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α-Vinyl azide–cysteine click coupling reaction enabled bioorthogonal peptide/protein modification†

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α-Alkyl and α-aryl vinyl azides were found to be able to couple with cysteine-derived alkyl thiols chemoselectively under mild conditions, providing the corresponding β-ketosulfides with simultaneous extrusion of N_2 and ammonia. This reaction was developed into an effective chemical platform for peptide and protein modification, which is completely cysteine selective and highly bioorthogonal, that avoids the interference from other native residues. The *in situ* formed ketone group can react specifically with alkoxyamines or hydrazines and therefore can serve as a versatile handle for a second modification. The modification of bovine serum albumin (BSA) with a dansyl fluorescent probe and the labeling of the genetically encoded fluorescent protein YPet-ECFP with biotin have been accomplished successfully through this platform.

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Chemical modifications of peptides or proteins, or alternatively bioconjugations, have been proved and continue to be a powerful means to construct bioconjugates with improved or desired properties and have thus been widely used not only in chemical biology to study cellular processes but also in the medical industry to prepare biopharmaceuticals.[1] Current trends in chemical modification emphasize on chemoselectivity, site-specificity and bioorthogonality.[2] Cysteine, a proteogenic amino acid that is relatively less abundant yet ubiquitously distributed among mammalian proteins, has a number of distinct chemical properties related to the characteristic sulfhydryl group. Collectively, these merits make cysteine arguably the most popular endogenous residue for protein bioconjugation, and a myriad of methods for selective cysteine ligation have been developed over the years.[3] Conventional cysteine modification reactions include disulfide bond formation,[4] S_N2 alkylation,[5] S_NAr (hetero)arylation[6] and the most practiced conjugate addition to an array of electrophilic alkenes and alkynes.[7] Recently, a number of creative methods have appeared, such as transition metal mediated cysteine arylation, alkenation, allylation and borylation,[8] hypervalent iodine and alkynyl dibenzothiophenium reagent mediated cysteine functionalizations,[9] light, strain or proximity promoted thiol–ene/yne reactions,[10] and others.[11] Overall, from the mechanistic perspective, the majority of the aforementioned modification methods involve the coupling of the nucleophilic cysteine thiol group with a particular electrophile under certain conditions, which can potentially be interfered by other biological nucleophiles (Fig. 1a). Disulfide exchange and retro-Michael addition also do harm to the stability, homogeneity and structural integrity of the relevant bioconjugates. On the other hand, thiol ene/yne reactions of inactivated alkenes/alkynes usually proceed with exclusive chemical or site selectivity to give robust products, but their applications are quite restricted probably because of the slow kinetics or the requirement for radical initiators (Fig. 1b).[10] Therefore, there is an unmet need for a chemoselective and bioorthogonal chemical cysteine-modifying protocol that can deliver robust bioconjugates. To this end, we report such a protocol based on an α-vinyl azide–cysteine click coupling reaction that affords stable β-ketosulfide adducts through a radical C–S bond forming event (Fig. 1c).

The reaction of aryl thiol with α-aryl vinyl azide to give a β-ketosulfide derivative was initially noted by Montevecchi and co-workers in 1997.[12] Recently, our group reinvestigated this coupling process and expanded the scope of azides to α-alkyl vinyl azides.[13] This thioether forming transformation takes place smoothly under benign conditions, without the need for extra additives and releasing ammonia and nitrogen as the only by-products. In addition, an aryl thiyl radical initiated radical-chain process was proposed and this process should

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†Electronic supplementary information (ESI) available: Experiment details, characterization of compounds, copies of compound NMR spectra. See DOI: https://doi.org/10.1039/d2qo00736c

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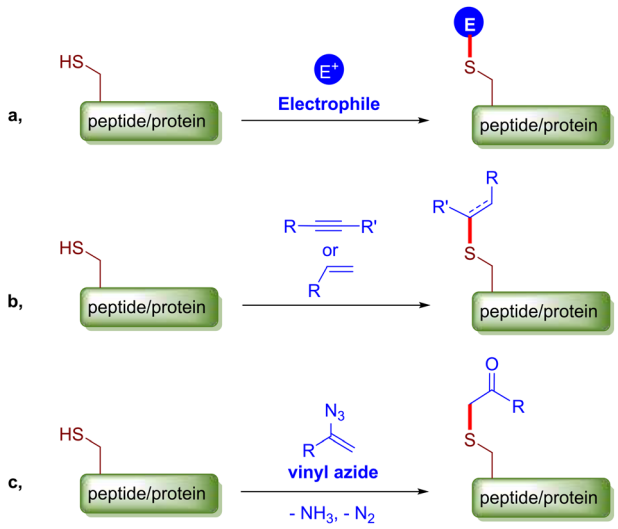

Fig. 1 Chemical modification of a peptide or protein at the cysteine residue.

tolerate aqueous medium due to its radical nature. These features prompted us to envisage that this reaction could evolve into an ideal platform for cysteine modification, based on the assumption that alkyl thiol is also able to participate in this radical event (Fig. 1c). To examine this premise, cysteine-derived alkyl mercaptan **2a** was reacted with α-phenyl vinyl azide **1a** (Table 1). To our delight, when mixed and stirred with 10 fold of **1a** in THF at 40 °C under air, the cysteine derivative **2a** disappeared in 30 min, as shown by TLC, and the desired β-ketosulfide **3aa** was isolated in 77% yield (entry 1). An additional compound was obtained and identified as **4a**, an oxidative homo-coupling product of **2a**. Cysteine containing dipeptide **2b** reacted similarly with **1a** to deliver conjugate **3ab** in 61% yield (entry 2). Furthermore, thioethers **3ac–an** were obtained successfully from the coupling of **1a** with the corresponding dipeptides **2c–n**, indicating the good compatibility of this reaction with various proteogenic side chains such as thioether, hydroxyl, phenol, indole, and primary amide groups (entries 3–14). Tripeptide **2o** with a cysteine residue in the middle was also a good substrate for this coupling reaction, affording adduct **3ao** in 61% isolated yield (entry 15). Vinyl azide **1b**, carrying a strong electron donating pivalamido group at the *para* position of the phenyl ring, reacted quickly with protected dipeptides **2b** and **2l** and tripeptide **2p** to give **3bb**, **3bl** and **3bp**, respectively. The relatively lower yields (30–40%) observed for these three reactions might be attributed to the use of a much less amount (2.0 *vs.* 10 equiv.) of vinyl azide (entries 16–18). With the restoration of the amount of vinyl azide to 10 equiv., the recovery in yields (50% for **3bj** and 61% for **3bk**) was observed for the reactions of **1b** with **2j** and **2k** (entries 19 and 20). Notably, although the reaction time varied broadly from less than 1 h to as long as 8 h, the yields were constantly moderate to good, and in all cases, the dimerization of the related alkyl thiols *via* S–S bond formation was more or less detected, which is a detrimental sideway that competes with the desired C–S bond forming event for the cysteine sulfhydryl group.

After realizing and evaluating the ligation of cysteine derivatives with α-aryl vinyl azides, we then turned to the reaction of the more stable and hence less reactive α-alkyl vinyl azides. (Benzo)imidazole substituted vinyl azides **1c–j** were readily obtained and reacted with **2a** under the above conditions. The reactions did occur, but we encountered considerable difficulty in separating the thioether products from the corresponding vinyl azides through silica gel column chromatography. To avoid this annoying problem, an excess of cysteine **2a** was employed to ensure complete consumption of vinyl azides, and the outcomes are presented in Table 2. Vinyl azides **1c–f**, each carrying an imidazole moiety, all coupled successfully with alkyl thiol **2a** to give rise to the related conjugates **3ca–3fa** in high yields (entries 1–4). The lower reactivity of alkyl vinyl azides was evidenced by the much longer reaction time needed for their full conversions (20–24 h). With their benzoimidazole congeners **1g–j**, even their corresponding adducts **3g–j** were collected in quantitative yields (entries 5–8, 90–99% yield). Two equivalents of **2a** were found to be capable of consuming the alkyl vinyl azide in all instances.

With this robust thioether-forming protocol in hand, our efforts were directed to evaluate its feasibility in selective cysteine modification employing phosphate buffered saline (PBS) as the solvent or cosolvent. Commercially available glutathione (GSH, **2q**), a cysteine-containing natural peptide antioxidant, was used as the model substrate (Table 3). In an open vial, a solution of vinyl azide **1a** (2.0 equiv.) in THF and a solution of glutathione (1.0 equiv.) in PBS (pH 7.4) were mixed and stirred at 40 °C. GSH **2q** disappeared in 4 h and the coupling product **3aq** was isolated in 75% yield through reverse phase column chromatography (entry 1). Without exception, α-aryl vinyl azides **1k–m** were all proven to be competent agents for cysteine ligation, furnishing the corresponding conjugates **3kq–mq** efficiently in longer reaction times (entries 2–4). Aliphatic vinyl azides **1n** and **1i** also demonstrated substantial potential in cysteine ligation (entries 5 and 6). For vinyl azides **1e**, **1f** and **1o–q** that can dissolve well in water, the coupling reactions were conducted in PBS buffer without the use of THF as the co-solvent. For these reactions, the yields were determined from their LC-mass spectra (entries 7–12). In PBS buffer, both imidazolyl vinyl azides **1e** and **1f** underwent this thiyl radical initiated process faster than parallel events in THF–PBS cosolvents, affording **3eq** and **3fq** in 71% and 88% LC-MS yields, respectively (entries 7 and 8). The benzoimidazolium functionalized homologues **1o** and **1p** also efficiently modified GSH at cysteine with high yields in PBS (entries 9 and 10). Glycopeptide **3qq** was constructed *via* the modification of GSH with **1q**, a glucoside functionalized with a vinyl azide moiety at the anomeric position (entry 11). As shown in entry 12, the use of water to replace PBS as the reaction medium is feasible as well, albeit with a slight decrease in the yield. Masking the thiol group with trityl protection resulted in no reaction with **1a** under the standard conditions even if stirred in air for 24 hours, indicating the inertness of the free

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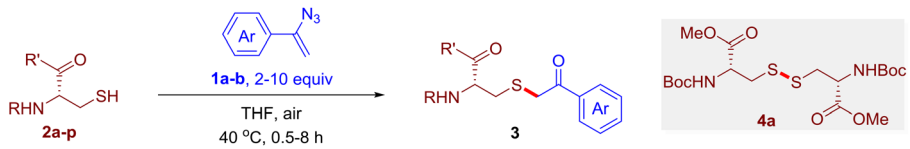
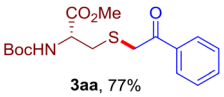
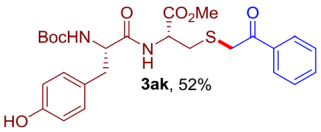
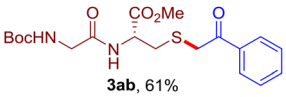
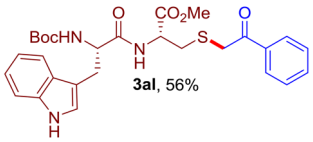
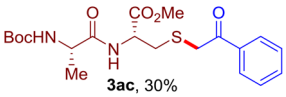
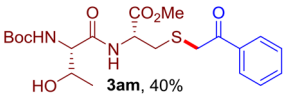
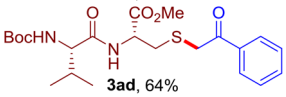
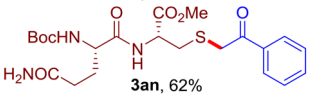
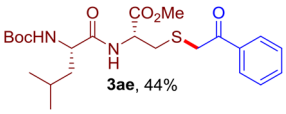
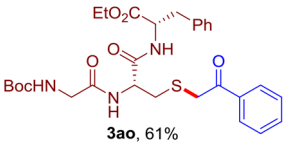
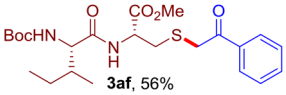
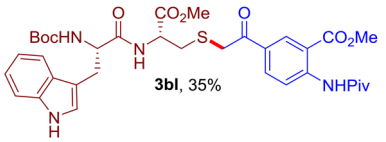
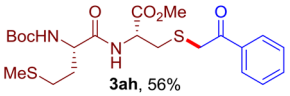
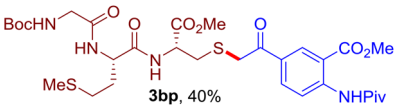
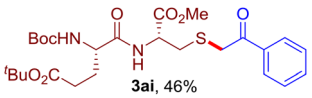
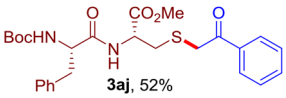
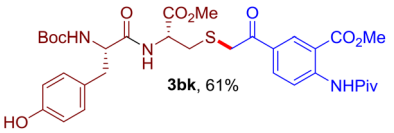
Table 1 Coupling reaction of cysteine derivatives with α-aryl vinyl azides[a]



Entry	1 (equiv.)	Time	Adduct (yield)	Entry	1 (equiv.)	Time	Adduct (yield)
1	**1a** (10)	30 min	 **3aa**, 77%	11	**1a** (10)	8 h	 **3ak**, 52%
2	**1a** (10)	50 min	 **3ab**, 61%	12	**1a** (10)	40 min	 **3al**, 56%
3	**1a** (10)	6 h	 **3ac**, 30%	13	**1a** (10)	40 min	 **3am**, 40%
4	**1a** (2.0)	40 min	 **3ad**, 64%	14	**1a** (10)	8 h	 **3an**, 62%
5	**1a** (10)	5 h	 **3ae**, 44%	15	**1a** (10)	8 h	 **3ao**, 61%
6	**1a** (10)	4 h	 **3af**, 56%	16	**1b** (2.0)	1.0 h	 **3bb**, 30%
7	**1a** (10)	3 h	 **3ag**, 54%	17	**1b** (2.0)	30 min	 **3bl**, 35%
8	**1a** (10)	8 h	 **3ah**, 56%	18	**1b** (2.0)	50 min	 **3bp**, 40%
9	**1a** (10)	8 h	 **3ai**, 46%	19	**1b** (10)	1.0 h	 **3bj**, 50%
10	**1a** (10)	3 h	 **3aj**, 52%	20	**1b** (10)	30 min	 **3bk**, 61%

[a] Conditions: **2** (0.2 mmol, 1.0 equiv.), **1** (2.0 or 10 equiv.), THF (3 mL), 40 °C, 0.5–8 h in air; isolated yields are reported.

amino and carboxyl groups toward this vinyl azide. At this point, the effectiveness of this reaction for selective chemical modification at cysteine has been firmly established. It is worth noting that in 2017, Chiba and colleagues realized site-specific modification at the cysteine residue in peptides and proteins using 2-azidoacrylates.[14] Although 2-azidoacrylates also fall in the class of vinyl azides, they serve as Michael acceptors and undergo 1,4-addition with free cysteinyl thiols, a mechanism totally different from the radical chain process for the present method.

To further investigate the applicability of the above system in protein chemical ligation, dansylated vinyl azide **1r** was obtained and incubated with bovine serum albumin (BSA, **2r**, 68 kDa) at 40 °C for 24 in air. In two solvent mixtures, *i.e.* 1/1

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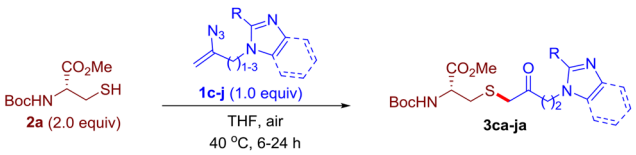
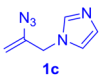
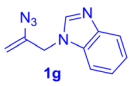
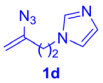
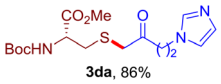
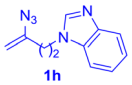
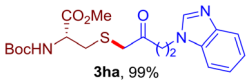
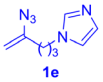
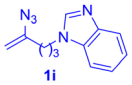
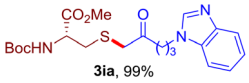
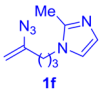
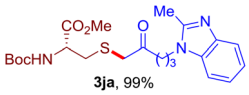
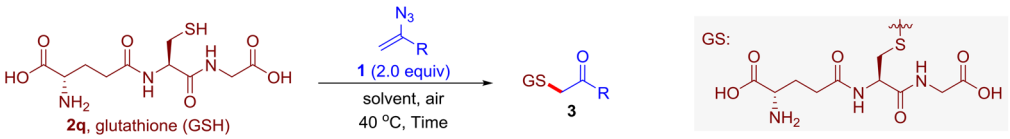
Table 2 Coupling reaction of BocNHCysOMe with α-alkyl vinyl azides[a]

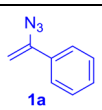
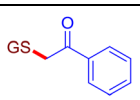
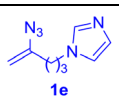
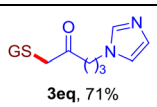
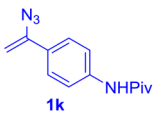
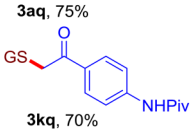
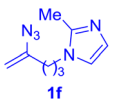
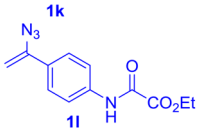
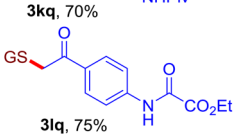
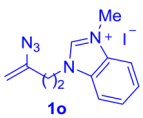
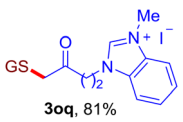
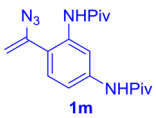
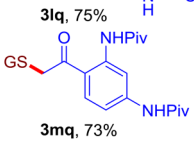
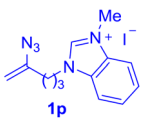
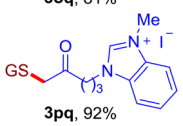
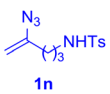
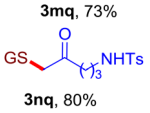
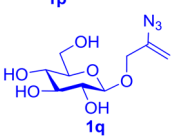
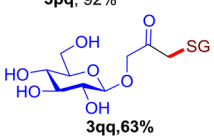
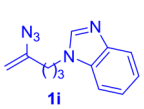
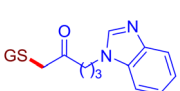


Entry	Vinyl azide	Time	Adduct, yield	Entry	Vinyl azide	Time	Adduct, yield
1	 1c	24 h	 3ca, 78%	5	 1g	6 h	 3ga, 90%
2	 1d	20 h	 3da, 86%	6	 1h	24 h	 3ha, 99%
3	 1e	20 h	 3ea, 90%	7	 1i	24 h	 3ia, 99%
4	 1f	24 h	 3fa, 80%	8	 1j	8 h	 3ja, 99%

[a] Conditions: **2a** (0.4 mmol, 2.0 equiv.), **1** (0.2 mmol, 1.0 equiv.), THF (3 mL), 40 °C, 6–24 h in air; isolated yields are reported.

Table 3 Bioconjugation of glutathione with α-vinyl azides[a]



Entry	Vinyl azide	Solvent Time	Adduct, yield	Entry	Vinyl azide	Solvent Time	Adduct, yield
1[b]	 1a	PBS/THF (1/1, v/v) 4.0 h	 3aq, 75%	7[c]	 1e	PBS 8.0 h	 3eq, 71%
2[b]	 1k	PBS/THF (1/1, v/v) 24 h	 3kq, 70%	8[c]	 1f	PBS 8.0 h	 3fq, 88%
3[b]	 1l	PBS/THF (1/1, v/v) 24 h	 3lq, 75%	9[c]	 1o	PBS 8.0 h	 3oq, 81%
4[b]	 1m	PBS/THF (1/1, v/v) 24 h	 3mq, 73%	10[c]	 1p	PBS 8.0 h	 3pq, 92%
5[b]	 1n	PBS/THF (1/1, v/v) 24 h	 3nq, 80%	11[c]	 1q	PBS 8.0 h	 3qq,63%
6[b]	 1i	PBS/THF (1/1, v/v) 8.0 h	 3iq, 78%	12[c]	1p	H_2O (pH 7.0) 30 h	3pq, 68%

[a] Unless otherwise noted, all reactions were carried out with 0.2 mmol GSH **2q** (1.0 equiv.) and 0.4 mmol vinyl azide **1** (2.0 equiv.) at 40 °C, using 2.0 mL PBS (pH 7.4) as the solvent or cosolvent. [b] Isolated yields are reported. [c] LC-MS yields are reported.

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and 1/3 EtOH/PBS (v/v), two parallel experiments were conducted, respectively (Fig. 2a). SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis of these reactions was carried out and the results are shown in Fig. 2b. For each reaction sample, a strong fluorescent-emission band appears around 70 kDa in the fluorescence image, which can also be visualized through Coomassie blue staining (lanes R1 and R2). As a comparison, no such band is observed in the fluorescence image but showed up upon staining for the control experiment for BSA (lane BSA). As expected, no protein band appears for the control with vinyl azide **1r** (lane **1r**). The fluorescent bands at the bottom correspond to the dansylated reagent **1r**. These experimental outcomes clearly demonstrate that BSA has been efficiently labeled by the dansyl fluorescent vinyl azide. And we claim with confidence that the current methodology can serve as an effective chemical tool for selective protein modification.

The validity of this biocompatible chemical tool in protein modification was further confirmed through the successful decoration of nickel NTA agarose beads (**Ni-NTA**) with a fluorescent protein (FP) carrying three fluorophores (Fig. 3a). Genetically encoded FP **YPet-ECFP** (**2s**),[15] a 6×His tagged bioconjugate comprising a yellow fluorescent protein (YPet) and an enhanced cyan fluorescent protein (ECFP) linked *via* a peptide CPKESCNLFVLKD that bears two cysteine residues, was incubated with excess biotin-derived vinyl azide ^{Biotin}**1s** at 37 °C under physiological reaction conditions for 40 h. This reaction mixture, presumably containing the biotinylated FP **3ss**, was then shaken with the Ni-NTA resin for 1 h and the biotin-grafted nickel particles **Ni-3ss** were obtained through

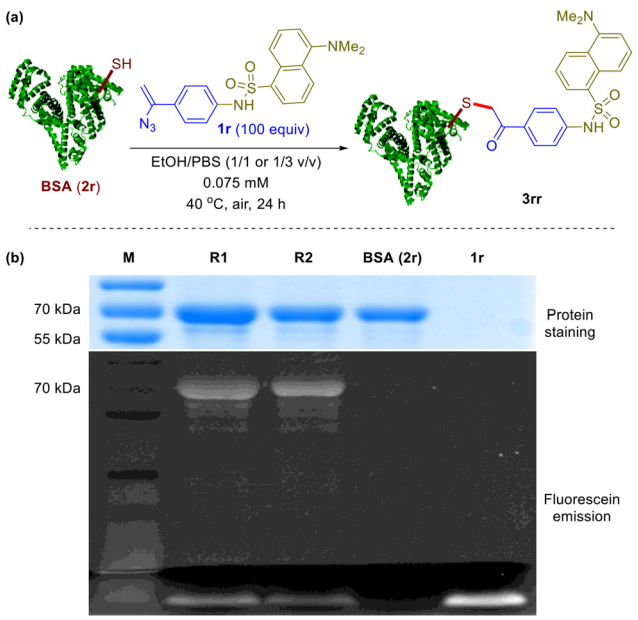

Fig. 2 Labeling of BSA with a dansyl fluorescent probe: (a) coupling reaction of BSA with **1r**; and (b) SDS-PAGE analysis (**M**: protein marker, **R1**: reaction in 1/1 EtOH/PBS (v/v), **R2**: reaction in 1/3 EtOH/PBS (v/v), **BSA**: control experiment without **1r**, **1r**: control experiment without BSA.

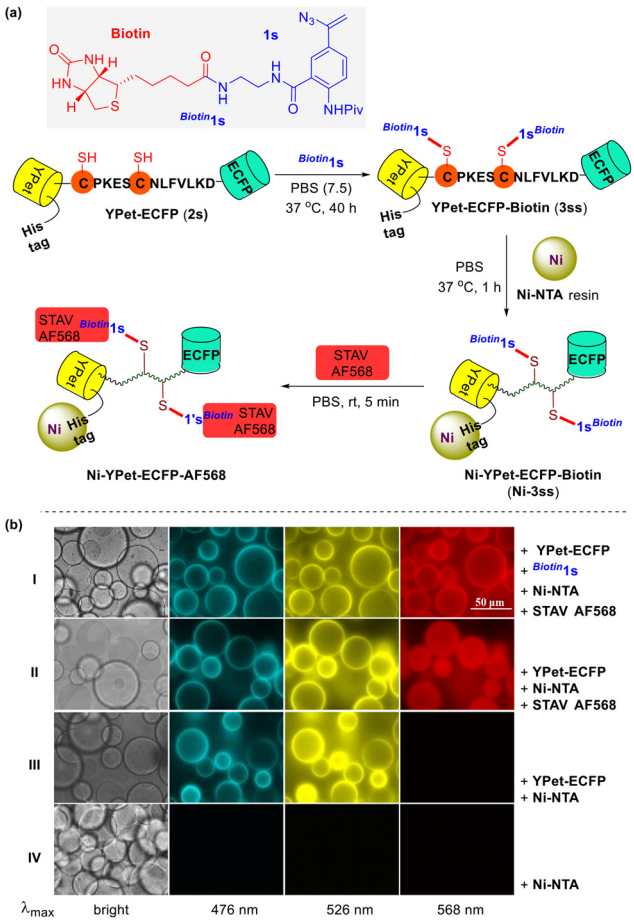

Fig. 3 (a) The labeling of Ni-NTA resin with 6×His-tagged FP conjugate **YPet-ECFP** and streptavidin-Alex Flor 568 dye **STAV AF568**; and (b) fluorescence imaging of labeled, unlabeled Ni-NTA resin and control samples.

subsequent centrifugation and washing. A 30 min treatment of these freshly made biotin-modified nickel beads with the dye Alexa Fluor® 568 streptavidin (**STAV AF568**) in PBS completed the installment of a third chromophore. After the removal of the unbound streptavidin dye molecules, the thus prepared nickel complex **Ni-YPet-ECFP-AF568** was suspended in PBS and visualized through fluorescence microscopy (Fig. 3b). The bright annuli in both ECFP and YPet emission images suggest the successful attachment of **YPet-ECFP** (**2s**) onto the surface of the Ni-coated NTA resin beads; the identical but red annuli in the Alex Flor 568 emission microscopy spectrum indicate an efficient labeling of the Ni-NTA resin surface with the streptavidin dye **STAV AF568** (Fig. 3b row I). These observations confirmed the successful installation of biotin segments on the nickel beads, which was accomplished *via* the coupling reaction of **YPet-ECFP** (**2s**) with biotin-conjugated vinyl azide ^{Biotin}**1s**. Following the same protocol but omitting the azide agent ^{Biotin}**1s**, a control sample was prepared and subjected to fluorescence imaging too (Fig. 3b row II). The green and yellow rings were present in its ECFP and YPet emissions, just like those in row I for **Ni-YPet-ECFP-AF568**. In contrast, in its Alex

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Fluor 568 emission spectrum, the otherwise red rings now become invisible but with even distribution of the red fluorescence in the beads. This sharp difference might be attributed to the staining of the whole Ni-NTA resin beads with the dye **STAV AF568** simply *via* kinetic diffusion when no specific biotin–streptavidin conjugation took place.

Conclusions

In summary, we have disclosed a coupling reaction of α-vinyl azides with cysteine-based alkyl mercaptans which gives stable β-ketothioethers. This reaction possesses several distinct merits that potentially make it a magnificent chemical platform for protein bioconjugation: (1) the intrinsic radical nature makes it completely feasible for use in aqueous solvent systems; (2) the non-electrophilic vinyl azide used prevents the attack from biological nucleophiles, especially other native amino acid side chains; (3) there is no need for extra reagents and the harmlessness of the by-products formed show its excellent biocompatibility; and moreover (4) the *in situ* formed stable β-ketothioether linkage might serve as a second ligating site specific for primary amine, hydrazine and alkoxylamine. These merits meet the characteristics of click reactions and we therefore would like to name this C–S bond forming process as vinyl azide–cysteine (VA–Cys) click coupling.[16] Using this click reaction, the chemical installation of β-ketothioether on glutathione at cysteine has been realized with an array of α-vinyl azides under (quasi)physiological conditions. The effective fluorescent labelling of BSA with a dansylated α-vinyl azide shows its effectiveness in protein modification. Its avidity is confirmed unequivocally through the successful introduction of a biotin functionality to an engineered FP **YPet-ECFP**. Based on these solid experimental data, we can claim confidently that a useful chemical platform for selective and bioorthogonal protein modification at the cysteine residue has been established. Further studies aiming to extend its application scenarios are ongoing in our laboratory.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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