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Thiol–yne radical reaction mediated site-specific protein labeling *via* genetic incorporation of an alkynyl-L-lysine analogue†

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Yiming Li,*[a,b] Man Pan,[a,c] Yitong Li,[a,c] Yichao Huang[a,c] and Qingxiang Guo[b]

Three alkyne-containing pyrrolysine derivatives were synthesized and genetically encoded into proteins by a mutant PylRS–tRNA pair with high efficiencies. With these alkyne handles, site-specific dual labeling of proteins can be achieved *via* a bioorthogonal thiol–yne ligation reaction.

Introduction

Site-specific labeling and modification of proteins have seen tremendous development in the last decade.[1] In addition to the well established bioorthogonal reactions such as Staudinger ligation, azide–alkyne cycloaddition, photo-inducible tetrazole-containing 1,3-dipolar cycloaddition and native chemical ligation,[2] the radical mediated thiol–ene and thiol–yne coupling (TEC and TYC) procedures have recently emerged as a new metal-free bioorthogonal reaction.[3] In principle, TEC and TYC are anti-Markovnikov regioselective reactions induced by photo-irradiation *via* a radical initiator. They have been successfully used for polymer–polymer conjugation, materials surface modification and peptide/protein labeling.[4] The fact that the TEC and TYC reactions can be performed with low-energy light at 365–400 nm and their robustness in aqueous buffer contribute to their promising applications, especially in biological studies.[5]

Among the two radical mediated coupling reactions, photo-induced hydrothiolation of alkynes (TYC) couples two thiols to one alkyne under UV (365 nm) irradiation at ambient temperature. Compared to TEC, TYC allows simple addition of two thiols to an alkyne for dual ligation and has been introduced for the development of a cross-linked polymer network.[3c] Moreover, TYC is usually more efficient when carried out with equimolar amounts of thiol reagents.[6] The radical mechanism of TYC also makes it a robust and versatile method that can tolerate a variety of functional groups.[7] In our previous work, the TEC reaction was used for site-specific dual labeling of proteins carrying two site-specifically incorporated alkene handles.[8] However, the incorporation efficiency of unnatural amino acids (UAA) was relatively low which limited its application. We thus anticipate that TYC may be more suitable for peptide/protein dual labeling and modification *via* the UAA approach.

Till now, very few TYC-mediated peptide/protein dual labeling reactions have been developed although these reactions are considered a good option. Recently, Dondoni *et al.* used TYC to achieve selective propargylation of cysteine-containing peptides by coupling with glycosyl thiols for post-translational dual glycosylation of peptides.[9] Moreover, Dondoni and Davies groups reported the TYC-mediated multi-glycoconjugation and fluorescent labeling of serum albumin induced by light at visible wavelength (365–405 nm).[10] Furthermore, the Boelens group demonstrated that thiol–yne addition with reduced peptidylcysteines is responsible for most of the azide-independent polypeptide labeling.[11] Despite these developments, we need to note that the previous TYC-based protein dual labeling strategies have a limitation, that is, the target protein should contain only one free cysteine residue unless one wants to conduct random labeling at multiple locations. To this end, redundant cysteine residues must be mutated to other amino acids, which may disturb the structure and/or activity of the labelled proteins. Furthermore, most of the TYC labeling reactions were carried out in organic solvents, which limited their application to native proteins.[6] Therefore, it is necessary to develop an alternative method for protein site-specific dual labeling *via* the thiol–yne reaction (Scheme 1).

To achieve the above idea of site-specific TYC-based protein dual labeling, we used a pyrrolysyl-tRNA synthetase (PylRS)–$tRNA^{Pyl}_{CUA}$ pair, encoding the 22nd naturally occurring amino acid-pyrrolysine in response to an amber codon in archaea species, for incorporation of alkynyl-L-lysines into proteins.[12]

[a]*School of Medical Engineering, Hefei University of Technology, Hefei 230009, P. R. China. E-mail: ymli@hfut.edu.cn; Tel: +86 0551-2904353*
[b]*Department of Chemistry, University of Science and Technology of China, Hefei 230026, P. R. China*
[c]*Department of Chemistry, Tsinghua University, Beijing 100084, China*
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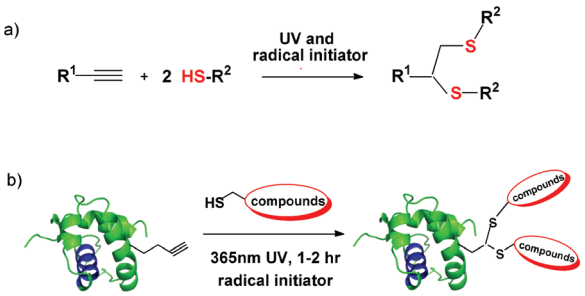
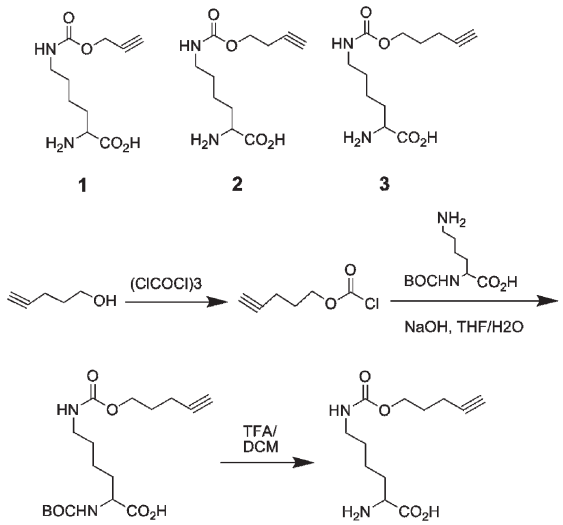


Scheme 1 Thiol–yne reaction mediated dual labeling of proteins. (a) Thiol–yne radical reaction; (b) site-specific thiol–yne labeling of proteins carrying an alkyne handle *via* genetically encoded alkynyl-pyrrolysine analogues.

The incorporation of an alkyne containing amino acid into proteins provides the ideal labeling site for the thiol–yne reaction. Note that the PylRS–tRNA pair has recently been adapted for incorporation of a variety of UAAs into proteins in bacteria, mammalian cells and living animals.[13] Inspired by our previously evolved ACPK-RS that can work in both bacterial and mammalian species with high incorporation efficiency,[14] we designed and synthesized three alkyne-containing pyrrolysine analogues and tested whether or not they can be recognized by ACPK-RS. After the incorporation was successful, we then utilized the ACPK-RS–tRNA pair to introduce compound **3** into an *E. coli* acid-chaperone HdeA. This site-specifically incorporated alkyne handle was subjected to a thiol–yne reaction with a thiol-containing *N*,*N*′-bis(dansyl) cystamine. To our best knowledge, this study presents the first thiol–yne mediated protein dual labeling *via* genetic incorporation of an alkynyl-L-lysine derivative as a general bioorthogonal handle.

Results and discussion

First, we synthesized three alkyne-containing pyrrolysine analogue compounds **1–3** (Scheme 2). To provide an example for the synthesis of compound **3**, 4-pentyn-1-ol (840 mg, 10 mmol) was added to a solution of triphosgene (2.9 g, 10 mmol) in dry THF (40 mL), stirred under an ice bath for 12 h, then the solvent was evaporated under vacuum. The collected chloroformate was dissolved in 5 mL THF and slowly added to a solution of Boc-Lys-OH (3.0 g, 12.2 mmol) in 1 M NaOH (25 mL)/THF (25 mL) solution in an ice bath, the reaction mixture was stirred at room temperature overnight. The solution was cooled to 0 °C, washed with ice-cold Et_2O (2 × 25 mL) and acidified with ice-cold 1 M HCl, and was extracted with EtOAc (2 × 30 mL). The combined organic layer was dried over Na_2SO_4, filtered and evaporated to give a white foam. The foam was dissolved in dry DCM (5 mL) and TFA (5 mL) was added slowly, the reaction mixture was stirred at room temperature for 1 h, the solvent was evaporated under vacuum, the product was precipitated with Et_2O, and the precipitate was collected and dried under vacuum, affording pure **3** (0.8 g, overall yield 31%). Compounds **1** (0.9 g, overall yield 37%) and **2** (0.8 g, overall yield 35%) were synthesized following similar synthetic procedures using 3-butyn-1-ol (700 mg, 10 mmol) and propargyl alcohol (560 mg, 10 mmol) as starting materials respectively.



Scheme 2 Synthesis of alkyne-containing pyrrolysine analogues **1–3**.

With compounds **1–3** in hand, we then compared the incorporation efficiency and fidelity of ACPK-RS on these compounds in *E. coli.* Protein expression was carried out in BL21-DE3 cells co-transformed with plasmids expressing the ACPK-RS–$tRNA^{Pyl}_{CUA}$ pair and previously optimized HdeA-V58TAG plasmid. It has been reported that HdeA is an essential acid-chaperone in supporting the acid-resistance of *E. coli* cells and it was suitable for being used as our model protein in this study.[15] Cells were grown in an LB medium with shaking overnight at 37 °C. After 1 : 100 dilution in LB medium, the culture was grown at 37 °C to an OD_{600} of ~0.6. Compounds **1–3** (100 mM for stock solution) were diluted by a factor of 1 : 100 to a final concentration of 1 mM and incubated with cell culture for 30 min. Protein expression was induced by the addition of arabinose and IPTG to the final concentration of 0.2% and 0.5 mM at 30 °C, respectively. After expression for 12–14 h, cells were harvested by centrifugation and re-suspended in lysis buffer. Bacterial lysate after sonication was further purified by a Ni-NTA column. Eluted proteins from a Ni-NTA column were analyzed by SDS-PAGE and ESI-MS. Fig. 1a indicates that HdeA-V58-**3** shows the best expression level, which was only slightly lower than wild type (WT) HdeA protein. The yield of HdeA-V58-**3** was estimated to be nearly 20 mg L^{-1}. We further confirmed the purity of expressed HdeA-V58-**3** by ESI-MS analysis (Fig. 1b).

We next performed site-specific radical thiol–yne coupling on HdeA with *N*,*N*′-bis(dansyl) cystamine after confirming the feasibility of obtaining a high yield of HdeA-V58-**3**. Fluorophores were synthesized as previously reported.[8] In theory, the protein should be labelled with two *N*,*N*′-bis-(dansyl) cystamines *via* two thiols containing compounds coupling to one alkyne handle.[3c] Similar to our recently reported thiol–ene reaction, about 1 mM HdeA-V58-**3** was dissolved in photo-irradiation buffer (0.2 M acetate buffer pH = 4

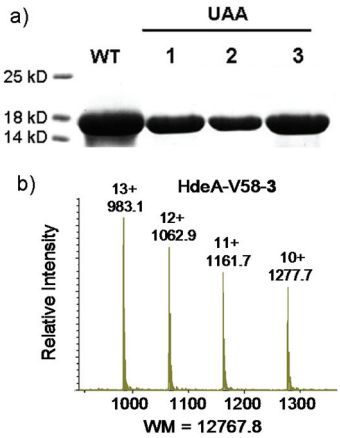


Fig. 1 Genetic incorporation of alkyne-bearing pyrrolysine analogues (**1–3**) with different side-chain lengths into proteins by an ACPK-RS–tRNA pair. (a) SDS-PAGE analysis demonstrating the incorporation efficiencies of compounds **1–3** into HdeA protein in *E. coli*; (b) ESI-MS analysis of the full length of HdeA-myc-His_6 protein containing **3** at residue V58 (*m*/*z* calculated: 12 768.5 Da, found: 12 767.8 Da).

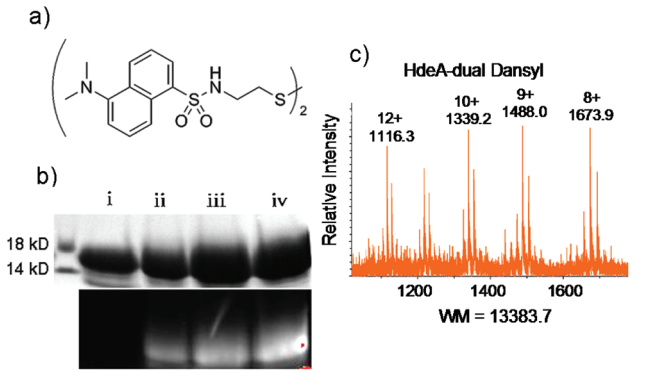


Fig. 2 Dual labeling of alkyne-bearing HdeA protein *via* a thiol–yne reaction. (a) The structure of *N*,*N*′-bis(dansyl) cystamine; (b) fluorescent labeling of HdeA-V58-3 with *N*,*N*′-bis(dansyl) cystamine by photo-irradiation for 15 min (ii), 0.5 h (iii) and 2 h (iv), purified WT HdeA (i) was used as a control; (c) ESI-MS analysis of HdeA-V58-**3** dual labeling with fluorophores by a thiol–yne reaction (*m*/*z* calculated: 13 384.8 Da, found: 13 383.7 Da). Note that the mass spectrometry data show a small fraction of different products besides dual labelled HdeA-*N*,*N*′-bis(dansyl) cystamine, we consider that the different products are the by-products of long period UV-irradiation. Admittedly, it was difficult to determine the structure of products by ESI-MS analysis.

or 0.2 M phosphate buffer pH = 6 or 7, the scale of reactions is about 300–500 μl) and reacted with a 5–10 fold amount of fluorophore *via* 365 nm UV light irradiation with 15 mM reduced glutathione. We first performed photo-irradiation by UV light (365 nm) using a CL-1000 ultraviolet cross-linker (UVP) installed with 365 nm wavelength UV lamps (80–100 V) at a distance of 3 cm at 25 °C. However, the labeling efficiency was relatively low after 2 h of irradiation. To increase the efficiency, we then used a high powered UV lamp (250 V mercury lamp) in front of a 365 nm optical filter and samples were placed in a cuvette cell at a distance of 1 cm at 25 °C for photo-irradiation. During the reaction, a photo-induced catalyst, such as 5 mM VA-044 or 2,2-dimethoxy-2-phenylacetophenone (DPAP, 10%), is necessary for the reaction. After irradiation, excess fluorophores were removed by passing through a desalting column followed by buffer exchange to NTA buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, pH 7.4). We used fluorescence SDS-PAGE gel to monitor the progress of the reaction. Fig. 2b clearly shows that alkyne containing protein was successfully labelled with *N*,*N*′-bis(dansyl) cystamine in the first 15 min and the labeling efficiency increased obviously after two hours of irradiation. Meanwhile, wild type HdeA protein was used as a control to make a fair comparison and failed to react with fluorophores under identical reaction conditions. ESI-MS analysis further verified that HdeA-V58-**3** was labelled with two *N*,*N*′-bis(dansyl) cystamines *via* a thiol–yne radical reaction only with a small fraction of by-products possibly induced by UV-irradiation. According to the peak intensity of products, we estimated that the yield of this reaction is about 60%.

To determine whether or not the photo-irradiation would disturb the structure of model protein, we tested the properties of HdeA by CD and native gel. During the reaction process, we first noted that the protein remained soluble, suggesting that no denaturation occurred. Moreover, the native PAGE analysis

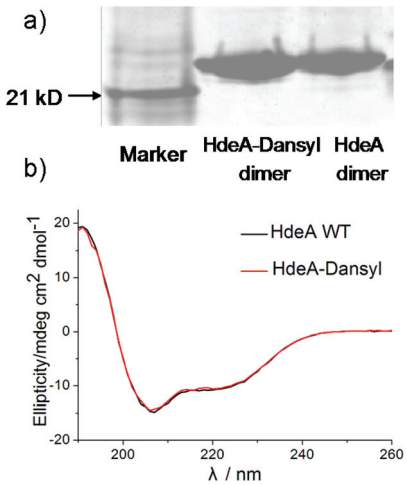


Fig. 3 (a) Native PAGE gel confirming the formation of WT HdeA and dual labelled HdeA-*N*,*N*′-bis(dansyl) cystamine; (b) CD spectrum analysis of the structure of WT HdeA and HdeA-*N*,*N*′-bis(dansyl) cystamine.

shows that fluorescent labelled *N*,*N*′-bis(dansyl) cystamine forms a dimer at pH 7.0 in the same way as recombinant HdeA. Furthermore, the CD spectra of both recombinant HdeA and fluorescence labelled HdeA superimpose well (Fig. 3), indicating that they fold to a similar three-dimensional structure. Thus, we concluded that the presence of an organic dye and a radical initiator or a long period of 365 nm UV irradiation did not disturb the structure of the folded HdeA protein.

Finally, we examined the biological activity of the dual labelled HdeA-*N*,*N*′-bis(dansyl) cystamine by a well established SurA protein assay. SurA has recently been identified as an *in vivo* client protein of HdeA. We evaluated the protein–protein interaction by measuring SurA's aggregation propensity

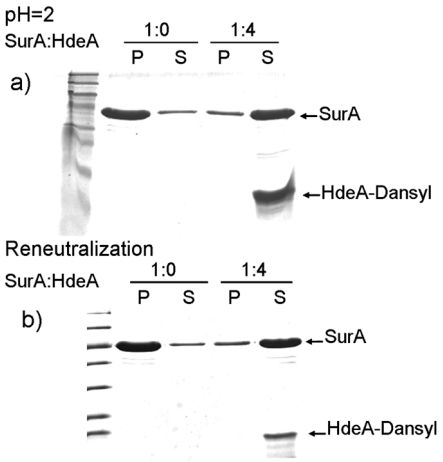


Fig. 4 HdeA suppresses the aggregation of its client protein-SurA at a low pH (a) and after the pH neutralization (b) SDS-PAGE analysis was followed by coomassie blue staining of the soluble supernatant (S) and aggregated pellet (P) protein in each sample.

in the presence and absence of HdeA-*N*,*N*′-bis(dansyl) cystamine. As shown in Fig. 4, a low pH value (pH = 2) caused SurA to fully aggregate in the absence of dual labelled HdeA. However, a significant fraction of SurA became soluble both at pH = 2 and after pH neutralization when dual-labelled HdeA was added. Because wild type HdeA proteins have been reported to carry out the same bio-function,[8,14b] we then concluded that the photo-irradiation did not disturb the biological activity of model protein HdeA.

Conclusions

In summary, three alkynyl-pyrrolysine analogues were successfully incorporated into a protein with high yields, especially for compound **3** (estimated yield = 20 mg mL^{-1}). Then, the alkyne-containing proteins were site-specifically dual-labelled with thiol containing fluorophores under 365 nm UV light irradiation. This metal-free radical induced thiol–yne coupling provides a promising strategy for protein dual labeling and modification. Moreover, the structure and biological activity assay demonstrated that the labelled protein possesses a similar enzymatic activity and conformation to the wild type HdeA. In current work, fluorophores were used as model compounds to confirm that site-specific dual labeling of proteins can be achieved *via* a thiol–yne ligation reaction. In future plans, this dual labeling strategy should be expanded to other protein modification, such as glycosylation. It has been reported that the peptide/protein double glycosylation can affect much more substantially than monoglycosylation the peptide/protein structure and biological activity.[9,10] Taken together, we conclude that this genetic-code expansion method in conjunction with the thiol–yne labeling may become a complementary approach to the growing arsenal of strategies for protein bioorthogonal ligation.

Experimental section

General information

All reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd or Alfa Aesar and were purified when necessary. All other commercially available reagents and solvents were used as received without further purification unless otherwise indicated. Analytical HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using an analytical column (Grace Vydac "Protein C18", 250 × 4.6 mM, 5 μm particle size, flow rate 1.0 mL min^{-1}, rt). Analytical injections were monitored at 214 and 254 nm. Semi-preparative HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using a semi-preparative column (Grace Vydac "Peptide C18", 250 × 10 mM, 10 μm particle size, flow rate 3.0 mL min^{-1}). MALDI-TOF mass spectra were measured on an Applied Biosystems 4700 Proteomics Analyzer 283. High-resolution ESI mass spectra were measured on a Bruker APEX IV Fourier Transform Ion Cyclotron Resonance Mass spectrometer. Normal ESI mass spectra were measured on a Bruker Daltonics Data Analysis 3.0 workstation. ^{1}H and ^{13}C NMR spectra were recorded on an Oxford 300 MHz spectrometer in deuteriochloroform ($CDCl_3$) or deuterium oxide (D_2O) with the solvent residual peak ($CDCl_3$: 7.26 ppm (^{1}H), 77.23 ppm (^{13}H), D_2O: 4.79 ppm (^{1}H)) as an internal reference unless otherwise stated. Data are reported in the following order: chemical shifts are given (δ); multiplicities are indicated as br (broadened), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), app (apparent); coupling constants, *J*, are reported (Hz); integration is provided. For molecular biology and biochemistry experiments, primers were ordered from Sangon Biotech (Shanghai) Co., Ltd. Enzymes were ordered from New England Biolabs (NEB). Bacterial cells were grown in LB (Luria–Bertani) broth or on LB agar medium (Sigma). Antibiotics were used at final concentrations of 100 μg mL^{-1} for ampicillin and kanamycin and at 50 μg mL^{-1} for chloramphenicol (Sigma). All pictures of protein gels including coomassie stained SDS-PAGE gel and native PAGE gel were taken on ChemDocXRS+ (Bio-Rad).

Protein expression and purification

In detail, HdeA protein expression was carried out in *E. coli* DH10B cells co-transformed with plasmids expressing the PylRS–$tRNA^{Pyl}_{CUA}$ pair and HdeA-V58TAG. Cells were grown in LB medium containing ampicillin (100 μg mL^{-1}) and chloramphenicol (50 μg mL^{-1}) with shaking overnight at 37 °C. After 1 : 100 dilution in LB medium containing ampicillin (100 μg mL^{-1}) and chloramphenicol (50 μg mL^{-1}), the culture was grown at 37 °C to an OD_{600} of ~0.5. Compounds **1–3** (100 mM for stock solution) were diluted by a factor of 1 : 100 to a final concentration of 1 mM and incubated with cell culture for 30 min. Protein expression was induced by the addition of arabinose and IPTG to the final concentration of 0.2% and 0.5 mM, respectively. After expression for 12 h, cells were harvested by centrifugation (10 000 *g*, 10 min), and resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl,

pH 7.0). Bacterial lysate after sonication was loaded onto a Ni-NTA column (Histrap 5 mL, GE healthcare). The column was washed with 30 mL washing buffer (20 mM Tris-HCl, 300 mM NaCl, pH 7.0 with 40 mM imidazole) and then eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.0 with 250 mM imidazole).

Proteins fluorescent labeling by thiol–yne reaction

The reaction was performed in the 0.2 M acetate buffer (pH = 4.0) or 0.2 M phosphate buffer (pH = 6 or 7). The final concentrations of the reactants were as follows: HdeA-V58-**3**: 1 mM; *N*,*N*′-bis(dansyl) cystamine: 20 mM. VA-044: 5 mM or 2,2-dimethoxy-2-phenylacetophenone (DPAP, 10%) as a radical initiator; reduced glutathione: 15 mM. The scale of this photo-irradiation reaction is about 300–500 μl. Photo-irradiation was performed by a high powered UV lamp (250 V mercury lamp) in front of a 365 nm optical filter and samples were placed in a cuvette cell at a distance of 1 cm at 25 °C. After irradiation, excess fluorophores were removed by passing through a desalting column and the buffer was exchanged to NTA buffer (20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 7.4).

SDS-PAGE, native PAGE gel

For SDS-PAGE, samples were loaded onto 12% SDS-PAGE gels and electrophoresed for 30 min at 80 V and 50 min at 150 V. The native PAGE gel was prepared using a Bio-Rad Mini-PROTEIN Tetra Electrophoresis System. SDS was removed from the ingredients of both the stacking gel (pH 6.8, 4%) and the resolving gel (pH 8.8, 15%). All the protein samples were prepared in Tris buffer containing bromophenol blue. Native running buffer (1 L) consists of 14.4 g glycine and 3.03 g Tris base dissolved in ddH_2O. Samples were run under ice-cold conditions (150 V, 400 mA, 60 min). A soybean trypsin inhibitor (from Beijing Biodee Biotechnology Co., Ltd, pI = 4.6, MW ~ 21 kDa) was used as the marker protein (pI of HdeA = 5.2).

CD spectroscopy

CD spectra were recorded on an Applied Photophysics Chirascan™ spectrometer from 260 to 190 nm in a 0.2 cm path length cell at room temperature. Each protein sample was dissolved in 20 mM phosphate buffer at pH 7.2 with a final concentration of 0.4 mg mL^{-1}. The result was recorded by averaging three scans and plotted as mean residue ellipticity $[\theta]$ (mdeg cm^2 $dmol^{-1}$).

SurA assay

SurA protein was incubated at a concentration of 8 μM in buffer A (8 mM H_3PO_4, 150 mM KCl, and 150 mM $(NH_4)_2SO_4$, pH 2.0) at 37 °C for 30 min in the absence or presence of 32 μM HdeA. After 30 min, the pH was neutralized by adding 0.133 volumes of buffer B (0.5 M sodium phosphate, pH 8). After 30 min at 37 °C, the soluble supernatant (S) and aggregated pellet (P) protein in each sample at the indicated HdeA : SurA ratios were separated by spinning at 12 000 *g* at 4 °C using a SIGMA 3–18 K centrifuge, and then analyzed by SDS-PAGE.

Acknowledgements

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Notes and references

1 (*a*) E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974–6998; (*b*) J. A. Prescher and C. R. Bertozzi, *Nat. Chem. Biol.*, 2005, **1**, 13–21.

2 (*a*) E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007–2010; (*b*) V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599; (*c*) L. Liu, Z. Y. Hong and C. H. Wong, *ChemBioChem*, 2006, **3**, 429–432; (*d*) N. K. Devaraj and R. Weissleder, *Acc. Chem. Res.*, 2011, **44**, 816–827; (*e*) W. J. Song, Y. Z. Wang, J. Qu, M. M. Madden and Q. Lin, *Angew. Chem., Int. Ed.*, 2008, **47**, 2832–2835; (*f*) E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa and T. Carell, *Angew. Chem., Int. Ed.*, 2012, **51**, 4466–4469; (*g*) K. Lang, L. Davis, J. Torres-Kolbus, C. Chou, A. Deiters and J. W. Chin, *Nat. Chem.*, 2012, **4**, 298–304; (*h*) S. Ohayon, L. Spasser, A. Aharoni and A. Brik, *J. Am. Chem. Soc.*, 2012, **134**, 3281–3289; (*i*) S. Liu, B. L. Pentelute and S. B. Kent, *Angew. Chem., Int. Ed.*, 2012, **51**, 993–999; (*j*) G. M. Fang, Y. M. Li, F. Shen, Y. C. Huang, J. B. Li, Y. Lin, H. K. Cui and L. Liu, *Angew. Chem., Int. Ed.*, 2011, **50**, 7645–7649; (*k*) J. S. Zheng, H. N. Chang, F. L. Wang and L. Liu, *J. Am. Chem. Soc.*, 2011, **133**, 11080–11083; (*l*) V. Y. Torbeev and S. B. Kent, *Org. Biomol. Chem.*, 2012, **10**, 5887–5891.

3 (*a*) A. Dondoni and A. Marra, *Chem. Soc. Rev.*, 2011, **40**, 338–351; (*b*) A. Dondoni, *Angew. Chem., Int. Ed.*, 2008, **47**, 8995–8997; (*c*) R. Hoogenboom, *Angew. Chem., Int. Ed.*, 2010, **49**, 3415–3417.

4 (*a*) A. A. Aimetti, A. J. Machen and K. S. Anseth, *Biomaterials*, 2009, **30**, 6048–6054; (*b*) C. E. Hoyle and C. N. Bowman, *Angew. Chem., Int. Ed.*, 2010, **49**, 1540–1573; (*c*) C. Wang, P. F. Ren, X. J. Huang, J. Wu and Z. K. Xu, *Chem. Commun.*, 2011, **47**, 3930–3932; (*d*) Y. Huang, Y. Zeng, J. Yang, Z. Zeng, F. Zhu and X. Chen, *Chem. Commun.*, 2011, **47**, 7509–7511.

5 (*a*) A. Dondoni, *Angew. Chem., Int. Ed.*, 2008, **47**, 8995–8997; (*b*) F. Floyd, B. Vijayakrishnan, J. R. Koeppe and B. G. Davis, *Angew. Chem., Int. Ed.*, 2009, **48**, 7798–7802; (*c*) Y. X. Chen, G. Triola and H. Waldmann, *Acc. Chem. Res.*, 2011, **44**, 762–773; (*d*) B. D. Fairbanks, T. F. Scott, C. J. Kloxin, K. S. Anseth and C. N. Bowman, *Macromolecules*, 2009, **42**, 211–217.

6 A. Massi and D. Nanni, *Org. Biomol. Chem.*, 2012, **10**, 3791–3807.

7 (*a*) M. T. Gokmen, J. Brassinne, R. A. Prasath and F. E. Du Prez, *Chem. Commun.*, 2011, **47**, 4652–4654;

(*b*) D. Konkolewicz, C. K. Poon, A. Gray-Weale and S. Perrier, *Chem. Commun.*, 2011, **47**, 239–241.

8 Y. M. Li, M. Y. Yang, Y. C. Huang, X. D. Song, L. Liu and P. R. Chen, *Chem. Sci.*, 2012, **3**, 2766–2770.

9 M. Lo Conte, S. Pacifico, A. Chambery, A. Marra and A. Dondoni, *J. Org. Chem.*, 2010, **75**, 4644–4647.

10 M. Lo Conte, S. Staderini, A. Marra, M. Sanchez-Navarro, B. G. Davis and A. Dondoni, *Chem. Commun.*, 2011, **47**, 11086–11088.

11 R. van Geel, G. J. Pruijn, F. L. van Delft and W. C. Boelens, *Bioconjugate Chem.*, 2012, **23**, 392–398.

12 (*a*) L. Wang, J. M. Xie and P. G. Schultz, *Annu. Rev. Biophys. Biomol. Struct.*, 2006, **35**, 255–249; (*b*) C. C. Liu and P. G. Schultz, *Annu. Rev. Biochem.*, 2010, **79**, 413–444; (*c*) Q. Wang, A. R. Parrish and L. Wang, *Chem. Biol.*, 2009, **16**, 323–336; (*d*) J. W. Chin, *EMBO J.*, 2011, **30**, 2312–2324; (*e*) L. Davis and J. W. Chin, *Nat. Rev. Mol. Cell Biol.*, 2012, **13**, 168–182.

13 (*a*) L. Wang, A. Brock, B. Herberich and P. G. Schultz, *Science*, 2001, **292**, 498–500; (*b*) P. R. Chen, D. Groff, J. T. Guo, W. J. Ou, S. Cellitti, B. H. Geierstanger and P. G. Schultz, *Angew. Chem., Int. Ed.*, 2009, **48**, 4052–4055; (*c*) S. M. Hancock, R. Uprety, A. Deiters and J. W. Chin, *J. Am. Chem. Soc.*, 2010, **132**, 14819–14824; (*d*) T. Mukai, T. Kobayashi, N. Hino, T. Yanagisawa, K. Sakamoto and S. Yokoyama, *Biochem. Biophys. Res. Commun.*, 2008, **371**, 818–822; (*e*) H. Neumann, S. Y. Peak-Chew and J. W. Chin, *Nat. Chem. Biol.*, 2008, **4**, 232–234; (*f*) Y. Huang, W. Wan, W. K. Russell, P. J. Pai, Z. Y. Wang, D. H. Russell and W. S. Liu, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 878–880; (*g*) J. K. Takimoto, N. Dellas, J. P. Noel and L. Wang, *ACS Chem. Biol.*, 2011, **6**, 733; (*h*) S. Greiss and J. W. Chin, *J. Am. Chem. Soc.*, 2011, **133**, 14196–14199.

14 (*a*) Z. Y. Hao, Y. Q. Song, S. X. Lin, M. Y. Yang, Y. J. Liang, J. Wang and P. R. Chen, *Chem. Commun.*, 2011, **47**, 4502–4504; (*b*) Y. M. Li, M. Y. Yang, Y. C. Huang, Y. T. Li, P. R. Chen and L. Liu, *ACS Chem. Biol.*, 2012, **7**, 1015–1022.

15 (*a*) K. S. Gajiwala and S. K. Burley, *J. Mol. Biol.*, 2000, **295**, 605–612; (*b*) W. Z. Hong, W. Jiao, J. Hu, J. Zhang, C. Liu, X. Fu, D. Shen, B. Xia and Z. Y. Chang, *J. Biol. Chem.*, 2005, **280**, 27029–27034.