

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 Fc¹ 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 ob-

tained 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 nonimmunogenic 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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¹ 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; NAG₃, N,N',N"-acetyl-chitotriose; scFv, single chain variable fragment; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Nmc-A, nonmetallo carbapenemase of class A; ELISA, enzyme-linked immunosorbent assay.

EXPERIMENTAL PROCEDURES

Cloning of Bispecific Antibodies—By standard DNA cloning techniques we assembled an expression vector (pKC6) to generate the bispecific proteins VHH1-LH-VHH2, tailed 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 *Nco*I and *Bst*EII. The C-terminal fragment is inserted using *Pst*I and *Not*I. 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- β -D-thiogalactopyranoside). The periplasmic proteins were extracted by osmotic shock (15) in the presence of protease inhibitors (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and leupeptin), and recombinant protein was purified by immobilized metal affinity chromatography (Ni-NTA Superflow, Qiagen). After concentrating on Vivaspin (Vivascience), recombinant protein was loaded on a Superdex 75 (16/60, Amersham Pharmacia Biotech) equilibrated with PBS. Fractions containing the proteins of the expected molecular weight were pooled, concentrated, and stored at -20°C until further use.

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/nitrobluetetrazolium (BCIP/NBT) substrate (Sigma).

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

ELISA—For ELISA experiments, the antigens (1 $\mu\text{g}/\text{ml}$ in PBS) were coated overnight at 4°C in a 96-well plate (Nunc Maxisorb). After blocking, bispecific proteins were added and detected 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 $\mu\text{g}/200\ \mu\text{l}$) 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 bispecificity, and to map the epitope.

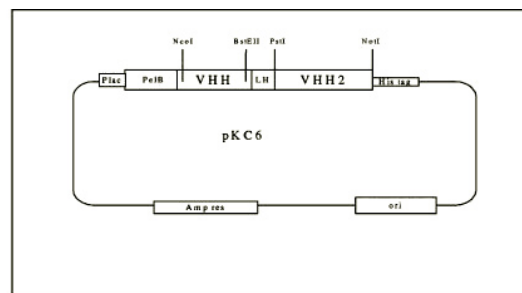
Affinity Measurements—Lysozyme and Nmc-A were immobilized (13) with a coupling efficiency around 1000 arc seconds. After equilibration of the cuvette with PBS, different concentrations of the bivalent or bispecific proteins (final concentration 10–70 nM) were directly added to the sample cuvette (each 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, K_d was calculated as k_d/k_a .

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 Na_2CO_3 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 $\mu\text{g}/\text{ml}$) or in mouse plasma (20 $\mu\text{g}/\text{ml}$) 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.



LH = llama IgG2 upper hinge =EPKIPQPQPKPQPQPKPQPKPQPKPEP

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<----- framework1 -----> <--- CDR1 ---> <----- framework2 ---->
cAbLA01 QLQLVESGGGVSQGGSLRLSCKVS GYGSTNCMG WFRQAPGKEREGVA
cAbAMD9 QVQLVESGGGVSQAGGSLSCAAS TYTDT -- VG WFRQAPGKEREGVA
cAbLys3 DVQLASGGGVSQAGGSLRLSCLAS GYTGIPYCMG WFRQAPGKEREGVA

<----- CDR2 -----> <----- framework3 ----->
cAbLA01 SLFTSGNTYYGDSVKG GRFTISEDNAKNTVSLQMNLSKPEDTAMYYCAT
cAbAMD9 AIYRRTGYTYSADSVKG RFTLSQDNKNTVYLQMNLSKPEDTGIYYCAT
cAbLys3 AINMGGGITYYADSVKG RFTLSQDNKNTVYLQMNLSKPEDTAIYYCAA

<----- CDR3 -----> <----- framework4 ---->
cAbLA01 SSVNGSDESGRKNTRQFVYTY QGGQTQVTVSS
cAbAMD9 GNSVRLASWEGYFY WGGQTQVTVSS
cAbLys3 DSTIYASYECGHLSTGGGYDYS WGGQTQVTVSS

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FIG. 1. Schematic representation of the expression vector with the unique restriction enzyme sites abutting the first and second VHH. The lower part of the figure shows the amino acid sequences of the hinge and of the camel VHHs used for constructing bispecific and bivalent antibodies.

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 K_d 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 K_d 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/

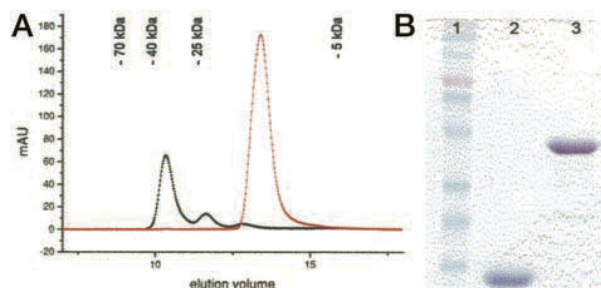


FIG. 2. A, size exclusion chromatography profile of a single-domain antibody fragment (red) and a bispecific antibody (black). The elution volumes of molecular weight standards for this column are given at the top; B, SDS-PAGE from a single-domain antibody (cAbLys3, lane 2) and the bispecific protein (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.

Gel Filtration Experiments—The functionality of the bispecific constructs (cAbLys3-cAbβLA01 or cAbβLA01-cAbLys3) was also demonstrated by size exclusion chromatography (Fig. 4A). Bispecific antibodies and antigens were applied individually or as mixtures on the column. The binding of lysozyme, Nmc-A, or both antigens to the bispecific protein reduced constantly the retention time in the column, in accordance with the increase of the molecular weight of the complex. The peak

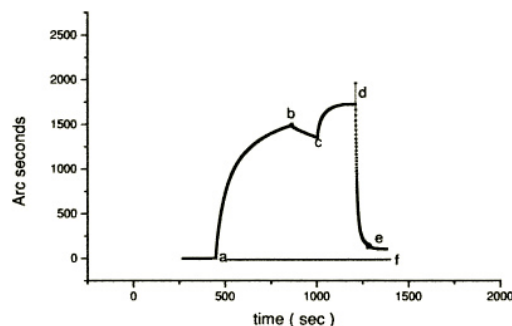


FIG. 3. Measurement of bispecificity of cAbLys3-cAbβLA01 in a biosensor with lysozyme immobilized on the cuvette. a and b, addition of saturating amounts of bispecific antibody; b and c, wash with PBS; c and d, addition of Nmc-A; and d and e, regeneration of the sample cuvette with 50 mM HCl. a–f, addition of Nmc-A in absence of bispecific construct.

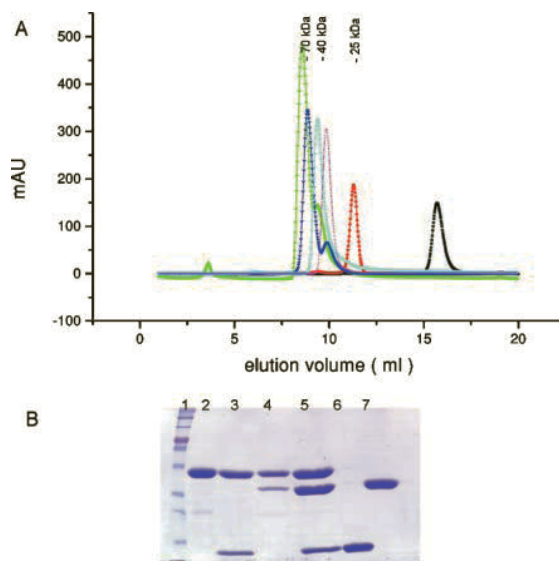


FIG. 4. A, determination of the functionality of the bispecific protein (cAbLys3-cAbβLA01) by size exclusion chromatography. Elution profile of 4.6 nmol of cAbLys3-cAbβLA01 (pink), cAbLys3-cAbβLA01 + lysozyme (cyan), cAbLys3-cAbβLA01 + Nmc-A (blue), cAbLys3-cAbβLA01 + lysozyme + Nmc-A (green), lysozyme (black), Nmc-A (red). B, 12% SDS-PAGE of the eluted peaks. Lane 1, molecular mass markers as in Fig. 2B; lane 2, cAbLys3-cAbβLA01; lane 3, cAbLys3-cAbβLA01 + lysozyme; lane 4, cAbLys3-cAbβLA01 + Nmc-A; lane 5, cAbLys3-cAbβLA01 + lysozyme + Nmc-A; lane 6, lysozyme; and lane 7, Nmc-A.

fraction of each loading was analyzed by SDS-PAGE to confirm the coelution of the bispecific antibody and the antigen(s) (Fig. 4B). Some chromatograms showed a peak with a minor tailing, possibly due to incomplete saturation of all binding sites, as no free antigen was seen, or to dissociation of the complex during chromatography. In any case, the elution profiles indicate that >90% of the construct is active in antigen binding in solution.

Combined, the ELISA, biosensor, and gel filtration experiments confirm the retention of the antigen specificity of each moiety within the bispecific proteins and the ability to bind both antigens simultaneously.

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-

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.

Protein	Cuvette coupled with	k_{on}	k_{off}	K_D
cAbLys3	Lysozyme	52	0.0030	57
cAbLys3-cAb β LA01	Lysozyme	50	0.0027	54
cAb β LA01-cAbLys3	Lysozyme	12	0.0026	215
cAbLys3-cAbLys3	Lysozyme	60	0.0007	11
cAbLys3-cAb β LA01	β -Lactamase	19	0.0005	26
cAb β LA01-cAbLys3	β -Lactamase	27	0.0006	22
cAb β LA01	β -Lactamase	62	0.0016	26

nomeric VHH by testing the inhibition of the enzymatic activity of the antigens.

Biosensor Measurements—The bispecific constructs were measured on a cuvette with immobilized lysozyme or Nmc-A. Native amylase could not be coupled, so the affinities of the cAbAMD9 containing bispecific constructs were not measured. Maximal binding signals of 580, 1300, and 750 arc seconds were obtained on the lysozyme cuvette after adding saturating amounts of cAbLys3, cAbLys3-cAb β LA01, and cAb β LA01-cAbLys3, respectively. Rate constants were determined at five different concentrations of the cAbLys3 and cAb β LA01 containing constructs on a lysozyme, as well as on a Nmc-A cuvette. For the C-terminal cAbLys3 moiety, a lower k_{on} value (five times lower) is noticed on lysozyme in comparison with cAbLys3 or cAbLys3-cAb β LA01, whereas their k_{off} values are identical within experimental errors (Table I). This leads to a lower affinity of the C-terminal cAbLys3 in comparison with the monomeric cAbLys3. It suggests that the extension at the N-terminal end, or the presence of the hinge itself, interferes with the optimal recognition of the antigen by cAbLys3. However, this impediment was less important for the cAb β LA01. Putting the cAb β LA01 at the C-terminal end of the bispecific construct resulted in a 3-fold lower k_{on} , but this was compensated by a lower k_{off} , so that the final affinity remained unchanged (Table I).

Epitope Mapping—It is essential to map the exact binding epitopes of the bispecific protein, especially since a lower k_{on} value is noticed for the C-terminal moiety. This can be tested for the cAbAMD9-cAbLys3, since each entity is an inhibitor of its antigen. A sensitive test is available to measure the inhibition of α -amylase by cAbAMD9 (13). This test demonstrated that the bispecific constructs harboring cAbAMD9 inhibit the enzymatic activity of α -amylase, both in absence and presence of saturating amounts of lysozyme. The epitope mapping of the cAbLys3 at the C-terminal end of the construct was measured by IAsys in a competition assay where a sample cuvette coated with lysozyme was first saturated with 1 mM NAG₃. NAG₃ binds into the catalytic site of lysozyme and thereby blocks the cAbLys3 epitope (16, 17). Saturating amounts of NAG₃ also abolish the lysozyme recognition by the C-terminal present cAbLys3 in the bispecific proteins, indicating that the cAbLys3 as mono- or bispecific construct shares an identical epitope.

Stability of the Bispecific Protein

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 76-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%

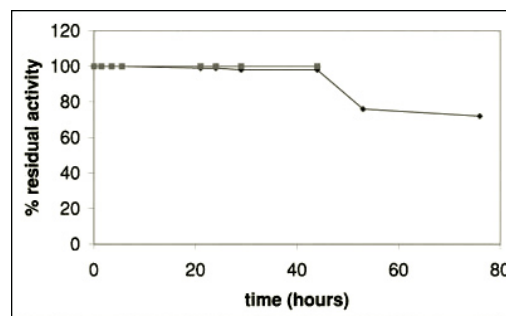


Fig. 5. Stability of the bispecific proteins at 37 °C in PBS (■) and in mouse plasma (◆).

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 proteolysis. 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.²

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 μ g/ml (which corresponds to 0.5 μ M bispecific protein and 1 μ M 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

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 and have superior properties such as simple purification, high solubility, and high stability that are beneficial for all kinds of manifold constructs (10). The molecular mass of a camel single-domain antibody is ~15,000, thus the bivalent or bispecific constructs are only ~30,000. This is half the size of a diabody and five times smaller than an intact antibody. The biodistribution and targeting potential of such minimal sized bispecific constructs might, therefore, exceed all other constructs based on scFvs (10).² More important, since the camel single-domain antibodies bind the antigen in a single-domain format, they avoid linker related problems except for the linker connecting the two entities.

We intentionally used the structural upper hinge of the llama (or dromedary) IgG2a isotype (14) for multiple reasons. The middle hinge containing the cysteines that links the two heavy chains (24) is removed to avoid oxidation with unwanted cysteine-containing proteins. The natural flexibility of the immunoglobulin hinge (25) ensures the independent movement of the connected variable domains in immunoglobulins in the natural antibody. The natural stability of the hinge in serum might also be an advantage. The linker is expected to space the two VHH fragments by ~70 Å, since it contains a repeated Pro-Xaa motif (Xaa = Lys, Gln, or Glu) also occurring in the Ton B protein of *E. coli* and in procyclin of trypanosomes, where it adopts a helix that functions as a rigid rod-like spacer (26, 27). Finally, the hinge allows a good expression, at least in B-cells, and preliminary experiments indicated that it is not immunogenic.

The performance of the bispecific and bivalent constructs was assessed with three different single-domain fragments in different combinations and order. Since unique restriction enzyme sites were introduced at the boundary of each VHH, simple cassette mutagenesis allows the exchange of the current VHHs. The bispecific and bivalent molecules were extracted as soluble proteins from the periplasm of *E. coli* in yields that are comparable with those of the composing monomeric VHHs. We therefore expect that the high quantities required for tumor therapy and diagnosis will be obtained easily. Extremely high expression levels might be attained more successfully in yeast expression systems, since 300 mg of VHH/liter are routinely produced in fermentors (28). The recombinant, bispecific, and bivalent constructs obtained by linking two camel, single-domain fragments are functional in all respects. This statement is based on the ELISA, biosensor, and gel filtration experiments revealing that the VHH entities within the bispecific constructs recognize the antigen(s), either individually or simultaneously. By various methods we estimated the bispecific protein that actively binds both antigens simultaneously to constitute >90% of the total protein. However, the C-terminal located binder might exhibit on-rates that are slightly reduced,

whereas the kinetics of the N-terminal moiety remain unchanged. This can lead to a reduced affinity (~4 times higher K_d in the case of cAbLys3) for the C-terminal moiety of this bispecific construct. This result points toward a more difficult access of the antigen, which is fair to assume, since the hinge connected to the N-terminal of this domain is in the immediate vicinity of the antigen-binding site. Nevertheless, the possible reduced affinity of the second binder within the construct does not interfere with the recognition of the correct epitope.

Multiple reports claim to observe a better tumor targeting with bivalent diabodies as compared with the monomeric scFv due to the avidity effects (18). We therefore made a cAbLys3-cAbLys3 bivalent construct and showed that the expression, stability, and solubility are similar to bispecific constructs with single-domain antibodies, which we described in this paper. An increase in functional affinity for our bivalent construct was noted, and the apparent K_d dropped by a factor ~5. The k_{on} rate is equivalent to the single-domain fragment, whereas the k_{off} rate is reduced and responsible for the enhanced binding strength. We believe that the selection of other antigen-specific VHHs that do not show the reduced intrinsic affinity when placed at the C-terminal of the bispecific protein might even increase the avidity effect.

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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