



## Sequential dual site-selective protein labelling enabled by lysine modification

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### ABSTRACT

Methods that allow for chemical site-selective dual protein modification are scarce. Here, we provide proof-of-concept for the orthogonality and compatibility of a method for regioselective lysine modification with strategies for protein modification at cysteine and genetically encoded ketone-tagged amino acids. This sequential, orthogonal approach was applied to albumin and a therapeutic antibody to create functional dual site-selectively labelled proteins.

### 1. Introduction

The ability to chemically install two different modifications into a protein of interest can dramatically expand a protein's functional capacity.<sup>1</sup> A number of methods are now available for chemical protein dual labelling at one site through the use of chemical scaffolds that display two different modifications and one handle for site-selective protein modification.<sup>2</sup> For example, Chudasama, Caddick and co-workers have developed dibromopyridazinedione reagents that enable simultaneous re-bridging of disulfide bonds and subsequent bioconjugation at two click-reactive handles.<sup>3</sup> However, methods that allow the precise installation of two different modifications on intact proteins at predefined sites are rare. In one example, Davis and co-workers incorporated two different post-translational modifications at pre-determined sites through two orthogonal reactions. More specifically, a mutant of thermophilic  $\beta$ -glycosidase from the archaeon *Sulfolobus solfataricus* (Ss $\beta$ G) was engineered by removal of all but one of the methionine residues, which were replaced by isoleucine with the remaining methionine site used for the incorporation of unnatural azido homoalanine by using auxotrophic strains, and a solvent-exposed cysteine was introduced by site-directed mutagenesis. Use of Cu(I)-catalyzed [3 + 2] cycloaddition reaction and disulfide formation allowed orthogonal introduction of two different modifications. This strategy was applied to create a reconstituted, functional mimic of a

naturally occurring protein, P-selectin glycoprotein ligand-1.<sup>4</sup> In another isolated example, Hackenberger and co-workers have combined residue-specific incorporation of azide-tagged unnatural amino acids with chemical oxidative aldehyde formation at the N-terminus to incorporate simultaneously biotin and  $\beta$ -linked galactose modifications to yield an enzymatically active thermophilic lipase.<sup>5</sup> Finally, Weil and co-workers have introduced in a site specific manner two different substitutions in peptides and proteins based on the different reactivity of cysteine residues in their thiol or disulfide forms.<sup>6</sup> These examples demonstrate the potential of installing synthetic groups at two distinct, pre-defined sites by using orthogonal chemical reactions to reconstitute active, post-translationally modified proteins. The potential of this strategy goes beyond enzyme mimicry; the development of new, efficient methods in drug-delivery, for example, may be realized.

### 2. Results and discussion

Our group has developed a sulfonyl acrylate reagent that reacts with lysine residues through stabilized H-bond-assisted conjugate-addition transition states.<sup>7</sup> For certain proteins in which a relatively acidic, reactive lysine is present, regioselective modification was achieved. This method generates, after *in situ* elimination of the sulfone moiety upon aza-Michael addition reaction, an acrylate electrophile at the most reactive lysine that can be subsequently modified. We started by

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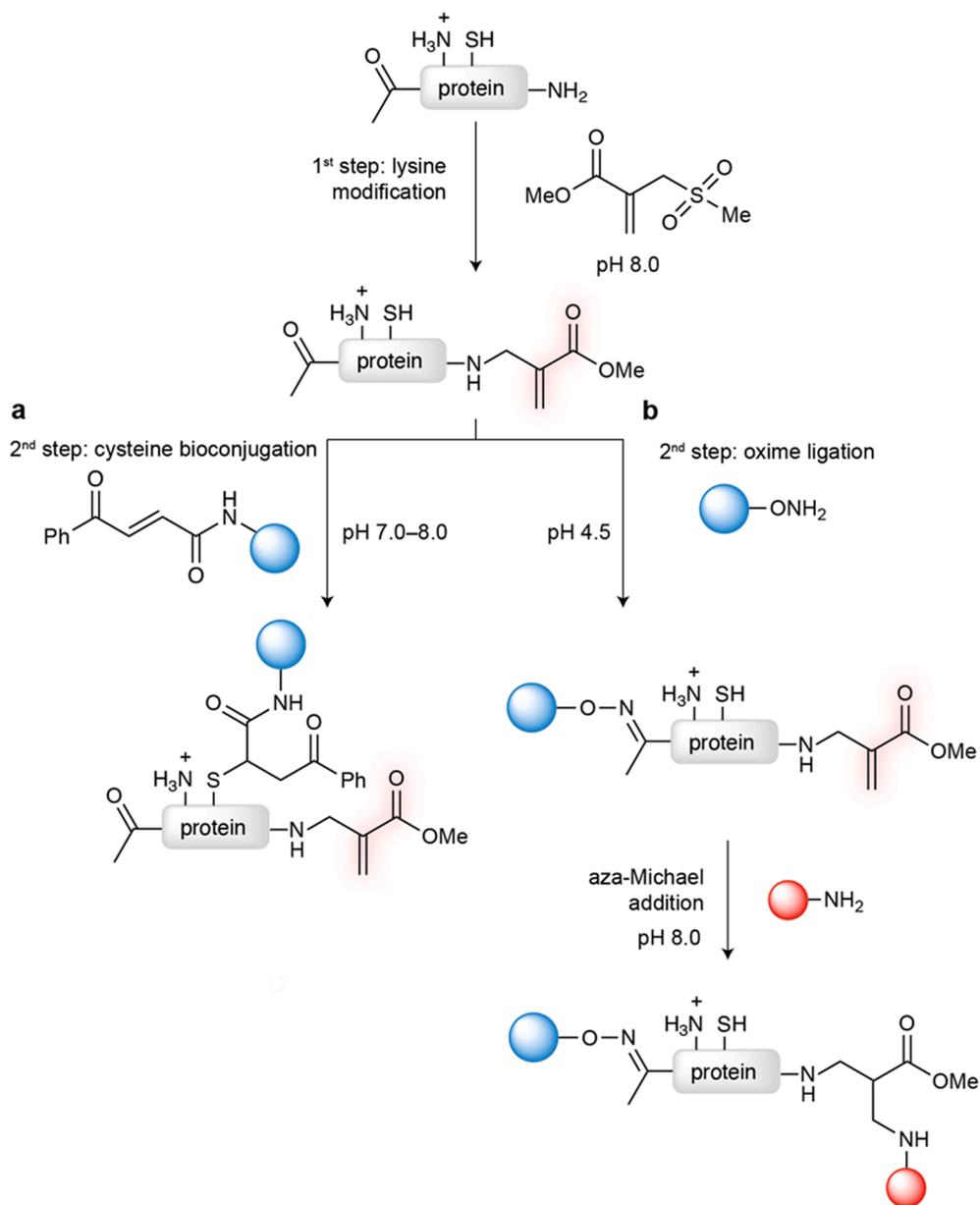
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exploring the conjugation between **1** and h38C2, a humanized catalytic IgG1 antibody. h38C2 contains a uniquely reactive lysine residue (Lys99) at its heavy chain that can facilitate the construction of highly homogeneous antibody-small molecule conjugates. The distinctive reactivity of the Lys99 residue arises from its positioning at the bottom of a deep hydrophobic pocket. Lys99 is deprotonated at physiological pH and is highly nucleophilic.<sup>8</sup> Antibody h38C2 was incubated with methyl 2-((methylsulfonyl)methyl)acrylate (**1**, 1 equiv. per heavy chain) for 5 h at 37 °C in sodium phosphate buffer (NaP<sub>i</sub>, 50 mM, pH 7.4), then treated with PNGase F enzyme (to remove *N*-glycosylation) and tris(2-carboxyethyl)phosphine (to reduce interchain disulfide bridges). Analysis by LC-MS indicated the formation of h38C2-1 (~75% conversion). The increase in mass occurred exclusively at the heavy chain and no multiple conjugation was detectable (Supporting Fig. 8). Further thiol-Michael addition at the newly formed electrophilic acrylate was investigated by reacting h38C2-1 with  $\beta$ -mercaptoethanol (100 equiv. per heavy chain) for 3 h, at room temperature. The product showed an increase in mass of 78 Da at the heavy chain, which corresponds to the addition of a single  $\beta$ -mercaptoethanol molecule. The reaction proceeded to approximately 60% conversion and no conjugated light chain

nor multiple conjugated heavy chain was detectable (Supporting Fig. 9). This data further demonstrates the specificity of **1** and the superior reactivity of Lys99 in h38C2.

We then thought to explore the orthogonality of this method with other bioconjugation procedures to target other amino-acid side chains to form dual site-selectively modified conjugates. The lysine modification method is particularly suited to achieve orthogonal modification because it introduces an acrylate electrophile that is not present in native proteins, which enables orthogonality in different labelling strategies, for example, based on the modification of nucleophilic side chains, such as the sulfhydryl group of cysteine. Moreover, and by pH switching, we were able to first modify lysine and then, at acidic pH, form an oxime at a ketone-tagged antibody (Fig. 1).

To provide proof-of-concept for the proposed dual site-selective protein modification strategy, we chose a therapeutically relevant recombinant human albumin Recombumun® (rHSA, Albumedix Ltd).<sup>7</sup> This protein features 59 potential reactive lysine residues and one free cysteine at position 34, which provides an ideal scaffold to perform sequential modification at lysine and cysteine residues, respectively.<sup>9</sup> We found that under mild conditions (Tris-HCl 20 mM pH 8.0, 37 °C, 1

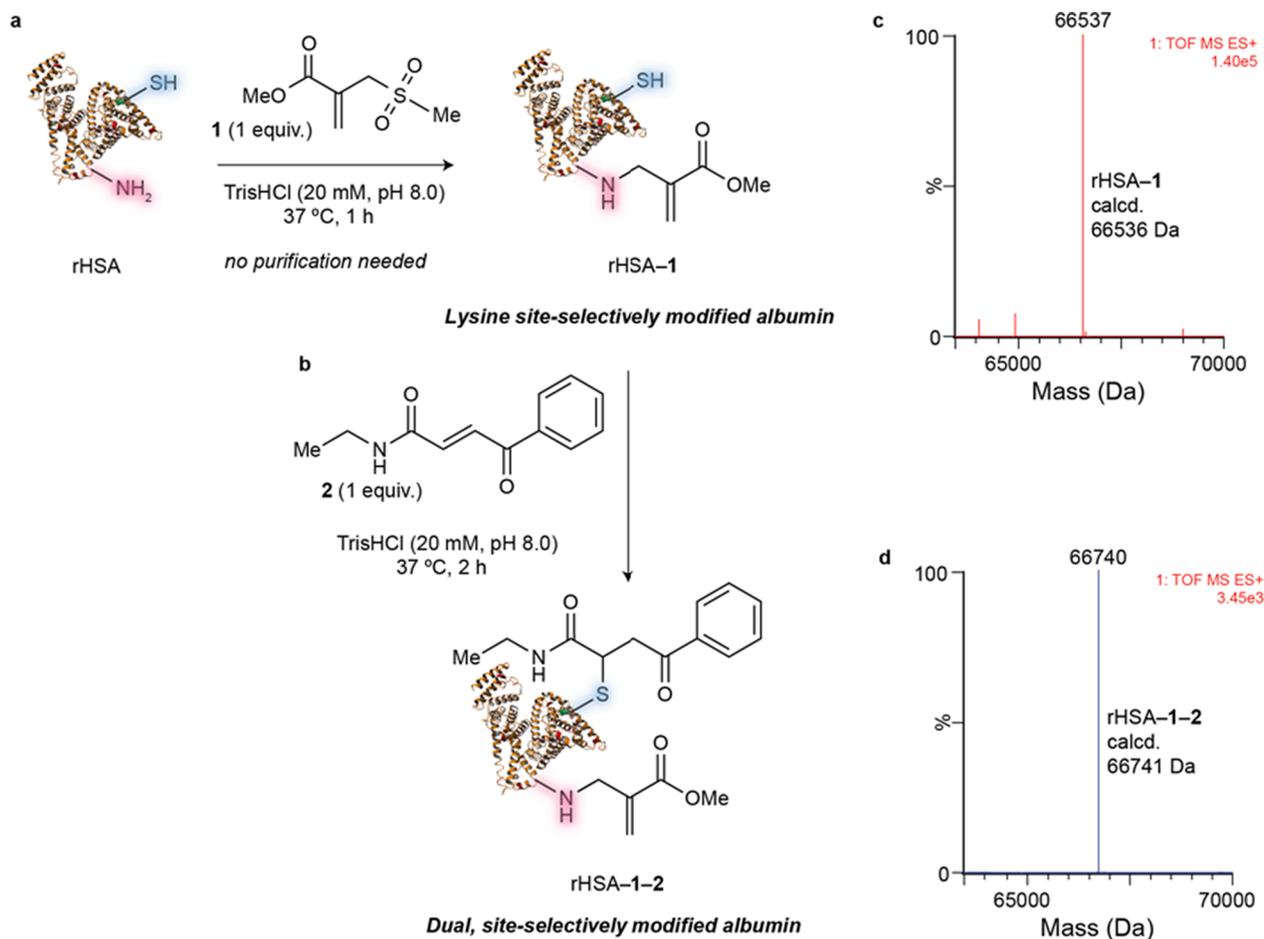


**Fig. 1.** Overview of synthetic strategy for the construction of dual site-selectively modified proteins. The first step consists of reaction with a sulfonyl acrylate reagent that is directed at the most nucleophilic lysine followed by subsequent bioconjugation either at (a) cysteine or at (b) genetically encoded *p*-acetylphenylalanine allows the construction of chemically defined dual site-selective conjugates. The newly created electrophilic acrylate can be used to add a second synthetic modification by thiol-Michael addition.

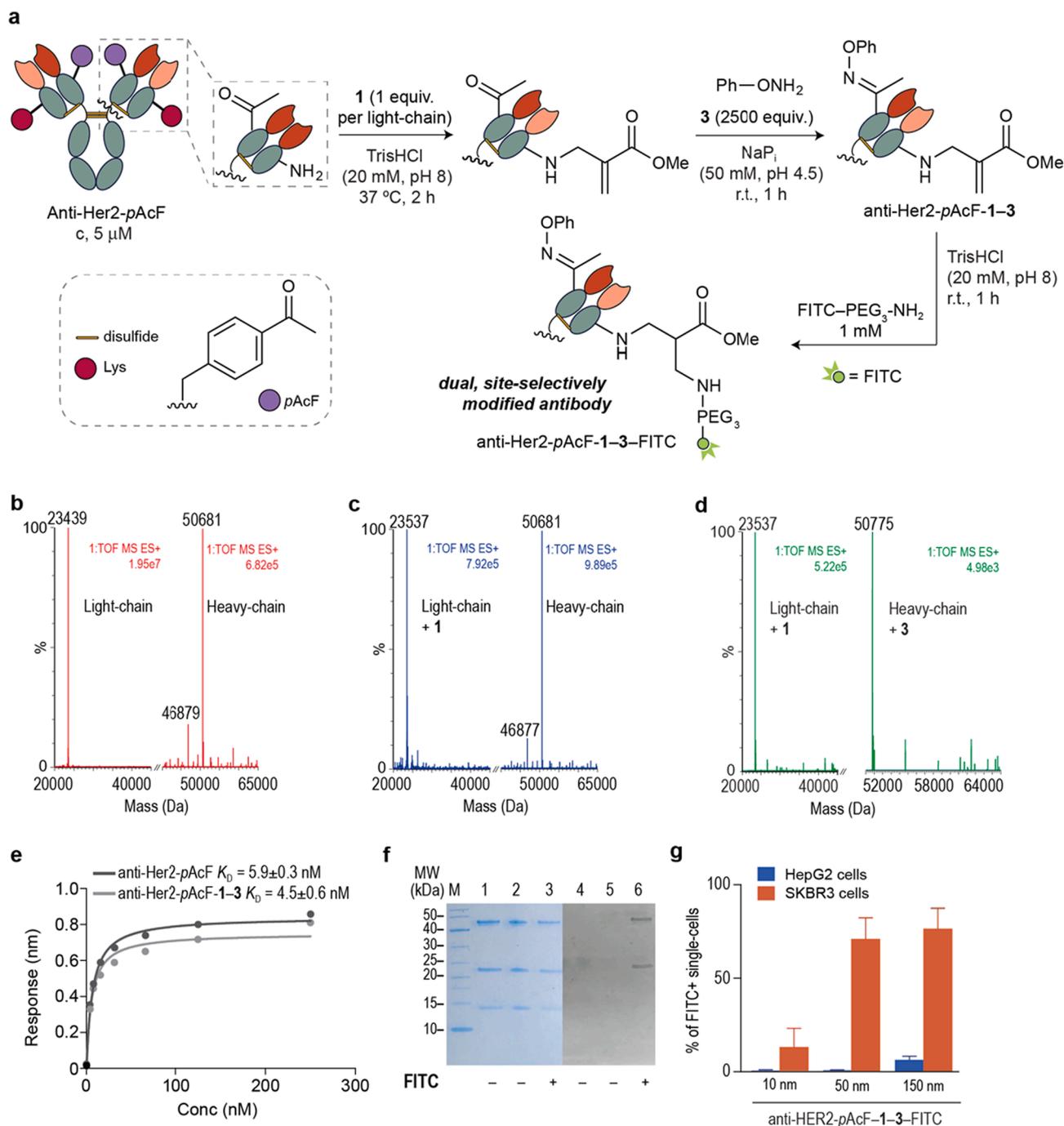
h) reaction of rHSA with methyl 2-((methylsulfonyl)methyl)acrylate (**1**, 1 equiv.) led to the formation of a chemically-defined conjugate rHSA-1. The product showed an increase in mass of 99 Da (Fig. 2a and b), which corresponds to the attack of the most nucleophilic amine to **1** followed by elimination of methanesulfinic acid. This result is in good agreement with our previously reported data.<sup>7,10</sup> The chemoselectivity of the acrylate reagent for lysine *versus* cysteine was corroborated by the rapid formation of a disulfide when rHSA-1 was reacted with Ellman's reagent [5,5-dithiobis(2-nitrobenzoic acid)] (Supporting Fig. 11). Next, we decided to react the free cysteine at position 34 on rHSA-1 with a carbonylacrylic derivative that is chemoselective for cysteine and provides stable thioether conjugates (Fig. 2c).<sup>10</sup> As a model reagent we used (E)-N-ethyl-4-oxo-4-phenylbut-2-enamide (**2**) and found that reaction of rHSA-1 with **2** in pH 8.0 buffer at 37 °C for 2 h led to complete conversion into dual site-selectively modified rHSA-1-2, as evidenced by a further increase of 201 Da in the mass of the conjugate (Fig. 2b and d). Importantly, and because equimolar amounts of reagents are used, no purification of the reaction product is needed after each step. Our data shows that lysine conjugation may be combined with cysteine-selective labelling methods to provide albumin dual site-selectively modified conjugates.

Recent studies have highlighted that the combination of antibody-drug conjugates with other small, untargeted chemotherapeutics may overcome differential drug sensitivities within heterogeneous tumour-cell populations.<sup>11</sup> Thus, the ability to attach two distinct and complementary payloads to an antibody at specific sites has the potential to advance the field of antibody targeted drug-delivery.<sup>1</sup> As proof-of-

concept, we demonstrated the orthogonality of our lysine modification with oxime ligation, a method previously used to produce therapeutic antibody conjugates.<sup>11</sup> For this purpose, we chose a Her2 targeting antibody that displays a genetically encoded *p*-acetylphenylalanine (*p*AcF) amino acid that may be site-specifically modified through oxime ligation when reacted with hydroxylamine-containing molecules.<sup>12,13</sup> First, the reaction of anti-Her2-*p*AcF with **1** (1 equiv.) in pH 8.0 buffer at 37 °C gave full conversion into lysine-modified antibody anti-Her2-*p*AcF-1 after 2 h, as evidenced by the increase of 99 Da in the mass of the light-chain of the antibody after the reaction (Fig. 3a-c). We next evaluated the stability of anti-Her2-*p*AcF-1 conjugate in human plasma and in the presence of 1 mM glutathione. Importantly, we observed that the conjugate did not degrade and remained intact in these physiological-like conditions, as demonstrated by LC-MS analysis (Supporting Figs. 16 and 17). The newly installed electrophilic acrylate on lysine was then demonstrated to undergo aza-Michael addition reactions. When anti-Her2-*p*AcF-1 was reacted with excess benzylamine, *o*-benzyl hydroxylamine and 2-(2-(2-methoxyethoxy)ethoxy)ethan-1-amine at pH 8.0 complete conversion to the corresponding conjugates was observed with the modification occurring exclusively in the antibody light-chain (Supporting Figs. 18-20). Next, and after buffer exchange and changing the pH from 8.0 to pH 4.5, anti-Her2-*p*AcF-1 was reacted for 1 h at room temperature in the presence of model hydroxylamine-containing molecule, phenylhydroxylamine **3** (12.5 mM). After size-exclusion purification and buffer exchange back to pH 8.0, analysis by LC-MS indicated complete conversion into corresponding dual site-selectively modified antibody anti-Her2-*p*AcF-1-3



**Fig. 2.** Dual site-selective modification of rHSA. (a) Scheme for the bioconjugation reaction between rHSA and sulfonylethyl acrylate **1**. General reaction conditions: rHSA reacted with **1** (1 equiv.) in Tris-HCl (20 mM, pH 8.0) at 37 °C for 2 h. (b) Scheme for the bioconjugation reaction between rHSA-1 and carbonylacrylic derivative **2**. General reaction conditions: rHSA-1 reacted with **2** (1 equiv.) in Tris-HCl (20 mM, pH 8.0) at 37 °C for 2 h. (c) Deconvoluted LC-MS data after aza-Michael addition to rHSA. (d) Deconvoluted LC-MS data after aza-Michael addition and thiol-Michael addition to rHSA-1.



**Fig. 3.** Dual site-specific modification of an anti-Her2 antibody. (a) Scheme for the sequential, dual site-specific modification of anti-Her2-pAcF IgG antibody. First, anti-Her2-pAcF was regioselectively modified at a lysine residue within its light chain through reaction with **1** at pH 8.0. Subsequent reaction with phenylhydroxylamine **3** at acidic pH 4.5 afforded the doubly modified antibody with oxime ligation occurring at the heavy chain at which the pAcF amino acid was encoded. Finally, reaction with FITC-PEG<sub>3</sub>NH<sub>2</sub> afforded a dual site-specifically, fluorescently labelled antibody. (b–d) Deconvoluted LC-MS data before (in red) and after aza-Michael addition (in blue) and oxime ligation (in green). Although lysine modification occurs in the light chain, oxime ligation happened at the engineered pAcF in the heavy chain as expected. (c) Calculated mass for the light chain, 23,536 Da. (d) Calculated mass for the light chain, 23,536 Da; calculated mass for the heavy chain, 50,777 Da. (e)  $K_D$  constants derived from BLI experiments for anti-Her2-pAcF and anti-Her2-pAcF-1-3 with FITC-PEG<sub>3</sub>NH<sub>2</sub> afforded a new fluorescent band as detected by SDS-PAGE that is consistent with site-specific incorporation of FITC. Lanes 1 to 3, coomassie staining. Lanes 4 to 6, fluorescence. From the bottom to the top: a lane between 20 and 25 kDa (light chain); a lane between 40 and 50 kDa (dimer light chain). (g) Analysis of specificity of anti-Her2-pAcF-1-3-FITC towards Her2 by flow-cytometry. Percentage of FITC-positive single cells, after treatment with fluorescently labelled or non-labelled anti-Her2-pAcF, both in HepG2 cells (blue) and SKBR3 cells (red). Controls were treated with non-conjugated anti-Her2-pAcF, whereas samples were treated with increasing concentrations of anti-Her2-pAcF-1-3-FITC (10, 50 and 150 nM). Superposition of contour plots in HepG2 and SKBR3 cells are in the Supporting Fig. 25.

(Fig. 3d). The increase in mass occurred at the heavy chain of the antibody, at which the pAcF non-canonical amino acid was engineered, while the light chain remained intact.

This data suggests that precise control of the site of installation of the modification is possible. We next assessed antigen binding by using biolayer interferometry (BLI) and confirmed that the dual-modified antibody anti-Her2-pAcF-1-3 retained its capacity to bind to Her2 ( $K_D = 4.5 \pm 0.6$  nM relative to  $K_D = 5.9 \pm 0.3$  nM for anti-Her2-pAcF; Fig. 3e). Because our lysine modification provides an alkene handle for site-specific aza-Michael ligation, we then conjugated fluorescein isothiocyanate (FITC)-PEG<sub>3</sub>NH<sub>2</sub> to afford a dual-modified, fluorescently labelled antibody as shown by SDS-PAGE (Fig. 3f). Importantly, we also show by flow-cytometry analysis and using fluorescently labelled antibody that the modified antibody retains its specificity towards SKBR3 cells, which express high levels of its target antigen (her2/c-erb-2), as opposed to HepG2 cells, which express low levels of its target at a concentration identical to anti-Her2 antibodies used in the clinic (Fig. 3g).<sup>14</sup>

### 3. Conclusion

In summary, we provide proof-of-concept that our platform for lysine bioconjugation can be used in an orthogonal, sequential manner with thioether and oxime ligation strategies to target cysteine and ketone residues, respectively. The simplicity of the protocol and availability of synthetic reagents bode well to routinely access dual site-selectively modified proteins and antibodies for basic biology and therapeutic applications.

## 4. Experimental section

### 4.1. General procedure for protein and antibody conjugation with sulfonyl acrylate 1

To an eppendorf tube with TrisHCl (20 mM, pH 8.0) and DMF (10% of total volume), an aliquot of a stock solution of protein (final concentration 10  $\mu$ M) was added. Afterwards, a solution of the acrylate derivative 1 (1 equiv.) in DMF was added and the resulting mixture was vortexed for 10 s. The reaction was mixed for 1 h at 37 °C. A 10  $\mu$ L aliquot of each reaction time was analysed by LC-MS and conversion to the expected product was observed.

### 4.2. LC-MS method for analysis of protein conjugation

LC-MS was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity Q6 UPLC BEH300 C4 column (1.7 mm, 2.1  $\times$  50 mm). Solvents A, a water with 0.1% formic acid and B, 71% acetonitrile, 29% water and 0.075% formic acid were used as the mobile phase at a flow rate of 0.2 mL min<sup>-1</sup>. The gradient was programmed as follows: 72% A to 100% B after 25 min then 100% B for 2 min and after that 72% A for 18 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L h<sup>-1</sup>. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions. To obtain the ion series described, the major peak(s) of the chromatogram were selected for integration and further analysis.

### 4.3. Analysis of protein conjugation by LC-MS

A typical analysis of a conjugation reaction by LC-MS is described below. The total ion chromatogram, combined ion series and deconvoluted spectra are shown for the product of the reaction. Identical analyses were carried out for all the conjugation reactions performed in this work. Conversion rates were determined by integration of the peak area

in liquid chromatogram.

### 4.4. Stability of bioconjugates in human plasma

A 20  $\mu$ L aliquot of the bioconjugate (10  $\mu$ M) in TrisHCl buffer (20 mM, pH 8.0) was thawed. 1  $\mu$ L of reconstituted human plasma was added at room temperature and the resulting mixture vortexed for 10 s. The resulting reaction mixture was then mixed at 37 °C overnight. After 1 and 48 h, a 10  $\mu$ L aliquot of each reaction mixture was analysed by LC-MS.

### 4.5. Stability of bioconjugates in the presence of GSH (1 mM)

A 20  $\mu$ L aliquot of the bioconjugate (10  $\mu$ M) in TrisHCl buffer (20 mM, pH 8.0) was thawed. 1  $\mu$ L of a 20 mM glutathione solution (6 mg glutathione dissolved in 1 mL of TrisHCl buffer (20 mM, pH 8.0)) was added at room temperature and the resulting mixture vortexed for 10 s. The resulting reaction mixture was then mixed at 37 °C overnight. After 1 and 48 h, a 10  $\mu$ L aliquot of each reaction mixture was analysed by LC-MS.

### 4.6. Protein gels

The incubation solution (5.0 mL) was transferred to tube, and NuPAGE LDS Sample Buffer (4x, 2.5 mL), NuPAGE Reducing Agent (10x, 1 mL), and H<sub>2</sub>O (1.5 mL) were added to the tube. The solution was heated at 70 °C for 10 min. The heated solution was loaded to NuPAGE Bis-Tris mini gel (10x 10 cm) with 4–12% gradient polyacrylamide concentration, and then the conjugation reaction was analysed by electrophoresis (200 V). The buffering system employed was 1x SDS Running Buffer (NuPAGE MES SDS Running Buffer, 20x, pH 7.3, 50–950 mL deionized water). For reduced samples, 500 mL of NuPAGE antioxidant was added to each 200 mL 1x SDS running buffer. After 35 min, the intensities of fluorescence were analysed. Then, the gel was stained with 0.5% of Ruby. The gel was mixed overnight at room temperature and read the day after. After wash the gel, coomassie (0.5%) was added and the gel was read 2 h after mixing at room temperature.

### 4.7. Enzymatic deglycosylation

Enzymatic deglycosylation was carried out using a ThermoFisher F Glycan cleavage kit. 50  $\mu$ g of the glycoprotein sample was added to a 1.5 mL Eppendorf tube and the total reaction was brought up to 35.5  $\mu$ L with Mili Q water. 3.5  $\mu$ L of PNGase F 10x buffer was combined with 1  $\mu$ L of PNGase F enzyme and the resultant mixture was transferred to the 1.5 mL Eppendorf tube. The reaction was incubated at 50 °C for 1 h then centrifuged briefly.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmc.2020.115783>.

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