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Chicken scFvs and bivalent scFv-C_H fusions directed against HSP65 of *Mycobacterium bovis*

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ABSTRACT

Two chicken single-chain variable region antibody fragments (scFvs) that recognised the 65 kDa heat-shock protein (HSP65) of *Mycobacterium bovis* were selected from a large semi-synthetic phage displayed library. Both recognised HSP65 in indirect enzyme-linked immunosorbent assay (ELISA) and immunoblots and retained their activity during storage. Neither, however, could function as the capture reagent in a sandwich ELISA when immobilised on polystyrene. To establish whether they could be engineered for general use in immunotests, the genes coding for these scFvs were subcloned in expression vectors that contained sequences encoding chicken IgY heavy-chain constant region domains. This resulted in larger bivalent constructs which more closely resembled IgY molecules. The engineered fragments were evaluated in ELISAs and gold-conjugated immunochromatographic tests (ICTs). In contrast to their previous behaviour as scFvs, the modified fragments (designated "gallibodies") could be used for immunocapture in ELISA and could be readily conjugated to colloidal gold nanoparticles. A sandwich ICT that could detect recombinant HSP65 was also devised. Although converting the recombinant single-chain monomeric antibody fragments to bivalent immunoglobulin-like molecules did not entirely 'standardise' the behaviour of the scFvs, this approach remains potentially useful for developing practical, robust, immunodiagnostic reagents.

1. Introduction

Bovine tuberculosis (BTB) is caused by the acid-fast, Gram-positive bacillus *Mycobacterium bovis* [1,2]. Although mainly a disease of cattle, BTB also affects wildlife [3] and humans [4]. The 65 kDa heat-shock protein (HSP65) from Mycobacterium is highly immunoreactive and since it is conserved in the tubercle bacilli, immunoassays targeting this antigen can be useful for the general diagnosis of mycobacterial infections [5]. For large scale screening (e.g. herds) and penside use, rapid and convenient assays such as enzyme-linked immunosorbent assays (ELISA) and lateral-flow immunochromatographic tests (ICTs) are regarded as being potentially more practical than the standard intradermal tuberculin test [6].

Recombinant antibody technology [7,8] allows immunoreagents to be derived, engineered and optimised using standard molecular biological methods. For immunodiagnostic applications, tests based on antibodies of which the encoding sequences are known can be more easily standardised than those derived from immune serum or a potentially unstable hybridoma. Single-chain variable fragments (scFvs) based on chicken immunoglobulins are particularly suitable as a basis for developing immunotests, not only because they are serologically distinct from mammalian immunoglobulins, but because the avian antibody repertoire can be accessed [9–11] more readily than that of any mammal other than the camelids [12]. Recombinant chicken antibodies derived either from large "universal" or dedicated immune repertoires have been shown to be eminently useful in a variety of immunotests aimed at detecting either antigens or antibodies [13–19]. This suggests that if judiciously applied, they could also play a role in the diagnosis of tuberculosis. While ELISAs are likely to be useful in the laboratory, for use in the field, ICTs [20–23] offer the advantages of economy and rapidity. So far, however, the use of chicken-derived antibody fragments in gold-conjugated ICTs has not been widely reported.

With the objective of evaluating a possible role for recombinant chicken antibodies in developing immunodiagnostic tests for bovine tuberculosis, we describe the derivation, modification and use of scFvs directed against dimeric recombinant HSP65 of *M. bovis*. Three different scFvs were obtained by screening a large semi-synthetic antibody library based on chicken immunoglobulin genes [11]. Two of these were converted into bivalent IgY-like molecules [24]. Originally called IgY-C_{H2-4} constructs because the first heavy-chain constant domain (C_{H1}) is omitted, for convenience

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we designated these modified antibody fragments as 'gallibodies', a word derived from the Latin binomial nomenclature for the domestic chicken, *Gallus gallus domesticus*. Their behaviour in various ELISA formats and in colloidal gold-based ICTs was evaluated. Depending upon the particular scFv and the system in which it was being used, converting it into a gallibody could markedly affect its suitability as an immunoreagent.

2. Materials and methods

2.1. Single-chain Fvs from the Nkuku® library

Immuno™ Tubes (Nunc Maxisorp, Roskilde, Denmark) were coated by overnight incubation at 4 °C with 10 μg/mL recombinant HSP65 from *M. bovis* BCG dissolved in phosphate-buffered saline (PBS). Single-chain Fvs were selected by panning a phage displayed repertoire derived from the immunoglobulin genes of the chicken (*Nkuku*® library) using methods described previously [11]. Phages displaying antibody fragments that bound to HSP65 were released at high pH and used to reinfect exponentially growing TG1 host cells. After overnight growth, the bacteria were collected by centrifugation and phage particles were rescued using M13KO7 helper phage. These phages were used as input for the next round of selection. For the first two rounds, the Immuno™ Tubes were washed 10 times. After three rounds of selection, single bacterial clones were selected, phage were rescued and tested in indirect ELISA to identify those that produced binders. These were then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to obtain soluble scFvs. DNA coding for the binders was sequenced to categorise individual clones. The deduced amino acid sequences were aligned [25] and numbered according to Kabat using published scFv sequences as a guideline [26]. For subsequent use, scFvs were expressed and immunoaffinity-purified via their c-myc tags by Ms J. Frischmuth (National Bioproducts Institute, Pinetown, South Africa).

2.2. Adding truncated C_H-region sequences to scFv genes

The scFv genes were recovered from the vector pHEN [27] by PCR amplification using primers to introduce a BsiW cleavage site (5′ GATCCGTACGGCCGTGACGTTGGACG 3′) and an AscI site (5′ GATCGGCGCGCCACCTAGGACGGTCAGGG 3′) (Inqaba Biotech, Pretoria, South Africa). The scFv-encoding inserts were then subcloned in the mammalian expression vector scFvIgY(C_{H2-4})His [24] at these restriction endonuclease sites. Ultracompetent M15 *Escherichia coli* (QIAGEN®, Hilden, Germany) were transformed to ampicillin resistance with the two constructs and 10 transformants from each subcloning experiment were selected and subjected to PCR using primers specific for the 5′ (5′ TAATACGACTCACTATAGGG 3′) and 3′ (5′ AGGAGGAGGGGTGGAGGACC 3′) ends of the scFv genes, to check for the presence of inserts. Plasmid DNA from clones bearing inserts of approximately 800 bp was sequenced and compared to the original template.

2.3. Gallibody expression and purification

After overnight growth in Luria-Bertani (LB) medium, plasmid DNA was extracted using the QIAfilter™ Plasmid Midi Kit (QIAGEN®, Hilden, Germany). Human embryonic kidney (HEK) 293-H cells (Invitrogen™, Carlsbad, USA) grown to 50–80% confluence in Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 31966-021, Gibco™, Carlsbad, USA), supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco™, Carlsbad, USA) were transfected with plasmid DNA using GeneJuice® Transfection Reagent (Merck, Darmstadt, Germany) or TransIT®-293 Transfection Reagent (Mirus Bio Products, Madison, USA). The cultures were grown at 37 °C in 5 % CO_2. Individual foci were expanded in fresh tissue culture plates containing DMEM, 10 % (v/v) FBS and Zeocin™ (Invitrogen™, Carlsbad, USA). Secreted gallibodies were purified using nickel-nitrilotriacetic acid (Ni–NTA) agarose according to the manufacturer's (QIAGEN®, Hilden, Germany) instructions and purity was verified on a 4–12 % SDS-PAGE gel (Criterion™ XT, Bio-Rad Laboratories, Hercules, USA) stained first with Coomassie blue, then destained overnight with 4 % (v/v) acetic acid. Specificity of the gallibodies for the target protein (HSP65) was confirmed by an immunoblot using these antibodies in cell culture medium for detection.

2.4. Stability studies of scFvs and gallibodies

Recombinant antibody samples were subjected to consecutive freeze–thaw cycles while suspended in LB medium (for scFvs) and DMEM (for gallibodies). After each cycle, an aliquot was removed and tested for its capacity to recognise the target antigen in indirect ELISA.

To determine the threshold temperature at which the recombinant antibodies lose binding activity, 12 aliquots (100 μL) of each of the scFvs and the gallibodies were incubated at different temperatures for 16 h using the temperature gradient programme on the Mastercycler® ep gradient S (Eppendorf, Hamburg, Germany) thermocycler. The heat-treated samples were tested in duplicate in indirect ELISA. To ascertain their long-term storage ability under various conditions, one millilitre samples were stored at −70 °C, −20 °C, room temperature (RT) and 4 °C for four weeks before testing in indirect ELISA. The storage media were supplemented with tetracycline to prevent microbial growth. To evaluate the propensity of the scFvs and gallibodies to refold after denaturation, recombinant antibodies were each exposed to 6 M guanidinium chloride (GdmHCl) overnight. Prior to testing in an indirect ELISA, the denaturant was removed by ultrafiltration (Vivaspin, Sartorius, Goettingen, Germany) replaced with three volumes of PBS and then concentrated to the original starting volume of approximately three millilitres.

2.5. ELISAs

For indirect ELISAs, Immuno™ Plate (Poly- or Maxisorp, Nunc™, Roskilde, Denmark) wells were coated overnight at 4 °C with 50 μL volumes of 10 μg/mL of HSP65 in single-strength PBS. Unoccupied binding sites were blocked for 1 h at 37 °C with 300 μL volumes of 2 % (w/v) milk powder dissolved in single-strength PBS (MPPBS). Recombinant antibodies in culture medium were then added (50 μL/well) and incubated for 1 h at 37 °C. Wells were then washed three times with PBS containing 0.05 % (v/v) Tween 20. Single-chain antibody fragments were detected by adding the anti-c-*myc* monoclonal antibody 9E10 [28] (produced in-house by Mr. W. van Wyngaardt, Onderstepoort Veterinary Institute, South Africa) diluted 1:1 with 4 % (w/v) MPPBS followed by rabbit anti-mouse IgG conjugated to horseradish peroxidise (HRP) (DakoCytomation, Glostrup, Denmark) diluted 1:1000 (v/v) in 2 % (w/v) MPPBS; while gallibodies were detected by adding HRP-conjugated anti-IgY Fc-region antibody (Rockland Immunochemicals, Gilbertsville, USA) diluted 1:5000 (v/v) in 2 % (w/v) MPPBS to the washed wells. To detect recombinant HSP65 directly, polyclonal rabbit anti-M. bovis antibody (DakoCytomation, Glostrup, Denmark) diluted 1:1000 (v/v) with 2 % (w/v) MPPBS was used, followed by polyclonal anti-rabbit antibody conjugated to HRP (DakoCytomation, Glostrup, Denmark) diluted 1:1000 (v/v) in 2 % (w/v) MPPBS. In all cases, after a final wash, 50 μL of 5 mg *o*-phenylenediamine (OPD) dissolved in 5 mL 0.1 M citrate buffer (pH 4.5) containing 2.5 μL 30 % (v/v) H_2O_2 was added to each well. The colour reaction was stopped using

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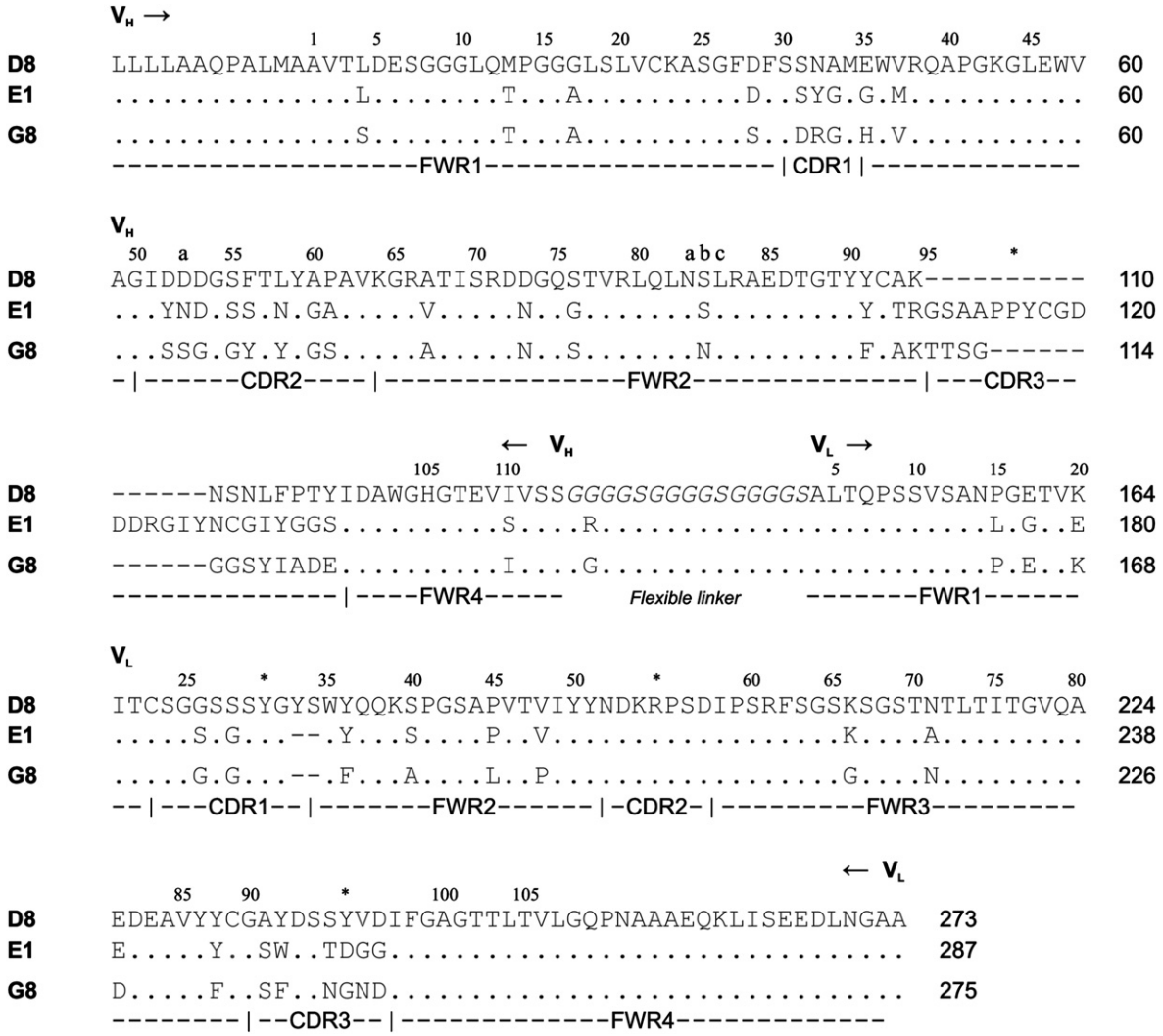

Fig. 1. Amino acid sequences coding for three unique anti-HSP65 scFvs derived from an antibody library based on the immunoglobulin genes of the chicken. Variable heavy- and light-chains (V_H and V_L) are indicated, as are framework regions (FWR).

50 μL of 1 M H_2SO_4 per well. Absorbance values were recorded at 492 nm using an Immunoskan MS (Labsystems, Helsinki, Finland) microplate reader. All determinations were performed in duplicate. Sandwich ELISAs were performed essentially in the same way except that scFv or gallibody capture antibodies (10 μg/mL in PBS; 50 μL/well) were first immobilised on the surface of the microplate well overnight at 4 °C, followed by the target antigen (10 μg/mL in PBS; 50 μL/well).

2.6. Immunoblotting

One microgram of recombinant HSP65 antigen was subjected to SDS-PAGE before electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Invitrogen™, Carlsbad, USA). Blocking and colorimetric detection of antibodies which bound to the antigen was carried out using the HisDetector™ Western Blot Kit (KPL, Gaithersburg, USA). For spot-blots one microlitre volumes of purified scFv or gallibody (10 μg/mL in PBS) were spotted onto a PVDF membrane, incubated with HSP65 (10 μg/mL in 2 % [w/v] MPPBS) which was then detected by adding polyclonal rabbit anti-M. bovis antibody diluted 1:1000 (v/v) with 2 % (w/v) MPPBS, followed by polyclonal HRP-conjugated anti-rabbit antibody diluted 1:1000 (v/v) in 2 % (w/v) MPPBS.

To detect gallibodies after purification, approximately two micrograms of protein (as determined spectrophotometrically) was separated using PAGE and transferred to a PVDF membrane. The HisDetector™ Western Blot Kit was used for blocking and colorimetric detection, according to the manufacturer's instructions, except that HRP-conjugated anti-IgY Fc-region antibody diluted 1:2500 (v/v) in blocking buffer replaced the anti-His antibody supplied with the kit.

2.7. Colloidal gold conjugates

Conditions under which gold-particles would conjugate to antibodies or antigen were determined using an 'ionic flocculation' assay according to proprietary methods (Vision Biotech, Cape Town, South Africa) based on published guidelines [29]. Essentially, a series of concentrations of recombinant antibody or antigen was cross-titrated against a series of 40 nm gold colloids (Vision Biotech, Cape Town, South Africa) that ranged in pH from 6.5 to 9.0 in increments of 0.5. The absorbance of the 40 nm gold colloid stock solution was 1.0 at 525 nm. Cross-titration conditions that yielded the closest absorbance to 1.0 after challenge with NaCl were regarded as optimal for production of lateral-flow reagents.

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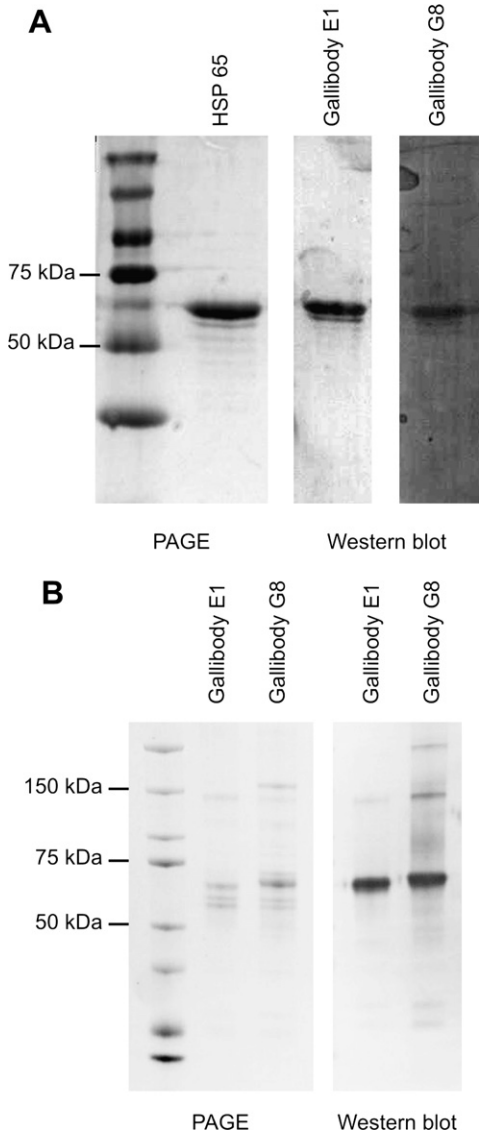

Fig. 2. (A) PAGE gels and Western blots illustrating the binding of secreted gallibodies to recombinant HSP65; (B) Immunoblot showing the binding of anti-chicken Fc-region antibodies to gallibodies after separation by PAGE under reducing conditions.

2.8. Immunochromatographic tests

A variety of nitrocellulose membranes (Vision Biotech, Cape Town, South Africa) were coated with narrow bands of either HSP65 antigen, purified scFv or purified gallibody (all 10 μg/mL in PBS). Assorted antibody–antigen assemblies were tested using these membranes and antibody- or antigen-labelled gold colloid. A commercially available ICT (Vision Biotech, Cape Town, South Africa) specific for Rift-Valley Fever (RVF) was used as a negative control to show that the gallibodies did not bind non-specifically or cross-react with any of the reagents in gold-conjugated lateral-flow test formats.

3. Results

3.1. Selection of scFvs

After three rounds of panning the *Nkuku*® recombinant antibody library on immobilised recombinant HSP65, a total of 90 bacterial clones were randomly picked and tested for the production of fusion phages that bound the target protein. Of these, 44 secreted HSP65-specific phage displayed antibodies, 37 of which also bound in the form of soluble scFvs. Only three distinct antibody fragments were represented in a random sample of 11 clones which were chosen for sequencing. These were designated D8, E1 and G8. (Fig. 1). E1 had a substantially (75 bp) larger third heavy-chain complementarity determining region (CDR3) than either G8 (36 bp) or D8 (24 bp). The light-chain CDRs of the three fragments were more similar to each other than those of the heavy chains. There were also several amino acid exchanges in the framework regions. Since E1 and G8 consistently produced the most intense signals when they were tested for binding to immobilised HSP65 in indirect ELISA during selection, they were chosen for further examination. Moreover, these two scFvs could withstand repeated freezing and thawing and remained functional after exposure to temperatures of 4 °C and 20 °C for up to three weeks (results not shown).

3.2. Expression of scFvs fused to chicken heavy-chain domains

The genes coding for the scFvs E1 and G8 were each subcloned in the mammalian expression vector scFvIgY(C_{H2-4})His which contained sequences coding for histidine-tagged chicken IgY heavy-chain constant region domains. The resulting constructs were used to transfect HEK 293-H cell cultures. Western blotting using antibodies against polyhistidine (Fig. 2A) revealed that fusion proteins which could recognise immobilised HSP65 antigen had been secreted into the culture medium. Transfected cultures were grown to confluence before expansion to establish stably transfected stocks. For subsequent use in immunoassays, the gallibodies were purified by metal–ion affinity chromatography. On average, Ni–NTA purification yielded 25–50 μg of protein per millilitre of cell culture supernatant. Despite numerous attempts, the final product could not be obtained as a single homogeneous band of approximately 63 kDa when analysed by reducing PAGE (Fig. 2B). Western blotting using antibodies to detect avian IgY Fc-regions revealed, however, that an estimated 30 % of the total protein analysed was IgY-derived (Fig. 2B). A few larger bands were also present on the blot and may have resulted from imperfectly reduced disulphides, incomplete denaturation or even degradation of the protein. Nevertheless, the purified product was subsequently found to be suitable for use as an immunoreagent.

3.3. Stability of scFvs vs. gallibodies

Stability and robustness are important factors when selecting reagents for immunoassay development. The scFvs and each of their corresponding gallibodies were therefore compared for their ability to survive freezing, thawing and elevated temperatures. In addition, they were tested for their capacity to refold after chemical denaturation. When suspended in bacterial culture medium, neither of the unmodified scFvs showed a significant decrease in binding capacity in indirect ELISAs after 10 freeze–thaw cycles. This resilience was not markedly altered after they had been remodelled as gallibodies (not shown). In thermal denaturation experiments, E1 no longer produced an ELISA signal after overnight heat treatment at around 50 °C, while G8 lost roughly 50 % of its binding capacity. In contrast, once converted to gallibodies the ability of both to bind after heating was reduced to a much lesser degree (Fig. 3). To evaluate their ability to regain activity after being chemically denatured, each was exposed overnight to 6 M GdmHCl. Once the denaturing agent had been removed by ultrafiltration, the scFv designated E1 regained only 10 % of its binding capacity, while G8 recovered over two-thirds of its binding. In contrast, when converted to a gallibody, E1 recovered 70% of its functionality in ELISA after removal of the GdmHCl. G8 on the other hand also

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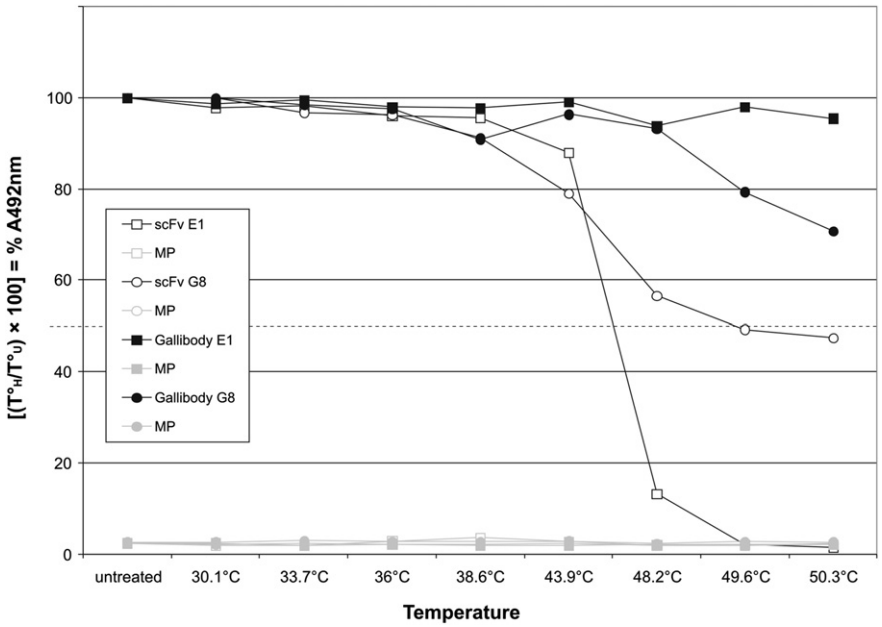

Fig. 3. Indirect ELISA illustrating the percentage change in A_{492nm} after scFvs and gallibodies were incubated overnight at various temperatures. T_H refers to heat-treated samples. T_U refers to untreated samples. MP are corresponding negative controls, in which HSP65 was replaced with 2% (w/v) MPPBS.

bound as a gallibody after the denaturant had been removed. However, it did not recover its activity as efficiently as the scFv from which it was derived (Fig. 4).

3.4. Single-chain Fvs and gallibodies as immunoassay components

Sandwich ELISAs in which the scFvs E1 and G8 were adsorbed to polystyrene in order to capture HSP65 from suspension produced no signals. In contrast, ELISA A_{492} values of over 2.5 were obtained when the corresponding gallibodies were used for immunocapture (Fig. 5). Using the gallibodies to capture the HSP65 dimer and the scFvs as detecting partners, was not successful (not shown). When used in analogous spot-blotting experiments for trapping HSP65, neither scFvs nor gallibodies produced visible spots when adsorbed to nitrocellulose or PVDF membranes (not shown).

Colloidal gold conjugates rely on the adsorbed proteins to prevent flocculation or "collapse" of the sol. When compared to the scFvs, the gallibody versions of E1 and G8 produced stable conjugates over a broader combination of conditions (pH vs. concentration). Stable conjugates were those with an A_{525} closest to that of the unconjugated colloidal gold (Fig. 6). In contrast to their behaviour in ELISA and dot-blots, E1 and G8 functioned as a capture and detecting antibody pair in ICTs provided that E1 (either as an scFv or a gallibody) was used for trapping and G8 scFv (gold-conjugated) was used for detecting the HSP65 dimer (Suppl. Fig. 1). Control ICTs were run to validate that the antibodies did not cross-

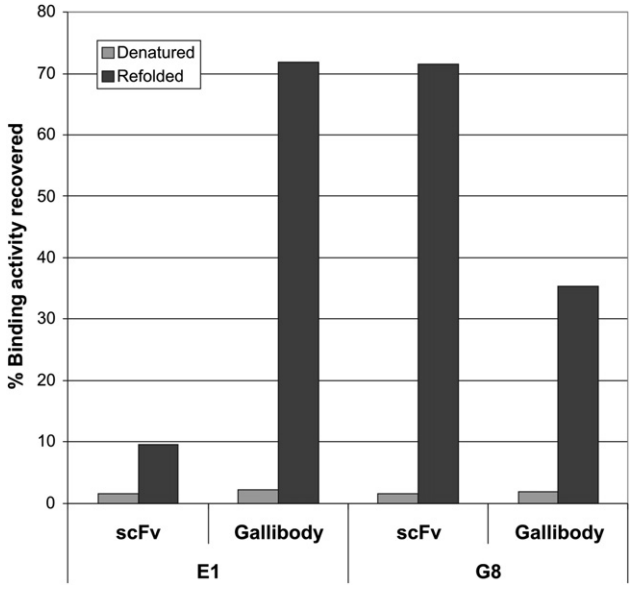

Fig. 4. Indirect ELISA comparing the propensity of scFvs and their respective gallibodies to regain their binding activity after denaturation with GdmHCl.

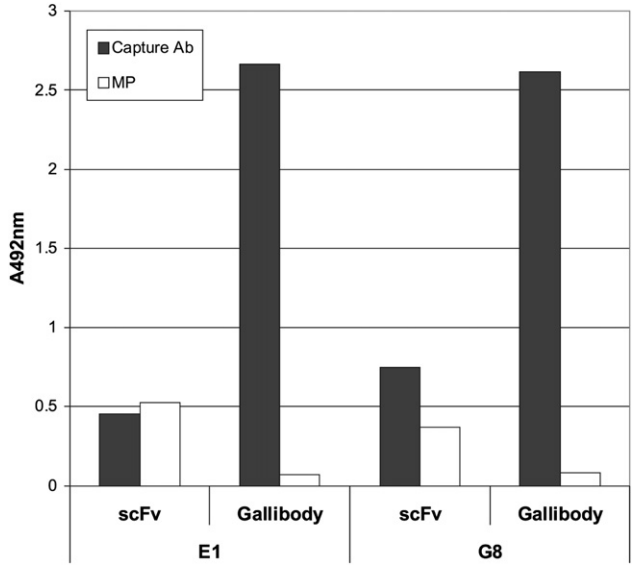

Fig. 5. Sandwich ELISA showing differences in the relative abilities of scFvs and their corresponding gallibodies to capture recombinant HSP65 antigen from suspension. Antigen was replaced by a 2% (w/v) MPPBS solution in the negative control wells (MP).

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react with any of the lateral-flow reagents and that they did not bind to unrelated antigen (Suppl. Fig. 2)

4. Discussion

A chicken recombinant antibody library which had been constructed earlier expressly to provide reagents for use in developing immunotests [11] was screened by panning against HSP65, a potentially important diagnostic antigen of both *M. bovis* and *Mycobacterium tuberculosis* [5]. Sequencing of a limited number of random clones showed that at least three different scFvs which recognised the target antigen had been obtained. Two of these (E1 and G8) were evidently strong binders which were sufficiently stable to be regarded as potentially useful immunodiagnostic reagents. During the process of selecting the two scFvs by panning, the library was exposed to recombinant HSP65 adsorbed to a plastic surface. Accordingly, both scFvs bound strongly to the immobilised target in indirect ELISA. Neither, however, could capture HSP65 from suspension when they themselves were adsorbed to a plastic surface, a finding which could restrict their use as diagnostic test components. Intact IgG molecules and other proteins can be deformed when adsorbed to a polystyrene or nitrocellulose solid phase [30,31] and small single-chain antibody fragments, in which the flexibly-linked variable heavy- and light-chain regions are kept in juxtaposition to form the paratope by hydrophobic interactions, are likely to be particularly susceptible. In contrast to the scFvs, the bivalent gallibodies derived from both E1 and G8 functioned well in ELISA as immunocapture reagents. Fusing an scFv to a single truncated C_H-region effectively doubles its size. This meant that even if the gallibodies bound in a random orientation to the solid phase, the actual binding regions of the gallibody would have a lower probability of being directly engaged in the interaction with the solid phase and hence the paratopes would have less chance of being distorted. In addition, the added hydrophobic Fc-region could conceivably help to orient the CDRs away from the polystyrene surface [32] thereby further preserving paratope integrity. Another factor is that as with an authentic IgY molecule, the modified fragments associate with each other via disulphide bonds to form a bivalent binding entity. Even if one of the scFv portions were to be distorted, the other would probably still be able to interact with its antigen.

The resilience of an antibody to physical and chemical stress may impact on how an immunoassay is produced, what its requirements for storage or shipping will be, its shelf-life, the need for or suitability of specific downstream reagents and in some cases, the type of sample that can be analysed. The scFv designated G8 largely retained its ability to bind HSP65 in ELISA after heating at temperatures up to 50 °C overnight. E1, however, lost more than 50% of its binding capacity once the temperature was increased above about 45 °C (Fig. 3). Resistance to GdmHCl, a factor which can be of importance e.g. recombinant antibodies which may form inclusion bodies, has been correlated with heat resistance of proteins [33]. When the relatively heat-resistant G8 was converted to a gallibody, it regained only a small portion of its activity once the chaotropic agent was removed. E1, on the other hand, which heat-denatured more readily, regained up to 70% of its binding capacity when converted to a gallibody. Renaturation after GdmHCl treatment can therefore not be seen as an infallible predictor of heat stability in the case of bivalent constructs such as the gallibody.

In murine and human immunoglobulin frameworks, specific amino acid exchanges at certain positions have been implicated in conferring stability [33–35]. While the framework regions of D8, E1 and G8 were not identical, there are as yet insufficient data in the case of chicken scFvs to draw conclusions about the effects of these exchanges. Chicken antibodies all have very similar framework regions resulting from the way in which their diversity is generated [9,10]. Any variation in their physical properties is therefore likely to be mainly as a result of amino acid sequence differences within in the CDRs which form a substantial part (roughly 28%) of the entire scFv. When fused to a single truncated chicken heavy-chain to form a gallibody, the CDRs comprise only approximately 12% of the molecule. Adding identical chicken C_H-domains to two different scFvs could therefore be expected to produce molecules with more closely related physical properties. When E1 and G8 were used as gallibodies in gold-conjugation experiments, however, they did not behave similarly; the gallibody based on the scFv G8 could not be used for immunocapture in lateral-flow ICTs whereas E1 could. Their performance was therefore still under the influence of the charge-composition of the individual scFv portions of the fusion proteins. In this instance, converting them into gallibodies did, however, bring them into a wider and more useable coupling pH and ionic strength range.

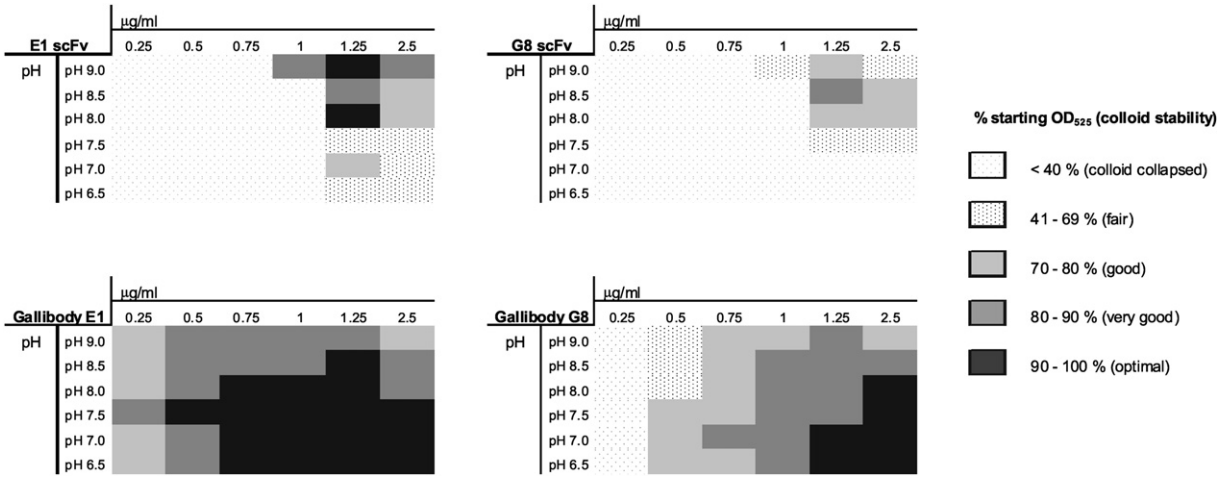

Fig. 6. Schematic representation of ionic flocculation assays using 40 nm colloidal gold and either scFvs or gallibodies. The conjugates are represented as percentages of the optical density of unconjugated 40 nm colloid.

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In sandwich ELISAs and dot-blots, it appeared that neither E1 nor G8, whether used in the form of an scFv or as a gallibody, could capture the target antigen such that the other could be used for detection. This implies that E1 and G8 recognised the same antigenic determinant on the HSP65 dimer, or alternatively, that they bind to regions located very near to each other. However, when used in ICTs, it was shown that the two antibodies could indeed function as a capture/secondary antibody pair, but only if E1 (either as an scFv or a gallibody) was used to capture HSP65 while G8, in scFv-form, was the detecting partner. It seems possible therefore that E1 and G8 do indeed recognise closely-situated sites but that simultaneous binding requires that the antigen be orientated in a particular way by E1 before G8 can bind. In the reciprocal situation, the E1 epitope may be concealed when the antigen is captured with G8. Additionally, in spot-blots and ELISAs, the captured antigen was detected by a primary and then a secondary or even tertiary, conjugated antibody, while in ICTs the antigen was detected directly by an antibody-coated gold particle. Differences in the degree of steric hindrance may have affected the binding of the respective detection systems.

Adding mammalian antibody domains has been used to alter the characteristics of scFvs from various sources [36–38]. The present study has shown that the vectors and approaches developed by Greunke et al. [24] to create larger bivalent molecules from monovalent chicken antibody fragments can be used for improving avian scFvs which under-perform in certain immunoassay applications. The physical behaviour of individual antibody fragments still plays a large part and it is not possible to unequivocally assume that all properties of an scFv will be improved by this process. Nevertheless, the resulting bivalent fusions more closely resemble authentic IgY than do scFvs. This should make them more amenable to being incorporated into a wider variety of standard assay formats than the unmodified antibody fragments.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biologicals.2010.02.002.

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