View Online / Journal Homepage / Table of Contents for this issue

Organic & Biomolecular Chemistry

www.rsc.org/obc

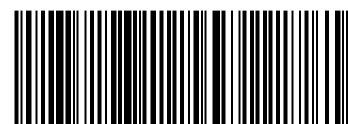
Volume 10 | Number 8 | 28 February 2012 | Pages 1469–1696

Downloaded on 09 February 2012
Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A

ISSN 1477-0520

RSC Publishing

FULL PAPER
Jacquelyn Gervay-Hague *et al.*
A general chemical synthesis platform for crosslinking multivalent single chain variable fragments



Organic & Biomolecular Chemistry

Dynamic Article Links
View Online

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 1521

www.rsc.org/obc

PAPER

A general chemical synthesis platform for crosslinking multivalent single chain variable fragments

Joan G. Schellinger,[a] Avinash Kudupudi,[b] Arutselvan Natarajan,[b] Wenjun Du,[c] Sally J. DeNardo[b] and Jacquelyn Gervay-Hague[a]

Received 28th December 2010, Accepted 20th October 2011
DOI: 10.1039/c0ob01259a

Downloaded on 09 February 2012
Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A

Multivalent single chain variable fragments (scFv) show increased affinity to tumor-associated antigens compared to monovalent scFv and intact monoclonal antibodies (mAb). Multivalent constructs can be derived from self-associating or covalent scFv with covalent constructs offering improved *in vivo* and *in vitro* stability. Covalent attachment of scFv can be achieved using genetically engineered expression vectors that afford scFv with site specific cysteine functionality. Expression vectors for di-scFv-C wherein the cysteine is located in the center of two scFv have also been developed for attaching chemically reactive linkers. In the example illustrated here, the di-scFv-C is derived from a mAb directed against the MUC1 epitope, which is presented on cancer cells. To achieve multivalency, a chemical crosslinking strategy utilizing various azide and multi-alkyne functionalized polyethylene glycol (PEG) linkers was implemented. Conjugation was achieved by attachment of these linkers to the scFv thiol functionality. Chemoselective ligation was employed to covalently link different protein conjugates *via* copper(I) catalyzed azide alkyne 1,3-dipolar cycloaddition reaction (CuAAC) chemistry. Ligations were achieved in >70% yield using a specific set of linkers as determined by SDS-PAGE and densitometry. ELISA showed increased tumor binding of a tetravalent scFv providing a versatile chemical crosslinking strategy for construction of multivalent and bi-specific immunoconjugates that retain biological activity and have potential application in pre-targeted radioimmunotherapy and imaging.

Introduction

Multivalency in biological recognition processes typically involves presentation of multiple ligands to surface receptors and often results in increased avidity.[1] In immunochemistry, early evidence of multivalent interactions was reported by Karush and co-workers who showed that the multivalent form of an antibody binds to antigenic surfaces more tightly and can provide immunity at lower concentrations compared to the monovalent form.[2] To overcome problems with poor pharmacokinetics (pk) and biodistribution brought by high molecular weight monoclonal antibodies (mAbs), single chain variable fragments (scFv) have been engineered.[3,4] ScFv are expressed as a single protein derived from the binding domain of the parent mAb in which the variable domains of the light (V_L) and heavy (V_H) chains are linked by a small peptide (Fig. 1). Although the smaller size of scFv offers advantages in terms of selectivity and rapid tumor penetration, decreased binding affinity and rapid clearance compared with intact mAbs is also observed.[4] To address this deficiency, multivalent antibody fragments have been developed with the ultimate goal of using these constructs in direct or pre-targeting radioimmunotherapy and radioimmunodetection for cancer.[5]

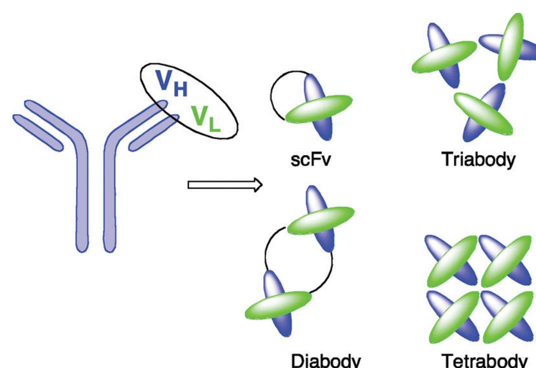


Fig. 1 ScFv and non-covalent multivalent forms.

A successful strategy to generate multivalent scFv is to manipulate the genetically encoded scFv linker length to enable direct self-assembly into diabodies (~55 kDa), triabodies (~80 kDa)

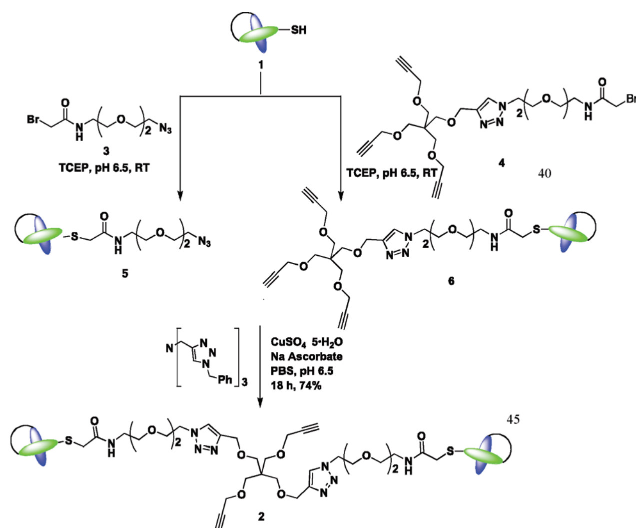
[a]*Chemistry Department, University of California Davis, One Shields Avenue, Davis, CA, 95616, USA. E-mail: Gervay@chem.ucdavis.edu; Fax: +1 530-754-6915; Tel: +1 530-754-9577*
[b]*Radiodiagnosis and Therapy Section, Internal Medicine, University of California Medical Center, Sacramento, CA, 95816, USA*
[c]*Department of Chemistry, Central Michigan University, Mount, Pleasant, MI, 48858, USA*

Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A
Downloaded on 09 February 2012

and tetrabodies (~110 kDa) (Fig. 1). In most cases, these non-covalent multimers show improved tumor penetration and faster blood clearance compared to the intact mAbs (~ 150 kDa).[6] For example, Scott and co-workers reported *in vitro* and *in vivo* biodistribution and avidity studies of multimeric scFv derived from anti-Lewis Y mAb hu3S193 directed against Lewis Y expressing cancer. Non-covalent multimers showed increased immunoreactivity and affinity compared to the parent mAb, F(ab')$_2$ and diabody.[7,8] Enhanced recognition was attributed to increased valency, although limited *in vitro* stability and higher renal localization was also noted. In an effort to maintain multivalency *in vivo*, chemical synthesis methodologies that covalently link scFv have been explored.[9]

We previously communicated a chemoselective ligation approach, based upon a strategy first reported by Tiefenbrunn and Dawson[10] that covalently links scFv to form divalent scFv (di-scFv).[11] DeNardo and co-workers engineered an expression vector for the addition of a free cysteine near the carboxy terminus of scFv that provided a specific site for thiol conjugation.[12] The cysteine-modified scFv (**1**, ~26 kDa), was targeted against tumor-associated MUC-1 antigen expressed on the surface of breast cancer cells, and proved to be accessible to covalent modification using different PEGylated small molecules. And importantly, biological activity was retained. PEGylation, which is the process of covalent attachment of a polyethylene glycol (PEG) molecule to a biomolecule, is a well established technique that renders favorable properties such as increased thermal and mechanical stability, enhanced water solubility, non-immunogenecity, reduced toxicity and decreased proteolysis.[13]

We designed and constructed di-scFv (**2**) by synthesizing PEG linkers containing a terminal α-bromoacetamide group for site selective thioether bond formation between the protein and the linker. The other end of the linker was functionalized with either an alkyne or an azide for chemoselective ligation of two proteins. Heterofunctionalized azide and trialkyne PEG bromoacetamide linkers, **3** and **4**, were then synthesized and conjugated to scFv (**1**).[11] Protein conjugates **5** and **6** were subsequently subjected to CuAAC for ligation to form di-scFv (**2**) in a satisfactory 74% yield (Scheme 1). Remarkably, the reaction was only successful when the alkyne was presented in multimeric form. In this instance, the concept of multivalency was being played out on the synthetic stage. With multiple copies of the alkyne, the probability of azide/alkyne cycloaddition increased due to an increase in effective concentration of the alkyne. It is noteworthy that the molar ratio of the protein conjugates did not significantly affect the extent of ligation. And no appreciable amounts of higher order constructs such as trimers or tetramers were observed. Moreover, the dimeric constructs showed enhanced immunoreactivity in ELISA and immunoblotting studies.



Scheme 1 Formation of di-scFv **1** *via* 1,3-dipolar cycloaddition.

Success in preparing di-scFv construct **2** has served as a basis for the studies reported herein, which are aimed at developing bispecific multivalent immunoconjugates for pre-targeted radioimmunotherapy and imaging.

Results and discussion

An extensive review of pre-targeting methods for antibody-based cancer therapy and its application in imaging and efficient delivery of radionuclides has been published by Sharkey *et al.*[14] In the pre-targeting approach, Ab fragment localization and radionuclide delivery are administered separately. The scFv conjugate is first introduced to cancer cells expressing tumor-associated antigens, then at the time of maximum tumor-scFv concentration, a radioactive compound that is specific to the scFv conjugate is brought in.[15] Wishing to maintain strong interactions between the antigen and scFv, we conceived of preparing a genetically engineered dimer of scFv (di-scFv-C) where two scFv are joined by a peptide unit that contains a cysteine residue for linker attachment (Fig. 2). Successful implementation of the CuACC ligation would result in a tetrameric molecule (**8**). With the capacity of di-scFv construct **8** to bind to both human prostate and breast cancer cells and to a smaller MUC1 antigenic peptide conjugated to a radionuclide carrier, this platform provides a viable approach to pre-targeted cancer therapy.

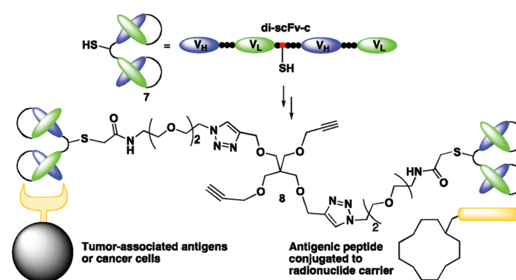


Fig. 2 Recombinant di-scFv-C and subsequent ligation for pre-targeting chemotherapeutic approaches.

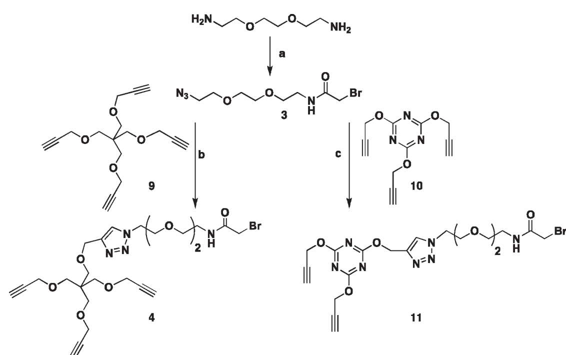
Studies in the DeNardo lab indicated that this novel anti-MUC1 di-scFv-C biomolecule with internal free cysteine could be efficiently produced, purified and conjugated by site-specific PEGylation without loss of immunoreactivity. The ability of the unmodified protein and the PEGylated di-scFv-C to bind MUC1 peptide, human prostate cancer cells (DU145) and human breast cancer cells (MCF-7) was also evaluated. Results showed that both engineered parent protein and PEGylated di-scFv-C bind cells that express MUC1 with comparable affinity.[16]

Copper-catalyzed azide–alkyne 1,3 dipolar cycloaddition (CuAAC) capitalizes on the chemoselectivity and highly reactive

 This journal is © The Royal Society of Chemistry 2012

nature of azide and alkyne functionalities.[17] Moreover, the triazole cycloaddition product of this bioorthogonal reaction has remarkable stability towards proteases providing an attractive chemoselective ligation approach.[18] Click chemistry has shown extensive utility in bioconjugation with peptides and small molecules[19] however, there are limited synthetic strategies that allow for the use of this strategy in chemical crosslinking of large macromolecules.[20]

We were concerned that the proposed conjugation with an internal thiol would present ligation problems due to steric hindrance, so we prepared alternative alkyne functionalized core structures. We were interested in *s*-triazine cores as versatile chemical handles,[21] and also for their potential to serve as copper chelating agents to accelerate click reactions.[22] At the same time, we wanted to further evaluate our hypothesis regarding the relationship between the number of reactive alkynes present and the probability for a facile reaction to occur. To meet this demand, we synthesized two linkers, trialkyne **4**[11] and dialkyne **11**, according to Scheme 2.

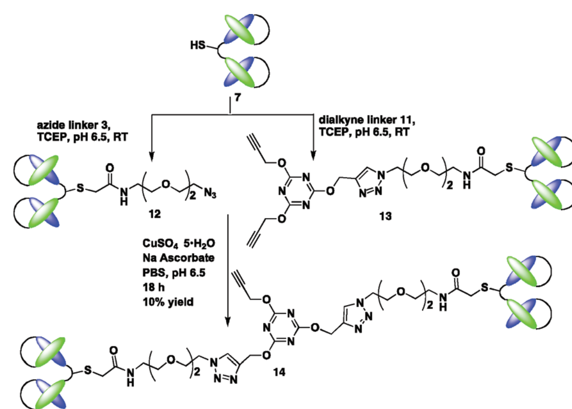


Scheme 2 *Reagents and conditions*: a) i. TfN_3, K_2CO_3, $CuSO_4$ $5{\cdot}H_2O$ (cat.), DCM/MeOH/H_2O, ii. bromoacetyl bromide, DCM/sat. $NaHCO_3$, 35% over two steps; b) tetra-alkyne core **9**,[11] sodium ascorbate, $CuSO_4$ $5{\cdot}H_2O$, DMF/H_2O, rt, overnight, 65%; c) tri-alkyne core **10**, sodium ascorbate, $CuSO_4$ $5{\cdot}H_2O$, DMF/H_2O, rt, overnight, 62%.

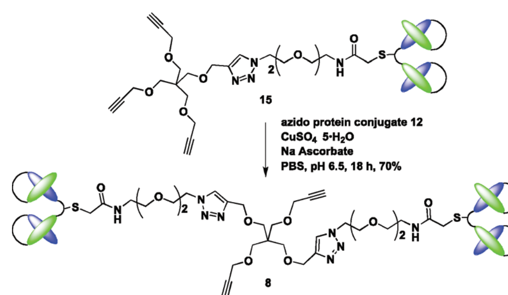
The synthesis of azide linker **3**[11] using a two-step one pot method, converting the two amino groups into an azide and α-bromoacetamide group (overall yield of 35%), allows for subsequent preparation of the different heterofunctionalized multi-alkyne PEG bromoacetamide linkers by CuAAC reaction (Scheme 2). The desired cycloaddition products, tri-alkyne linker **4**[11] and di-alkyne linker **11**, can be prepared in modest yields between 60–65%. Although formation of di- or tri-"clicked" side-products cannot be avoided, the strategy nevertheless allows for the preparation of various multi-functionalized linkers utilizing various multi-alkyne cores.[23]

After site-specific thiol conjugation to di-scFv-C (**7**) to form azide and di-alkyne conjugated di-scFv-C **12** and **13** respectively, conjugated proteins were subjected to CuAAC in the presence of ligand and (tris(2-carboxyethyl)phosphine) TCEP at room temperature to give tetravalent scFv (**14**) in low ligation yield of ~10% (Scheme 3). In contrast, CuAAC of azido protein conjugate **12** and trialkyne protein conjugate **15** in 2 : 1 molar ratio (Scheme 4) afforded tetravalent scFv **8** in a high ligation yield of ~70%.

The success of the CuAAC reaction was confirmed by SDS-PAGE (Fig. 3). The band at higher molecular weight



Scheme 3 Formation of tetravalent-scFv **14** using heterofunctionalized di-alkyne and azide PEG linkers.



Scheme 4 Formation of tetravalent-scFv using heterofunctionalized tri-alkyne and azide PEG linkers.

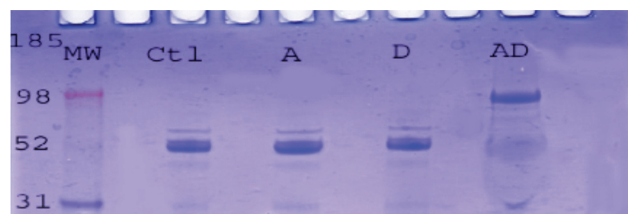


Fig. 3 SDS-PAGE of tetravalent scFv **8** cycloaddition product of protein conjugates **12** and **15** in the presence of TCEP. MW: Protein standard molecular weight marker; Ctl: di-scFv-C **1**; Lane A: trialkyne protein conjugate **15**; Lane D: azide protein conjugate **12**; Lane AD: tetravalent scFv **8**.

around 100 kDa indicated crosslinking of the two di-scFv-C. Addition of excess TCEP to lanes A and D established that the band is due to the cycloaddition product by CuAAC reaction and not by disulfide oxidation between two proteins. SDS-PAGE is not very useful for characterizing PEG-protein conjugates with narrow molecular size differences (Fig. 3, Control, lane A and D) and identification of PEG by iodine was not possible due to the low quantity of PEG relative to the protein (data not shown). Nevertheless, the ligation results support the hypothesis that by increasing the number of reactive groups, in this case from two to three alkyne groups, ligation efficiency is significantly increased presumably due to increased probability of the reaction centers meeting. Taken together, these results indicate that the use of tri-alkyne linker **4**[11] from our previous work gives optimal ligation efficiency for the formation of tetravalent scFv proteins.

Downloaded on 09 February 2012
Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A

View Online

Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A
Downloaded on 09 February 2012

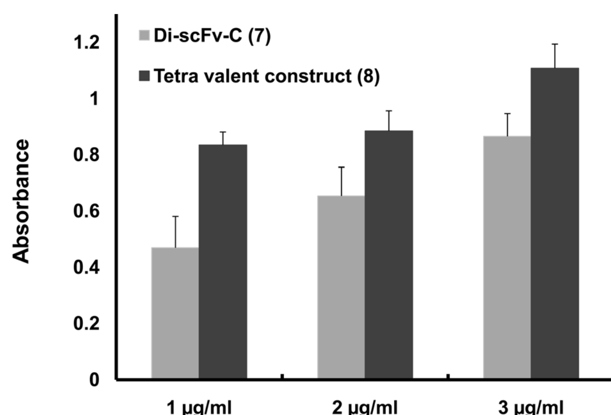


Fig. 4 ELISA of di-scFv-C 7 (52 kDa) and tetravalent scFv 8 (~100 kDa) tested against varying concentrations (1–3 μg mL^{-1} per well) of MUC1 peptide (n = 3; Absorbance values are mean ± STD). t Test of 1, 2 and 3 μg mL^{-1} per well experiments shows p values = 0.015, 0.033, and 0.021 respectively.

ELISA was used to evaluate the binding affinity of unmodified di-scFv-C (**7**) and the cycloaddition product, tetravalent scFv (**8**) to MUC1, a synthetic 100-mer peptide comprised of tandem repeats of 20 amino acids (PDTRPAPGSTAPPAHGVTSA).[16] As shown in Fig. 4, there is increased binding of **8** compared to the parent protein **7** at varying concentrations indicating nearly a 2-fold increase in binding towards the antigen MUC1 peptide using concentrations as low as 1 μg mL^{-1} of the tetravalent scFv.

Conclusion

We have demonstrated a general chemical crosslinking method for higher molecular weight genetically engineered di-scFv-C to form multivalent immunoconjugates. The trialkyne and azide heterofunctionalized PEG bromoacetamide linkers were synthesized and conjugated to an anti-MUC-1 di-scFv-C antibody fragment. Protein conjugates were crosslinked at high ligation yields using a CuAAC reaction to provide multidentate constructs with significant tumor binding affinity toward MUC1 compared to the unmodified di-scFv-C.

The versatility of the chemical crosslinking strategy reported herein allows for the construction of homo- or hetero-multimeric Ab fragments, wherein one arm could be conjugated to a another protein like an Ab fragment specific to a radiochelate compound.[24] Formation of tetravalent scFv **8** serves as a platform for the design of bispecific tetravalent radioimmunoconjugates with pharmacological advantages for pre-targeted Ab-based cancer therapy.[25]

Experimental

General

Reagents obtained from commercial sources were used without further purification. Solvents in capped DriSolv™ bottles were purchased and used directly without further purification and stored under argon. All glassware was flame-dried or oven dried prior to use. Glass-backed TLC plates (Silica Gel 60 with a 254 nm fluorescent indicator) were used and stored over desiccant. TLC visualization was accomplished with a short-wave UV lamp, by heating plates dipped in ammonium molybdate/cerium(IV) sulfate solution and/or by staining with an $KMnO_4$ solution. Flash column chromatography (FCC) was performed using silica gel (32–63 μm) employing a solvent polarity correlated with TLC mobility. NMR experiments were conducted on either 600, 400 or 300 MHz instrument using $CDCl_3$ (99.9% D) as a solvent. Chemical shifts were referenced to the solvent peak. Low resolution mass spectra were acquired using ThermoQuest SurveyorTM LC/MS or Qtrap LC/MS. High resolution mass spectra were recorded at the UC Davis Molecular Structure Facility. Infrared spectra were acquired using an ATR-FTIR spectrometer

Synthesis of azido-PEG-bromoacetamide linker

***N*-(2-(2-(2-Azidoethoxy)ethoxy)ethyl)-2-bromoacetamide (3).** **3** was synthesized using similar methods from our previous work with slight modifications.[11] TfN_3 was prepared according to Wong's method.[26] To 1.75 g (12 mmol) of diamine was added 10 mL of MeOH in a 250 mL oven-dried round bottom flask. Then 2.5 g of K_2CO_3 (18 mmol) in 8 mL H_2O and 1.2 mL of 0.1 M $CuSO_4\ 5{\cdot}H_2O$ (0.12 mmol) was added to the solution. Then 20 mL of TfN_3 in DCM (6 mmol) was cannulated into the solution over a period of 1 h. The reaction mixture was allowed to stir at rt overnight. It was then concentrated to dryness and redissolved in 20 mL of (1 : 1) DCM/sat. $NaHCO_3$ mixture with pH = 8–9 at 0 °C. Then 2 mL (24 mmol) of bromoacetyl bromide was added dropwise and the reaction was stirred for 2 h. The crude mixture was extracted with DCM (3×). The organic layer was collected and washed with brine solution and dried over anhydrous sodium sulfate and concentrated to give a yellow oil. The solution was concentrated and the residue was further purified by FCC using (40 : 1) DCM/MeOH (R_f 0.25) to give a pure **3** as a colorless oil (590 mg, 35% yield over two steps). δ_H(600 MHz, $CDCl_3$) 3.40 (2 H, t, J 6.0), 3.50 (2 H, dd, J 10.6, 5.3), 3.62–3.59 (2 H, m), 3.68–3.64 (4 H, m), 3.71–3.69 (2 H, m), 3.87 (2 H, s), 6.83 (1 H, br s); δ_C(151 MHz, $CDCl_3$) 29.3, 40.1, 50.9, 69.7, 70.4, 70.6, 70.8, 165.9; m/z (100%, ESI-HRMS) 295.0354, 297.0326 (M^+ + H. $C_8H_{16}BrN_4O_3^+$ requires 295.0406, 297.0385); ν_{max}/cm^{-1} 3301 (N–H str, br), 2103 (N≡N str), 1659 (C=O str), 1119 (C–O str).

Synthesis of alkyne-PEG-bromoacetamide linker

3-(3-(Prop-2-ynyloxy)-2,2-bis((prop-2-ynyloxy)methyl) propoxy) prop-1-yne (9). Synthesis of **9** follows the procedure from previous works and characterization data matches the reported work,[11] (45% yield) δ_H(600 MHz, $CDCl_3$) 2.40 (1 H, t, J 2.4), 3.53 (2 H, s), 4.12 (2 H, s, J 2.4); δ_C(151 MHz, $CDCl_3$) 44.9, 58.9, 69.2, 74.2, 80.2.

2,4,6-Tris(prop-2-ynyloxy)-1,3,5-triazine (10). Synthesis of **10** follows the procedure from previous works and showed matched characterization data for NMR and MS.[22,27] (82% yield) δ_H(600 MHz, $CDCl_3$) 2.52 (1 H, t, J 2.4), 5.02 (2 H, d, J 2.4); δ_C(151 MHz, $CDCl_3$) 56.2, 76.2, 77.5, 172.7.

 This journal is © The Royal Society of Chemistry 2012

Downloaded on 09 February 2012
Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A

General procedure

Alkyne linkers were synthesized through CuAAC following similar conditions developed by Sharpless and co-workers.[28] In a flame-dried, pear-shaped flask was added the alkyne core (3 or 4 equiv.) and TBTA ligand (0.01 equiv.) with DMF as the solvent. Sodium ascorbate (0.1 equiv.) and $CuSO_4\ 5{\cdot}H_2O$ (0.01 equiv.) in H_2O were added and the reaction mixture was allowed to stir. Azide linker **3** (1 equiv.) in DMF was then added dropwise to give a total of 0.05 M (4 : 1) DMF/H_2O concentration. After stirring overnight, the reaction mixture was concentrated *in vacuo*, dissolved in dichloromethane and filtered to remove any undissolved salts. Crude yellow oil was purified by FCC in 50 : 1 (DCM/MeOH) R_f 0.25 to give a colorless oil in moderate yield.

2-Bromo-*N*-(2-(2-(2-(4-((3-(prop-2-ynyloxy)-2,2-bis((prop-2-ynyloxy)methyl)propoxy)methyl)-1*H*-1,2,3-triazol-1yl)ethoxy)ethoxy)ethyl)acetamide (tri-alkyne linker 4). (65% yield) δ_H(600 MHz, $CDCl_3$) 2.41 (3 H, t, *J* 2.3), 3.47 (2 H, dd, *J* 10.5, 5.3), 3.55–3.49 (12 H, m), 3.61–3.56 (4 H, m), 3.89 (2 H, s), 3.91 (2 H, t, *J* 5.2), 4.10 (6 H, d, *J* 2.4), 4.53 (2 H, t, *J* 5.2), 4.63 (2 H, s), 6.89 (1 H, br s), 7.68 (1 H, s); δ_C(151 MHz, $CDCl_3$) 29.3, 40.1, 45.2, 50.4, 58.9, 65.4, 69.2, 69.5, 69.6, 69.7, 70.5, 70.7, 74.3, 80.3, 123.5, 145.8, 165.8; *m*/*z* (100%, ESI-HRMS) 605.1488, 607.1467. (M^+ + H. $C_{25}H_{35}BrN_4NaO_7{}^+$ requires 605.1587, 607.1566)

***N*-(2-(2-(2-(4-((4,6-Bis(prop-2-ynyloxy)-1,3,5-triazin-2-yloxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)-2-bromoacetamide (di-alkyne linker 11).** (62% yield) δ_H(600 MHz, $CDCl_3$) 2.54 (2 H, t, *J* 2.4), 3.48 (3 H, dd, *J* 10.6, 5.3), 3.54 (2 H, t, J 5.2), 3.59 (5 H, ddd, *J* 8.3, 6.1, 2.8), 3.91–3.87 (4 H, m), 4.58–4.54 (2 H, m), 5.04 (5 H, d, J 2.4), 5.60 (2 H, s), 6.98 (1 H, s), 7.89 (1 H, s); δ_C(151 MHz, $CDCl_3$) 29.4, 40.1, 50.6, 56.1, 62.1, 69.5, 69.6, 70.4, 70.7, 76.1, 125.1, 142.1, 165.9, 172.6, 172.9 *m*/*z* (100%, ESI-HRMS) 538.1045, 540.1018 (M^+ + H. $C_{20}H_{25}BrN_7O_6{}^+$ requires 538.1050, 540.1029); ν_{max}/cm^{-1} 3278 (C–H str), 2127 (C≡C str), 1668 (C═O str), 1128 (C–O str).

Di-scFv-C engineered protein

Expression and purification of di-scFv-C protein with a free cysteine was adopted from previous works.[16,29,30,31]

General crosslinking method to form tetravalent-scFv

Di-scFv PEG-conjugated azides and alkynes were synthesized *via* site-specific thiol conjugation of the protein with different azide and alkyne–PEG–bromoacetamide linkers following the conditions described from our previous work.[11]

Gel electrophoresis and densitometry

SDS-PAGE (Novex XCell II, Invitrogen) of di-scFv and click constructs were performed with a 4–12% Bis-Tris NuPAGE gel and MES running buffer was used according to manufacturer's direction. Protein bands were detected by Coomassie blue staining. In order to analyze the ratio of ligated to unligated protein, SI Densitometer (Personal Densitometer S1, model PDS1, Molecular Dynamics Inc., Sunnyvale, CA), which creates image files that can be transferred to compatible graphic programs. Image analysis software provides data sets of the amount of protein concentration at each band. Ligation efficiencies were calculated from quantitation of the initial di-scFv-C compared to the resulting cycloaddition product as tetravalent-scFv conjugates *vs.* unmodified di-scFv-C. The relative amounts of each species in each reaction were obtained by calculations of digital information from densitometry performed on the scanned gel images and protein standards.[32]

Enzyme-linked immunosorbant assay (ELISA)

ELISA was used to compare the binding affinities of di-scF-C (**7**) *vs.* tetravalent scFv (**8**) against MUC1 peptides. The 96 well ELISA assay plate was coated with MUC 1 peptide at varying concentrations (1–3 μg mL^{-1} per well). The assays were performed in triplicate, following the approach described previously.[32] As soon as the colorimetric reaction had developed, the plate was read at A_{405} and the absorbance was recorded. *t* Test *p* value = 0.05.

Acknowledgements

This work was supported by National Cancer Institute NCI grant PO1-CA47829 and National Science Foundation CHE-0196482. The NSF CRIF program (CHE-9808183), NSF grant OSTI 97-24412 and NIH grant RR11973 provided funding for the NMR spectrometers used during this project.

Notes and references

1 M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794.
2 C. L. Hornick and F. Karush, *Immunochemistry*, 1972, **9**, 325–330; P. V. Gopalakrishnan and F. Karush, *J. Immunol.*, 1974, **113**, 769–778.
3 A. M. Wu and P. D. Senter, *Nat. Biotechnol.*, 2005, **23**, 1137–1146.
4 S. K. Batra, M. Jain, U. A. Wittel, S. C. Chauhan and D. Colcher, *Curr. Opin. Biotechnol.*, 2002, **13**, 603–608.
5 A. Goel, D. Colcher, J. Baranowska-Kortylewicz, S. Augustine, B. J. Booth, G. Pavlinkova and S. K. Batra, *Cancer Res.*, 2000, **60**, 6964–6971; A. Goel, J. Baranowska-Kortylewicz, S. H. Hinrichs, J. Wisecarver, G. Pavlinkova, S. Augustine, D. Colcher, B. J. Booth and S. K. Batra, *J. Nucl. Med.*, 2001, **42**, 1519–1527; U. A. Wittel, M. Jain, A. Goel, S. C. Chauhan, D. Colcher and S. K. Batra, *Nucl. Med. Biol.*, 2005, **32**, 157–164; B. E. Power, L. Doughty, D. R. Shapira, J. E. Burns, A. M. Bayly, J. M. Caine, Z. Liu, A. M. Scott, P. J. Hudson and A. A. Kortt, *Protein Sci.*, 2003, **12**, 734–747.
6 P. Holliger and P. J. Hudson, *Nat. Biotechnol.*, 2005, **23**, 1126–1136.
7 M. P. Kelly, F. T. Lee, K. Tahtis, B. E. Power, F. E. Smyth, M. W. Brechbiel, P. J. Hudson and A. M. Scott, *Cancer Biother. Radiopharm.*, 2008, **23**, 411–423.
8 A. A. Kortt, O. Dolezal, B. E. Power and P. J. Hudson, *Biomol. Eng.*, 2001, **18**, 95–108.
9 P. E. Dawson and S. B. Kent, *Annu. Rev. Biochem.*, 2000, **69**, 923–960; T. W. Muir, *Annu. Rev. Biochem.*, 2003, **72**, 249–289; A. Brik and C. H. Wong, *Chem.–Eur. J.*, 2007, **13**, 5670–5675; M. Francis, *New methods for protein bioconjugation in: Chem. Biol.* Wiley-VCH Verlag GmbH & Co. KGaA, 2007, vol. 2, pp. 593–634; K. L. Heredia and H. D. Maynard, *Org. Biomol. Chem.*, 2007, **5**, 45–53; J. Kalia and R. T. Raines, *Curr. Org. Chem.*, 2010, **14**, 138–147.
10 T. K. Tiefenbrunn and P. E. Dawson, *Biopolymers*, 2010, **94**, 95–106.

11 A. Natarajan, W. Du, C. Y. Xiong, G. L. DeNardo, S. J. DeNardo and J. Gervay-Hague, *Chem. Commun.*, 2007, 695–697.
12 H. Albrecht and S. J. DeNardo, *Cancer Biother. Radiopharm.*, 2006, **21**, 285–304.
13 A. P. Chapman, *Adv. Drug Delivery Rev.*, 2002, **54**, 531–545; M. J. Roberts, M. D. Bentley and J. M. Harris, *Adv. Drug Delivery Rev.*, 2002, **54**, 459–476; F. M. Veronese and J. M. Harris, *Adv. Drug Delivery Rev.*, 2002, **54**, 453–456; R. Duncan, *Nat. Rev. Drug Discovery*, 2003, **2**, 347–360.
14 R. M. Sharkey, H. Karacay, T. M. Cardillo, C. H. Chang, W. J. McBride, E. A. Rossi, I. D. Horak and D. M. Goldenberg, *Clin. Cancer Res.*, 2005, **11**, 7109s–7121s.
15 D. A. Goodwin, C. F. Meares, M. J. McCall, M. McTigue and W. Chaovapong, *J. Nucl. Med.*, 1988, **29**, 226–234.
16 C. Y. Xiong, A. Natarajan, X. B. Shi, G. L. Denardo and S. J. Denardo, *Protein Eng., Des. Sel.*, 2006, **19**, 359–367.
17 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021; C. W. Tornoe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064; V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599; W. G. Lewis, L. G. Green, F. Grynszpan, Z. Radic, P. R. Carlier, P. Taylor, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 1053–1057; V. P. Mocharla, B. Colasson, L. V. Lee, S. Roper, K. B. Sharpless, C. H. Wong and H. C. Kolb, *Angew. Chem., Int. Ed.*, 2005, **44**, 116–120; M. Whiting, J. Muldoon, Y. C. Lin, S. M. Silverman, W. Lindstrom, A. J. Olson, H. C. Kolb, M. G. Finn, K. B. Sharpless, J. H. Elder and V. V. Fokin, *Angew. Chem., Int. Ed.*, 2006, **45**, 1435–1439.
18 J. A. Prescher and C. R. Bertozzi, *Nat. Chem. Biol.*, 2005, **1**, 13–21; C. W. Tornoe, S. J. Sanderson, J. C. Mottram, G. H. Coombs and M. Meldal, *J. Comb. Chem.*, 2004, **6**, 312–324; A. Brik, J. Alexandratos, Y. C. Lin, J. H. Elder, A. J. Olson, A. Wlodawer, D. S. Goodsell and C. H. Wong, *ChemBioChem*, 2005, **6**, 1167–1169; V. D. Bock, D. Speijer, H. Hiemstra and J. H. van Maarseveen, *Org. Biomol. Chem.*, 2007, **5**, 971–975.
19 H. C. Kolb and K. B. Sharpless, *Drug Discovery Today*, 2003, **8**, 1128–1137; Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn, *J. Am. Chem. Soc.*, 2003, **125**, 3192–3193; J. E. Moses and A. D. Moorhouse, *Chem. Soc. Rev.*, 2007, **36**, 1249–1262; Y. L. Angell and K. Burgess, *Chem. Soc. Rev.*, 2007, **36**, 1674–1689.
20 S. S. Gupta, J. Kuzelka, P. Singh, W. G. Lewis, M. Manchester and M. G. Finn, *Bioconjugate Chem.*, 2005, **16**, 1572–1579; I. v. Baal, H. Malda, S. A. Synowsky, J. L. J. v. Dongen, T. M. Hackeng, M. Merkx and E. W. Meijer, *Angew. Chem., Int. Ed.*, 2005, **44**, 5052–5057; J. S. Foot, F. E. Lui and R. Kluger, *Chem. Commun.*, 2009, 7315–7317; J. Xiao and T. J. Tolbert, *Org. Lett.*, 2009, **11**, 4144–4147; N. S. Hatzakis, H. Engelkamp, K. Velonia, J. Hofkens, P. C. Christianen, A. Svendsen, S. A. Patkar, J. Vind, J. C. Maan, A. E. Rowan and R. J. Nolte, *Chem. Commun.*, 2006, 2012–2014.
21 A. Chouai and E. E. Simanek, *J. Org. Chem.*, 2008, **73**, 2357–2366; C. Courme, N. Gresh, M. Vidal, C. Lenoir, C. Garbay, J. C. Florent and E. Bertounesque, *Eur. J. Med. Chem.*, 2010, **45**, 244–255.
22 Y. Lu and J. Gervay-Hague, *Carbohydr. Res.*, 2007, **342**, 1636–1650.
23 Go, J.G. Ph.D. Dissertation, University of California, Davis, 2011.
24 J. Xiao and T. J. Tolbert, *Org. Lett.*, 2009, **11**, 4144–4147.
25 R. M. Sharkey, T. M. Cardillo, E. A. Rossi, C. H. Chang, H. Karacay, W. J. McBride, H. J. Hansen, I. D. Horak and D. M. Goldenberg, *Nat. Med.*, 2005, **11**, 1250–1255.
26 P. B. Alper, S. C. Hung and C. H. Wong, *Tetrahedron Lett.*, 1996, **37**, 6029–6032; P. T. Nyffeler, C. H. Liang, K. M. Koeller and C. H. Wong, *J. Am. Chem. Soc.*, 2002, **124**, 10773–10778.
27 P. Wu, A. K. Feldman, A. K. Nugent, C. J. Hawker, A. Scheel, B. Voit, J. Pyun, J. M. Frechet, K. B. Sharpless and V. V. Fokin, *Angew. Chem., Int. Ed.*, 2004, **43**, 3928–3932.
28 T. R. Chan, R. Hilgraf, K. B. Sharpless and V. V. Fokin, *Org. Lett.*, 2004, **6**, 2853–2855.
29 M. D. Winthrop, S. J. DeNardo, H. Albrecht, G. R. Mirick, L. A. Kroger, K. R. Lamborn, C. Venclovas, M. E. Colvin, P. A. Burke and G. L. DeNardo, *Clin. Cancer Res.*, 2003, **9**, 3845S–3853S.
30 H. Albrecht, P. A. Burke, A. Natarajan, C. Y. Xiong, M. Kalicinsky, G. L. DeNardo and S. J. DeNardo, *Bioconjugate Chem.*, 2004, **15**, 16–26.
31 H. Albrecht, G. L. DeNardo and S. J. DeNardo, *J. Immunol. Methods*, 2006, **310**, 100–116.
32 A. Natarajan, C. Y. Xiong, H. Albrecht, G. L. DeNardo and S. J. DeNardo, *Bioconjugate Chem.*, 2005, **16**, 113–121.

Downloaded on 09 February 2012
Published on 25 October 2011 on http://pubs.rsc.org | doi:10.1039/C0OB01259A

This journal is © The Royal Society of Chemistry 2012