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# Improving the characteristics of a mycobacterial 16 kDa-specific chicken scFv

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ABSTRACT

Recombinant antibodies can be engineered to improve their binding or other characteristics. A chicken single chain variable fragment (scFv) phage display library was panned against the mycobacterial 16 kDa antigen. Three fusion phages which bound specifically to the antigen were selected, each of which produced low signals in ELISA when secreted as a soluble scFv. One scFv was therefore chosen to be modified in an attempt to improve its binding. Firstly, a mutant sublibrary was created by random mutagenesis. High stringency panning of this sublibrary yielded binders which produced ELISA signals up to eleven times higher than the parent scFv. An increase in the intrinsic affinity was confirmed by surface plasmon resonance. Secondly, the flexible linker between the heavy and light chains of the parent scFv was either shortened to one glycine residue or deleted entirely. No ELISA signal was obtained when the linker was absent, but the glycine-linked scFv showed enhanced binding. Size exclusion chromatography revealed that the enhanced binder had aggregated to form tetramers. This study confirms that the strategies used to improve the binding of human and mouse scFvs can also enhance chicken scFvs.


## 1. Introduction

*Mycobacterium tuberculosis* and *Mycobacterium bovis*, both of which are in the *M. tuberculosis* (TB) complex, each possess an identical 16 kDa protein (accession numbers P30223 and P0A5B8) [1] which is synonymous with heat shock protein hspX, hsp16.3 and the 14 kDa protein [2–4]. This antigen is considered to be a potentially useful diagnostic target for serodiagnosis of TB in humans [5] and can also be used to stimulate lymphocytes in the gamma interferon test for bovine TB [6]. Antibodies directed against this protein are therefore likely to be useful in research and diagnosis.

Unlike polyclonal antibodies in immune serum, recombinant antibodies are a renewable resource which can be characterised by their encoding DNA sequence. This in turn makes it possible to standardise assays with reproducible reagents and the antibodies can even be recovered by constructing a synthetic gene if necessary. A further advantage of recombinant antibodies is that their physical and chemical properties can be changed using standard recombinant DNA methods such as mutagenesis [7–13], multimerisation [14,15], chain and DNA fragment shuffling [16–18]. Affinity maturation by random mutagenesis, followed by increased selection pressure mimics somatic mutation *in vitro*, a process which often allows antibodies with higher affinity and specificity to be derived [7]. Moreover, more stable scFvs [19] and an increase in bacterial expression of an scFv have been obtained in this way [20].

ScFvs consist of the variable heavy ($V_H$) and variable light ($V_L$) domains of immunoglobulins, most commonly joined by a flexible polypeptide linker [21,22]. The sequence and the length of this linker can influence the properties of the scFv [15,23]. For example, mouse scFvs with linkers between 12 and 15 amino acids occur mostly as monomers while those joined with between 5 and 11 residues occur as dimers [14,24,25]. Variable domains joined either directly to each other or with linkers of up to four residues occur as trimers and tetramers [15]. This multimerisation results in an increase in cooperative binding and hence the avidity of the scFv. While the antibody engineering methods described above have been shown to be effective in improving mouse and human scFvs, they have so far not been widely used with scFvs from other species. Using an scFv specific for the 16 kDa *M. tuberculosis* protein this study to explores whether random mutation and manipulating linker length can also be used to improve chicken scFvs.

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## 2. Materials and methods

### 2.1. Antibody selection by panning

The *Nkuku*® library [26] was panned against 20 μg/ml of the *M. tuberculosis* 16 kDa antigen. The purified 16 kDa antigen of *M. tuberculosis* was obtained from Europa Bioproducts (Cambridge, England). Four rounds of panning were performed followed by screening by monoclonal phage and scFv ELISA as described previously [26].

### 2.2. Mutant sublibrary construction

The first strategy was to attempt to improve the characteristics of the scFv B4 by error-prone PCR. Random mutations were introduced into the gene coding for the scFv using the Diversify ™ PCR Random Mutagenesis kit as described by the manufacturer (BD Biosciences, Palo Alto, CA, USA). Plasmid DNA (1 ng) containing the scFv gene and 0.4 pmol/μl Sfi1L and LCNOT1 primers [26] were added to the PCR reaction. Conditions were used to obtain seven mutations per 1000 base pairs. The PCR products were digested overnight (ON) with 40 U of *SfiI* (Roche Diagnostics, Mannheim, Germany) at 50 °C followed by another ON digestion with 40 U *NotI* (Roche) at 37 °C. The digested products were purified with the QIAquick PCR kit (QIAGEN, Hilden, Germany). Primers Sfi1L and LCNOT1 (0.4 pmol/μl) were used to allow cloning into the *SfiI* and *NotI* sites of the phage display vector pHEN1 (1 μg) which was digested with 40 U of the same restriction enzymes and purified with the QIAquick PCR kit. To remove the stuffer fragment the vector was further purified with a crystal violet gel [27]. The genes were ligated into the vector with the Rapid DNA Ligation Kit (Roche). The ligations were electroporated into *Escherichia coli* TG1 and plated as described before [26]. Serial dilutions of $10^{-1}$–$10^{-4}$ were plated onto TYE agar with 100 μg/ml ampicillin (amp) to determine the size of the library. The plates were incubated ON at 30 °C. The next day the colonies were scraped off the plates in 2x TY and the bacteria were stored in 15% glycerol at −70 °C.

### 2.3. Sequencing

To check for the presence of mutations, DNA inserts of individual clones were sequenced. Single *E. coli* TG1 transformant colonies were grown in 5 ml 2x TY supplemented with 100 μg/ml amp and 2% (w/v) glucose (2x TY-AG) at 30 °C, 240 rpm. Phagemid DNA was isolated using the QIAprep Spin Miniprep plasmid purification kit (QIAGEN). Sequencing primers OP52 and M13rev were used [26]. Automated sequencing was done by the Molecular Biology Division, Onderstepoort Veterinary Institute. Sequences were analysed using the Staden and BioEdit software packages [28,29].

### 2.4. Screening mutant library

The mutant library was panned using stringent conditions to select for scFvs with higher affinities than the parent scFv. Panning was done as described previously with some minor modifications [26]. For the first two rounds of panning 20 μg/ml of 16 kDa *M. tuberculosis* antigen in PBS was used to coat the wells of a microtitre plate (Nunc, Maxisorp) and for the third round 2 μg/ml was used. For the first round the wells were washed 19 times with PBS containing 0.1% Tween-20 (Tween-PBS) (Merck, Schardt, Germany) followed by a long 20 min wash on a rocker. Thereafter the wells were washed 20 times with PBS. For the second and third rounds the wells were washed 20 times with 0.5% Tween-PBS followed by 20 washes with PBS. The phages were eluted with 0.1 N HCl for all rounds. In order to determine input phage titres dilutions of $10^{-9}$–$10^{-11}$ phages were made in PBS. From each phage dilution 10 μl was added to 40 μl *E. coli* TG1 cells ($OD_{600}$ 0.4–0.6) and 50 μl 2x TY. This was incubated at 37 °C for 30 min, plated on TYE plates containing 100 μg/ml amp and incubated at 30 °C ON. The eluted phages (output) were titred by plating ten-fold dilutions ($10^{-1}$–$10^{-4}$) and the rest plated on a 16 cm petri dish.

### 2.5. Production and purification of scFvs

To produce scFvs, individual TG1 transformant colonies were inoculated into 5 ml 2x TY-AG and incubated ON at 30 °C at 240 rpm. A $^{1}/_{100}$ dilution on the ON culture was made in the same medium and grown until the $OD_{600}$ was 0.9. The culture was centrifuged at 2000× *g* for 10 min and the pellet resuspended in a fifth volume 2x TY with 100 μg/ml amp and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce scFv production. The final induction volumes of 10 ml or 2 ml were shaken at 240 rpm ON at 30 °C. Bacteria were removed by centrifuging the culture at 2000× *g* for 15 min. The supernatant fluids containing the secreted antibodies were used directly in ELISAs. Alternatively, scFvs were affinity purified from the supernatant fluid using an anti-c-myc tag monoclonal antibody 9E10 as described previously [26]. The scFv samples were stored at 4 °C or in 2% sucrose solution at −20 °C.

### 2.6. ELISA

The binding of the mutant scFvs to the antigen was characterised by ELISA. An immunoplate (Nunc, Maxisorp) was coated ON at 4 °C with 10 μg/ml of 16 kDa *M. tuberculosis* antigen diluted in PBS. Apart from blocking and washing steps, all the reactions volumes were 50 μl per well. The plate was blocked with 300 μl per well of 2% (w/v) bovine serum albumin (BSA) in PBS at 37 °C for 1 h followed by three washes with 0.05% Tween-PBS. The scFvs in the supernatant fluid were diluted 1:1 with 4% BSA–PBS and incubated at 37 °C for 1 h followed by three washes. An anti-c-myc tag monoclonal antibody 9E10 [30] in Dulbecco's Modified Eagles Medium (GIBCO, Grand Island, USA) diluted 1:1 in 4% BSA–PBS was added, incubated at 37 °C for 1 h and washed three times with 0.05% Tween-PBS. For detection a $^{1}/_{1000}$ dilution of polyclonal rabbit anti-mouse immunoglobulins conjugated to HRP (DakoCytomation, Ely, UK) in 2% BSA–PBS was added and incubated at 37 °C for 1 h and washed as in the previous step. After the final wash 50 μl of substrate made up of 1 mg/ml o-phenylene diamine and 0.5 μl/ml of 30% ($^{v}/_{v}$) $H_2O_2$ in 0.1 M citrate buffer (pH 4.5) was added and left at room temperature (RT) for 40 min. The enzyme reaction was stopped with 50 μl 2N $H_2SO_4$ and the absorbance measured at 492 nm. All scFv samples in the study were tested in duplicate. An additional ELISA with a short 10 min incubation time was used to determine whether a shorter reaction time would reduce the ELISA signal. The scFvs were diluted 1:1 with 4% BSA–PBS and incubated at 37 °C for 10 min for the short ELISA and washed as above. An ELISA with harsh washing conditions was also used to determine whether the scFvs could still bind to the antigen. Here the scFvs diluted 1:1 with 4% BSA–PBS were incubated at 40 °C for 1 h and washed 10 times with 0.05% Tween-PBS at 40 °C, followed by 30 min incubation with 0.05% Tween-PBS at 40 °C.

### 2.7. Surface plasmon resonance (SPR)

SPR was used to compare the binding kinetics of the mutant scFvs on a Biacore X (Biacore, Uppsala, Sweden). Experiments were performed at 25 °C using HBS-EP running buffer. The 16 kDa protein of *M. tuberculosis* was covalently bound to the dextran surface of a CM5 chip via its primary amine groups (BIApplications handbook, Biacore). A volume of 35 μl of 16 kDa (50 μg/ml in 10 mM acetate buffer, pH 4) was injected and un-reacted ester groups were blocked with 1 M ethanolamine-HCL, pH 8.5. These conditions resulted in 3, 300 RU being immobilized. The control flow cell was left empty. For

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mutant scFv and short linker analysis dilutions of the scFvs B4, B4m1, B4m2, B4m3 and B4sL in HBS-EP were passed over the chip at a flow rate of 30 μl/min for 70 s and allowed to dissociate for the same time. Bound scFvs were removed with 10 μl of 0.1 M glycine, pH 2.

### 2.8. Linker length manipulation

To reduce the linker to a single glycine residue, the $V_H$ and $V_L$ domains of the scFv B4 were amplified by PCR separately using 2.5 U of Faststart High Fidelity enzyme (Roche) in a 50 μl reaction using 1 ng of scFv DNA and 0.4 pmol/μl of each primer. The primer pairs Sfi1L and Hend1 and LStart1 and LCNOT1 (Table 1) were used for the heavy and light chain reactions respectively. The reaction was allowed to proceed for 30 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s. An initial denaturing step of 94 °C and a final extension step of 4 min were included for all PCRs. HyperLadder (Bioline, London, UK) DNA standards were used to determine the size of the PCR products. The PCR products were separated by electrophoresis in an agarose gel supplemented with 1.5% ethidium bromide using 1x Tris-acetate-EDTA buffer at 100 V for 20 min and purified using TaKaRa Recochips (TaKaRa, Kyoto, Japan) as per manufacture's instructions. To add overlapping sequences, the recovered products were used in the second PCR using the primer pairs Sfi1L and HendG1 for the heavy chain and LStart G1 and LCNOT1 for the light chain reaction (Table 1). Thermal cycling conditions were the same as above for the heavy chain reaction and the melting temperature (Tm) was 47 °C for the light chain reaction. The $V_H$ and $V_L$ (each 100 ng) were joined by using splicing by overlap extension (SOE) [31]. The reaction was allowed to proceed for 15 cycles of 95 °C for 30 s, 47 °C for 30 s and 72 °C for 30 s. Amplification of the joined genes after SOE was performed using the primers Sfi1L and LCNOT1 and the reaction was allowed to proceed for 25 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. To remove the linker and the terminal serines from the heavy chain, the primers Sfi1L and Hend2 were used for the heavy chain reaction. For the light chain reaction the primers LStart1 and LCNOT1 were used. For the second PCR that adds overlapping sequences, the primer pairs Sfi1L, Hend2-SOE (Tm 47 °C; Table 1), LStart-SOE (Table 1), LCNOT1 (Tm 55 °C) were used for the heavy and light chain reactions respectively. Joining and amplification of $V_H$ and $V_L$ were performed as above except with a Tm of 65 °C. The PCR products were purified using a PCR purification kit (QIAGEN). The cloning of the shortened and "no-linker" constructs into the vector pHEN1 and transformation of *E. coli* were performed as described previously [26]. To confirm that the correct constructs were made, DNA was extracted and sequenced as before.

### 2.9. Size exclusion chromatography

To determine the extent of multibody formation, purified scFvs were separated by gel filtration using a Superdex 75 HR10/30 (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Samples were compared with gel filtration molecular mass markers (Blue Dextran, 2000 kDa; Amylase, 200 kDa, Alcohol dehydrogenase, 150 kDa; Albumin, 66 kDa; Cytochrome c, 12.4 kDa) (Sigma, Missouri, USA) to estimate the size of the molecules.

**Table 1**
Nucleotide sequences of DNA primers.

| Primer | Sequence |
|---|---|
| Hend1 | 5′ CCG GAG GAG ACG ATG ACT TCG G 3′ |
| Lstart1 | 5′ GCG CTG ACT CAG CCG TCC TCG G 3′ |
| HendG1 | 5′ CGG CTG AGT CAG CGC TCC GGA GGA GAC GA 3′ |
| LstartG1 | 5′ TCG TCT CCT CCG GAG CGC TGA CTC AGC CG 3′ |
| Hend2 | 5′ ACG ATG ACT TCG GTC CCG TGG 3′ |
| Hend2-SOE | 5′ CGG CTG AGT CAG CGC TAC GAT GAC TTC GGT C 3′ |
| Lstart-SOE | 5′ CCG AAG TCA TCG TAG CGC TGA CTC AGC CG 3′ |

## 3. Results

### 3.1. Antibody selection by panning

The *Nkuku*® scFv repertoire was panned against the recombinant 16 kDa *M. tuberculosis* antigen. After four rounds of panning, three scFvs which bound specifically to the antigen were obtained. In phage displayed format all the clones produced $OD_{492}$ signals of more than 0.5 in ELISA. When expressed as soluble scFvs, however, the signals were less than 0.5 (Fig. 1). Since B4 yielded the lowest ELISA signal in phage displayed format and it functioned as a soluble scFv, it was chosen as a model for the engineering of recombinant chicken antibodies.

### 3.2. Mutant anti-16 kDa scFvs

A mutant sublibrary (mB4) of $3.3 \times 10^7$ clones was created by introducing random mutations by means of PCR into the entire gene sequence coding for the scFv using conditions that mutated approximately seven per 1000 bases. The DNA sequences of five clones from the mutant sublibrary confirmed that mutations had been introduced (results not shown). The sublibrary was then panned for three rounds against the 16 kDa MkDa *M. tuberculosis* antigen. Only a single binding clone (B4m1) was obtained from the last round of selection. Screening of clones from the first round yielded two more binders, namely, B4m2 and B4m3.

The three mutant scFvs obtained after selection contained between one and six nucleotide substitutions. All had point

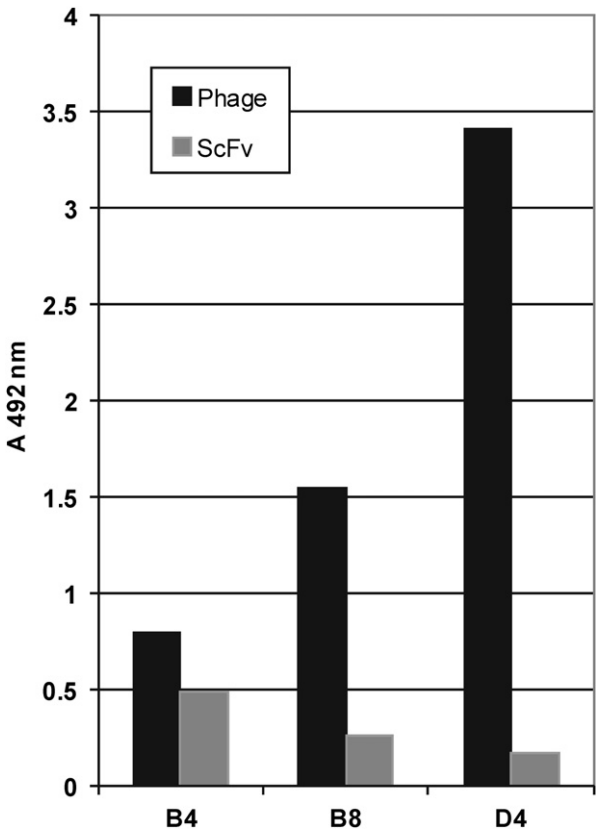


**Fig. 1.** ELISA of scFvs from *Nkuku*® library in phage displayed and soluble scFv formats reacting to the 16 kDa antigen of *M. tuberculosis*.

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mutations, none of which occurred in the same position in any of the three. In the B4m1 mutant, asparagine (N) was replaced by serine (S) in the heavy chain CDR1 (Fig. 2). B4m3 had arginine (R) instead of glutamine (Q), aspartic acid (D) was replaced by glycine (G) and threonine (T) by alanine (A) in its heavy chain framework region, light chain CDR2 and light chain framework region. Mutant B4m2 had three amino acid exchanges, from T to A, lysine (K) to R, and K to R in the heavy chain framework, heavy chain CDR2 and light chain framework regions respectively. In addition, B4m3 had one, and B4m2, two silent mutations (nucleotide sequence not shown).

### 3.3. Mutant binding analyses

The binding of the mutant scFvs was tested in ELISA and SPR. In ELISA, scFvs B4m1, B4m2 and B4m3 yielded signals that were 10.8, 6.9 and 9.6 times higher than that of the parent antibody B4 (Fig. 3). An ELISA with a short incubation time of 10 min and one with more stringent washing showed that B4m3 bound more rapidly to the antigen than the other scFvs. When comparing the results with that of a normal ELISA with 1 h incubation periods, B4m1 and B4m2 emerge as poor performers (Fig. 4). SPR, which shows the binding kinetics in real time, in contrast to the equilibrium state ELISA, supported the ELISA (Fig. 5). For this analysis, purified scFvs at defined concentrations were used. The mutations had no significant effect on the yield, with B4 producing 8 mg per litre of bacterial culture and the mutants 7.6–8.9 mg per litre. In the Biacore analyses, all three mutant scFvs gave a higher response than the parent B4, with B4m3 showing that its binding had not reached equilibrium after 70 s, unlike B4 and the other two mutants. This mutant also

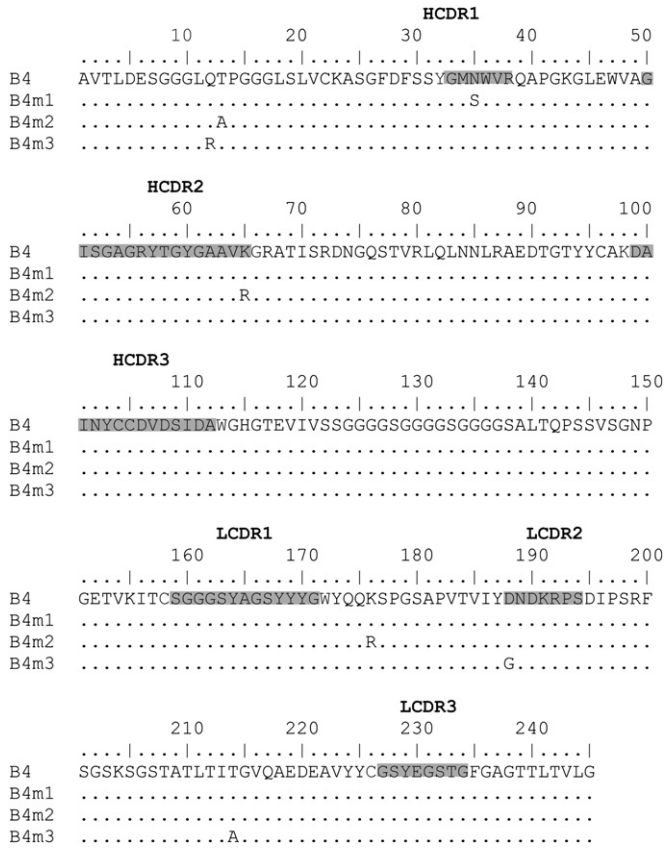


**Fig. 2.** Amino acid sequence alignment showing the changes in the scFv genes compared to parent scFv B4. Dots indicate amino acids identical to that of the parent scFv B4. The highlighted areas represent the CDRs.

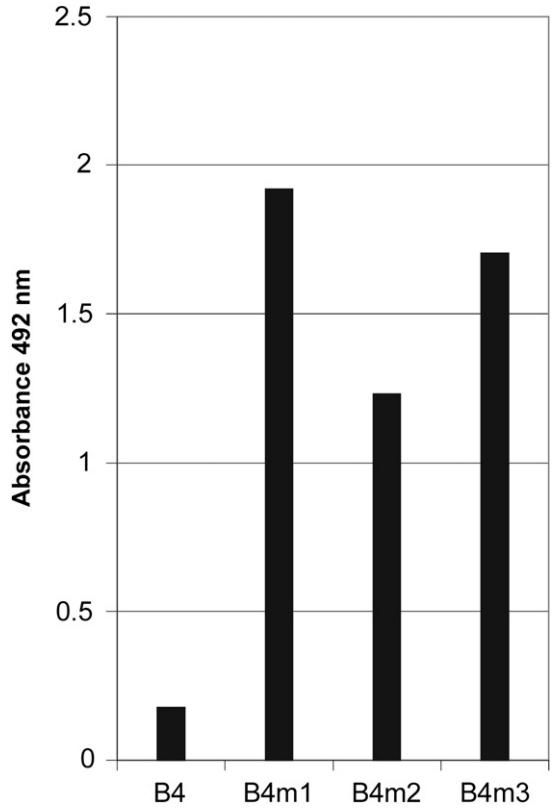


**Fig. 3.** An ELISA showing the binding of mutant scFvs derived from the scFv B4 to the 16 kDa antigen of *M. tuberculosis*.

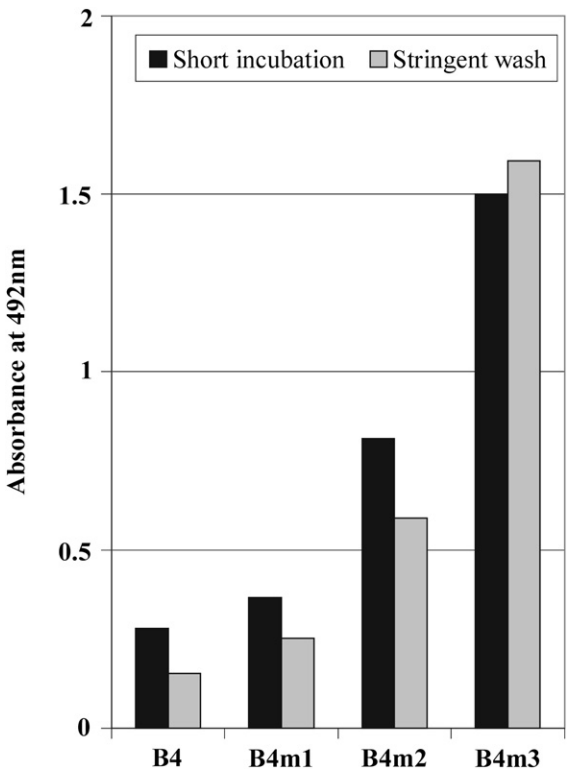


**Fig. 4.** An ELISA showing the binding of mB4 mutant scFvs to the 16 kDa antigen under short incubation times or with stringent washing conditions.

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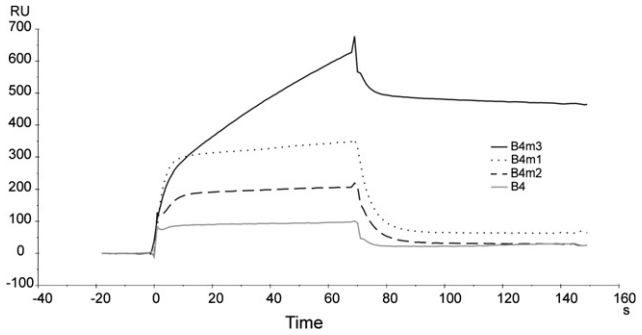


**Fig. 5.** Sensogram of purified scFvs injected over the *M. tuberculosis* 16 kDa protein immobilised on the surface of a CM5 biacore chip. The response is measured in resonance units (RU).

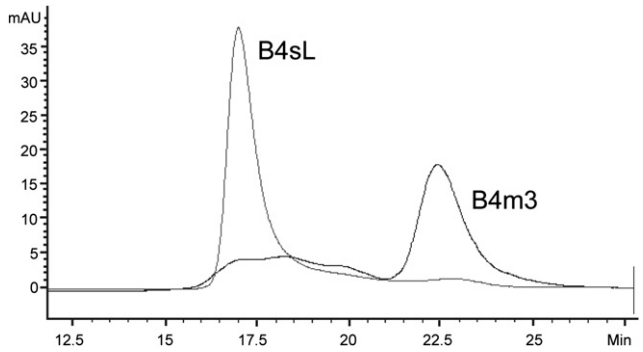


**Fig. 7.** Size exclusion chromatograph depicting the clone [1]B4m3 as a monomer (29–31 kDa), and B4sL as a tetramer (116 kDa). [1]B4m3 is used for illustration purposes to depict the chromatograph of a monomer.

showed a marked difference in the rate at which it dissociated; after a washing step using the same flow rate as injection, 75% of the B4m3 scFvs remained bound to the protein whereas B4, B4m1 and B4m2 were released more rapidly. The remaining resonance signal of B4m3 was 18-fold higher than that of the parent antibody fragment. This confirmed the ELISA results which showed that the scFv remained bound after extended washing.

### 3.4. Effect of linker length

The effect of different lengths of linkers on the conformation of the chicken scFv B4 was investigated. Shortening the linker to one glycine residue (B4sL) resulted in an increase in the apparent functional affinity of the antibody fragment, as was shown by ELISA in which the signal was almost 14-fold higher compared to that of the parent antibody B4 (Fig. 6). Size exclusion chromatography revealed that B4sL had formed a 116 kDa tetramer (Fig. 7). B4m3 was used for comparative purposes as it occurred mainly as monomers with very few dimmers being present, while the parent B4 preparation comprised of a mixture of monomers and dimers (not shown). No reaction in ELISA was obtained using the construct where the two variable domains were joined directly. The association kinetics of the tetrameric scFv (B4sL), with its higher valency, was greatly improved compared to the parent as shown by SPR (Fig. 6). The binding of the parent to the immobilised antigen reached equilibrium in less than 10 s while the tetramer had not yet reached equilibrium after 70 s. The off-rate of the tetramer was slower than that of the parent. After 140 s a greater amount still remained antigen bound as compared to the parent.

## 4. Discussion

Three single chain recombinant antibodies that recognized the mycobacterial 16 kDa protein were selected from a semi-synthetic single chain Fv phage display library based on chicken immunoglobulin genes. The resulting soluble scFvs reacted relatively poorly in ELISA and as a consequence, would probably be unsuitable as research or diagnostic reagents. One of the scFvs was subjected to methods previously shown to improve the binding characteristics of human-and mouse-derived scFvs. Firstly, to mimic somatic mutation, the gene coding for the scFv was subjected to random mutagenesis. Conditions favouring a relatively low frequency of mutation were used since this mimics the natural process of evolution while a high mutation frequency is more likely to result in a large number of non-functional genes [9]. After selection by panning on the 16 kDa protein, three mutants with between one and three amino acid exchanges were obtained, all of which produced ELISA signals higher than the original scFv. Mutant B4m1 showed a ten-fold increase in

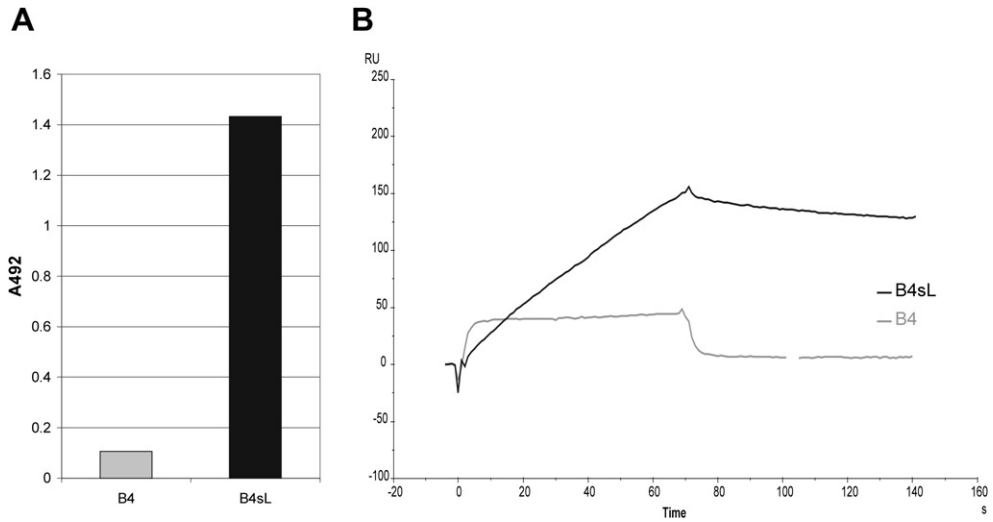


**Fig. 6.** ELISA and sensogram showing the difference in binding of a 15 amino acid linker scFv B4 compared to B4sL with a single amino acid residue linker. The SPR response is measured in resonance units (RU).

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ELISA signal compared to its parent. This clone had an S instead of an N in its heavy chain CDR1. These residues both have polar side chains and such substitutions have been found to be well tolerated in the V domains of immunoglobulins [32]. B4m1 was the result of three rounds of panning. It was selected using relatively stringent panning conditions, but when tested in ELISA under similarly stringent conditions, it did not perform well, although immunoblotting revealed that the bacterial clone apparently secreted more scFvs than the parent (not shown). Clones selected after the first round of panning, B4m2 and B4m3, produced much higher ELISA signals compared to the unmutated B4. B4m2 had six base substitutions resulting in three amino acid exchanges and three silent mutations. B4m3 had four base substitutions leading to three amino acid exchanges and one silent mutation.

Mutant B4m3 yielded the highest resonance signal in SPR and had a slower off-rate compared to both the parent clone B4 and the other two mutants, B4m1 and B4m2. The SPR results were supported by the short and stringent ELISAs where after 10 min of incubation, B4m3 produced signals similar to those obtained with an hour's incubation. B4m3 also maintained its binding after extended washing at elevated temperatures.

The second strategy investigated as a way of improving binding was to shorten the linker between the two variable domains of the scFv. Chicken scFvs with a 15 amino acid linker $(Gly_4Ser)_3$ have been found to occur mostly as monomers and a mixture of lesser amounts of multimers. Shortening the linker of the weak 16 kDa mycobacterial binder significantly improved its signal in ELISA owing to the formation of tetrameric "multibodies". The formation of trimers from a scFv comprising a linker with a single glycine residue has been reported previously for mouse scFvs while other such scFvs formed a mixture of dimers, trimers and tetramers [24,33,34]. Different scFvs and different linker compositions may therefore differ in their propensity to form dimers, trimers or tetramers. This is unlike the strict transitions observed when the linker used in constructing an anti-neuraminidase mouse scFv (NC10) was reduced to one glycine residue for [24].

In both mouse and chicken derived scFvs, the amino acids VSS are found at the C-terminus of the heavy chain [14,22,26]. In mouse scFvs, directly joining the last residue of the heavy chain $V_HS^{113}$ to the first residue of the light chain $V_LD^1$ resulted in a mixture of trimers and tetramers [35,36]. Another study reported the formation of trimers exclusively when $V_HS^{112}$ was fused to $V_LD^1$ and tetramers when $V_HS^{113}$ was ligated to $V_LD^1$ [34]. In the case of the anti-16 kDa TB scFv, both the terminal serine residues of the heavy chain were removed and the light chain was directly coupled to a valine residue (Fig. 2). This abrogated all binding in ELISA. This finding supports the notion that linker dependent oligomerisation of scFvs may be affected by the sequence of the variable domains [35]. Removal of the terminal serine residues might therefore have prevented the variable domains of the multibodies from forming functional antigen binding sites.

As expected, SPR analyses of the tetramer B4sL confirmed an increase in the on-rate and a reduced off-rate. Affinity constants (SPR) were not determined since the scFvs occurred as multibodies and thus the 1:1 Langmuir model could not be used. This is because SPR analyses of multibodies is influenced by the multiplicity of binding, amount of antigen immobilized on the biosensor chip, the multibody alignment and its orientation with respect to the exposure of Fv portions to the immobilized antigen [37]. Nevertheless the SPR data could still be used as a convenient way to rank scFvs by comparing the apparent dissociation rates. Together with ELISA these results have shown that random mutagenesis and shortening the linker can improve the binding characteristics of chicken scFvs. Since the 16 kDa TB protein is known to be a useful diagnostic target for serodiagnosis and cellular immunoassays [5,6], the engineered scFvs described here are likely to be useful research tools and in diagnostics development.

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