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ScFv multimers of the anti-neuraminidase antibody NC10: shortening of the linker in single-chain Fv fragment assembled in V_L to V_H orientation drives the formation of dimers, trimers, tetramers and higher molecular mass multimers

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Synthetic genes encoding single-chain variable fragments (scFvs) of NC10 anti-neuraminidase antibody were constructed by joining the V_L and V_H domains with linkers of fifteen, five, four, three, two, one and zero residues. These V_L–V_H constructs were expressed in *Escherichia coli* and the resulting proteins were characterized and compared with the previously characterized NC10 scFv proteins assembled in V_H–V_L orientation. Size-exclusion chromatography and electron microscope images of complexes formed between various NC10 scFvs and anti-idiotype Fab′ were used to analyse the oligomeric status of these scFvs. The result showed that as the linker length between V_L and V_H was reduced, different patterns of oligomerization were observed compared with those with V_H–V_L isomers. As was the case for V_H–V_L orientation, the scFv-15 V_L–V_H protein existed mainly as a monomer whereas dimer (diabody) was a predominant conformation for the scFv-5, scFv-4 and scFv-3 V_L–V_H proteins. In contrast to the V_H–V_L isomer, direct ligation of V_L to V_H led to the formation of predominantly a tetramer (tetrabody) rather than to an expected trimer (triabody). Furthermore, the transition between dimers and higher order oligomers was not as distinct as for V_H–V_L. Thus reducing the linker length in V_L–V_H from three to two residues did not precisely dictate a transition between dimers and tetramers. Instead, two-residue as well as one-residue linked scFvs formed a mixture of dimers, trimers and tetramers.

Keywords: antibody/diabody/single-chain Fvs/tetrabody/triabody

Introduction

The antigen-binding portion of an antibody molecule (Fv fragment) is formed by the association of the heavy chain variable region (V_H) and the light chain variable region (V_L). Fv fragments are the smallest entities that consistently maintain the binding specificity of the whole antibody. Although recombinant DNA techniques have facilitated individual Fv domain production, the non-covalently associated V_L and V_H domains in an individual Fv fragment tend to dissociate from one another (Glockshuber *et al.*, 1990). To improve stability, recombinant single chain Fv fragments (scFvs) have been engineered with two variable domains covalently joined via a flexible peptide linker (Bird *et al.*, 1988; Huston *et al.*, 1988), length form stable monomers, which usually exhibit similar antigen binding affinity to the parent antibody (Skerra *et al.*, 1991; Kortt *et al.*, 1994). However, scFv monomers have also been observed to form active dimers and higher molecular mass multimers upon freezing at high protein concentrations (Kortt *et al.*, 1994).

To provide increased avidity to target antigens, recent attention has focused on the design of linkers which generate multivalent scFv molecules (reviewed by Hudson, 1999). Multivalent scFv molecules are sufficiently large to avoid the fast clearance through the kidneys observed for scFv monomers and thereby have potential application for tumour imaging and radiotherapy (Adams *et al.*, 1998; Colcher *et al.*, 1998; Wu *et al.*, 1999). Construction of stable multimeric scFv molecules can be achieved by the shortening of the linker length to <12 residues such that the V_H domain is unable to associate with its attached V_L domain and thus generate a monomeric Fv fragment. Instead, V_H and V_L domains from one scFv molecule associate with V_H and V_L domains from a second scFv molecule to form a bivalent dimer, termed a diabody (Hollinger *et al.*, 1993). When the linker is shortened to <3 residues or when V_H and V_L domains are directly ligated to each other, scFv molecules associate to form a trimer, termed a triabody (Iliades *et al.*, 1997; Kortt *et al.*, 1997).

ScFvs of both V_H–linker–V_L and V_L–linker–V_H orientation have been produced by various research groups (Huston *et al.*, 1991). Our laboratory has to date almost exclusively produced scFvs with the V_H domain at the amino terminus (Malby *et al.*, 1993, Lilley *et al.*, 1994; Coia *et al.*, 1997; Iliades *et al.*, 1997). In particular, NC10 anti-neuraminidase scFv antibody, in a V_H–linker–V_L orientation, has been used extensively to elucidate the oligomeric nature of scFvs with reduced linker lengths (Kortt *et al.*, 1997; Atwell *et al.*, 1999). To investigate the oligomerization phenomenon further, the NC10 scFv fragment was assembled in a reverse, V_L–linker–V_H, orientation. The observed result showed that as the linker length between V_L and V_H was reduced, different patterns of oligomerization were observed compared with the original V_H–linker–V_L orientation. Furthermore, the direct ligation of V_L to V_H led to the formation of a tetramer, rather than to a trimer as observed for direct ligation of V_H to V_L.

Materials and methods

Sequence numbering

Antibody residues were numbered according to Kabat *et al.* (1991) and for NC10 correspond exactly to Malby *et al.* (1993). Residues in the V_L domain of the scFv were superscripted with L and the residue number; for example, Arg^{L107} signifies arginine in position 107 of the V_L domain. Similarly residues in the V_H domain of the scFv were superscripted H and the residue number.

General cloning procedures

Unless stated otherwise, all DNA manipulations were carried

with reagents purchased from New England Biolabs. All polymerase chain reactions (PCRs) were performed with *Pfu* DNA polymerase (Stratagene). The PCR-amplified DNA fragments were digested with appropriate restriction enzymes, run on 1% (w/v) agarose gel and purified from the gel using BRESAclean purification kit (Bresatech). The purified DNA fragments were ligated into similarly prepared expression vectors using reagents and protocols supplied by Gibco-BRL. The ligation mixtures were transformed by the electroporation method (Dower *et al.*, 1988) into *Escherichia coli* XL 1-Blue MRF′ cells (Stratagene) and recombinant clones were identified by colony PCR using primers complementary to 5′ and 3′ ends of recombinant gene inserts. All DNA sequences of various scFv constructs were verified using Dye Terminator Cycle Sequencing kits with AmpliTaq (PE Applied Biosystems).

Construction of NC10 scFv gene fragments with 0 and 15 residue linkers

The previously described pPOW–NC10 scFv-15 (V_H–V_L) gene construct (Malby *et al.*, 1993) was used as a source of V_L and V_H gene fragments. To generate the NC10 scFv-0 (V_L–V_H) synthetic gene, the V_L and V_H gene fragments were PCR-amplified using primers N4311 and N4341 for V_L, and N4342 and N4293 for V_H (Table I). The resulting V_L and V_H PCR products were gel-purified and joined into an scFv-0 using PCR overlap extension (Horton *et al.*, 1989). To create the NC10 scFv-15 (V_L–V_H) synthetic gene, V_L and V_H gene fragments were PCR-amplified using primers N4311 and N4535 for V_L, and N4534 and N4293 for V_H (Table I). The resulting V_L and V_H gene fragments each contained part of the linker sequence at the 5′ and 3′ ends, respectively, as well as the *Bam*HI restriction site which allowed for correct in frame ligation of these V fragments via a linker sequence encoding 15 amino acid $(GGGGS)_3$. The NC10 scFv-0 and scFv-15 (V_L–V_H) synthetic genes were then digested with *Nco*I and *Not*I restriction enzymes and cloned separately into a likewise digested pGC-4C2 *E.coli* vector (Coia *et al.*, 1997) to create pGC-NC10 scFv-0 and scFv-15 plasmids (Figure 1a-i). This pGC-4C2 vector backbone incorporates two, rather than one, C-terminal FLAG peptide epitopes (Hopp *et al.*, 1988) for improved purification efficacy of FLAG-fusion proteins. The resulting NC10 scFv-0 and scFv-15 gene constructs encoded N-terminal cleavable pelB periplasmic targeting signal followed by NC10 scFv and two C-terminal FLAG peptide epitope tags which were linked to V_H domain at the C-terminus by three alanine residues and separated from one another by two alanine residues (Figure 1a-iii). The NC10 scFv-15 and scFv-0 (V_L–V_H) gene fragments were also inserted into a heat-inducible pPOW vector (Power *et al.*, 1992). This was done by digesting the above described pGC-NC10 scFv-15 and scFv-0 (V_L–V_H) plasmids with *Nco*I and *Eco*RI restriction enzymes and ligating the scFv synthetic genes into a likewise digested pPOW vector. Similarly as for pGC constructs, the resulting gene fragment (Figure 1a-ii) was designed to express pelB signal sequence followed by NC10 scFv and two C-terminal FLAG tags.

Construction and cloning of NC10 scFv gene fragments with shorter linkers

The pGC-NC10 scFv-0 (V_L–V_H) gene construct (Figure 1a) was used for insertion of linkers of increasing length. This vector was digested simultaneously with *Xho*I and *Pst*I restric-

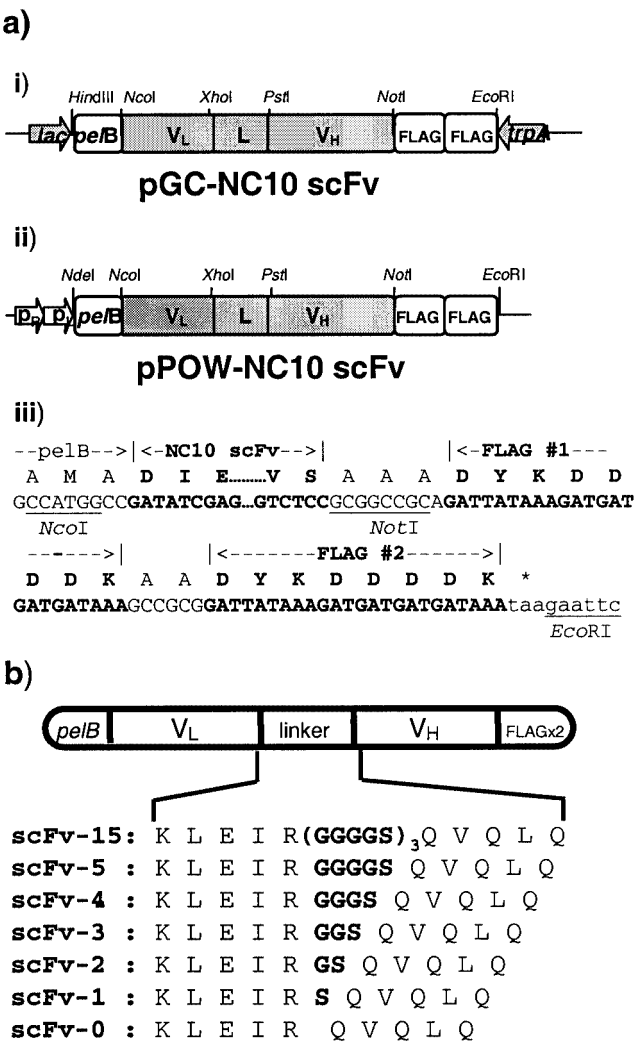

Fig. 1. (**a**) Schematic diagram of the NC10 scFv (V_L–V_H) expression unit in pGC (**i**) and pPOW (**ii**) vectors. (**iii**) Important parts of nucleotide and amino acid sequences of the NC10 scFv (V_L–V_H) unit in pGC and pPOW, outlining restriction sites for cloning (underlined), C-terminal region of *pel*B sequence, N-terminal sequence of V_L gene and C-terminal sequence of V_H gene terminal (in bold), and two C-terminal octapeptide FLAG epitopes (in bold). (**b**) Amino acid sequences of the C-terminus of the V_L domain, N-terminus of the V_H domain and of the linker peptide (in bold) used in each of the NC10 scFv constructs.

using agarose gel. Five sets of synthetic oligonucleotides (Table I) were phosphorylated at the 5′ termini by incubation at 37°C for 30 min with 0.5 units of T4 polynucleotide kinase and 1 mM rATP (Biotech International) in 1× PNK buffer. Pairs of complementary phosphorylated oligonucleotides were pre-mixed in equimolar ratios to form DNA duplexes that encoded linkers of increased length. The one-residue linker construct was designed to encode a Ser residue in between codons for C-terminal V_L Arg^{L107} and N-terminal V_H Gln^{H1}, whereas the two-, three-, four- and five-residue linker constructs were designed to encode additional glycine residues immediately preceding the Ser linker residue (Figure 1b). All five of these duplexes were designed to contain a ‘sticky end’ overlap compatible with *Xho*I and *Pst*I restriction enzyme sites at the 5′ and 3′ ends, respectively. This allowed for direct cloning of these duplexes into *Xho*I/*Pst*I restricted pGC-NC10 scFv-0

Table I. Synthetic oligonucleotides used for construction of NC10 scFv V_L–V_H gene fragments

scFv–15 and scFv–0	
N4311:	*AAA ACC ATG GCC* GAT ATC GAG CTC ACA CAG
N4341:	AGA CTG CTG CAG CTG CAC CTG TCT TAT CTC GAG CTT GGT
N4342:	GGG ACC AAG CTC GAG ATA AGA CAG GTG CAG CTG CAG CAG TCT
N4293:	*TTT TGC GGC CGC* GGA GAC GGT GAC CGT GGT CCC
N4535:	*TTT GGA TCC ACC TCC ACC GCA ACC GCC TCC ACC* TCT TAT CTC GAG CTT GGT CCC
N4534:	*AAA GGA TCC GGT GGA GGC GGT TGC* CAG GTG CAG CTG CAG CAG
scFv–5	
N5006:	TCG AGA TAA GA*G GTG GAG GTG GAT CCC* AGG TGC AGC TGC A
N5009:	GCT GCA CCT G*GG ATC CAC CTC CAC C*TC TTA TC
scFv–4	
N5007:	TCG AGA TAA GA*G GAG GTG GAT CCC* AGG TGC AGC TGC A
N5010:	GCT GCA CCT G*GG ATC CAC CTC C*TC TTA TC
scFv–3	
N5008:	TCG AGA TAA GA*G GTG GAT CCC* AGG TGC AGC TGC A
N5004:	GCT GCA CCT G*GG ATC CAC C*TC TTA TC
scFv–2	
N5011:	TCG AGA TAA GA*G GAT CCC* AGG TGC AGC TGC A
N5003	GCT GCA CCT G*GG ATC C*TC TTA TC
scFv–1	
N5005	TCG AGA TAA GA*T CCC* AGG TGC AGC TGC A
N5002	GCT GCA CCT G*GG A*TC TTA TC

Oligonucleotide sequences are listed 5′ to 3′. Linker- and vector-related gene sequences are in italics.
Important restriction sites are underlined.

Expression and purification of the scFvs

NC10 scFv-15 and scFv-0 gene constructs (V_L–V_H) were initially expressed in a heat-inducible pPOW vector (Power *et al.*, 1992). Each pPOW-NC10 scFv construct was expressed in 1 l of 2YT/amp_{100} as described by Malby *et al.* (1993), using *E.coli* strain TOP 10F′ (Invitrogen) as host. The scFv-15 and scFv-0 proteins (V_L–V_H) were located in the periplasm as insoluble aggregates associated with the membrane fraction, as found previously for NC10 scFv V_H–V_L proteins (Malby *et al.*, 1993; Kortt *et al.*, 1997). Recombinant scFv proteins were purified using a modified protocol of Kortt *et al.* (1997). Cells were disrupted by sonication in 100 ml of phosphate-buffered saline, pH 7.4 (PBS), centrifuged and the insoluble aggregates of NC10 scFvs were solubilized in 100 ml of 6 M guanidine hydrochloride, 0.1 M Tris–HCl, pH 8.0. The resulting mixture was then dialysed against PBS and insoluble material removed by centrifugation. Recombinant scFvs were purified from soluble fractions (supernatant) using an M2 anti-FLAG IgG-Sepharose column (5×1 cm; Brizzard *et al.*, 1994). The affinity column was equilibrated in PBS and bound proteins were eluted with 100 mM glycine, pH 3.0. The eluted proteins were neutralized with 1/10 volume of 1 M Tris–HCl (pH 8.0) and dialysed extensively against PBS–0.02% (w/v) sodium azide. Proteins samples were concentrated by ultrafiltration (Amicon) over a 10 kDa cut-off membrane (YM10, Diaflo) to ~1 mg/ml and stored at 4°C.

Expression and purification of NC10 scFvs (V_L–V_H) with shorter linkers

Each pGC-NC10 scFv (V_L–V_H) construct was expressed in 500 ml of 2YT/amp_{100} + 0.1% (w/v) D-glucose as described in Dolezal *et al.* (1995), using *E.coli* strain TOP 10F′ cells as host. Expression experiments were terminated 3 h post-induction and proteins were isolated from the periplasmic space using a modified method of Minsky *et al.* (1986). Briefly, cells were centrifuged at 5000 *g* for 10 min and supernatant was were resuspended in 20 ml of ice-cold spheroplast buffer (100 mM Tris–HCl, pH 8.0, 0.5 M sucrose, 2 mM EDTA, 100 µg/ml lysozyme). After incubating the cell mixture on ice for 20 min, ice-cold half-strength spheroplast buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.25 M sucrose) was added to a final volume of 100 ml. Cells were mixed gently and left on ice for a further 30 min. Periplasmic proteins were harvested by centrifugation at 10 000 *g*. The supernatant (100 ml) was sonicated briefly to shear the remaining DNA/RNA and filtered through 0.45 µm filter. An Amicon concentrator unit (with YM10 membrane) was used to concentrate the periplasmic fraction to ~15 ml. Recombinant NC10 scFvs (V_L–V_H) proteins were affinity purified from the periplasmic fraction using an M2 anti-FLAG IgG-Sepharose column as described in the previous section. Eluted scFvs proteins were dialysed against PBS–0.02% (w/v) sodium azide, concentrated by ultrafiltration (YM10, Diaflo) to ~1 mg/ml and stored at 4°C.

Biochemical and biophysical characterization of NC10 scFv proteins

The purity of the NC10 scFvs was monitored by SDS–PAGE and Western blot analysis as described previously (Kortt *et al.*, 1994). The concentrations of the scFv fragments were determined spectrophotometrically, using values for the extinction coefficient ($\varepsilon^{0.1\%}$) at 280 nm of 1.66 for V_L–V_H scFvs and 1.70 for V_H–V_L scFvs calculated as described by Gill and von Hippel (1989). The relative molecular mass of each affinity-purified NC10 scFv was estimated using size-exclusion chromatographic columns (Superose 12 HR10/30 and/or Superdex 200 HR 10/30, Pharmacia) on an HPLC system (Bio-Rad Model 700) at 21°C in PBS which were previously calibrated with Bio-Rad Gel Filtration Standard proteins. The flow-rate was 0.5 ml/min, and the absorbance of the effluent stream was monitored at both 214 and 280 nm. Elution times of various V_L–V_H scFv oligomers were compared with those already established for V_H–V_L scFv monomers, dimers and trimers

Formation of complexes with 3–2G12 anti idiotype Fab′

Fab′ fragments of the NC10 anti-idiotype antibody, 3–2G12, were prepared as described in Kortt *et al.* (1997). Purified NC10 scFv-0, scFv-1, scFv-2 and scFv-5 (V_L–V_H) proteins were mixed with a small molar excess of 3–2G12 anti-idiotype Fab′, as described previously by Kortt *et al.* (1997). The complexes were separated from excess Fab′ by size-exclusion chromatography on Superdex 200 (HR 10/30) in PBS, pH 7.4, with a flow-rate of 0.5 ml/min. The column had previously been calibrated with uncomplexed scFvs and 3–2G12 Fab′.

Electron microscopy

Complexes of scFv-0 and scFv-1, scFv-2 and scFv-5 with 3–2G12 Fab′ and the complex of influenza virus neuraminidase (soluble tetrameric extracellular domain) with NC10 Fab′ (Malby *et al.*, 1994) were examined by electron microscopy (EM). EM imaging and data analysis were performed as described previously (Lawrence *et al.*, 1998; Atwell *et al.*, 1999).

Molecular modeling

Computer-generated models of NC10 scFv triabodies and tetrabodies were constructed using Fv modules that corresponded to the coordinates of the NC10 Fv domain in PDB entry 1NMB (Malby *et al.*, 1994, 1998). Fv modules were manipulated as rigid bodies with the O molecular graphics package (Jones *et al.*, 1991). Triabody structure corresponded to the model described by Kortt *et al.* (1997) comprising three Fv modules with threefold symmetry. Tetrabodies comprised four Fv modules with fourfold symmetry. No attempt was made to model conformational changes in the Fv domains.

Results and discussion

Expression of NC10 scFv-15 and scFv-0 constructs

For direct comparison with the original NC10 scFv-15 and scFv-0 (V_H–V_L) constructs (Malby *et al.*, 1993; Kortt *et al.*, 1997), the NC10 scFv-15 and scFv-0 (V_L–V_H) gene fragments were initially constructed and expressed in a heat-inducible expression vector pPOW (Figure 1a-iii). As for V_H–V_L orientation, in the V_L–V_H construct of the NC10 scFv, the V_L domain was linked to the V_H domain using the classical linker design of Huston *et al.* (1988, 1991), whereby the codon for C-terminal V_L Arg^{L107} was linked to the codon for N-terminal V_H Gln^{H1} via a 15 amino acid residue linker ($[G_4S]_3$; Figure 1b). The Arg^{L107} was defined from the NC10 scFv crystal structure to be the last residue in the V_L that made intra-domain contacts within V_L and additional residues therefore formed a true linker (Malby *et al.*, 1998). In case of scFv-0, the C-terminal V_L Arg^{L107} was ligated directly to V_H Gln^{H1} (Figure 1b).

The pPOW/NC10 scFv-15 and scFv-0 (V_L–V_H) constructs were expressed under the same conditions as those used previously for the V_H–V_L constructs (Malby *et al.*, 1993; Kortt *et al.*, 1997). As was the case for the V_H–V_L proteins, the majority of expressed scFv-15 and scFv-0 (V_L–V_H) proteins were located in the periplasm as insoluble protein aggregates which were solubilized by extraction with 6 M GuHCl and on dialysis refolded into soluble, functional scFv entities. Furthermore, there was no apparent difference in expression levels between the V_L–V_H and V_H–V_L scFvs proteins. The resulting soluble scFvs were purified by affinity chromatography on an M2 anti-FLAG IgG-Sepharose column. Yields of shake-flask culture were typically obtained for scFv-15 and scFv-0, respectively.

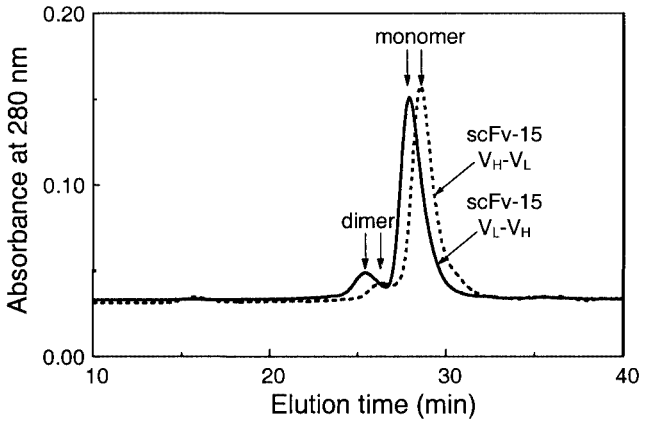

Fig. 2. Size-exclusion chromatography on a calibrated Superose 12 HR 10/30 column of affinity-purified NC10 scFv-15 proteins. Superimposed on the same scale are runs for scFv-15 V_L–V_H (solid line) and scFv-15 V_H–V_L (dashed line). The V_L–V_H protein eluted as monomer at 28.0 min and dimer at 25.5 min. These elution times are consistent with calculated molecular masses of 28.5 and 57 kDa, respectively. The V_H–V_L protein eluted as monomer at 28.6 min and dimer at 26.2 min. These elution times are consistent with calculated molecular masses of 27 and 54 kDa, respectively. The column was equilibrated in PBS, pH 7.4, and the flow-rate was 0.5 ml/min.

Biophysical analysis of NC10 scFv-15 and scFv-0 proteins

Samples of affinity-purified scFv-15 proteins (V_L–V_H and V_H–V_L) were shown by SDS–PAGE to comprise essentially homogeneous scFv preparations with a main protein band at ~27.0 kDa for V_H–V_L and ~28.5 kDa for V_L–V_H (data not shown). This apparent difference in protein mobility on SDS–PAGE was attributed to the additional sequence associated with the second FLAG epitope that has been added to the C-terminus of scFv V_L–V_H proteins (see Materials and methods and Figure 1a). Size-exclusion chromatography on a calibrated Superose 12 column showed that the oligomeric status of the NC10 V_H–V_L and V_L–V_H scFv-15 proteins was similar in solution (Figure 2). Gel filtration of affinity-purified scFv-15 V_L–V_H protein showed the presence of two main peaks with apparent molecular masses of ~28.5 kDa (major peak) and ~57.0 kDa (minor peak), corresponding to scFv-15 monomer and dimer, respectively. The scFv-15 V_H–V_L protein when analysed on the same column eluted mainly as monomer at ~27.0 kDa with a small amount of dimer at ~54.0 kDa. The relative differences in elution times for V_L–V_H and V_H–V_L monomers and dimers were again attributed to the additional FLAG sequence at the C-terminus of scFv-15 V_L–V_H protein. Both scFv-15 protein samples also contained traces of higher order oligomers, but because of small quantities of these species it was not possible to assign their oligomeric status unequivocally. Similarly as shown for NC10 scFv-15 V_H–V_L protein (Kortt *et al.*, 1994), the formation of NC10 scFv-15 V_L–V_H dimers and higher molecular mass multimers was induced by storing the sample at higher concentrations (>1 mg/ml) or by repeated freezing and thawing (data not shown).

In contrast to scFv-15 proteins, size-exclusion chromatography of scFv-0 (V_L–V_H and V_H–V_L) proteins demonstrated a significant difference in elution profiles (Figure 3a). The chromatography of the NC10 scFv-0 (V_L–V_H) protein yielded

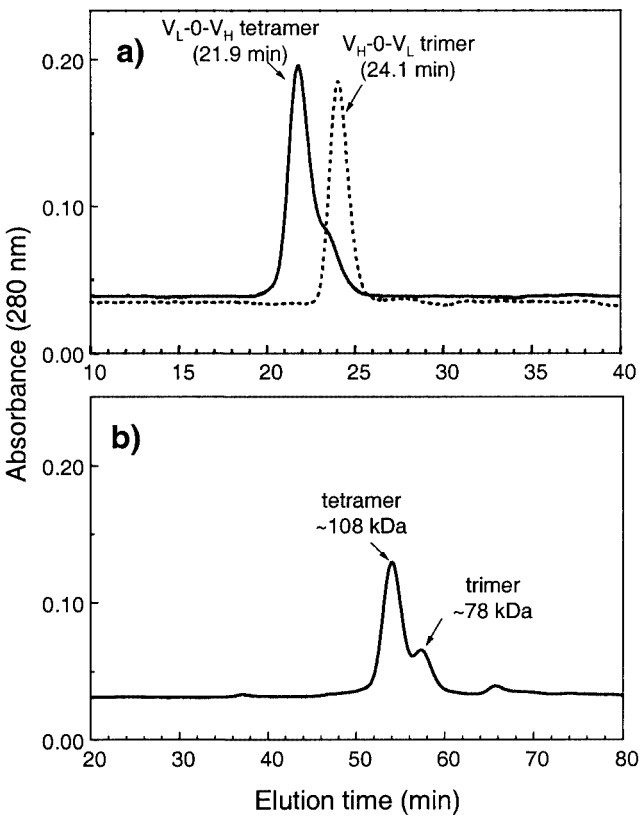

Fig. 3. Size-exclusion chromatography on a calibrated Superdex 200 HR 10/30 column of affinity-purified NC10 scFv-0 proteins. Columns were equilibrated in PBS, pH 7.4, and the flow-rate was 0.5 ml/min. (**a**) Shows the scFv-0 V_L–V_H tetramer (solid line) eluting at 21.9 min with a trimer shoulder on the trailing edge. Superimposed on the same scale is a run for scFv-0 V_H–V_L trimer (dashed line) eluting at 24.1 min. (**b**) Shows separation of scFv-0 tetramer from scFv-0 trimer using two Superdex 200 HR 10/30 columns linked in tandem.

shoulder on the trailing edge of the peak. The NC10 scFv-0 (V_H–V_L) protein, previously demonstrated to be a trimer (Kortt *et al.*, 1997), eluted as a single peak at 24.1 min on this column. The components of NC10 scFv-0 (V_L–V_H) protein were resolved by gel filtration on two Superose 12 HR10/30 columns linked in tandem, yielding two protein peaks with apparent molecular masses of ~108 and ~78 kDa (Figure 3b). In contrast to NC10 scFv-0 (V_H–V_L), the tandem gel filtration indicated that NC10 scFv-0 (V_L–V_H) forms not only the expected trimer (~78 kDa) but also a tetramer (~108 kDa). Attempts to isolate homogeneous NC10 scFv-0 tetramer were only partially successful, as isolated tetramer rapidly interconverted into a mixture of tetramer and trimer (data not shown). This observation indicated a relatively rapid equilibrium between NC10 scFv-0 V_L–V_H tetramer and trimer with the tetramer being the predominant species as shown in Figure 3.

Expression of NC10 V_L–V_H scFvs with variable linker length

To investigate further the effect of linker length upon multimerization of NC10 scFvs in reverse (V_L–V_H) orientation, seven different NC10 scFv constructs with linkers from 15 to 0 amino acid residues were assembled in pGC secretion vector (Coia *et al.*, 1997) as described in Materials and methods. This vector was chosen for construction and expression of

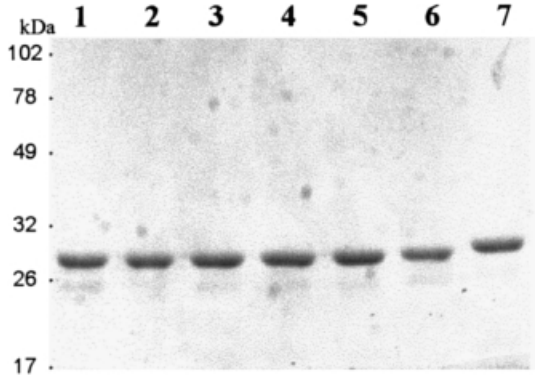

Fig. 4. Reducing SDS–PAGE of affinity-purified NC10 scFvs (V_L–V_H) stained with Coomassie Brilliant Blue G-250. Lanes: 1, scFv-0; 2, scFv-1; 3, scFv-2; 4, scFv-3; 5, scFv-4; 6, scFv-5; 7, scFv-15.

vector because of its capacity to produce soluble and active scFv proteins in the bacterial periplasm. A schematic diagram depicting the general outline of these short-linkered NC10 scFv expression units in pGC is shown in Figure 1b. The pGC NC10 scFv V_L–V_H constructs were expressed in *E.coli* TOP 10F′ cells using the expression protocol of Dolezal *et al.* (1995). The expressed scFv products were isolated from the periplasmic fraction by affinity chromatography as described in Materials and methods. The yield of soluble affinity-purified protein decreased progressively as the linker length was shortened, from 2 mg/l bacterial culture for scFv-15 to 0.5 mg/l bacterial culture for scFv-0.

Molecular mass analysis of scFv proteins with shorter linkers

SDS–PAGE analysis of affinity-purified NC10 scFv-0, scFv-1, scFv-2, scFv-3, scFv-4, scFv-5 and scFv-15 (V_L–V_H) protein samples showed that the scFvs comprised predominantly a single component of apparent molecular mass ~27.5–28.5 kDa, as expected for this series of proteins (Figure 4). When these scFvs were subjected to analysis by size-exclusion chromatography on a calibrated Superdex 200 column, a number of major protein peaks were observed with a significant variation in elution times consistent with the presence of scFv oligomers (Figure 5). Elution profiles for scFv-5, scFv-4 and scFv-3 (Figure 5a) showed the presence of a single major peak with an elution time consistent with a molecular mass of ~57 kDa expected for a dimer. This peak eluted at the same time as the dimer peak in scFv-15 (V_L–V_H) preparations (Figure 5b). The minor higher molecular mass species observed in these three profiles (Figure 5a) eluted at the same elution times as the zero-linked NC10 V_L–V_H trimer and tetramer peaks (Figure 5b). These findings are consistent with those observed for the NC10 scFv-5, scFv-4 and scFv-3 (V_H–V_L) proteins which mainly formed dimer and a small amount of trimer (Atwell *et al.*, 1999). However, no tetramer species was observed for scFv-5, -4 and -3 (V_H–V_L) proteins. Interestingly, in the case of scFv-5 (V_L–V_H) protein, consistently larger yields of tetramer species were observed and this allowed for the purification and subsequent characterization of this tetramer by gel filtration on a Superdex 200 column and by electron microscopy (see below). This scFv-5 tetramer was, however, relatively unstable as it partially reverted back to dimer (40% dimer after 24 h at 4°C). Similarly, a small amount of purified scFv-5 dimer species converted to tetramer (~5–10% after

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