Construction of di-scFv through a trivalent alkyne–azide 1,3-dipolar cycloaddition†

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Heterofunctional azide and alkyne PEG-linkers have been synthesized and site specifically conjugated to scFv *via* a reactive thiol functionality; two scFv were coupled by copper catalyzed 1,3-dipolar cycloaddition to make divalent scFv (di-scFv) with an inter-scFv distance defined to provide divalent binding; antigen binding was maintained for the di-scFv construct and increased several times compared to that of the parent scFv; the cycloaddition reaction reported herein represents an important ligation strategy to covalently link macromolecular proteins and retain sensitive structural conformations.

Intact monoclonal antibodies (MAb) that provide a high binding affinity and specificity towards tumor-associated antigens have been successfully applied in cancer diagnosis and treatment.[1] However, several factors have limited clinical applications of MAb therapy, including poor bio-distribution and long persistence in the blood pool.[2] Single chain fragments (scFv) derived from the binding domain of the parent MAb offer advantageous pharmacokinetics[3] but they typically exhibit decreased affinity and specificity compared to their parent MAb.[3c,4] Multivalent scFv constructs have been prepared that have 2–3 orders of magnitude lower off-rates and increased k_a relative to monovalent scFv. These conjugates show improved pharmacokinetics and target cancer cells more efficiently.[5] However, producing multivalent scFv has proved to be challenging and methods are limited. In this paper, we describe a novel chemical cross-linking method to construct divalent scFv (di-scFV) through small molecule linkers using azide–alkyne 1,3-dipolar cycloaddition chemistry.

Typically scFv are genetically engineered through bacterial or phage display libraries.[5a–d,5f,6] In this approach, a heavy chain (V_H) and a light chain (V_L) of scFv are covalently linked through a polypeptide linker, which can dictate the valency. For example, when the peptide length is less than five amino acids, the short linker precludes V_H–V_L association but drives dimerization to produce non-covalent di-scFv (Fig. 1). Shorter peptide lengths, *e.g.* three amino acids, can result in tri-scFv.[5a,5c,6,7] Although folding is distorted and specificity can be compromised, this method is the one most commonly used to generate multivalent scFv.[8]

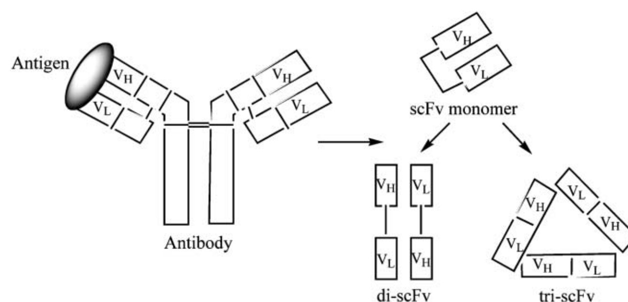

Fig. 1 scFv are derived from the V_H and V_L regions of the antibody. The length of the linker determines multiplicity.

Chemical cross-linking methods using bi-functional linkers have also been reported.[9] For example, DeNardo and co-workers identified a scFv, **1**, against tumor-associated MUC-1 antigen expressed on the surface of breast cancer cells,[10] and constructed a covalent di-scFv by site-specific PEGylation using a maleimide–PEG–maleimide linker (Fig. 2).[9c] Low divalent ligation yields (10–30%) have limited the application of this technology in clinical settings, prompting us to explore alternative chemical coupling methods.

Azide–alkyne 1,3-dipolar cycloaddition chemistry is an attractive alternative approach because it involves reactive functional groups that are orthogonal to biomolecules.[11] Initially, we hypothesized that the PEG technology could be improved by simply modifying the linker with azide and alkyne end groups (Scheme 1). Linkers **2** and **3** were first synthesized and conjugated with scFv to produce **4** and **5**. The subsequent azide–alkyne 1,3-dipolar cycloaddition was catalyzed by Cu(I) in the presence of ligand **6**,[12] but unfortunately the yields were too low to be useful.

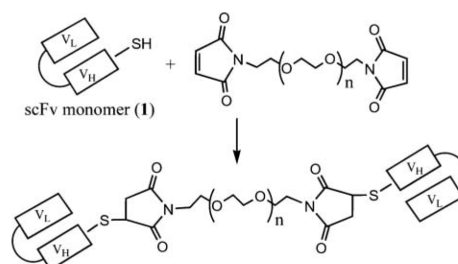

Fig. 2 Chemical ligation of scFv, using an engineered cysteine residue to attack a bis-maleimide PEG.

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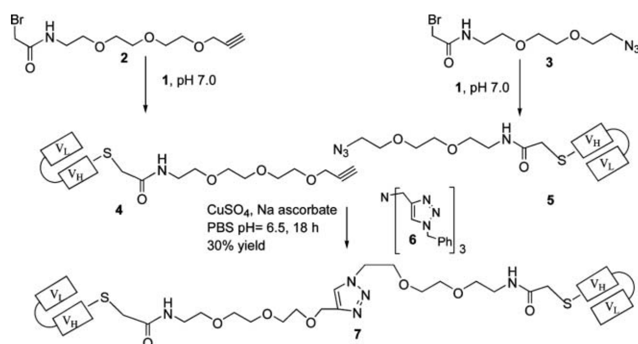
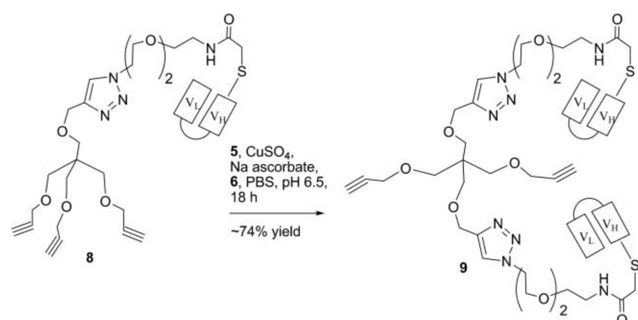


Scheme 1 1,3-Dipolar cycloaddition of functionalized scFv.

The best yields (30%) were achieved using a 1 : 5 molar ratio of **4** : **5**. We were assured that the preparation of **4** and **5** was efficient, as we were able to react rhodamine-azide with the protein conjugates, and the amount of protein-containing fluorophore could be quantified.† This reaction showed that, in principal, the cycloaddition was efficient, suggesting that issues specific to **4** and **5** were the problem. Reasoning that steric hindrance may be the cause, we attempted variations of this protocol, including several longer linker lengths, but yields were never better than 33%.

At this point, we began to consider other possible factors that could impede the process, and it occurred to us that the reaction suffered intrinsically. scFv is a 28 kDa protein with only one unpaired cysteine per macromolecule, to which is linked only one reactive group. Its macromolecular size reduces the probability of productive encounters between the azide and alkyne, relative to small molecules, due to differences in diffusion and rotation rates. We hypothesized that increasing the number of reactive groups on one protein would increase the probability of the two reaction centers meeting. To test this hypothesis, we synthesized a scFv containing tri-alkyne linker **8** (Scheme 2) and subjected it to a Cu(I)-catalyzed cycloaddition with **5** in the presence of **6**. To our satisfaction, di-scFv **9** readily formed, and the extent of reaction was not significantly affected by the ratio of the reactants; reaction of **8** and **5** in a 1 : 1 ratio gave a 58% yield, 1 : 2 gave 74% and 1 : 3 gave 66% (Fig. 3A, lanes 1–3 and the ESI†). We attribute the increased reactivity to statistics, however we cannot rule out the possibility that the trialkyne is intrinsically more reactive than the monoalkyne at this time.

The band at 56 kDa in lane 0 of Fig. 3A is attributed to non-covalent di-scFv formation and not disulfide formation. The presence of sodium ascorbate as a reducing agent limits disulfide formation, as evidenced by the gel in Fig. 3B, in which the reaction mixture resulting from the cycloaddition was treated with 5 equiv. of tri(carboxyethyl)phosphine (TCEP), a reducing agent commonly used to break disulfide bonds. Under these conditions, the 56 kDa band in lane 0 is still present. The faint bands at approximately 84 kDa in lanes 1–3 may result from either covalent trimerization through cycloaddition or non-covalent aggregation. From the outset, we were not overly concerned about forming significant amounts of trimer or higher order constructs since, according to our hypothesis, di-scFv with two reactive alkynes would be far less reactive than scFv with three reactive alkynes. Indeed, less than 5% of higher molecular weight bands corresponding to trimer (84 kDa) were observed.



Scheme 2 Trivalent alkyne–azide dipolar cycloaddition.

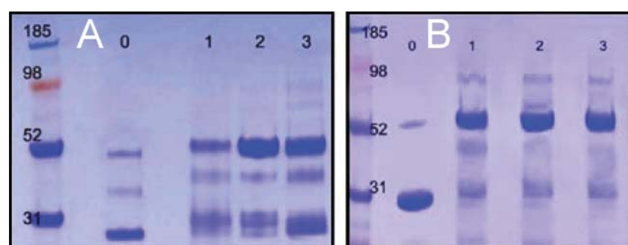


Fig. 3 A: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of azide–alkyne 1,3-dipolar cycloaddition; lane 0: scFv only, lanes 1–3: **8** + **5** (in 1 : 1, 1 : 2 and 1 : 3 molar ratios). B: Treatment of the cycloaddition reaction mixture with 5 × TCEP indicates that the 56 kDa band in lanes 1–3 is due to cycloaddition and not disulfide bond formation.

Having solved the problems associated with efficient ligation, we next conducted preliminary biological studies on di-scFv **9** using enzyme linked immunosorbant assay (ELISA) and immunohistochemistry (IHC). Since the triazole ligation product consists of two identical scFv against MUC-1 peptide found on cancer cells, IHC and ELISA could be used to compare the binding of single scFv *vs.* di-scFv constructs. As shown in Fig. 4, di-scFv maintained a high affinity with the antigen after ligation, and also demonstrated increased binding of MUC-1 peptide and cancer cells relative to scFv. The IHC of purified **9** on human prostate cancer cells (DU145) and human breast cancer cells (MCF7),

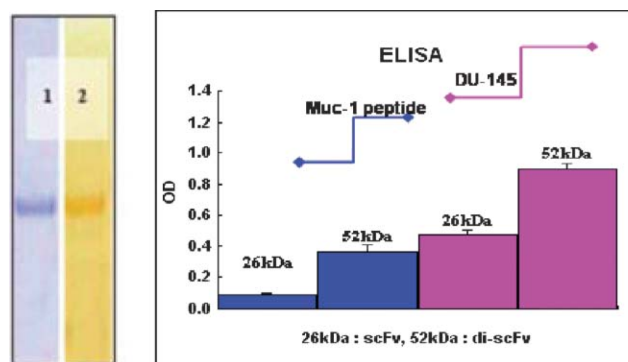


Fig. 4 Left: PAGE of purified di-scFv **9** stained for protein (lane 1) and PEG (lane 2). Right: ELISA of scFv (26 kDa, 1 mg 100 mL^{-1} $well^{-1}$) and di-scFv (52 kDa, 1 mg 100 mL^{-1} $well^{-1}$) tested against synthetic MUC-1 peptide (1 mg 100 mL^{-1} $well^{-1}$) and DU145 cells (1×10^6 cells 100 mL^{-1} $well^{-1}$). Error bars represent the standard deviations from triplicate experiments.

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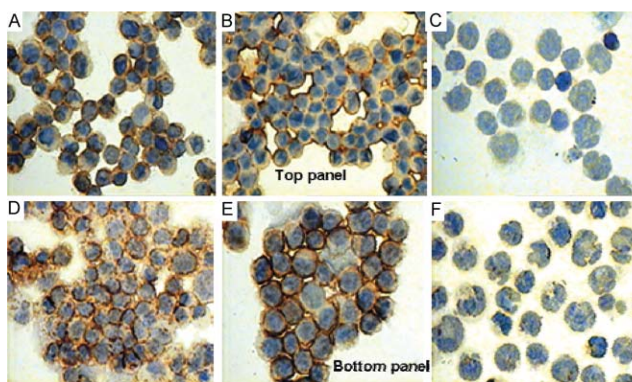

Fig. 5 Di-scFv (bottom panels) was tested on sections of human breast (MCF7) and prostate tissue (DU145) by IHC. The brown membrane staining can be easily detected on the cells with scFv and di-scFv proteins, and increased binding was observed for di-scFv (bottom panels) relative to scFv (top panels). Panel A: scFv bound to MCF7. Panel D: Di-scFv bound to MCF7. Panel B: scFv bound to DU145. Panel E: Di-scFv bound to DU145. Panel C: scFv does not bind Jurkat cells. Panel F: Di-scFv does not bind Jurkat cells.

demonstrated that the di-scFv had 2–4 times increased binding to cancer cells compared to scFv, whilst the negative control (Jurkat lymphoma cells) did not stain (Fig. 5).

The multivalent 1,3-dipolar cycloaddition strategy described herein provides a general approach for modular design applications. Azide–alkyne 1,3-dipolar cycloaddition has been established as an effective chemistry for the covalent modification of biomolecules such as proteins,[13,14] DNA,[15] carbohydrates,[16] virus particles[11,17] and bacterial surfaces.[18] In virtually all of these cases, a large macromolecule and a small molecule were "clicked" together. Since small molecules can freely diffuse, a critical reactant concentration can be achieved, and the reaction proceeds efficiently. As far as we are aware, azide–alkyne 1,3-dipolar cycloaddition has not been successfully applied to the ligation of two large macromolecules, each possessing a single reaction site.¶ Here we show that the efficiency of such a reaction is limited by macromolecular reaction dynamics, which can be overcome by the site-specific introduction of a polyfunctional small molecule.

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Notes and references

¶ Finn and co-workers have ligated large proteins onto viral particles, but in this case, the virus has several reactive sites and approximately 80% efficiency was observed. See ref. 17.

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