

Triabodies: single chain Fv fragments without a linker form trivalent trimers

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Received 25 March 1997; revised version received 11 April 1997

Abstract A single chain Fv fragment (scFv) of the murine monoclonal antibody 11-IG10 was constructed by directly joining the C-terminal residue of the V_H domain to the N-terminal residue of V_L. 11-IG10 is an anti-idiotypic and competes with the antigen, influenza virus neuraminidase (NA), for binding to the NC41 antibody. The scFv formed stable trimers with three active antigen combining sites for NC41 Fab fragments. We propose that trimeric scFvs may be the preferred conformation for directly linked V_H-V_L molecules, which contrasts the formation of scFv dimers (diabodies) when the V_H and V_L domains are joined by short flexible linkers of between 5–10 residues. BIAcore biosensor binding experiments showed that the trimeric scFv showed an expected increase in binding affinity, due to avidity, compared to the monomeric 15-residue linked scFv. The increase in avidity of scFv trimers offers advantages for imaging and immunotherapy.

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Key words: Antibody; Dimer; Trimer; Single chain Fv; scFv; Antigen complex

1. Introduction

Recombinant single chain variable fragments (scFvs), in which the two variable domains are covalently joined via a flexible peptide linker, have been shown to fold in the same conformation as the parent Fab [1,2]. ScFvs with linkers greater than 12 residues in length can form stable monomers which usually exhibit similar antigen binding affinity compared to the parent antibody [1,3]. Reduction of the linker length to shorter than 12 residues prevents the monomeric configuration and forces two scFv molecules into a dimeric conformation, termed diabodies [4–6]. The increased tumour to blood ratio reported for these bivalent scFv dimers over monomers [7], presumably from higher avidity and reduced clearance rates, offers advantages for imaging, diagnosis and therapy. Bispecific diabodies have been produced using bicistronic vectors to express two different scFv molecules *in situ*, V_HA-linker-V_LB and V_HB-linker-V_LA, which associate to

form the parent specificities of A and B [5,8,9]. The 5 residue linker sequence chosen for these bispecific diabodies, Gly₄Ser, provided a flexible and hydrophilic hinge.

ScFv molecules have been designed without a linker (scFv-0), by direct ligation of the C-terminal residue of V_H to the N-terminal residue of V_L [4,10]. Although these scFv-0 structures have been modelled as dimers with V_H domains joined back-to-back [4], the structure is severely constrained and cannot be accommodated in the crystal structure of a 5-residue linked dimeric scFv [11]. In contrast, we have recently demonstrated that an scFv-0 derived from antibody NC10, specific for influenza virus neuraminidase (NA), was a trimer with three active antigen combining sites [12]. We initially thought that this trimeric conformation was unique to NC10 scFv, perhaps due to steric clashes between V-domains which prevented the dimeric association. However, we show in this report that a second scFv, constructed from the anti-idiotypic 11-IG10 antibody, is also a trimer and also possesses trivalent specificity. The parent antibody, murine 11-IG10 competes for binding to the murine NC41 antibody with the original target antigen, influenza virus N9 neuraminidase (NA) [13]. We now propose that the propensity to trimerise might be a general property of scFv-0 molecules constructed by direct ligation of V_H-V_L domains.

2. Materials and methods

2.1. Construction of 11-IG10 scFv-0

The V_H and V_L genes were amplified by PCR from the parent 11-IG10 hybridoma [P. Iliades, in preparation], and joined into an scFv-0 gene by ligation between codons for C-terminal V_H-Ser¹¹³ and N-terminal V_L-Gln¹ by PCR overlap-extension. Residues are numbered according to the Kabat nomenclature as described [1]. The scFv-0 gene was cloned into the *SfiI*-*NotI* sites of the expression vector pGC using methods described previously [14]. The vector pGC provides an N-terminal pelB leader sequence and C-terminal FLAG octapeptide tag tail [14]. The entire DNA sequence of the cloned scFv-0 insert was determined using DNA purified by alkaline lysis and sequencing reactions performed using the PRISM Cycle Sequencing Kit (ABI).

2.2. Expression and purification of 11-IG10 scFv and NC41 Fab fragments

HB101 *E. coli* containing the scFv-0 gene in pGC were grown in 2×YT supplemented with 100 µg/ml ampicillin and 1% glucose at 37°C overnight and then subcultured in the absence of glucose at an A₆₀₀ of 0.1, and grown at 21°C until A₆₀₀ was 1.0. Expression was induced by addition of IPTG to 1 mM and cells cultured for 16 h at 21°C. Cells and culture supernatant were separated by centrifugation and samples of cell pellet and supernatant were analysed on a 15% SDS-PAGE gel followed by Western blot analysis using M2 anti-FLAG antibody [15] and goat anti mouse IgG (H+L)^{HRP} (Bio-Rad) as the second antibody to visualise the expressed product.

The expressed scFv-0 was purified from supernatant by precipitation with ammonium sulphate to 70% saturation at 21°C followed by

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Abbreviations: BIAcore, Pharmacia BIAcore 1000 biosensor apparatus; FPLC, fast protein liquid chromatography; IPTG, isopropyl-β-thiogalactoside; NA, influenza neuraminidase; *M_r*, molecular mass; PCR, polymerase chain reaction; scFv, single chain Fv molecule; SDS-PAGE, electrophoresis in a 15% polyacrylamide gel comprising 1% SDS; RU, resonance units; V_H, variable region from antibody heavy chain; V_L, variable region from antibody light chain; YT, yeast tryptone medium

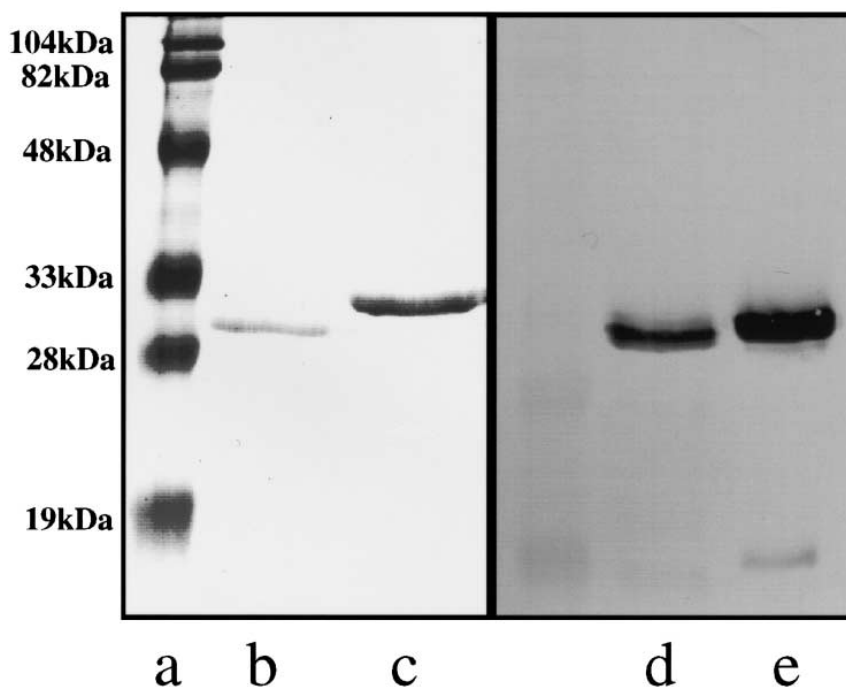


Fig. 1. SDS-PAGE analysis of scFv-15 and scFv-0 and Western Transfer detection using anti-FLAG M2 antibody; lanes on Coomassie stained gel (a) Biorad Low M_r standards, (b) scFv-0, (c) scFv-15 and corresponding Western blot of (d) scFv-0 and (e) scFv-15. The theoretical M_r of scFv-15 is 28427 Da and scFv-0 is 26466 Da.

centrifugation at $10\,000\times g$ for 15 min. The aqueous phase was discarded, and the pellet resuspended and dialysed in PBS (phosphate-buffered saline, pH 7.4) at 4°C overnight. Insoluble material was removed by centrifugation at $70\,000\times g$ and the supernatant was filtered through a 0.22 μm membrane and affinity purified on either an M2 anti-FLAG antibody affinity column [15] or an NC41 Fab Sepharose 4B affinity column. The affinity resin was equilibrated in TBS (0.025 M Tris-buffered saline, pH 7.4) and bound protein was eluted with gentle elution buffer (Pierce). The scFv-0 was concentrated to ~ 1 mg/ml, dialysed against TBS and stored at 4°C. SDS-PAGE, Western Blot analysis and N-terminal sequence analysis of the affinity purified material were performed as described previously [1].

For the other proteins described in this study, 11-1G10 scFv-15 (comprising a 15 residue linker in the orientation V_H -(Gly₄Ser)₃- V_L) was synthesised under similar conditions to the scFv-0 described above [P. Iliades, in preparation]. The 11-1G10 scFv-15 was isolated by gel filtration as a 27-kDa monomer and shown to be stable at 4°C for several weeks, similar to previous studies with different scFv-15 fragments [1–6,12]. NC41 and 11-1G10 Fab fragments were prepared by proteolysis from the parent hybridoma IgG as described previously [12,16].

2.3. Size exclusion FPLC chromatography, molecular mass determination and binding analysis

The affinity purified scFv-0 and scFv-15 was fractionated by size exclusion FPLC on either a Superdex 75 HR10/30 column or a Superose 12 HR10/30 column (Pharmacia) in PBS to determine the molecular size and aggregation state. The complexes formed between 11-1G10 scFv and NC41 Fab were analysed and isolated by size exclusion FPLC on a Superose 12 column in PBS (flow rate 0.5 ml/min). The FPLC columns were calibrated with standard proteins as described [1]. The molecular mass of each isolated complex was determined by sedimentation equilibrium on a Beckman model XLA centrifuge as described previously [1,12] using partial specific volumes calculated from amino acid compositions. An upgraded Pharmacia BIAcore 1000 was used for analysis of the binding of monomeric 11-1G10 scFv-15 and trimeric 11-1G10 scFv-0 to immobilised NC41 Fab as described [12,17]. The resulting binding curves were analysed with BIAevaluation 2.1 software (Pharmacia Biosensor), to obtain values for the apparent dissociation rate constants [17].

3. Results

DNA sequencing confirmed that the 11-1G10 scFv-0 gene comprised a direct ligation between codons for the C-terminal V_H -Ser¹¹³ and N-terminal V_L -Gln¹. Expression of the scFv-0 gene was performed in *E. coli* strain HB101 for 16 h at 21°C under conditions which release the contents of the periplasmic space into the culture supernatant, presumably by cell lysis, to yield soluble and biologically active scFv [14].

3.1. ScFv purification and solution properties

Fully active scFv-0 was purified at 0.5 mg/L from the culture supernatant by affinity chromatography on an NC41 Fab-Sepharose affinity column. SDS-PAGE analysis of the affinity purified scFv-0 revealed a single protein band of 27 kDa which on Western analysis reacted with the anti-FLAG M2 antibody (Fig. 1). N-terminal sequence analysis of the 27-

Table 1
Sedimentation equilibrium data for complexes of 11-1G10 scFv-15 monomer and scFv-0 trimer with NC41 Fab

Sample	Calculated	Experimental
Monomer+NC41 Fab 28427+47273	75700	78600
Trimer	79398	85000
Trimer+NC41 Fab 79398+141819	221217	262000

The complexes of NC41 Fab with either scFv-15 monomer or scFv-0 trimer were isolated by size exclusion FPLC chromatography and analysed by sedimentation equilibrium in a Beckman Model XLA ultracentrifuge. The molecular mass was determined experimentally by the method described in [12] at 20°C. The calculated M_r of NC41 Fab is 47273 Da, scFv-15 is 28427 Da, and scFv-0 is 26466 Da.

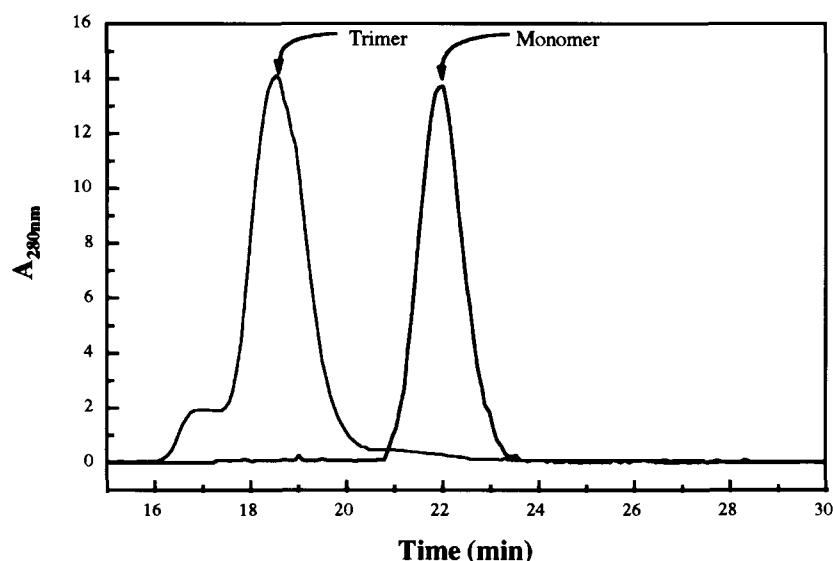


Fig. 2. Size exclusion FPLC on a calibrated Superdex 75 HR10/30 column (Pharmacia) showing overlaid profiles of scFv-15 monomer and scFv-0 trimer with peaks eluting at times corresponding to $M_r \sim 27$ kDa and ~ 85 kDa, respectively. The column was equilibrated with PBS (pH 7.4) and run at a flow rate of 0.5 ml/min.

kDa protein gave the expected sequence for the N terminus of the 11-1G10 V_H domain and confirmed that the pelB leader sequence had been correctly cleaved (data not shown).

Gel filtration of affinity purified scFv by FPLC on either a Superdex 75 column (Fig. 2) or a Superose 12 column (Fig. 3) revealed a single peak of $M_r \sim 85$ kDa consistent with the calculated molecular mass of a trimer (calculated M_r 79.2 kDa). Gel filtration of the scFv-0 preparation showed no evidence of monomers and dimers and no evidence of proteolytic degradation to single V-domains. Sedimentation equilibrium analysis indicated that the scFv-0 migrated as a distinct species with $M_r \sim 85$ kDa (Table 1) consistent with a trimeric conformation and there was no evidence for a dimeric spe-

cies which might exist in rapid equilibrium with the trimer species.

In comparison, the scFv-15 fragment of 11-1G10 (comprising a 15 residue linker in the orientation V_H -(Gly₄Ser)₃- V_L) was also synthesised using the pGC vector in HB2151 *E. coli* cells and then purified as a stable monomer with a $M_r \sim 27$ kDa determined by gel filtration and sedimentation equilibrium (Figs. 1 and 2). Previous gel filtration and sedimentation equilibrium studies of NC10 scFv fragments [12] had revealed that scFv-15 monomers possessed an $M_r \sim 27$ kDa, scFv-5 dimers $M_r \sim 54$ kDa and scFv-0 trimers $M_r \sim 70$ kDa [12]. Thus, the calculated and experimental M_r of ~ 27 kDa for monomeric scFv-15 derived from both 11-1G10

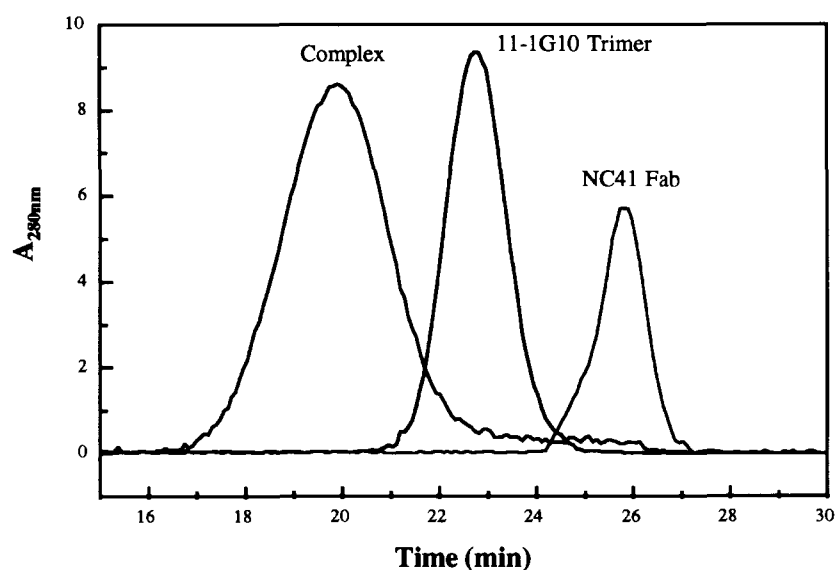


Fig. 3. Size exclusion FPLC on a calibrated Superose 12 HR10/30 column (Pharmacia) showing overlaid profiles of the isolated 11-1G10 scFv-0 trimer, NC41 Fab and scFv/Fab complex formed on the interaction of scFv-0 and NC41 Fab premixed in 1:3 molar ratio. The column was equilibrated with PBS (pH 7.4) and run at a flow rate of 0.5 ml/min.

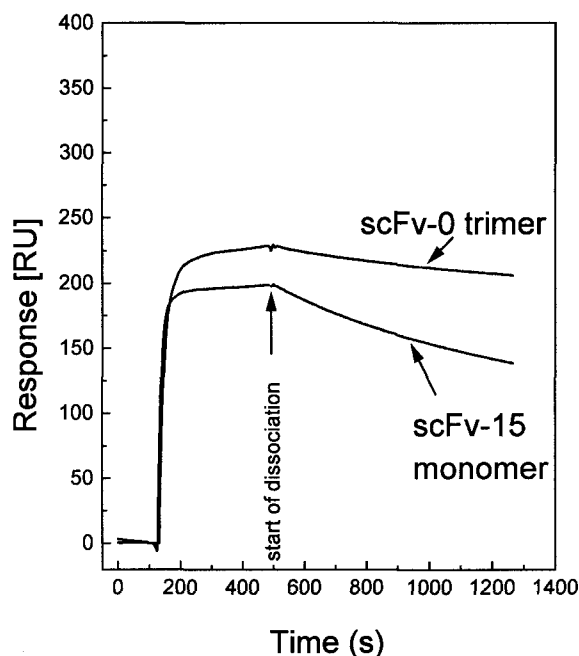


Fig. 4. BIAcore sensorgrams showing the association and dissociation of scFv-15 monomer and scFv-0 trimer each at a concentration of 222 nM, to immobilised NC41 Fab. An injection volume of 30 μ l and a flow rate of 5 μ l/min were used. The surface was regenerated with 10 μ l of 10 mM sodium acetate, pH 3.0 after each binding experiment.

and NC10 antibodies were almost identical, whereas scFv-0 from 11-1G10 exhibited a $M_r \sim 85$ kDa slightly larger than that predicted for a trimer (79 kDa) and scFv-0 from NC10 a $M_r \sim 70$ kDa slightly smaller than a trimer.

3.2. Interaction between 11-1-G10 scFv and NC41 Fab fragments

Gel filtration analysis by FPLC on a Superose 12 column showed that all the scFv-0 interacted with NC41 Fab to form a stable complex of $M_r \sim 245$ kDa (Fig. 3) whilst scFv-15 monomer interacted with NC41 Fab to form a stable complex of $M_r \sim 79$ kDa (not shown). The molecular mass of these complexes were determined by sedimentation equilibrium analysis to be 262 kDa and 78.6 kDa, respectively (Table 1). The linearity of the $\ln c$ versus r^2 plots [12] of the sedimentation data showed that both complexes were homogeneous with respect to molecular mass and indicated that discrete and stoichiometric complexes were formed. Furthermore, both isolated complexes were stable to dilution, and freezing (data not shown). These data are consistent with the trimeric scFv-0 binding three Fab molecules whilst the monomeric scFv-15 formed a 1:1 complex with Fab. Comparison of the binding of scFv-15 monomer and scFv-0 trimer to immobilised NC41 Fab by BIAcore (Fig. 4) showed that the apparent dissociation rate of the scFv-0 trimer/ NC41 Fab complex ($k_d \sim 8.2 \times 10^{-5} \text{ s}^{-1}$) was approximately 4-fold slower than that for the scFv-15 monomer/NC41 Fab complex ($k_d \sim 3.2 \times 10^{-4} \text{ s}^{-1}$). These dissociation rates are only approximate values since the sensorgram analysis required a 1:1 interaction model [17]. The 4-fold reduced apparent dissociation rate for the 11-1G10 scFv-0 trimer is similar to our previous analysis on the NC10 scFv-0 trimer [12] and can be

attributed to multivalent binding which results in the increased avidity for both scFv-0 trimers.

4. Discussion

Linkers less than 12 residues are too short to permit pairing between V_H and V_L domains on the same chain and have been used to force an intermolecular pairing of domains into dimers, termed diabodies [4,6,11,12]. However, in only a few studies [11,12] have accurate molecular mass analyses for scFvs with shorter linkers been reported. Holliger et al. [4,5] described a model of scFv diabodies with V_H domains joined back-to-back and suggested these structures required a linker of at least one or two residues. This model was confirmed in a crystal structure of a 5-residue diabody [11], but it was noted that scFv-0 could not be fitted to this conformation, even with severe rotations of the V_H domains. Desplancq et al. [18] described a series of scFvs with linkers of 10, 5 and zero residues and showed by FPLC analysis that these scFvs were predominantly dimers with minor amounts of monomer. Alftan et al. [19] also showed that scFvs with a small linkers down to 2 residues in length formed dimers. McGuinness et al. [10] claimed that bispecific scFv-0 molecules were diabodies and could be displayed and selected from bacteriophage libraries, but precise molecular size determination was not performed on the expressed soluble products.

We have recently reported that scFv-5 and scFv-10 molecules derived from an NC10 antibody (with 5 and 10 residues linking the V_H and V_L domains, respectively) formed stable dimers, but that the NC10 scFv-0 formed a stable trimer [12]. In the design of the trimeric NC10 scFv-0 residues Ser¹¹² and Asp¹ were ligated as a direct fusion of V_H - V_L domains and, presumably, the absence of a flexible linker prevents the dimeric configuration [12]. The C-terminal residue Ser¹¹² was chosen from precise structural data, obtained by crystallographic analysis [20], as the last constrained residue in the V_H domain framework before the start of the flexible hinge region. Similarly, Asp¹ of V_L was known to be hydrogen bonded to the V-domain framework and was close, but not involved, in antigen binding. The NC10 scFv molecules were fully active since the scFv-5 and scFv-10 were bivalent and scFv-0 was trivalent. In contrast, Holliger et al. [4] provided an additional linker residue (V_H -Ser¹¹³) which might have provided the additional flexibility required for their diabody conformation.

For the design of 11-1G10 scFv-0 we included, like Holliger et al. [4], an additional residue V_H -Ser¹¹³ for the direct ligation to V_L -Gln¹. The 11-1G10 scFv-0 exclusively formed trimers (Fig. 2), which were shown to be fully active and trivalent for Fab binding by complex formation in solution (Fig. 3). In contrast, the 11-1G10 scFv-15 preferentially formed monomers with a small percentage of dimers, consistent with most previous observations of scFv-15 structures [1–6]. The slight difference between calculated and experimental molecular masses determined by gel filtration and sedimentation equilibrium is within the usual error range for these analytical methods (Table 1). As expected, binding experiments with the immobilised NC41 Fab on the BIAcore biosensor showed that the trimer had a slower dissociation rate compared to the monomer and can be attributed to the increased avidity of multivalent binding (Fig. 4).

ScFv-0 molecules can be easily modelled into a symmetric

trimeric conformation without interdomain steric constraints [12]. In this model of NC10 scFv-0, and in preliminary electron micrograph images (P. Tulloch, unpublished), the Fab arms of the trimer/Fab complex are not extended in planar configuration, but are angled together in one direction and appear as the legs of a tripod. Obviously, alternative configurations can be modelled, guided by steric constraints which limit both the flexibility of Fv modules and the proximity of three binding antigens. Unfortunately, protein chemical data alone cannot differentiate between symmetric or non-symmetric trimer configurations.

It is tempting to speculate that the trimeric conformation (triabodies) will be preferred over dimers (diabodies) for other scFv-0 molecules, although this property may be dependent on the particular antibody chosen and the precise choice of residues for the V_H - V_L linkage. The gain in affinity through avidity makes trimeric scFvs attractive for in vivo imaging as an alternative reagent to diabodies [7] and multivalent chemical conjugates [21–24]. It is also tempting to speculate that the increased avidity and tumour penetration of these small multivalent scFv molecules will offer advantages for immunotherapy [21–25]. Furthermore, the construction of trispecific scFv-0 reagents with applications in cell recruitment and activation. The flexibility of trimers is unknown and may limit their ability to cross-link two cells via their surface receptors.

Acknowledgements: The authors thank Profs. D. Metzger and R. Webster for providing the 11-1G10 and NC41 antibodies, Dr. D. Hewish for preparing the antibodies, John Burns and Geoff Oddie for assistance with protein purification and BIAcore analysis, Dr. Geoff Howlett for analytical ultracentrifugation, and Drs. Greg Coia and John Atwell for their critical review of the manuscript. P. Iliades was supported by a CSIRO scholarship.

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