shortening the linkers to produce diabodies does not always guarantee success, since the linkers can induce an erroneous angle between the VH-VL pair, thus forming less functional molecules (9).

In principle, the camel single-domain antibodies should be perfect building units for bivalent and bispecific proteins. The camel single-domain antibodies with desired antigen specificity can be selected by phage display (12, 23). They are very robust camel single-domain antibodies with desired antigen specificity molecules (9).

By various methods we estimated the bispecific protein that can be selected by phage display (12, 23). They are very robust camel single-domain antibodies with desired antigen specificity molecules (9).

Finally, our bifunctional constructs stay intact and active in plasma at 37 °C for a long period of time, again a requirement for good candidates in therapeutic and diagnostic applications.

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