Precise installation of diazo-tagged side-chains on proteins to enable in vitro and in-cell site-specific labelling

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ABSTRACT: The chemistry of diazocompounds has generated a huge breadth of applications in the field of organic synthesis. Their versatility combined with their tuneable reactivity, stability and chemoselectivity makes diazo compounds desirable reagents for chemical biologists. Here, we describe a method for the precise installation of diazo-handles on proteins and antibodies in a mild and specific approach. Subsequent 1,3-cycloaddition reactions with strained alkynes enable both bioimaging through an in-cell 'click' reaction and probing of the cysteine proteome in cell lysates. The selectivity and efficiency of these processes makes these suitable reagents for chemical biology studies.

Diazo-containing natural products have been shown to exhibit antitumor and antimicrobial activities at nanomolar–picomolar concentrations, which makes diazo compounds valuable from a biological perspective.[1,2] Already, diazo compounds have been used to investigate mechanisms of DNA cleavage in vitro,[3,4] in which the diazo moiety is likely the active pharmacophore directly involved in the damage pathway.[5,6] Some natural diazo-amino acids isolated from *Streptomyces* cultures have also found application in medicine. Indeed, the broadly active glutamine antagonist 6-diazo-5-oxo-*L*-norleucine entered early phase I/II clinical trials based on its activity against carcinomas, lymphomas and Hodgkin's disease.[7]

Although commonly observed in small natural products derived from microorganisms, the diazo moiety is non-existent in higher organisms. This feature enables their use as bioorthogonal probes.[8] The number of functional groups with biocompatibility as chemical reporters is still limited. In this context, azido groups are dominant and widely used in chemical biology because of their chemical stability and excellent reactivity with strained alkynes[9] and non-strained alkynes when catalysed by copper.[10,11] Similarly to azides, the diazo group can undergo uncatalysed Huisgen addition reactions with unstrained dipolarophiles, such as terminal alkenes and alkynes.[12] The diazo group has even better characteristics than those of analogous azido groups, such as improved kinetics for cycloaddition reactions[13] and a greater range of reactivity in a variety of thermal, photochemical and metal-catalysed reactions. Insertion of diazocarbonyl compounds into C–H and X–H (X = N, O, S, P, Se) bonds, cyclopropanation reactions, dipolar cycloaddition reactions, ylide formation, alkylations, ring expansions and the Wolff rearrangement are examples of these transformations.[14,15] In fact, functional groups that perform as broad as range of different reactions as diazo groups are relatively rare. Moreover, the diazo group is more electron-rich and has an increased HOMO energy relative to the analogous azido, which displays superior reactivity in normal-electron-demand cycloaddition reactions with electron-deficient dipolarophiles.[16] However, and as reported by Reines et al. diazoketones, diazoesters and diazoamides are electron-deficient dipoles and, therefore, tipically undergo inverse-electron demand cycloadditions.[13] Lastly, the tolerance of stabilised diazo groups towards cellular metabolism is also similar to that of azido groups.[17]

The earliest application of diazo reagents for covalent modification of proteins was the direct esterification of carboxylic acids. Diazomethane and stabilised compounds such as α-diazoacetamides were used to label different proteins and nucleic acids to study their structural and functional aspects.[8,18] New applications of diazo compounds in chemical biology aim to use their unique reactivity to access ligations between biomolecules that cannot be obtained through other approaches. For this, a robust and site-precise method for the installation of diazo groups on proteins and antibodies surfaces still requires development. So far, there are two non-specific reagents described for this purpose (Fig. 1a).

The diazoacylating reagent **1** (Fig. 1a) was first reported in 1993 by Badet and co-workers based on *N*-hydroxysuccinimide (NHS) esters.[19] This reagent was later used to modify several small molecules and biomolecules, which included bovine serum albumin.[20] Leeper and co-workers reacted α-diazo NHS ester **1** with lysine residues on lysozyme (6 Lys, no free Cys, 4 disulfides).[21] Following the incorporation of the diazo functionality, a fluorescein complex equipped with a tetramethoxydibenzocyclooctyne TMDIBO-strained cyclooctyne was reacted with the diazo-protein by means of a [3+2] cycloaddition reaction. NHS esters usually react with any exposed primary amine, such as the α-amino group of *N*-terminal amino acid and ε-amino groups of lysines present in a protein leading to heterogenous conjugates. More recently, Gaunt and co-workers demonstrated the use of hypervalent iodine reagents **2** (Fig. 1a) to target methionine residues to install an electrophilic diazo group on peptides and small proteins.[22] Once incorporated into the target biomolecule, the diazo handle was removed by reduction with a Hantzch ester to generate trialkylsulfonium motifs, as exemplified for

peptides and small protein thioredoxin. Although remarkable in terms of methionine modification, all the peptides and proteins used had no free Cys (Cys were previously protected with maleimides or were present as non-reactive disulphide bridges). Moreover, the approach uses additives such as thiourea, TEMPO, formic acid, and non-biological conditions that can limit the scope of therapeutic proteins/antibodies that can be modified.

Our group has recently reported the design of a new class of Michael-acceptor reagents, carbonylacrylic derivatives, that undergo very rapid, chemoselective thiol addition with Cys residues on proteins and antibodies.[23,24] In an attempt to provide a robust reagent capable of site-selectively introducing a diazo handle on protein and antibodies, we developed carbonylacrylic derivative α,β -unsaturated diazoketone **3** (Fig. 1c),[15] which reacts in high yields with Cys under biological conditions at near neutral pH. The afforded diazo-conjugates showed resistance to degradation in aqueous media and did not react with natural thiols, such as glutathione (GSH), which opens opportunities for further bioorthogonal functionalisation in chemical biology.

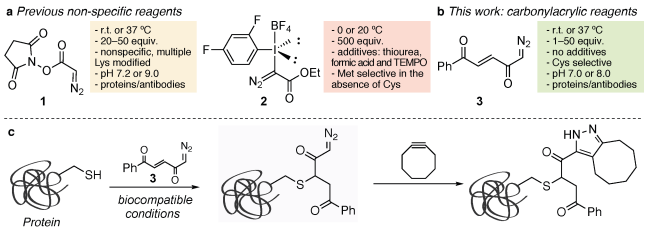

Figure 1. The development of a cysteine (Cys)-selective reagent for the incorporation of diazo handle on biomolecules. (**a**) Reagents described for the modification of peptides and proteins (**1** and **2**) and (**b**) our proposed diazocarbonylacrylic reagent (**3**). (**c**) Once introduced into target biomolecules, the diazo group can be tagged with a wide range of reactive probes. In this case, we used 1,3-dipolar cycloadditions with strained alkynes.

RESULTS AND DISCUSSION

We started our work by preparing diazocarbonylacrylic reagent **3** by means of a Horner–Wadsworth–Emmons (HWE) olefination reaction between diazophosphonate **4**[25] and the commercially available aldehyde phenylglyoxal **5**. Different HWE conditions and bases were evaluated for the formation of α,β-unsaturated diazoketone **3** (Table S1). By using NaH[25], the desired *E*-olefin was obtained in low to moderate yields (Table S1, Entries 2, 3, 6, 7 and 16). The best results were obtained with *N,N*-diisopropylethylamine (DIPEA) as base and lithium chloride as additive. In this case, the isolated yield was 78% with 3:1 selectivity for the desired *E* isomer that can be easily separated by flash chromatography (Table S1, Entry 17). Importantly, diazo **3** can be stored at –20 °C for 12 months in the absence of light without degrading. Next, we evaluated the reaction between the *tert*-butyloxycarbonyl (Boc)/benzyloxycarbonyl (Cbz)-protected amino acid Cys-methyl ester with stoichiometric amounts of diazocarbonylacrylic **3**. The reaction proceeded smoothly in DMF and was complete in < 10 min at room temperature to give adducts **7a**/**7b** in 90% yield. The same result was obtained with a 30% DMF solution in sodium phosphate buffer (NaP_i pH 8.0, 50 mM) as solvent in a reaction open to the air (Fig. 2). The stability of the Cys-adduct in DMF/NaP_i buffer (pH 7.4 and 8.0, 50 mM) was evaluated by liquid chromatography-mass spectrometry (LC-MS) analysis after 24 h at room temperature. No reaction was observed after incubation with 1.5 equiv. of GSH (Fig. **2c** and Fig. S1a–1d). Importantly, this result shows the diazo group remained intact in the presence of reduced GSH, which corroborates previous reports in the literature.[13] The reactivity of diazoketones **7b** and **10** with equimolar amounts of commercially available alkyne (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (**8**) was then confirmed (Fig. 2d and 2e). The cycloaddition was complete after 24 h at room temperature with a mixture of DMF/NaP_i buffer (pH 8.0, 50 mM). Pyrazoles **9** and **11** were obtained in 70 and 99% yield, respectively. In the case of diazoacetophenone **10**, the starting material was fully converted (>99%) into cycloaddition product **11** and no by-products were observed (Fig. 2f and Fig. S2).

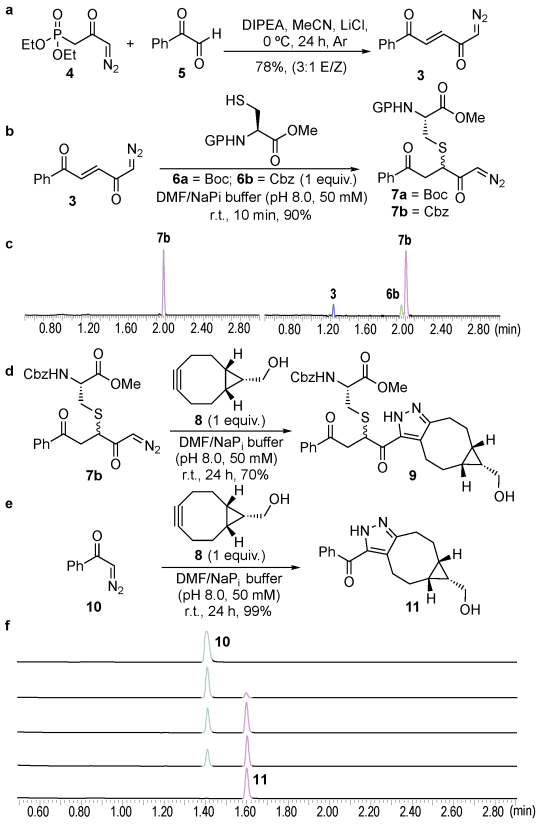

Figure 2. Diazocarbonylacrylic for Cys modification. (**a**) Preparation of α,β-unsaturated diazoketone **3** from diazophosphonate **4** and commercially available phenylglyoxal **5**. (**b**) Equimolar reaction of **3** with *N*-Boc-Cys-OMe **6a** and *N*-Cbz-Cys-OMe **6b** provides diazo thioether product **7a**/**7b** in 90 and 88% yield, respectively, after 10 min. (**c**) Stability of **7b** in the presence of reduced GSH by HPLC after 24 h at room temperature (NaP_i buffer pH 7.4, 50 mM). In this case, 7% of **3** and **6b** is observed and no reaction of the diazo group with GSH was detected (analysis at pH 8 and Full details in the Fig. S1). (**d** and **e**) Cycloaddition reaction of **7b** and **10** with strained alkyne **8.** (**f**) Reaction progress from **10** to **11**. RT = room temperature, Ar = Argon.

This well-known enhanced reactivity of strained alkynes[26] was corroborated by quantum mechanical calculations (Fig. 3). Thus, the lower activation barriers calculated for the cycloadditions between strained bicyclononyne (**8'**) and dipoles diazoacetone (**7'**) and diazoacetophenone (**10**) ($\Delta G^{\ddagger}$ = 27.0 and 26.5 kcal mol^{-1}, respectively), revealed a much higher reactivity in comparison to the same reaction with unstrained dimethylacetylene (**A**) ($\Delta G^{\ddagger}$ = 41.3 and 41.5 kcal mol^{-1}, respectively, see SI). Analogously, cycloadditions

between **7'** and **10** with strained dibenzocyclooctyne (DBCO) **12'** (see experiments below) showed much lower calculated activation barriers ($\Delta G^{\ddagger}$ = 25.8 and 25.9 kcal mol^{-1}, respectively) than those for the reactions with linear diphenylacetylene (**B**) ($\Delta G^{\ddagger}$ = 39.7 and 39.0 kcal mol^{-1}, respectively, SI). Such billion- to nearly trillion-fold acceleration makes the reaction to proceed at room temperature within 24 h, as corroborated experimentally.

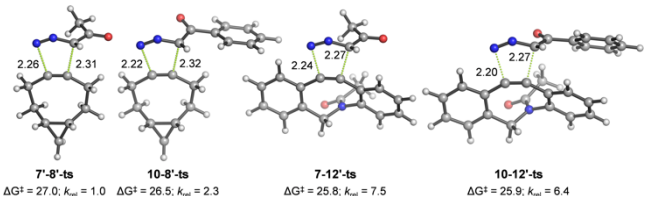

Figure 3. Lowest-energy structures calculated with PCM(H_2O)/M06-2X/6-31+G(d,p) for the 1,3-dipolar cycloaddition reactions between diazo-compounds **7'**, and **10** with model strained alkynes **8'** and **12'**. Distances are given in angstrom (Å) and free energies (ΔG) in kcal mol^{-1}. Relative kinetic rate constants (k_{rel}) were derived from the corresponding activation free energies ($\Delta G^{\ddagger}$) using the Eyring equation at 298 K.

Based on the calculated Frontier Molecular Orbitals (FMO, see ESI), diazoketones are electron-deficient reagents and undergo inverse-electron demand (IED) cycloadditions with either linear or strained alkynes. The same reactivity was previously reported for the cycloaddition of diazoacetophenone and strained cyclooctyne DIBONE.[13] However, despite the $LUMO_{diazo\text{-}compound} - HOMO_{alkyne}$ energy gap being the smallest calculated one, the more appropriate symmetry of the LUMO+1 and LUMO+2 orbitals of diazoacetone (i.e. having bigger coefficients at the diazo carbon atom), which lead to effectively larger HOMO–LUMO energy gaps, suggests that diazoketones are ambiphilic dipoles with no clear preference for NED (Normal Electronic Demand) or IED (Inverse Electron Demand) pathways. The inadequacy of simple FMO energies to quantitatively predict the outcome of these strain-promoted cycloadditions is reflected by the fact that the ΔE_{IED} ($LUMO_{diazo\text{-}compound} - HOMO_{alkyne}$) energy gaps calculated for the very slow reactions between **7'** and **10** with linear diphenylacetylene (**B**) are *smaller* (ΔE_{IED} = 156.8 and 144.8 kcal mol^{-1}) than those calculated for the much faster reactions between the same dipoles and the analogous strained alkyne **12'** (ΔE_{IED} = 158.9 and 146.9 kcal mol^{-1}).

On the other hand, it is widely accepted that the enormous acceleration observed for strain-promoted cycloadditions lies on the much lower energy needed to distort the strained alkynes into the geometry of the transition state (i.e. reduced distortion energies) compared to the linear alkyne analogues (SI for the calculated values).[27]

After this new class of Cys-specific diazo reagents had been prepared, we explored their use for bioconjugation on different proteins (Fig. 4a). Diazocarbonylacrylic **3** was initially evaluated for labelling the engineered version of ubiquitin Ub-K63C.[28] This protein contains a solvent-exposed Cys residue at position 63. Complete conversion (>95%) into the expected product with 25 equiv. of **3** at room temperature after shaking for 3 h was confirmed by LC-MS analysis (Fig. 4b). With the engineered variant of the C2A domain of synaptotagmin-I (C2Am), a protein used as an apoptosis imaging agent that contains a free Cys at position 95, we found that 25 equiv. of **3** were sufficient to obtain full conversion (>95%) into the expected product after 1 h at room temperature (Fig. 4c). Annexin V, which is another apoptosis imaging agent,[29] has a hindered Cys at position 316. The protein was converted completely into the expected product (>95%) with 50 equiv. of **3** after 1 h reaction at 37 °C (Fig. 4d). Chemical controls that involved blocking annexin V-Cys 316 with Ellman's reagent prior to reaction with **3** showed complete selectivity for Cys and no cross-reactivity with Lys (Fig. S13–15). These results are in complete agreement with the previous data obtained for carbonylacrylic reagents.[23] In the case of an engineered human albumin variant V0354, a protein used for drug delivery applications,[30,31] 5 equivalents were used to obtain full conversion into the expected product after 2 h at room temperature (Fig 3e). These results indicate that the required amount of **3** is dependent on the reactivity, chemical microenvironment and local availability of the Cys residues). The Cys-selectivity of diazocarbonylacrylic reagent **3** was corroborated for C2Am by using trypsin digestion followed by peptide mapping with mass spectrometry (Fig. 4f and Fig. S16). In addition, circular dichroism (CD) analysis of albumin and modified albumin-**3** showed no alterations in the secondary structural content (Fig. 4g and Fig. S17), which reflects the mildness and efficiency of the conjugation process (for CD analysis of C2Am and C2Am-**3**, see Fig. S18).

Once introduced into target biomolecules, the diazo group was confirmed by using highly selective strain-promoted [3+2] cycloaddition reactions (Fig. 5a). Depending on the protein, 100–700 equiv. of strained alkyne (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (**8**) was applied. Full conversion into the expected pyrazole was observed after 3–18 h. In the case of diazo-ubiquitin, 700 equiv. of **8** was used and the reaction was complete after 18 h at room temperature in sodium phosphate buffer, pH 8.0, 50 mM (Fig. 5b). The same conditions were applied to C2Am to obtain >95% conversion after reaction for 6 h (Fig. 5c). Diazo-Annexin V can be fully converted with a reduced amount of **8** (100 equiv.) after reaction for 18 h at room temperature (Fig. 5d). With 700 equiv., full conversion of annexin V can be obtained after just 3 h (Fig. S23). For albumin, the best conditions were 100 equivalents and 18 h at room temperature. It is important to mention that all of these reactions can be done in one pot, which obviates the need to purify the diazo-protein before cycloaddition with the cyclooctyne. For instance, C2Am can be first reduced with TCEP (20 equiv., RT, 30 min,), conjugated with **3** (25 equiv., RT, 1–2 h,) and then reacted with cyclootyne **8** (700 equiv., RT, 18 h) without any purification step (the same results were obtained by purifying each step with a spin desalting column or buffer exchange columns).

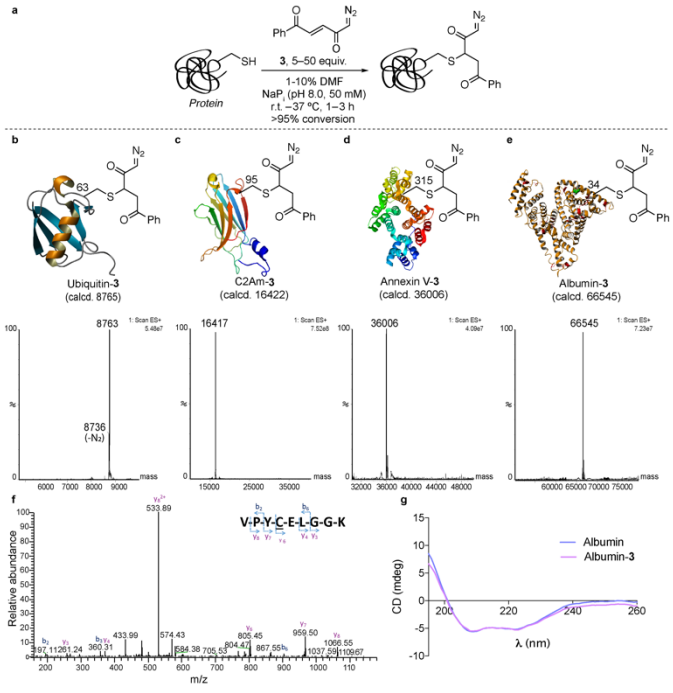

Figure 4. Protein scope for Cys bioconjugation. (**a**) Schematic of the reaction of Cys-tagged proteins with **3**. (**b–e**) Electrospray ionization (SI)-MS spectra of conjugates (**b**) ubiquitin-**3**, (**c**) C2Am-**3** (**d**) annexin V-**3** and (**e**) albumin-**3**. Full spectra and details are presented in the SI. (**f**) MS/MS spectrum of the m/z 583.27 doubly charged ion of tryptic peptide YPY**C**ELGGK, which contains the diazo modification at the Cys residue 95 on C2Am. (**g**) CD of albumin and albumin-**3**.

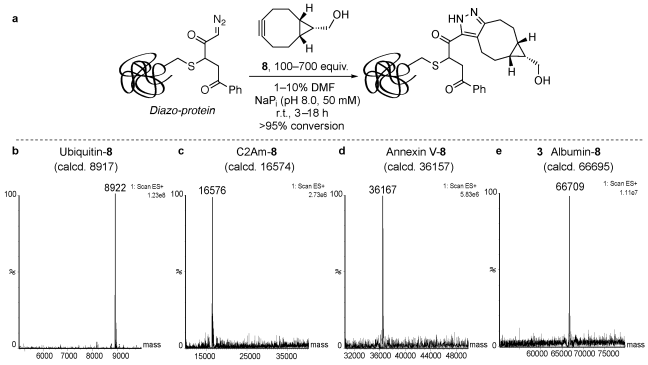

Figure 5. Confirming the diazo on protein. (**a**) Schematic of the cycloaddition reaction with **8** (**b–e**) ESI-MS spectra of conjugates (**b**) ubiquitin-**8** (700 equiv., 18 h), (**c**) C2Am-**8** (700 equiv., 6 h) (**d**) Annexin V-**8** (100 equiv., 18 h or 700 equiv., 3 h) and (**e**) Albumin-**8** (100 equiv., 18 h). Full spectra and details are presented in Fig. S19–23.

Encouraged by the simplicity of the method and the stability of the protein diazo-conjugates, we evaluated the potential application of diazocarbonylacrylic reagent **3** to modify Cys-tagged antibodies, specifically the smaller recombinant fragment nanobody 2Rb17c, an internalising antibody with one engineered Cys targeting the Her2 antigen.[32] Initial treatment with TCEP (20–50 equiv., 1 h, room temperature) afforded the free surface-exposed Cys at position 17 (Fig. 5a) in >95% conversion. Complete conversion into the Cys-tagged diazo-nanobody was then obtained with 10 equiv. of **3** after 3 h at room temperature in sodium phosphate buffer (pH 8.0, 50 mM NaP_i) in a reaction open to the air (Fig. 6a). Deconvoluted mass spectra of the site-selectively modified antibody is shown in Fig. 6b and Fig. S25, and indicates the starting material was fully consumed, and a new diazo-product was formed with a precisely installed modification at the intended Cys residue. The diazo group showed total stability in sodium phosphate buffer after incubation with human plasma for 24 h at room temperature (Fig. S26). Importantly, the diazo-nanobody can be stored for 4 months at –20 °C without degrading (Fig. S27). Next, we decided to produce fluorescently labelled antibodies by means of 1,3-dipolar cycloaddition reactions with strained alkynes. Pleasingly, the reaction proceeded to completion in the presence of 100 equiv. of DBCO-Cy5 click dye **12** after 18 h at room temperature to give a well-defined, fluorescently labelled antibody conjugate (Fig. 6c and 6d). SDS-PAGE analysis of the native and fluorescent nanobody-Cy5 conjugate is described in Fig. S28.

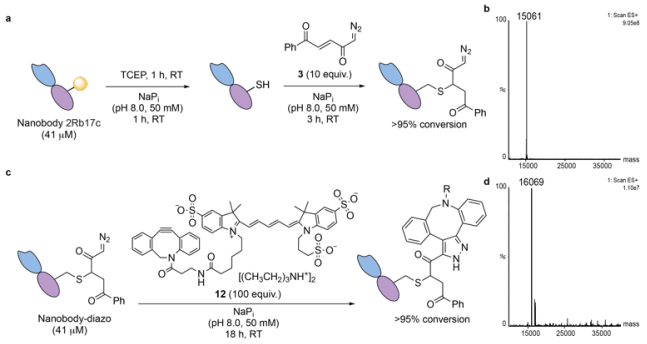

Figure 6. Production of diazo-antibodies for click chemistry. (**a**) ESI ionization-MS spectra of diazo-conjugate. Full spectra and details are presented in the SI. (**c**) Diazo-nanobody 1,3-dipolar cycloaddition reaction with DBCO-Cy5. (**d**) ESI-MS spectra of fluorescent nanobody-conjugate that shows all the starting material was converted into the expected pyrazole product.

Next, we decided to demonstrate the utility of our diazo-nanobody conjugate for bioimaging by means of an in-cell 'click' reaction (Fig. 7). Live-cell microscopy was performed with SKBR3 cells that express high levels of HER2 receptors in contrast to MCF-7 cells that express low levels of this receptor as a control. The cells were initially treated with the diazo-nanobody for 4 h at 37 °C followed by incubation with (DBCO)-Cy5 dye for an additional 1 h at the same temperature. After washout of the unbound dye, the cells were immediately imaged with a Leica SP5 inverted confocal microscope. Fluorescence intensity appeared greater inside the cells pre-treated with 2Rb17c-**3** than cells treated with dye alone. The same results were observed when analysing the images of low-expressing MCF-7 cells; i.e. no fluorescence staining was detected. This experiment demonstrates the retained capability of the modified nanobody to bind to the HER2 receptor and the effectiveness of the diazo group for in-cell 'click' reactions in an analogous manner to the commonly used azide functionality. Further experiments also suggested that the click reaction occurred inside the cell upon nanobody internalisation (Fig. S29 and 30).

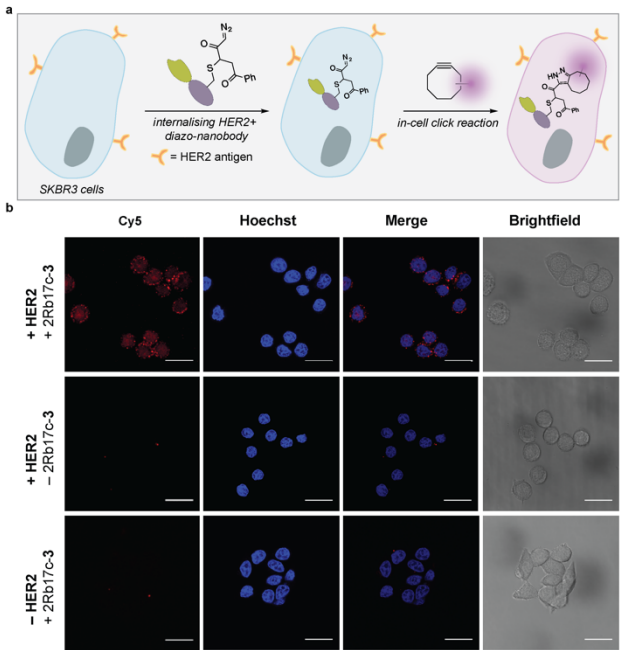

Figure 7. (**a**) Click imaging of HER2 targeting nanobody 2rb17c. (**b**)Confocal fluorescence images of SKBR3 cells that express high levels of HER2+ incubated with or without 2Rb17c-**3** conjugate then incubated with DBCO-Cy5 **12** click dye. Images show Cy5, Hoechst-33342 nuclear stain and merge. Scale bar: 25 μm.

With the strategy validated on protein/antibody models in vitro and in live cells, we next evaluated the feasibility of the diazocompound as probe for the detection of cysteines in cell lysates. For this, we tested the diazoketone **3** as well as a the relatively similar diazoester **13** for comparison (Fig. 8). The activation barriers calculated for the cycloadditions between model strained bicyclononyne (**8'**) and DBCO (**12'**) and methyl phenyldiazoacetate (**13**) ($\Delta G^{\ddagger}$ = 25.6 and 24.1 kcal mol^{-1}, respectively), were very similar to those of the diazoketones used in the previous essays, which recommended they use as efficient labelling reagents in proteins and cell lysates (SI).In protein level, these compounds showed equivalent kinetics (Fig. S31-36), stability in the presence of GSH (Fig. S37-S43) and reactivity for cycloadditions reactions with DBCO and BCN reagents (Fig. S44-S49). To determine optimal labelling conditions in the lysates, the concentrations of probes **3** and **13** were systematically varied. Accordingly, HeLa cell lysates were treated with the diazo probes **3** and **13** (5 to 50 mM) for 2 h at room temperature. Samples were analysed directly by SDS-PAGE and Western blotting after ligation of the diazo to an alkyne-tagged biotin derivative prior to SDS-PAGE. After the incubation with the diazo probes for 2 h, the lysates were treated with biotin-PEG-DBCO (100 mM) and biotin-PEG-alkyne (100 mM) in the presence or absence of $CuSO_4$ (Fig. 8, **b** and **c**) for an additional 2 h. Labelling profiles were also compared varying the amount of DBCO/alkyne (5 – 100 mM), maintaining the amount of diazo probes **3** and **13** at 2.5 mM (Fig. 8, **a** and **b** and Fig. S50). These results indicate that the diazo compounds **3** and **13** are compatible for cycloaddition reactions in Hella lysates. The strain-promoted cycloaddition reaction provided superior protein labelling efficacy in comparison with the copper catalysed alkyne control. Nonetheless, compound **13** was able to react with the non-strained alkyne in the absence of copper, providing cysteine labelling equivalent to the copper catalysed reaction (Fig. 8d).

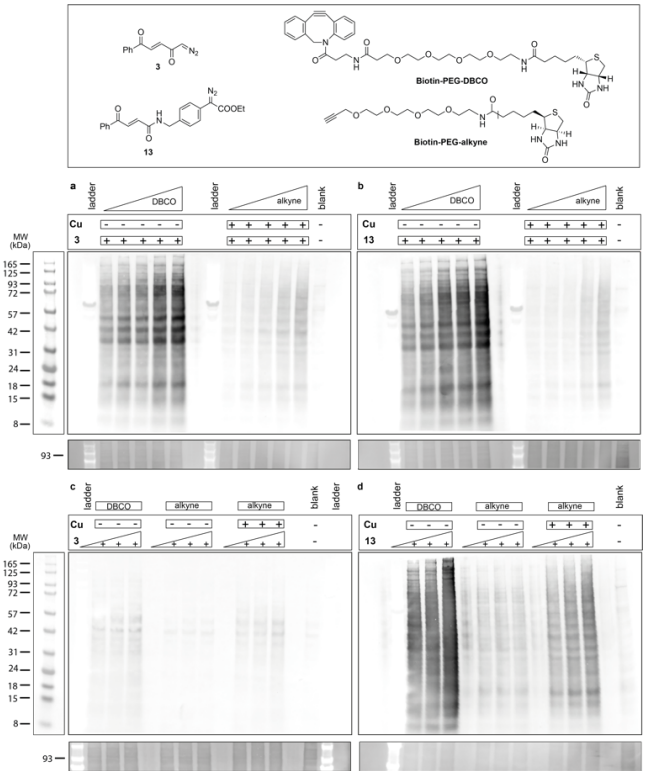

Figure 8. Assessment of 3 and 13 as probes for cysteine labelling in HeLa cell lysates. Probes (at the desired concentration) were added to the lysates as solutions in DMSO before the addition of the cycloaddition reagent in DMSO. Probe **2** and **13** were modified with a biotin-containing alkyne and DBCO before SDS-PAGE in the presence or absence of $CuSO_4$. Western blots were developed using an Alexa Fluor 555 streptavidin conjugate. **(a)** Assessment of diazoketone **3** (2.5 mM) with DBCO-biotin (5 to 100 mM) and alkyne-biotin (5 to 100 mM). **(b)** Assessment of diazoester **13** (2.5 mM) with DBCO-biotin (5 to 100 mM) and alkyne-biotin (5 to 100 mM). **(c)** and **(d)** Variation in the concentration of **3** and **13** (5, 10 and 50 mM) with fixed amounts of DBCO-biotin (100 mM), alkyne-biotin (100 mM), see supporting information for detailed conditions and protocols.

CONCLUSIONS

In summary, we have developed a rapid and site-specific strategy for the installation of a diazo functionality on proteins and antibodies. The reaction proceeds through a thiol-Michael addition reaction of Cys to diazocarbonylacrylic reagent **3.** We applied this mild method to modify various Cys-containing proteins, such as ubiquitin, C2Am, Annexin V and albumin variant V0354 with full conversion, and showed these modified proteins remained stable and retained their secondary structure. Antibodies, such as nanobody 2Rb17c, was also modified successfully. We confirmed the presence of the diazo moiety through a 1,3-cycloaddition reaction with strained alkynes to give homogeneous conjugates in high yields. Cell microscopy studies proved the effectiveness of the diazo group for in-cell 'click' reactions in an analogous manner to the commonly used azide functionality. We also showed the diazoketone **3**, together with the diazoester **13** can be used as probes to label cysteines in cell lysates using strained and non-strained alkynes. This is the first example of installation of a diazo handle on proteins and antibodies in a site-selective manner and, as such, provides opportunities to explore new biorthogonal reactions in chemical biology.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed methods, characterization data and additional figures (PDF).

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Nawrat, C. C.; Moody, C. J. (2011) Natural Products Containing a Diazo Group. *Nat. Prod. Rep. 28* (8), 1426–1444.

(2) Herzon, S. B.; Woo, C. M. (2011) The Diazofluorene Antitumor Antibiotics: Structural Elucidation, Biosynthetic, Synthetic, and Chemical Biological Studies. *Nat. Prod. Rep. 29* (1), 87–118.

(3) Laufer, R. S.; Dmitrienko, G. I. (2002) Diazo Group Electrophilicity in Kinamycins and Lomaiviticin A: Potential Insights into the Molecular Mechanism of Antibacterial and Antitumor Activity. *J. Am. Chem. Soc. 124* (9), 1854–1855.

(4) Colis, L. C.; Hegan, D. C.; Kaneko, M.; Glazer, P. M.; Herzon, S. B. (2015) Mechanism of Action Studies of Lomaiviticin A and the Monomeric Lomaiviticin Aglycon. Selective and Potent Activity Toward DNA Double-Strand Break Repair-Deficient Cell Lines. *J. Am. Chem. Soc. 137* (17), 5741–5747.

(5) Arya, D. P.; Jebaratnam, D. J. (1995) DNA Cleaving Ability of 9-Diazofluorenes and Diaryl Diazomethanes: Implications for the Mode of Action of the Kinamycin Antibiotics. *J. Org. Chem. 60* (11), 3268–3269.

(6) Colis, L. C.; Woo, C. M.; Hegan, D. C.; Li, Z.; Glazer, P. M.; Herzon, S. B. (2014) The Cytotoxicity of (−)-Lomaiviticin A Arises from Induction of Double-Strand Breaks in DNA. *Nat. Chem. 6* (6), 504–510.

(7) Lemberg, K. M.; Vornov, J. J.; Rais, R.; Slusher, B. S. (2018) We're Not "DON" Yet: Optimal Dosing and Prodrug Delivery of 6-Diazo-5-Oxo-L-Norleucine. *Mol. Cancer Ther. 17* (9), 1824–1832.

(8) Mix, K. A.; Aronoff, M. R.; Raines, R. T. (2016) Diazo Compounds: Versatile Tools for Chemical Biology. *ACS Chem. Biol. 11* (12), 3233–3244.

(9) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. (2004) A Strain-Promoted [3 + 2] Azide–Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. *J. Am. Chem. Soc. 126* (46), 15046–15047.

(10) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. (2002) A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. *Angew. Chem. Int. Ed. 41* (14), 2596–2599.

(11) Tornøe, C. W.; Christensen, C.; Meldal, M. (2002) Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J. Org. Chem. 67* (9), 3057–3064.

(12) Aronoff, M. R.; Gold, B.; Raines, R. T. (2016) Rapid Cycloaddition of a Diazo Group with an Unstrained Dipolarophile. *Tet. Lett. 57* (22), 2347–2350.

(13) McGrath, N. A.; Raines, R. T. (2012) Diazo Compounds as Highly Tunable Reactants in 1,3-Dipolar Cycloaddition Reactions with Cycloalkynes. *Chem. Sci. 3* (11), 3237–3240.

(14) Ford, A.; Miel, H.; Ring, A.; Slattery, C. N.; Maguire, A. R.; McKervey, M. A. (2015) Modern Organic Synthesis with α-Diazocarbonyl Compounds. *Chem. Rev. 115* (18), 9981–10080.

(15) Burtoloso, A. C. B.; Dias, R. M. P.; Bernardim, B. (2015) α,β-Unsaturated Diazoketones as Useful Platforms in the Synthesis of Nitrogen Heterocycles. *Acc. Chem. Res. 48* (4), 921–934.

(16) Aronoff, M. R.; Gold, B.; Raines, R. T. (2016) 1,3-Dipolar Cycloadditions of Diazo Compounds in the Presence of Azides. *Org. Lett. 18* (7), 1538–1541.

(17) Andersen, K. A.; Aronoff, M. R.; McGrath, N. A.; Raines, R. T. (2015) Diazo Groups Endure Metabolism and Enable Chemoselectivity in Cellulo. *J. Am. Chem. Soc. 137* (7), 2412–

(18) McGrath, N. A.; Andersen, K. A.; Davis, A. K. F.; Lomax, J. E.; Raines, R. (2014) T. Diazo Compounds for the Bioreversible Esterification of Proteins. *Chem. Sci. 6* (1), 752–755.

(19) Ouihia, A.; Rene, L.; Guilhem, J.; Pascard, C.; Badet, B. (1993) A New Diazoacylating Reagent: Preparation, Structure, and Use of Succinimidyl Diazoacetate. *J. Org. Chem. 58* (7), 1641–1642.

(20) Friscourt, F.; Fahrni, C. J.; Boons, G.-J. (2015) Fluorogenic Strain-Promoted Alkyne–Diazo Cycloadditions. *Chem. Eur. J. 21* (40), 13996–14001.

(21) Josa-Culleré, L.; Wainman, Y. A.; Brindle, K. M.; Leeper, F. J. (2014) Diazo Group as a New Chemical Reporter for Bioorthogonal Labelling of Biomolecules. *RSC Adv. 4* (94), 52241–52244.

(22) Taylor, M. T.; Nelson, J. E.; Suero, M. G.; Gaunt, M. J. (2018) A Protein Functionalization Platform Based on Selective Reactions at Methionine Residues. *Nature, 562* (7728), 563–568.

(23) Bernardim, B.; Cal, P. M. S. D.; Matos, M. J.; Oliveira, B. L.; Martínez-Sáez, N.; Albuquerque, I. S.; Perkins, E.; Corzana, F.; Burtoloso, A. C. B.; Jiménez-Osés, G.; *et al.* (2016) Stoichiometric and Irreversible Cysteine-Selective Protein Modification Using Carbonylacrylic Reagents. *Nat. Commun. 7*, 13128.

(24) Bernardim, B.; Matos, M. J.; Ferhati, X.; Compañón, I.; Guerreiro, A.; Akkapeddi, P.; Burtoloso, A. C. B.; Jiménez-Osés, G.; Corzana, F.; Bernardes, G. J. L. (2019) Efficient and Irreversible Antibody–Cysteine Bioconjugation Using Carbonylacrylic Reagents. *Nat. Protoc. 14* (1), 86.

(25) Pinho, V. D.; Burtoloso, A. C. B. (2011) Preparation of α,β-Unsaturated Diazoketones Employing a Horner–Wadsworth–Emmons Reagent. *J. Org. Chem. 76* (1), 289–292.

(26) Jewett, J. C.; Bertozzi, C. R. (2010) Cu-free Click Cycloaddition Reactions in Chemical Biology. *Chem. Soc. Rev. 39* (4), 1272–1279.

(27) Bickelhaupt, F. M.; Houk, K. N. (2017) Analyzing Reaction Rates with the Distortion/Interaction-Activation Strain Model. *Angew. Chem. Int. Ed. 56* (34), 10070–10086.

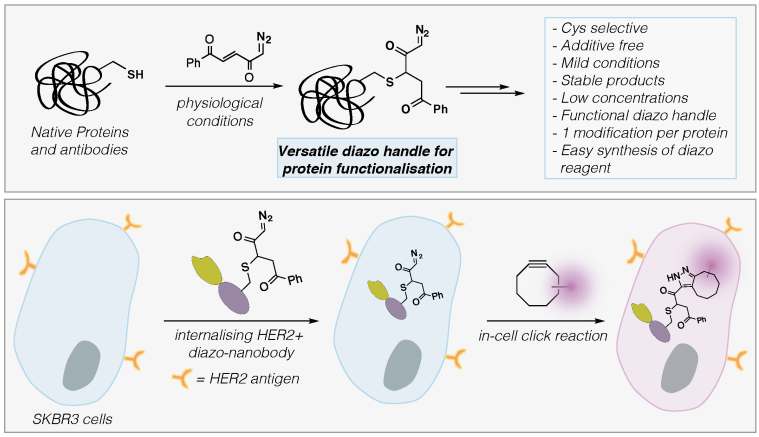
(28) Lee, B.; Sun, S.; Jiménez-Moreno, E.; Neves, A. A.; Bernardes, G. J. L. (2018) Site-Selective Installation of an Electrophilic Handle on Proteins for Bioconjugation. *Bioorganic & Medicinal Chemistry 26* (11), 3060–3064.

(29) Logue, S. E.; Elgendy, M.; Martin, S. J. (2009) Expression, Purification and Use of Recombinant Annexin V for the Detection of Apoptotic Cells. *Nat. Protoc., 4* (9), 1383–1395.

(30) Schelde, K. K.; Nicholls, K.; Dagnæs-Hansen, F.; Bunting, K.; Rawsthorne, H.; Andersen, B.; Finnis, C. J. A.; Williamson, M.; Cameron, J.; Howard, K. A. (2019) A New Class of Recombinant Human Albumin with Multiple Surface Thiols Exhibits Stable Conjugation and Enhanced FcRn Binding and Blood Circulation. *J. Biol. Chem., 294* (10), 3735–3743.

(31) Kratz, F.; Elsadek, B. (2012) Clinical Impact of Serum Proteins on Drug Delivery. *J. Control Release, 161* (2), 429–445.

(32) Vaneycken, I.; Devoogdt, N.; Van Gassen, N.; Vincke, C.; Xavier, C.; Wernery, U.; Muyldermans, S.; Lahoutte, T.; Caveliers, V. (2011) Preclinical Screening of Anti-HER2 Nanobodies for Molecular Imaging of Breast Cancer. *The FASEB J. 25* (7), 2433–2446.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