

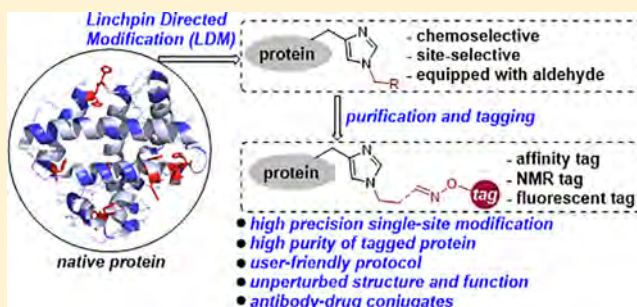
Single-Site Labeling of Native Proteins Enabled by a Chemoselective and Site-Selective Chemical Technology

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Supporting Information

ABSTRACT: Chemical biology research often requires precise covalent attachment of labels to the native proteins. Such methods are sought after to probe, design, and regulate the properties of proteins. At present, this demand is largely unmet due to the lack of empowering chemical technology. Here, we report a chemical platform that enables site-selective labeling of native proteins. Initially, a reversible intermolecular reaction places the “chemical linchpins” globally on all the accessible Lys residues. These linchpins have the capability to drive site-selective covalent labeling of proteins. The linchpin detaches within physiological conditions and capacitates the late-stage installation of various tags. The chemical platform is modular, and the reagent design regulates the site of modification. The linchpin is a multitasking group and facilitates purification of the labeled protein eliminating the requirement of additional chromatography tag. The methodology allows the labeling of a single protein in a mixture of proteins. The precise modification of an accessible residue in protein ensures that their structure remains unaltered. The enzymatic activity of myoglobin, cytochrome C, aldolase, and lysozyme C remains conserved after labeling. Also, the cellular uptake of modified insulin and its downstream signaling process remain unperturbed. The linchpin directed modification (LDM) provides a convenient route for the conjugation of a fluorophore and drug to a Fab and monoclonal antibody. It delivers trastuzumab-doxorubicin and trastuzumab-emtansine conjugates with selective antiproliferative activity toward Her-2 positive SKBR-3 breast cancer cells.



INTRODUCTION

The human quest for understanding and steering the properties of biological systems has fueled advancements in chemical biology and bioconjugate chemistry. Through post-translational modifications, Nature inspired the use of covalent labeling as a regulatory tool to steer the protein function. The intricate machinery of the natural system is challenging to replicate. However, protein-modification chemistry has facilitated the covalent labeling to probe or modulate its function.¹ In this perspective, initial successes have been demonstrated using amber codon suppression,^{2–7} enzymatic protocols,^{8–10} synthetic,^{11,12} and semisynthetic methods.^{13,14} These methods require incorporation of unnatural amino acids or fragments in the protein during its expression. The engineered functionality serves as the site of labeling through a chemoselective transformation. A few examples also showcase the potential of engineered Cys or a sequence of natural amino acids in selective recognition and labeling.^{15–20} Even though these methods are not applicable to native proteins, they provided a significant advance for applications revolving around selective protein labeling.^{21,22} The areas of biophysical chemistry,²³ biologics,²⁴ protein trafficking, and translocation²⁵ benefited immensely by elimination of artifacts emerging from the

heterogeneous installation of drugs and probes. A chemical technology that could include the large repository of native proteins within the purview of single-site labeling would similarly have an incredible impact on chemical biology.

In the past, the occasional attempts of selective labeling of native proteins have been met with very limited success. For example, chemoselective targeting of low-frequency residues such as Cys,²⁶ Trp,²⁷ and Tyr²⁸ in a protein often results in a heterogeneously labeled mixture of proteins. The credentials of N-terminus α -amine as a reactivity hotspot have been established over the past few years.^{29–37} Also, a few amino acids such as Cys³⁸ and Ser³⁹ have been able to drive selective labeling when primed at the N-terminus.⁴⁰ Beyond the N-terminus, strategies for native protein modification remain limited to selective ligand–protein interaction driven labeling^{41–45} and identification of the most reactive Lys residue.^{46–49} The absence of a general approach for site-selective labeling of proteins emerges from a diverse set of challenges. This work details a systematic approach to

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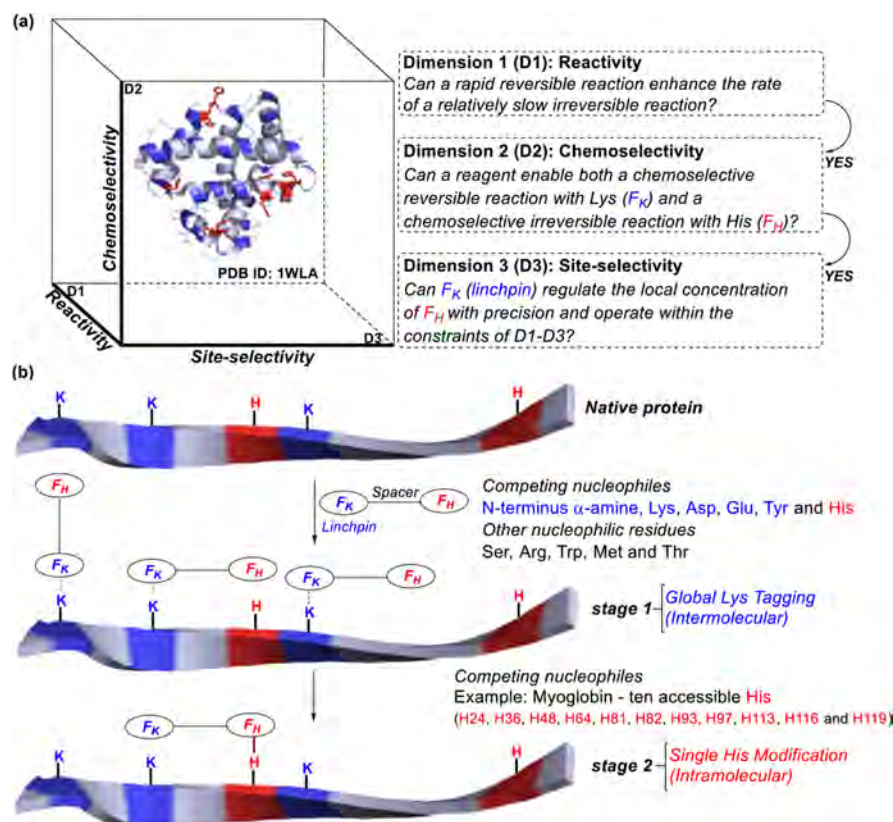


Figure 1. Site-selective labeling of a native protein. (a) Deconvolution of the challenges associated with the protein labeling. A chemical platform needs to operate in a confinement of three dimensions where the methodology can offer high reactivity (D1) along with chemoselectivity (D2) and site-selectivity (D3). (b) The sequential targeting of Lys (or N-terminus) and His can regulate the site-selectivity in protein modification. The global and reversible labeling of Lys residues (stage 1) by F_K can direct the selective labeling of His by F_H (stage 2).

understanding these questions and finding an answer in the form of a chemical technology.

In general, a protein acts as a multifunctional nucleophile-rich molecule in a chemical transformation. It offers a combination of challenges as a substrate for site-selective modification. The effective concentration of proteins is typically lower by 10^3 – 10^4 times when compared to the well-established protocols for an organic transformation. This disparity compromises the kinetics of a chemical reaction when applied to a protein (D1, Figure 1a). Excess electrophile can overcome this limitation but at a cost to selectivity, resulting in a mixture of heterogeneously labeled proteins. The chemoselectivity delineates the second challenge where an electrophile is desired to react with a single type of functional group (D2, Figure 1a). Next, a chemoselective electrophile has to identify a single residue in the presence of its multiple copies for site-selectivity (D3, Figure 1a). An additional complexity is to identify principles that would operate over a spectrum of structurally diverse proteins. These obstacles culminate due to a wide range of nucleophilicities^{50–52} of a given residue under the influence of variable microenvironments.

Even under ideal circumstances, targeting a single residue at a given time would be limited to the most reactive residue.^{46–49} We hypothesized that the reactivity order could be circumvented by aiming at pairs of residues. This can be achieved by taking advantage of the relative orientation of two functional groups (Figure 1b). The first group (F_K , linchpin) is desired to react rapidly, reversibly, and chemoselectively with all the accessible Lys residues (stage 1, D1 and D2).

Subsequently, the other functional group (F_H) should render a relatively slow, irreversible, and chemoselective reaction with a His residue (D1 and D2). Tuning the reagent with the spacer could precisely match the relative orientation of F_K – F_H with the Lys and His residue pair to accelerate selective labeling (stage 2, D1, D2, and D3). Such a linchpin-directed modification (LDM) would regulate the local concentration of F_H near a specific His and would administer a protein with covalently linked F_H . The other end of the reagent, F_K , would enable purification of the labeled protein and its late-stage modification. The differential rates of the linchpin (F_K)-directed intramolecular reaction of F_H in comparison to its undirected intermolecular analog would eliminate the participation of background reactions.

From this perspective, here we report a new chemical methodology for single-site labeling of native proteins hinged on the linchpin-directed installation of an electrophile. An aldehyde group (F_K , linchpin) coupled with an epoxide (F_H) through a spacer satisfied the design elements of the desired reagent. The method allows chemoselective and site-selective labeling of His residues of multiple native proteins. The reagent can be devised to alter the site of modification with exclusive selectivity. The technology can be further applied to label a single protein in the presence of other proteins. The methodology does not require site-selective reaction or coordination of the linchpin as a prerequisite. In contrast to the protocols utilizing ligand-proximity driven labeling,⁴⁴ our method relies on the global formation of imine with all the accessible primary amines. Importantly, the genesis of site-

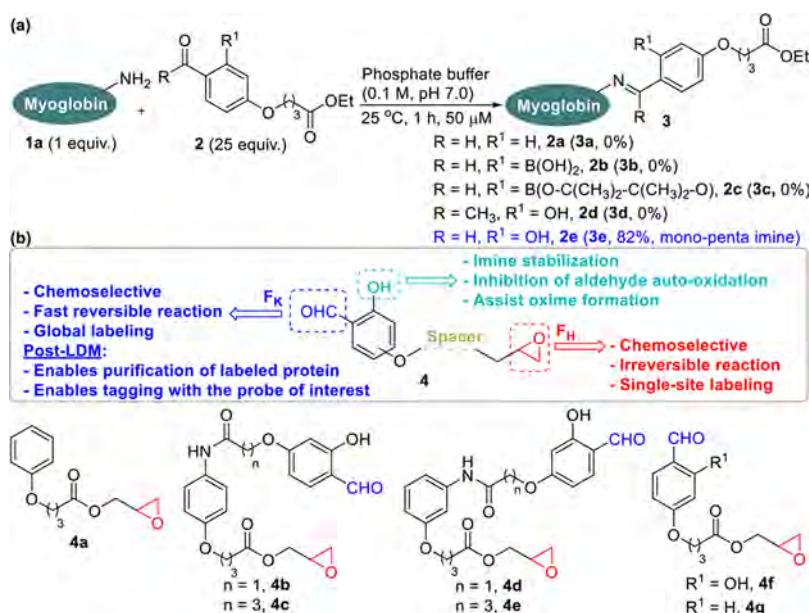


Figure 2. LDM reagent design. (a) Screening of carbonyl compounds 2a–2e for identification of F_K . % Conversion to products 3a–3e is analyzed by MALDI-ToF-MS. The aldehyde 2e (1.25 mM) results in 82% conversion of 1a (50 μ M) into a heterogeneous mixture of mono-, bis-, tris-, tetra-, and penta-imine. (b) Multiple roles played by the functional groups in the LDM reagent 4 (4b–4f). The set of analytically pure reagents 4a–4g has been synthesized in 3–8 steps (section 3b, Supporting Information). The LDM reagents are stable at +4 °C for more than six months.

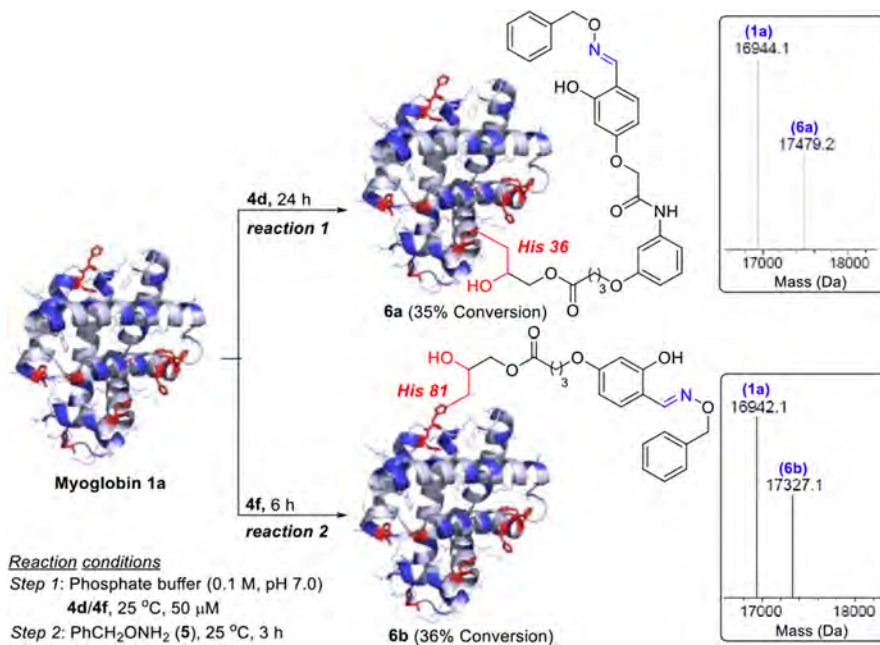


Figure 3. LDM reagent can regulate the site of modification. The LDM reagents 4d (1.25 mM) and 4f (1.25 mM) results in labeling of distinct sites in myoglobin 1a (170 μ g, 10 nmol, 50 μ M). The control reagent 4a leads to the preferential labeling of H64. ESI-MS estimates % conversion of the labeled myoglobin 6a and 6b. The deconvoluted ESI-MS spectrum is given in the inset.

selectivity shifts from an intermolecular to an intramolecular step. This unique attribute is essential and serves as the basis for application of this methodology to a diverse set of proteins.

RESULTS AND DISCUSSION

Design of the LDM Reagent. Initially, the three functional components, F_K , F_H , and the spacer, were optimized within the purview of three dimensions (D1–D3). F_K was devised to target Lys, a high-frequency residue in the proteome.⁵³ Additionally, the primary amine residue is

extensively protonated in the physiological conditions resulting in their high average relative accessibility.⁵⁴ For an appropriate F_K , we required an aromatic carbonyl compound that can form stable Schiff bases and yet offer convenient reversibility on demand. Additionally, the carbonyl group should be stable toward oxidation under physiological conditions over the period of reaction time. After an initial screening (2a–2e, Figure 2a and Figure S13), we found that the hydroxylaldehyde 2e core renders all the desired attributes.

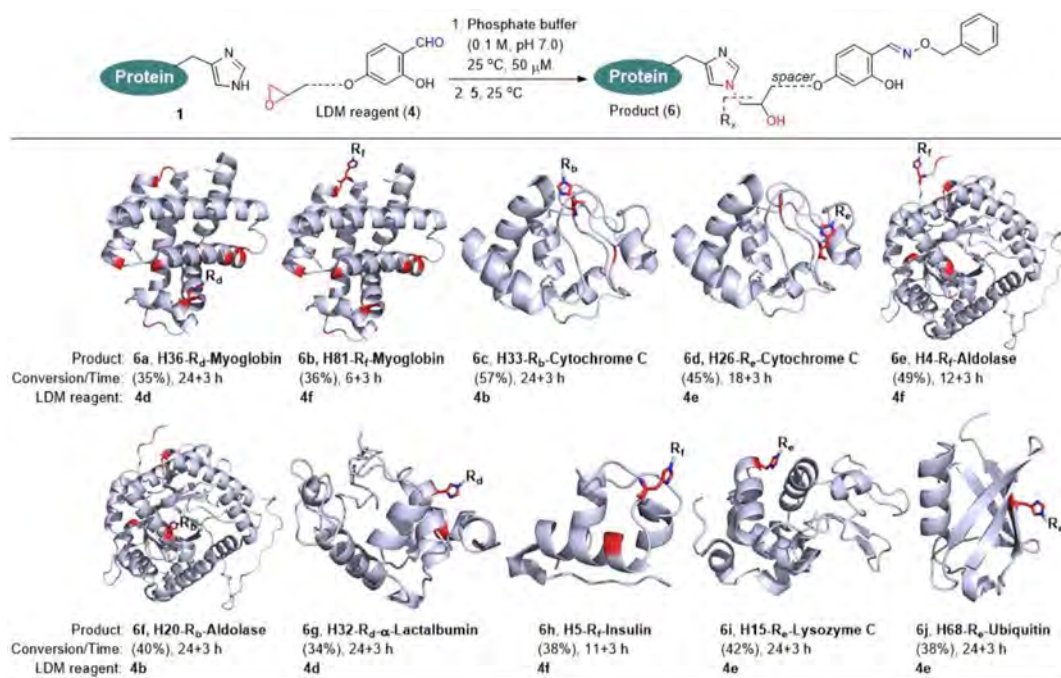


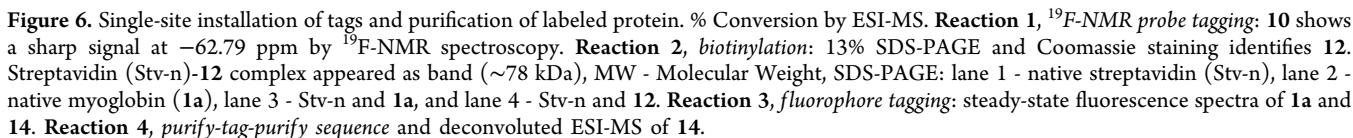
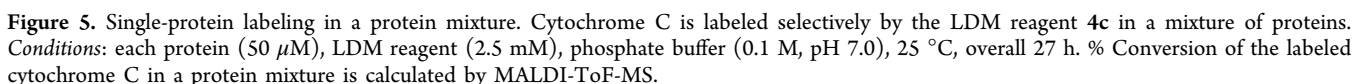
Figure 4. Single-site native protein modification enabled by LDM chemistry. MS estimates the overall % conversion for two steps (ESI-MS for **6a**–**6d**, **6g**, and **6j**; MALDI-ToF-MS for **6e**, **6f**, **6h**, and **6i**). R_x is the label from LDM reagent after oxime formation, and x varies as per the LDM reagent. The reactions were performed at 37 °C for **6h** and **6i**. For MS and MS-MS data, see [Figures S18–S25](#), [S27](#), and [S28](#).

On the other hand, F_H targets His, a nucleophilic amino acid with a pK_a in the physiological range and average relative accessibility. We screened α -iodoacetamide,⁵⁵ enal,⁵⁶ aryl sulfonate ester,⁵⁷ and epoxide⁴¹ (1.25 mM each) with myoglobin **1a** (50 μ M) ([Figures S14 to S16](#) and [Table S1](#)). The epoxide was identified as an optimal F_H due to its chemoselectivity and low reactivity in an intermolecular reaction. The third component of the reagent (*i.e.*, spacer) allowed us to position F_K and F_H variably ([Figure 2b](#)). Keeping these aspects in the background, we designed a set of bifunctional reagents with F_K (hydroxylaldehyde) and F_H (epoxide) tethered with modular spacers ([Figure 2b](#)). Next, we reasoned that the determination of “stoichiometry threshold” of the LDM reagent could be of paramount importance. The linchpin-directed intramolecular reaction of F_H (imine directed alkylation) would be operational at this concentration without appreciable competition from analogous intermolecular reactions with His (*i.e.*, the background reaction, direct alkylation).

Labeling, Tagging, and Purification of Proteins. Initially, we screened the stoichiometry of control reagent **4a** with myoglobin **1a** ([Table S1](#) and [Figure S17](#)). No reaction was observed with 15 equiv of **4a** whereas an additional 10 equiv resulted in the labeling of H64 (12% conversion) in 24 h. When myoglobin **1a** was vortexed with the reagent **4c** (25 equiv, [Figure 2b](#)), we did not observe any protein modification over 27 h ([Figure S43](#)). It was pleasing to note that single-site labeling and subsequent oxime formation resulted in labeled myoglobin **6a** (35% conversion, reaction 1, [Figure 3](#) and [Figure S18b](#)) after 27 h with LDM reagent **4d** ([Figure 2b](#)). The oxime formation follows the transimination pathway and results in >99% conversion. The reaction with **5** also ensures that the reagents bound to protein through imine formation are removed conveniently during the spin concentration. Digestion of **6a** with α -chymotrypsin confirmed the homoge-

neity and led to the identification of tagged peptide (labeled TGHPELT, T34-L40, m/z 1288.6 [$M + H$]⁺). MS-MS of the sequence confirmed the site-selective and homogeneous labeling of the His residue (H36, reaction 1, [Figure 3](#) and [Figure S18c](#)). At this point, we were intrigued to know whether reorientation of F_K and F_H in LDM reagent can lead to the labeling of another His residue. After an initial screening, we vortexed **4f** ([Figure 2b](#)) with myoglobin (**1a**). The monolabeled myoglobin **6b** was formed site-selectively (36% conversion, reaction 2, [Figure 3](#) and [Figure S19b](#)) in 9 h. No side products or higher order labeling were observed under the optimized conditions. We were pleased to note a distinct site of labeling in this case (H81, reaction 2, [Figure 3](#) and [Figure S19c](#)). The homogeneity of the H81-labeled myoglobin **6b** highlights the strength of this chemical platform. The neighboring residue (H82) remains unperturbed under these conditions. It is remarkable that two distinct sites (H36 and H81) undergo modification with LDM reagent **4d** and **4f**.

Next, we applied this chemical platform to modify other native proteins. Cytochrome C **1b** (104 residues, 19 Lys, 3 His) was selected to challenge the methodology with two His residues in near vicinity (H26 and H33, ~ 10.6 Å, PDB ID: 1HRC). When protein **1b** is vortexed with the reagent **4c** ([Figure 2b](#)), it results in monolabeled product (10% conversion, [S55](#), [Figure S45](#)). The LDM reagent **4b** results in an over 5-fold improvement to render monolabeled cytochrome C **6c** (H33, 57% conversion, [Figure 4](#) and [Figure S20](#)). Subsequently, LDM reagents were screened for selective labeling of the second His residue (H26) in cytochrome C. The reagent **4e** ([Figure 2b](#)) resulted in H26-labeled cytochrome C **6d** (45% conversion, [Figure 4](#) and [Figure S21](#)). After establishing the strength of methodology in distinguishing geometrically close residues (H26 and H33), we selected a few structurally diverse proteins to examine the generality of methodology. Aldolase **1c** (363 residues, N-



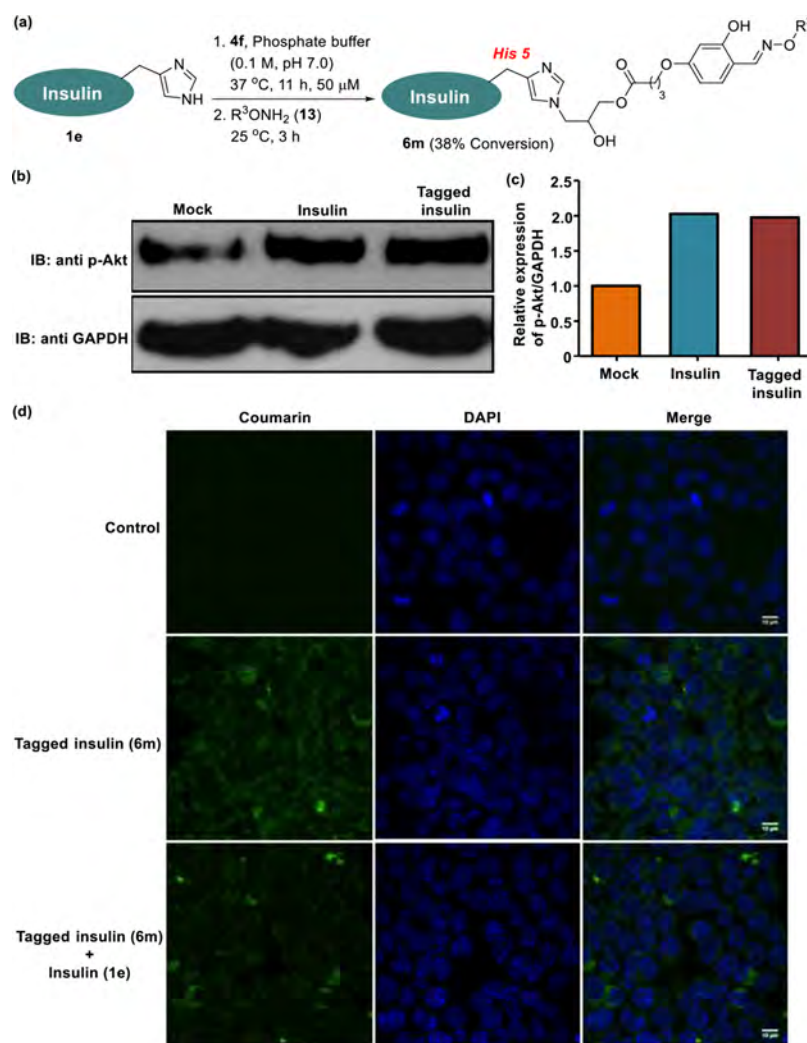


Figure 7. (a) Single-site fluorophore tagging of insulin **1e**. (b) Western blot analysis of pAkt and GAPDH in HEK293T cells lysates. (c) Quantification of pAkt signal relative to GAPDH. (d) Uptake of tagged insulin (green) and mixture of tagged insulin and insulin in cells. Chromatin (blue) (scale bar: 10 μm).

terminus α -amine, 26 Lys, 11 His) offers 11 histidines and 8 competing cysteine residues along with other nucleophilic proteinogenic amino acids. When LDM reagent **4f** was vortexed with aldolase **1c**, it resulted in monolabeled product **6e** (H4, 49% conversion, Figure 4 and Figure S22). The spatial reorganization of the F_K/F_H pair (**4b**) led to the single-site modification of H20 (40% conversion, Figure 4) in aldolase with absolute site-selectivity (Figure S23). Screening of LDM reagents for α -lactalbumin **1d** (123 residues, N-terminus α -amine, 12 Lys, 3 His) modification revealed that reagent **4d** facilitates its single-site labeling (H32, **6g**, 34% conversion, Figure 4 and Figure S24). The labeling of insulin **1e** (51 residues, two N-terminus α -amines, 1 Lys, 2 His) with the LDM reagent **4f** results in the H5-labeled insulin **6h** (38% conversion, Figure 4 and Figure S25). On the other hand, lysozyme C **1f** (129 residues, N-terminus α -amine, 6 Lys, 1 His) was unreactive toward LDM reagent **4c** (0% conversion, Figure S46). However, the spatial reorganization of F_K/F_H in the LDM reagent (**4e**) led to single-site labeled lysozyme C **6i** (42% conversion, Figure 4 and Figure S27). A protein with a profound presence in all tissues, ubiquitin **1g** (76 residues, N-terminus α -amine, 7 Lys, 1 His), was selected for the next experiment. In the initial screening, **4b**, **4c**, and **4d** (Figure 2b)

led to 0%, 3%, and 16% conversion respectively (Figures S47 to S49). Interestingly, an extension of the spacer in LDM reagent (**4e**) renders the H68-labeled product **6j** with 38% conversion (Figure 4 and Figure S28). Subsequently, the labeling experiment was performed for the mutant (H68C) ubiquitin **1h** with the LDM reagent **4e**. Interestingly, the Cys residue in the mutant ubiquitin exhibited 2.5-fold improvement (**6k**, 95% conversion, Figure S29) compared to His in its wild-type counterpart (**6j**, 38% conversion, Figure 4). These results indicate that the LDM platform could be conveniently extended for the modification of an appropriately placed Cys. The secondary structure of all the labeled proteins **6a–6j** remains unaltered after modification as suggested by circular dichroism spectroscopy (Figure S69). The enzymatic activity of distinct single-site labeled myoglobin (**6a** and **6b**), cytochrome C (**6c** and **6d**), aldolase (**6e** and **6f**), and labeled lysozyme C (**6i**) remains conserved and highlights the mild nature of the reaction conditions (Figures S70–S73).

Next, we challenged the LDM technology to identify a unique residue pair (Lys-His) and label a single protein in a mixture of proteins (ubiquitin, cytochrome C, RNase A, α -lactalbumin, lysozyme C, myoglobin, and α -chymotrypsinogen A). In the initial experiments with the LDM reagents **4b** and

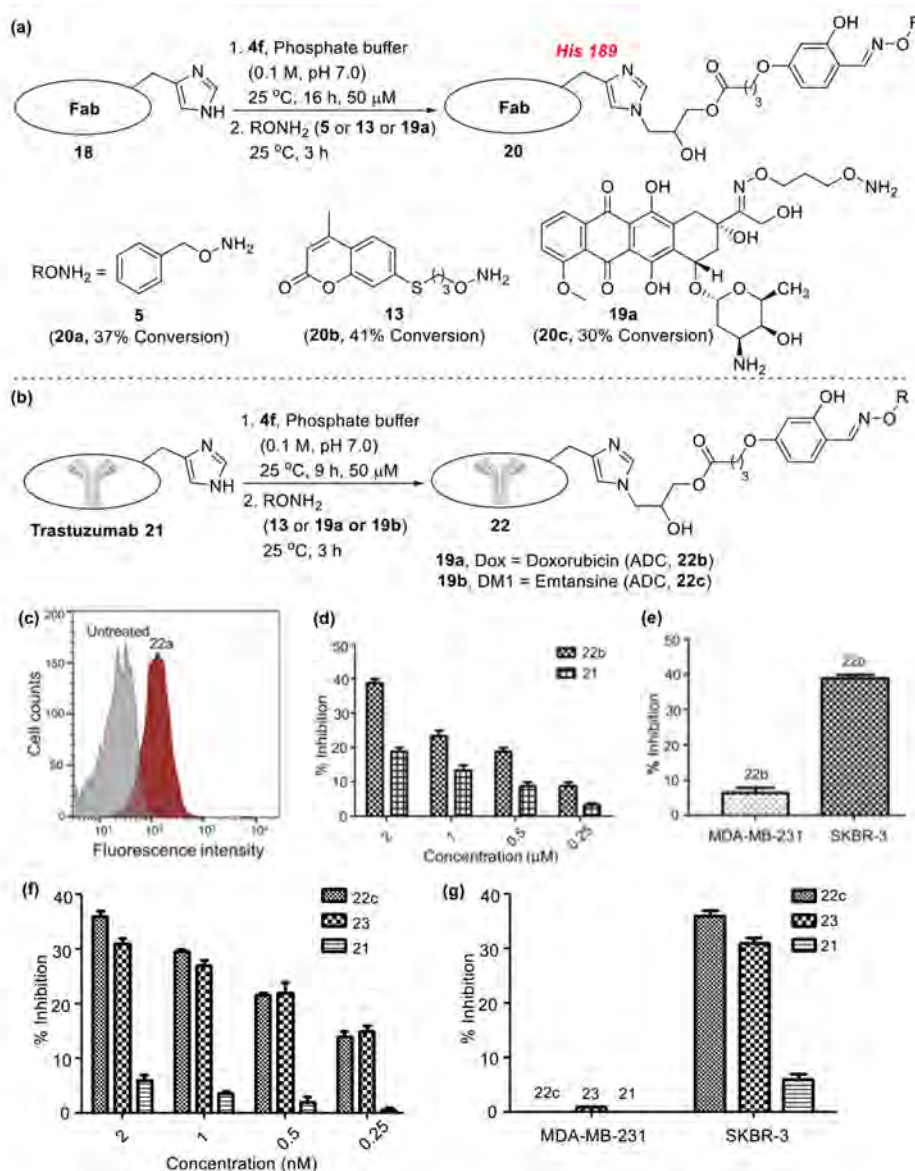


Figure 8. Site-selective trastuzumab-Fab and trastuzumab modification enabled by LDM reagent 4f. (a) Single-site modification of trastuzumab-Fab followed by the late-stage tagging with derivatives of *O*-hydroxylamine. (b) Modification of trastuzumab followed by tagging with fluorophore (coumarin) and drug (doxorubicin and emtansine). (c) The binding of tagged trastuzumab 22a to the Her-2 expressing human SKBR-3 cell line using flow cytometry. Untreated cells were used as a negative control. (d) Inhibition of cell proliferation by ADC (22b) as compared to trastuzumab (21) in SKBR-3 (Her-2 positive) cancer cell line. The percentage inhibition was calculated using untreated cells as control. (e) Inhibition of cell proliferation by ADC (22b, 2 μ M) in Her-2 positive SKBR-3 as compared to Her-2 negative MDA-MB-231 cells. (f) Inhibition of cell proliferation by ADC (22c) and T-DM1 (23) as compared to trastuzumab (21) in SKBR-3 (Her-2 positive) cancer cell line. (g) Inhibition of cell proliferation by ADC (22c), T-DM1 (23, Kadcyla), and trastuzumab (21) at 2 nM concentration in Her-2 positive SKBR-3 as compared to Her-2 negative MDA-MB-231 cells.

4e, ubiquitin, RNase A, and myoglobin also result in monolabeled products in addition to cytochrome C (Figures S30 and S31). Excitingly, the LDM reagent 4c leads to exclusive labeling of cytochrome C (34% conversion, Figure 5 and Figure S32). The single-site labeling of native proteins enabled by LDM technology offers an orthogonal functional group, cyclic imine 7af (or aromatic aldehyde 8af, Figure 6 and Figure S19a). This group provides a site for orthogonal late-stage modification. The potential of this transformation is showcased by attachment of an NMR tag, affinity tag, and fluorophore through oxime formation. The *O*-hydroxylamine derivative 9, having six magnetically equivalent ^{19}F nuclei, was attached to the protein (reaction 1, Figure 6). ^{19}F NMR probe

labeled myoglobin 10 shows a sharp signal at -62.79 ppm by ^{19}F NMR spectroscopy (TFA, internal standard at -75.45 ppm, Figures S33 and S66). The incubation of biotinylated myoglobin 12 and streptavidin (Stv-n) at 25 °C for 15 min results in a sharp band at ~ 78 kDa for the Stv-12 complex in SDS-PAGE (reaction 2, Figure 6, Figures S34 and S67). Next, a fluorophore 13 was installed on labeled myoglobin (7af/8af, reaction 3, Figure 6, Figure S35). The fluorescence spectra of coumarin tagged myoglobin 14 show emission at 428 nm upon excitation at 336 nm (Figure S68).

The rich chemistry of the F_K again comes to the fore in our attempts to integrate a purification protocol to the LDM technology. Initially, we immobilized the labeled protein 7af

(8af) on hydrazide activated resin via hydrazone formation catalyzed by *p*-phenylenediamine (*p*-PDA, reaction 4, Figure 6). Subsequently, treatment with derivatives of hydroxylamine enabled transoximization and released the tagged protein (Figure 6). In this process, late-stage tagging accompanies the purification of end product 17 (Figure S36). The sample concentration allows the complete removal of excess tagging reagent. The overall protocol delivers analytically pure (ESI-MS) coumarin tagged protein 14 with high efficiency (Figure S37).

Insulin Bioactivity Assay. We examined the activity of insulin in cell-based assays using its coumarin-tagged derivative 6m (Figure 7a and Figure S26) through its ability to activate the insulin receptor (IR) mediated signaling. The insulin binding to its receptor and subsequent activation of downstream signaling cascade provides a measure of its biophysical and biochemical functions. The circular dichroism data confirmed that the installation of coumarin at a single site does not alter the structure of insulin (Figure S69k). The functional engagement of insulin to its receptor is determined through the fluorescence signal of coumarin inside cells. Next, we probed the activation of IR mediated signaling by monitoring the increase in phosphorylated Akt (pAkt) levels. The signaling cascade activation is detected by pAkt specific antibodies recognizing pSer-473 of Akt by Western blotting. The untreated (mock) cells exhibit basal pAkt activity most probably due to contribution from other pathways. The treatment of cells with insulin, tagged (6m) or untagged (1e), increases the pAkt levels (Figure 7b). The quantitation of pAkt signals (Figure 7b) relative to the loading control GAPDH is represented in Figure 7c. It is noteworthy that the cells treated with 6m display coumarin signal accumulation throughout the cells indicating their regular uptake in the cellular assays (Figure 7d, first column, middle panel). In a subsequent experiment, the untagged insulin outcompeted the intracellular coumarin signal from tagged insulin during the cellular uptake (1e:6m, 3:1; Figure 7d, first column, down panel). Together, the enhanced pAkt levels and cellular uptake upon treatment with the labeled insulin suggest that biochemical and biophysical properties of proteins are unperturbed. These results reaffirm that the LDM chemistry provides access to the single-site installation of a probe to a protein while conserving its structure and bioactivity.

Antibody–Drug Conjugates. Site-selective conjugation of cytotoxic drugs and biophysical probes to antibodies is important for therapeutics and diagnostics.^{58–62} We aimed to apply the LDM technology for the synthesis of homogeneous antibody–drug conjugates as well as antibody–fluorophore conjugates. We chose trastuzumab-Fab 18 for the initial investigations. The vortexing of LDM reagent 4f and trastuzumab-Fab 18 renders monolabeled trastuzumab-Fab 20a (37% conversion, Figure 8a and Figure S38a) over 19 h. Enzymatic digestion of 20a followed by peptide mapping confirmed the identity of the tagged peptide (labeled EKHKVY, E187-Y192, light chain). MS-MS of the tagged peptide confirmed the site-selective and homogeneous labeling of H189 (Figure S38b). Subsequently, the reaction of labeled trastuzumab-Fab with an *O*-hydroxylamine derivative of coumarin results in single-site fluorophore tagged 20b (Figure 8a and Figure S39a). The fluorescence spectrum of coumarin tagged trastuzumab-Fab 20b maintains a maximum emission at 434 nm upon excitation at 333 nm (Figure S39c).

Along the same lines, vortexing of trastuzumab 21 with LDM reagent 4f and subsequent late-stage installation of fluorophore result in coumarin tagged trastuzumab 22a (Figure 8b). Fluorescence of the band at ~150 kDa confirms late-stage tagging of coumarin to the labeled trastuzumab (Figure S41). The SDS-PAGE exhibits the fluorescent band for light chain and heavy chain protein after the reduction of 22a by DTT. Next, we synthesized the *O*-hydroxylamine derivative of the doxorubicin (Dox) 19. This drug was conveniently installed on trastuzumab-Fab 18 and trastuzumab 21 to render Fab-Dox conjugate 20c and antibody–drug conjugate (ADC) 22b (Figure 8a and 8b and Figures S40 and S42a). The trastuzumab-Dox conjugate 22b shows a drug to antibody ratio (DAR) of 4.0. To demonstrate the efficacy of the method, we evaluated the antiproliferative activity of the antibody–drug conjugate (ADC, 22b) toward the Her-2 expressing SKBR-3 breast cancer cells. In the initial experiments, a cell-binding study using the flow cytometric analysis confirms the binding of tagged trastuzumab 22a to the Her-2 expressing cell line (Figure 8c). Next, the MTT assay was performed using Her-2 positive and Her-2 negative cancer cell lines, SKBR-3 and MDA-MB-231 respectively. The ADC (22b) demonstrated a significant dose-dependent inhibition of SKBR-3 cancer cell proliferation (Figure 8d). The inhibitory effect of the ADC (22b) was considerably higher with respect to trastuzumab 21. Subsequently, we demonstrated that the ADC (22b) mediated inhibition was specific to Her-2 positive SKBR-3 and showed minimal effect in Her-2 negative MDA-MB-231 cells (Figure 8e). This confirms the high selectivity of the ADC (22b) for the Her-2 receptor. Finally, we decided to compare the ADC (trastuzumab-emtansine conjugate, T-DM1) from LDM platform (22c) with the FDA approved drug Kadcyla (23). We installed emtansine (19b, DM1) to the labeled trastuzumab to render the ADC 22c (Figure 8b and Figure S42b). In the MTT assay, ADC 22c exhibited 37% growth inhibition of SKBR-3 cells at 2 nM dose in comparison to 32% by Kadcyla, 23 (Figure 8f). Both the conjugates (22c and 23) exhibited remarkable specificity toward Her-2 positive cells in comparison to Her-2 negative MDA-MB-231 cells (Figure 8g).

CONCLUSION

The LDM technology offers a platform for single-site labeling of native proteins. The key is to regulate the desired pathway through an intramolecular route, while the undesired pathway traverses through an intermolecular route (background reaction). In the process, we enhanced the rate of a desired slow irreversible reaction through a fast reversible reaction exclusively through the linchpin-directed pathway. Here, F_K (*o*-hydroxybenzaldehyde) plays a crucial role in achieving the above-mentioned tasks. Apparently, the rapid reaction of F_K is also responsible for bringing down the effective concentration of free LDM reagent available for intermolecular background reaction. The assistance of the proximal hydroxyl group to the aldehyde is critical in enhancing the stability of imine. It also inhibits the aerobic auto-oxidation of aldehyde that can generate an unwanted electrophilic formate and radical species. In parallel, we identified epoxide as a suitable functional group that can impart reproducible chemoselectivity toward His. The collaborative effect of F_K and linker translates a high local concentration of F_H near a specific His residue while eluding others. The interplay of all the parameters might appear complex at first glance. However, the systematic, step-by-step approach delivers a chemical platform that follows an

operationally convenient protocol. Single-site labeling of proteins is achieved by simply mixing an LDM reagent with the protein. The F_K enables easy purification of the labeled protein and eliminates the requirement of an additional purification tag. This multitasking group (F_K) also facilitates the late-stage bio-orthogonal attachment of an NMR tag, an affinity tag, and a fluorophore. Importantly, the structure and function of the labeled proteins remain unaltered. We believe that invention of the LDM technology will offer a launchpad to chemical methods for single-site labeling of proteins. It also holds promise for delivering homogeneous antibody conjugates for biologics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b10490.

Experimental methods, additional discussion, figures, schemes, tables, and characterization data (PDF)

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Notes

The authors declare the following competing financial interest(s): V.R. is the scientific founder of Plabeltech Private Limited. A patent application has been filed on "multi-functional chemical agents and the method for protein modification" with V.R. and S.R.A. as inventors.

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Traceless cysteine-linchpin enables precision engineering of lysine in native proteins

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The maintenance of machinery requires its operational understanding and a toolbox for repair. The methods for the precision engineering of native proteins meet a similar requirement in biosystems. Its success hinges on the principles regulating chemical reactions with a protein. Here, we report a technology that delivers high-level control over reactivity, chemoselectivity, site-selectivity, modularity, dual-probe installation, and protein-selectivity. It utilizes cysteine-based chemoselective Linchpin-Directed site-selective Modification of lysine residue in a protein (LDM_{C-K}). The efficiency of the end-user-friendly protocol is evident in quantitative conversions within an hour. A chemically orthogonal C-S bond-formation and bond-dissociation are essential among multiple regulatory attributes. The method offers protein selectivity by targeting a single lysine residue of a single protein in a complex biomolecular mixture. The protocol renders analytically pure single-site probe-engineered protein bioconjugate. Also, it provides access to homogeneous antibody conjugates (AFC and ADC). The LDM_{C-K}-ADC exhibits highly selective anti-proliferative activity towards breast cancer cells.

The functional well-being of a living organism thrives on the seamless coordination of diverse biomolecules. Hence, molecular-level intervention becomes vital for rescue whenever a component malfunctions. For instance, the ongoing development of inhibitors^{1–4} and degraders^{5–7} provides access to an extended landscape of undruggable proteome. Assisted by chemoproteomics, the leads often emerge from the top-down screening of a ligand library against the proteome. Such activity-based protein profiling also provides vital insight into the off-target modifications responsible for multiple failures. Hence, controlling the precision while targeting a site in a complex protein-derived ecosystem needs urgent attention. Initially, it requires a profound knowledge of operating principles in the bioconjugation of model peptides^{8,9} or isolated proteins. Subsequently, we can translate the concepts while understanding the impact from additional layers of complexity. Alongside, such platforms are desired to probe biological

systems¹⁰ and meet the requirements of protein-based healthcare tools and therapeutics^{11,12}. For example, the challenge of heterogeneity and ambiguous conjugation sites has been the most significant roadblock to developing antibody-drug conjugates (ADCs) for directed cancer chemotherapeutics. A chemical platform that empowers the regulation of homogeneity, drug-to-antibody ratio, and site-of-drug-conjugation is of immense value in this perspective.

The biochemical single-site engineering of chemically orthogonal handles such as carbonyls creates an excellent opportunity for their precise conjugation^{13,14}. However, controlling the selectivity in the chemical modification of a genetic manipulation-free protein faces a spectrum of challenges. The magnitude depends on whether the protein is isolated or accompanied by other biomolecules. For example, we and others demonstrated that the labeling of reactivity hotspots provides efficient tools for chemoselective and site-selective

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modification of isolated proteins^{15–27}. However, such methods are not equipped to deal with the drastic increase in competing functional groups presented by a complex mixture of proteins. Besides redefining the chemoselectivity and site-selectivity, it also presents the question of protein-selectivity for achieving absolute precision. In this perspective, the non-covalent ligands bound to an electrophilic warhead have been able to offer an interesting case^{28,29}. Besides, residue-specific labeling emerged as a robust tool for targeting N-Cys^{30–33} and N-Gly³⁴. The impact could be multifold if a chemical technology can exhibit a combination of chemoselectivity, site-selectivity, modularity, and

protein-selectivity to constitute a platform that can address the precision engineering of proteins in a complex mixture.

We recently demonstrated that a chemical method could deliver single-site protein modification irrespective of the residue's reactivity order. Here, a lysine-derived linchpin-directed modification (LDM[®]) results in the labeling of a His (LDM_{K-H}, LDM_{K-X}), Lys (LDM_{K-K}), and Asp (LDM_{K-X})^{35–37}. The high-frequency Lys residues regulate the number of linchpins and direct the modification of the other residue. It is noteworthy that Lys-derived linchpin would offer excellent opportunities with an isolated protein (Fig. 1a). As we increase the number of proteins

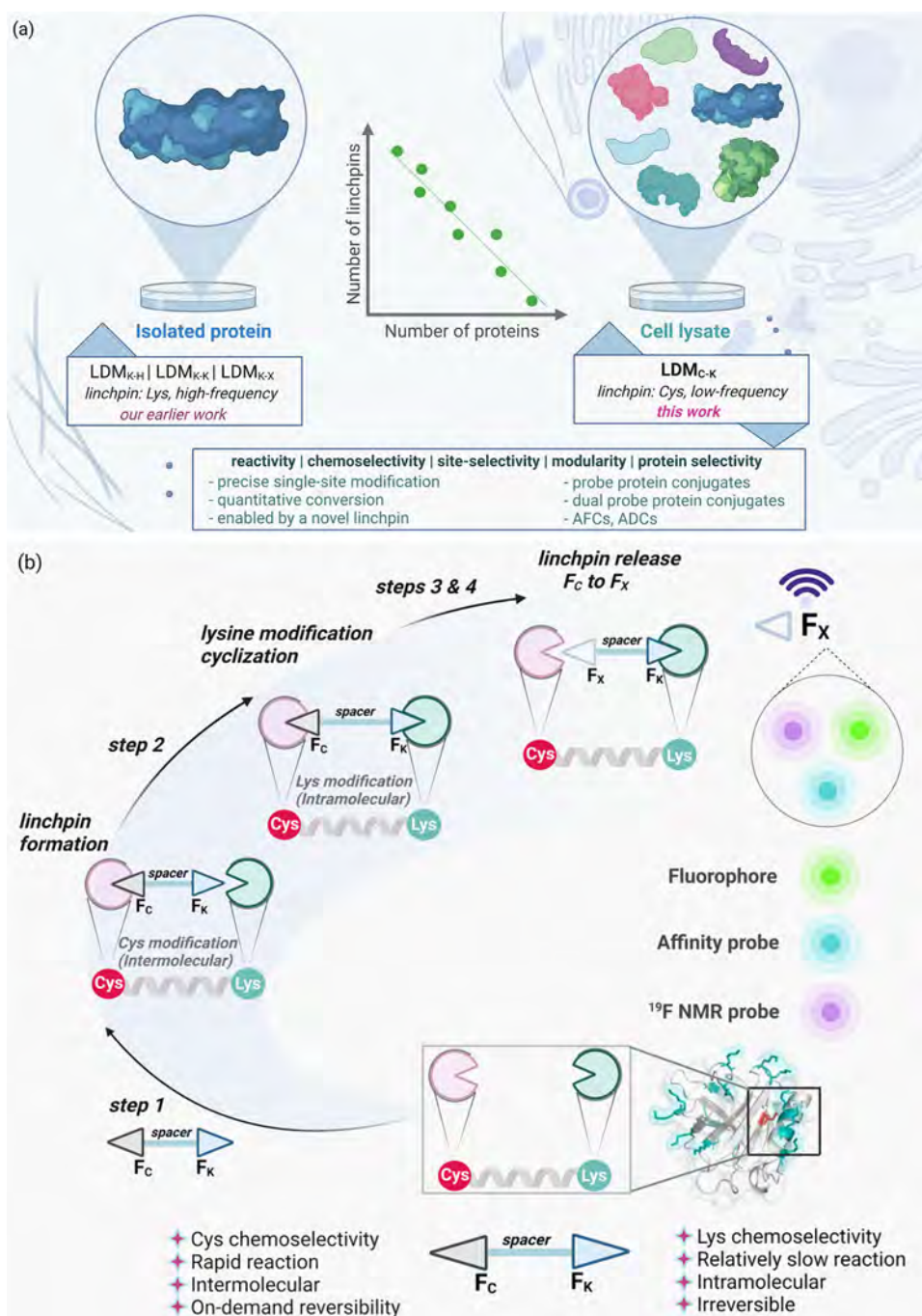


Fig. 1 | Chemical technology for precision engineering of native proteins.

a Requirement: low-frequency residue-derived proximity regulator to create a unique targetable landscape and opportunity for simultaneous regulation of reactivity, chemoselectivity, site-selectivity, modularity, and protein-selectivity.

b Hypothesis: Cys-linchpin-directed modification of Lys residue (LDM_{C-K}). Step 1,

linchpin formation: rapid, intermolecular, chemoselective reaction of F_C with Cys; step 2, cyclization: intramolecular, irreversible, site-selective reaction of F_K with Lys; step 3, F_C to F_X: sequential C-S and C-C bond dissociation; step 4, late-stage modification.

in the mixture, it becomes exceedingly challenging to identify the LDM reagent suitable for precise modification of a single protein. In this perspective, we hypothesized that a method could be valuable if it derives linchpin from a low-frequency residue to modify a high-frequency site (steps 1–4, Fig. 1b). Such an expedition would require the development of a chemoselective electrophile that could enable a rapid reaction with the identified residue at low micromolar concentration. We argued that cysteine could be an ideal residue to anchor the linchpins with F_C if we can solve a combination of two non-trivial challenges. At first, the linchpin must be released under physiological conditions after the completion of the bioconjugation. Secondly, the linchpin-released functional group must render a chemically unique handle for late-stage modifications. We realized that the popular cysteine-selective electrophiles would not serve the purpose³⁸. It is not surprising as they evolved to deliver enhanced stability of the thiol-adduct. On the contrary, there are only a few electrophilic reagents where reversibility was pursued in addition to chemoselectivity³⁹. Hence, it became necessary to develop one that can meet the requirements of our hypothesis.

Here, we report a Cys-derived linchpin-directed single-site modification of lysine (LDM_{C-K}) to meet the technological demands. The success of site-selective LDM_{C-K} required an appropriate multifunctional reagent (F_C -spacer- F_K , Fig. 1b). At first, we developed nitroolefin (F_C) as an electrophile capable of rendering a rapid chemoselective reaction with cysteine (>99% conversion). Subsequently, we identified an acylating group (F_K) for a relatively slow, irreversible, and chemoselective reaction with lysine. However, the success of LDM_{C-K} required us to develop another methodology for reverting the nitroolefin thio-Michael adduct. Also, such a C-S bond dissociation should render a functional group amenable to orthogonal late-stage modification. In this perspective, we developed a one-pot sequential protocol for retro-Michael/retro-Henry reaction that operates under mild conditions with remarkable efficiency over two steps. Importantly, it offers an aromatic aldehyde to enable the capture, enrichment, purification, and late-stage installation of probes. At first, we demonstrate that LDM_{C-K} offers chemoselectivity and site-selectivity to render single-site modification of a protein. The simulation results gave insight into how the design, length, and adaptable rigidity of the spacer regulate the bioconjugation site. Subsequently, we demonstrate that the method translates efficiently to the protein mixture or cell lysate and addresses the question of protein selectivity. Besides, we present a user-friendly protocol for isolating analytically pure protein tagged with the probe of interest at a single site. In this modular platform, the reagent design enables modification of a lysine beyond protein-defined reactivity order. We demonstrate that LDM_{C-K} could deliver the single-site installation of various biophysical probes. The chemical and functional space expansion for selective targeting has also opened gateways for dual-probe bioconjugates. We established it through LDM_{C-K}-enabled installation of a fluorophore, affinity tag, or NMR probe coupled with chemoselective single-site installation of the second tag at the linchpin site to ensure proximal precision dual-labeling of the protein. Further, we demonstrate that the methodology enables the synthesis of homogeneous ADC capable of highly selective anti-proliferative activity towards HER-2 expressing SKBR-3 breast cancer cells.

Results

Design and synthesis of LDM_{C-K} reagent

The design of the LDM_{C-K} reagent involved three critical building blocks: F_C , F_K , and the spacer. We initiated by searching for an electrophile that can result in a rapid chemoselective reaction with cysteine (F_C). Besides, it must offer the attribute of on-demand C-S bond dissociation to render an orthogonally maneuverable functional group. In this perspective, we selected a set of soft electrophiles (**1a–1l**) bearing polarized double bonds and diffused electron densities for

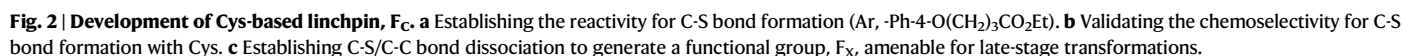
screening with thiol-based reagent **2** (Fig. 2a and Supplementary Figs. 15–26, also see Supplementary Table 1). A few electrophiles (**1a–c**, **1f–i**) were non-reactive under the reaction conditions. Interestingly, vinyl sulfone **1e** resulted in 60% conversion (**3e**). While acrylonitrile (**1j**) has shown better reactivity (**3j**, 83% conversion), another vinyl sulfone derivative, **1d** led to quantitative yields. Keeping the synthetic maneuverability constraints in mind, we furthered our screening. The maleimide (**1k**) also worked well to result in high conversions to the thio-Michael adduct. However, it is challenging to trigger retro-Michael addition or nucleophilic substitution in this case without interference from side reactions, particularly hydrolysis³⁸. To our delight, nitroolefin (**1l**) resulted in quantitative conversions (Fig. 2a). Further, we mixed twenty amino acids with unprotected side-chain and found it to exhibit exclusive Cys-selectivity (Fig. 2b and Supplementary Figs. 27–28, also see Supplementary Table 2). Hence, we selected the thio-Michael adduct with nitroolefin (**3l**) to test it for the C-S bond dissociation reaction under mild aqueous conditions (Fig. 2c).

The C-S bond dissociation proceeded with moderate efficiency through retro-Michael pathway over time or in the presence of DABCO, TMG, and urea (Fig. 2c, also see Supplementary Table 3 and Supplementary Figs. 29–37). The regenerated nitroolefins can be used for late-stage installation of probes with nucleophilic thiol handles. However, such a system is more suited for applications involving reversibly installed tags but not for biologics such as ADCs. Hence, it was important for us to develop a method for the transformation of F_C to another chemically orthogonal handle (F_X) that can result in stable probe conjugates. The subsequent transformation of F_C , such as retro-Henry pathway involving C-C bond dissociation, remained elusive in the previous cases. However, DBU, DIPEA, and TEA enabled the retro-Henry reaction to form aldehyde in moderate conversions (14–22% in 24 h, Supplementary Table 3). After a thorough screening, we were delighted to establish a one-pot sequence of reactions involving both C-S and C-C bond dissociation under mild conditions (55% within 12 h, bicarbonate buffer, pH 8.5, Supplementary Table 3). The chemoselectivity with thiol bioconjugation and chemically orthogonal dissociation rendering F_X made nitroolefin the preferred group to be examined as the F_C component.

The aryl group in these test studies offers the synthetic maneuverability for subsequent installation of F_K and spacer. This feature is vital as the screening of the second electrophilic group, F_K , needs to be performed in conjugation with the F_C and spacer. This leap of faith was necessary to validate the chemoselectivity and site-selectivity along with the relative reactivity order of F_C versus F_K . In this perspective, we identified acylating groups to serve the latter's purpose. Next, we synthesized LDM_{C-K} reagents (**9a–9d**) with a range of leaving groups to establish the right combination of functional groups (Fig. 3a; for multistep synthesis of reagents, see Supplementary Figs. 1–5). We selected β -lactoglobulin A (BLGA, **8a**, PDB ID: 3BLG, Supplementary Fig. 39) with one free Cys and fifteen Lys residues as the model protein to validate the hypothesis (Fig. 3a). The NHS-ester derivative (**9a**) was highly reactive and resulted in heterogeneous labeling of proteins (Supplementary Figs. 38 and 40). Upon replacing the leaving group to bring down the reactivity, the tribromophenoxide derivative (**9b**) resulted in mono-labeled BLGA (**10b**, 9% conv., Supplementary Fig. 41). Interestingly, substituting one bromine with a morpholine amide (**9c**) led to improved conversions (**10c**, 30%, Supplementary Fig. 42) while retaining the homogeneity. Finally, we were delighted to note quantitative yields (**10d**, >99%, Supplementary Fig. 43) with tetrafluorophenoxide (**9d**) as the leaving group. With desired F_C and F_K in hand, we synthesized a set of LDM_{C-K} reagents for further evaluation (**9e–9h**, Fig. 3b and Supplementary Figs. 6–9).

Investigation with peptides

At this point, we designed a set of experiments with peptides to validate the reactivity and selectivity attributes of LDM_{C-K} (Fig. 4 and



that such competing residues would not leave traces after the C-S bond dissociation. Next, we established the efficiency of the LDM-C_K workflow with a similar peptide equipped with two additional arginine residues for enhanced solubility (Fig. 4h). The rapid LDM-C_K results in the cyclic peptide (**14i**) within 15 minutes. The subsequent C-S bond dissociation, thiol interception with maleimide, and retro-Henry reaction allow quantitative aldehyde generation (**15b**, >99%). Finally, the oxime formation proceeds in excellent conversions (>99%) to render the dual-labeled peptide **15c** (Fig. 4h).

After validating the hypothesis and a clear understanding of reactivity and selectivity parameters, we were all set to establish the capability of LDM_{C-K} with a native protein. At first, we re-validated the chemoselectivity of the nitroolefin-based control reagent (**11**) with BLGA (**8a**). The proteolytic digestion of **11**-labeled BLGA with α -chymotrypsin, peptide mapping (labeled VCQCL; residues 118–122; m/z 844.7 [M + H]⁺), and MS-MS confirmed the chemoselective labeling of C121 (Supplementary Fig. 52). In a control experiment, the proteins devoid of Cys did not result in any irreversible bioconjugation (Supplementary Table 5 and Supplementary Fig. 51). Now, we had to establish the efficiency of the C-S bond dissociation protocol (Fig. 5a). For this purpose, we selected the optimized example (Fig. 3) and synthesized mono-labeled BLGA (**10d**) with LDM_{C-K} reagent (**9d**) in >99% conversion (step 1, Fig. 5a). In a control experiment, the

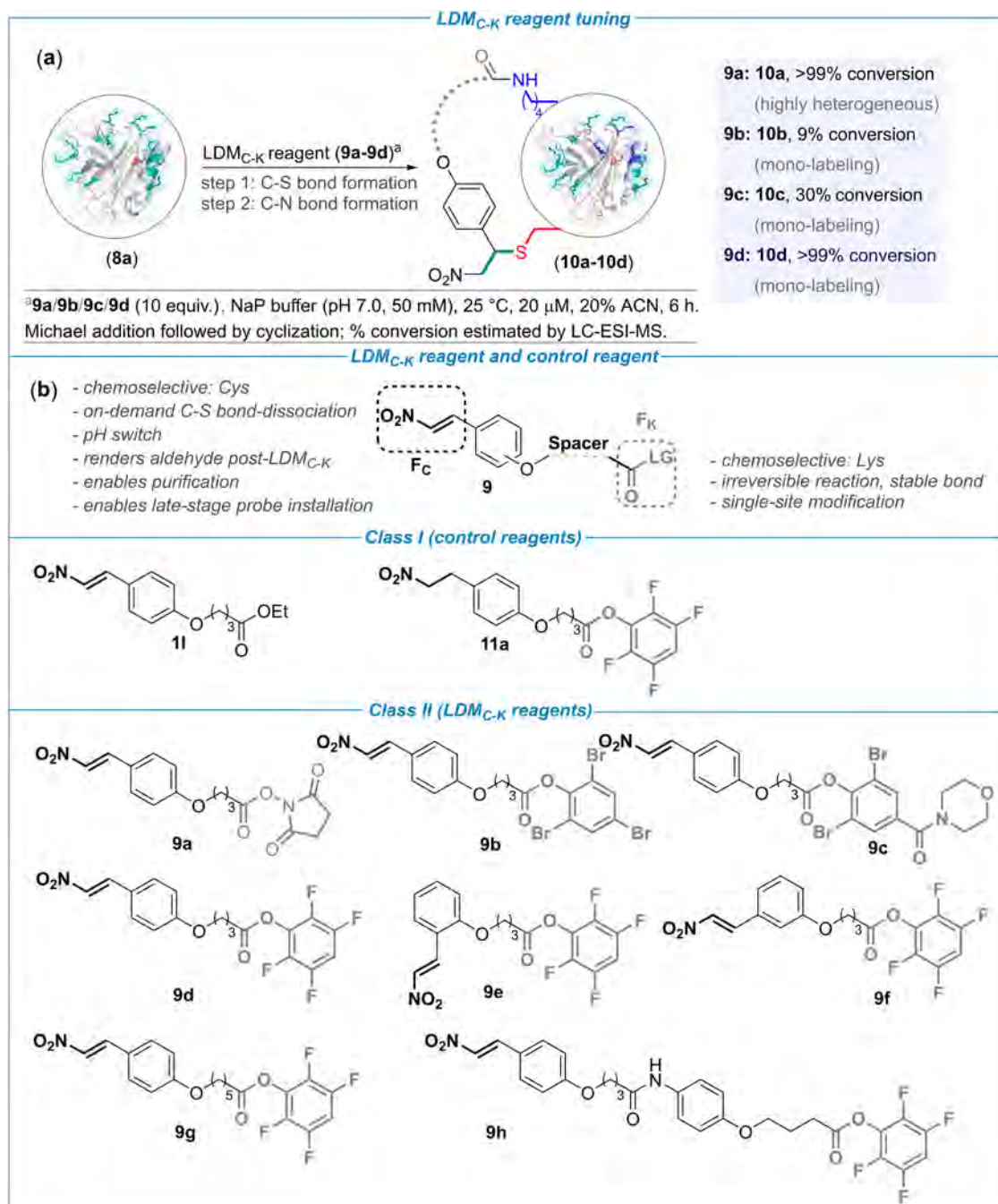


Fig. 3 | Development of LDM_{C-K} reagents. a Initial exploration with BLGA to establish the design of F_K and relative reactivity with F_C. **b** The design and synthesis of potential LDM_{C-K} and control reagents (for synthesis, see Supplementary Figs. 1–10).

BLGA (**8a**) pre-treated with maleimide does not result in bioconjugation with **9d** (Supplementary Fig. 53). The non-reactivity of **9d** with proteins devoid of free Cys revalidates the case (Supplementary Table 6 and Supplementary Figs. 58–64). Also, the reduced nitroolefin derivative of LDM_{C-K} reagent **9d** (**11a**) leads to heterogeneous modification of BLGA, potentially through Cys-based thioester (Supplementary Fig. 54). Further, it was interesting to note that our findings with the small molecule model system (Fig. 2c) translated for the C-S bond dissociation in BLGA bioconjugate (**10d**). We trapped the retro-Michael reaction-led nitroolefin (path I, Fig. 5a and Supplementary Fig. 55) immediately by a thiol to avoid the reversible reaction. We were delighted to note the efficient translation of one-pot C-S and C-C bond dissociation to proteins for transforming F_C (nitroolefin) to F_X (aldehyde) under operationally simple conditions

(path II, Fig. 5a and Supplementary Fig. 56). To enable unambiguous protein sequencing, we further treated the BLGA bioconjugate (**17**) with hydroxylamine (**18**). The subsequent mono-labeled product (**20d**) was digested with α-chymotrypsin enabling the identification of labeled KKY in peptide mapping (residues 100–102, *m/z* 643.3 [M+H]⁺). The MS-MS confirmed the site of modification as K101 (Supplementary Fig. 66). Besides multiple other Lys residues, the other proximal Lys (K100) does not undergo any modification highlighting the method's capability in distinguishing closely placed targets.

After establishing the concept unambiguously, we were equipped with all the tools of LDM_{C-K} technology to test it further. We selected the LDM_{C-K} reagents (**9e** and **9f**) with ortho- and meta-disubstituted aromatic ring-based spacer in place of para-disubstituted aryl ring in **9d**.

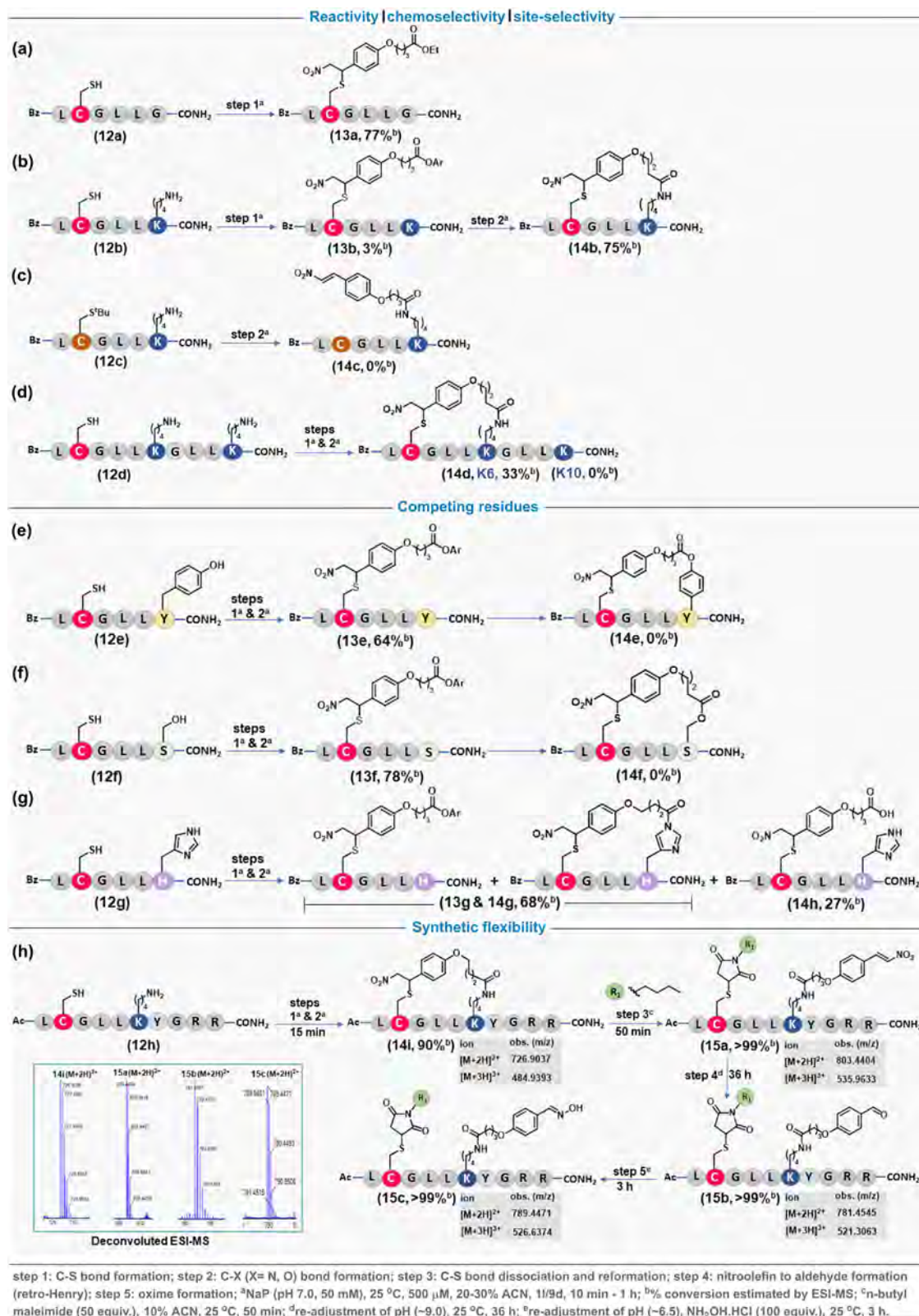


Fig. 4 | Peptide modification. **a** Control reagent **11** with Fc and Cys-containing peptide **12a** results in efficient C-S bond formation. **b** LDM_{C-K} reagent **9d** mixed with peptide **12b** having Cys and Lys at *i* and *i* + 4 positions result in C-S bond followed by C-N bond formation rendering the cyclic peptide **14b** within 1 h. **c** LDM_{C-K} reagent **9d** with peptide **12c** having thiol protected Cys and Lys at *i* and *i* + 4 positions result in no conversion within 1 h. **d** LDM_{C-K} reagent **9d** with peptide **12d** having Cys at *i*

and two Lys residues at *i* + 4/*i* + 8 positions result in C-S bond formation followed by site-selective C-N bond formation at *i* + 4 position. Control experiments to examine competition with e Tyr, f Ser, and g His. **h** Establishing the complete LDM_{C-K} workflow. The selected MS for **14i**, **15a**, **15b**, and **15c** are given in the inset. Also see Supplementary Figs. 102–106 for detailed data, full XIC, and MS spectra.

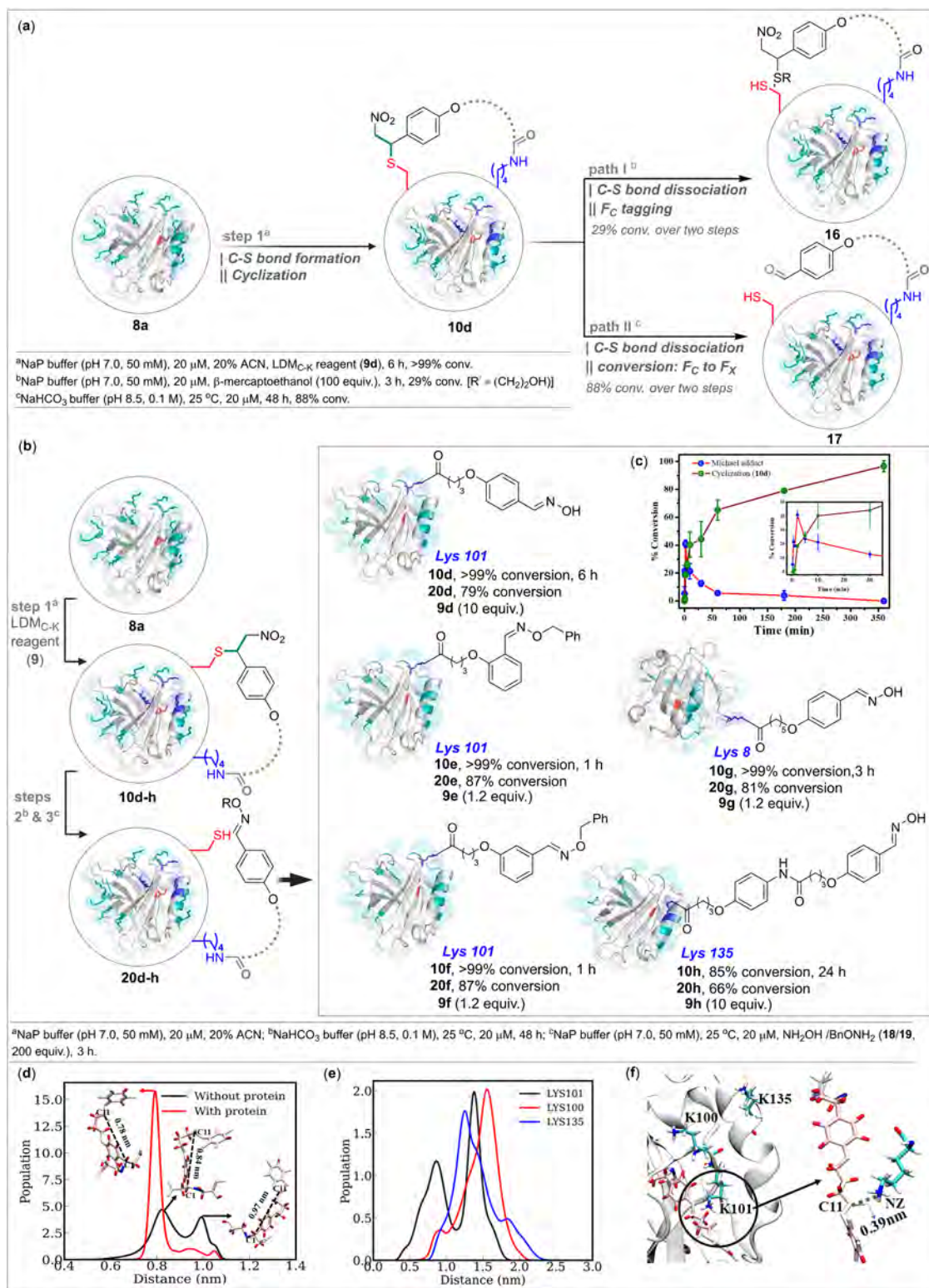


Fig. 5 | LDMC-K technology. **a** Workflow for C-S bond formation, cyclization, C-S/C-C bond dissociation, and F_C to F_X transformation. **b** The chemoselective, site-selective, and modular single-site labeling of a native protein (β -lactoglobulin, BLGA, **8a**). **c** The plot of % conversion versus time highlights the progress of Michael addition and cyclization with BLGA in the presence of LDMC-K reagent **9d**. Data are presented as mean values (\pm SD), $n = 3$ independent experiments. **d** Adaptable rigidity of LDMC-K reagent: Probability distribution for the reagent (**9d**)

and end-to-end distance between electrophilic C1 and C11 atoms in water with (red) or without (black) BLGA. The most probable conformations corresponding to peaks are shown. **e** Probability distribution of the distance between electrophilic C11 atom of the LDMC-K reagent **9d** and nearby lysine residues (NZ atom) in BLGA. **f** Interaction of C11 in **9d** with the nearby lysine residues. Inset highlights the interaction of Lys101 with the C11 (**9d**).

Interestingly, eight-fold less bioconjugation reagent **9e** was enough for six-fold improvement in reaction time to render mono-labeled BLGA within 1 h (Fig. 5b and Supplementary Fig. 67). Also, the LDM_{C-K} reagent (**9f**) turned out to be remarkable and delivered mono-labeled BLGA (**8a**) within an hour (Fig. 5b and Supplementary Fig. 68). We released the linchpin for these two bioconjugates by C-S/C-C bond dissociation and the subsequently formed aldehyde was captured with benzoyloxamine (**19**). These adducts were then subjected to protease degradation followed by peptide mapping. Both **20e** and **20f** resulted in labeled KKY (residues 100–102, m/z 733.3 [M + H]⁺) that upon subsequent MS-MS confirmed the site-of-modification as K101 (Supplementary Figs. 67 and 68). To gain further insights into the sequence of steps, we performed a time-dependent investigation of LDM_{C-K}-enabled bioconjugation (Fig. 5c and Supplementary Fig. 65). Here, mixing BLGA and LDM_{C-K} reagent **9d**, quenching it with dilution after ten seconds, and subjecting it immediately to MS confirmed a rapid C-S bond formation with F_C. At this point, no acylation is observed. Within the next twenty seconds, the increase of linchpin formation by thio-Michael addition (Cys-F_C) is accompanied by cyclization (Lys-F_K), while the latter becomes more prominent after 2 minutes. The Michael adduct disappears gradually and renders >99% cyclic adduct within 6 h.

With these detailed studies in the background, the next step was to establish modular capabilities. The initial noteworthy result in this perspective came with >99% conversion to bioconjugate **10g** within 3 h when we treated BLGA with the re-designed LDM_{C-K} reagent **9g** (Fig. 5b). Upon subsequent steps involving C-S/C-C bond dissociation followed by oxime formation, we subjected the mono-labeled product **20g** to protein sequencing. The proteolytic digestion and peptide mapping led to the identification of labeled KGL (residues 8–10, m/z 550.4 [M + H]⁺). The MS-MS confirmed the labeling of site K8 and the modularity of LDM_{C-K} technology (Supplementary Fig. 69). Interestingly, the closely placed K91 and K141 do not get labeled at all. We further challenged the method to label another unique Lys residue in the vicinity of Cys121. Gratifyingly, the LDM_{C-K} reagent **9h** rendered the chemoselective and site-selective modification of K135 (EKF, residues 134–136, m/z 806.4 [M + H]⁺). Again, the neighboring Lys residues (K138 and K141, Supplementary Fig. 70) do not compromise the bioconjugation selectivity. Overall, these results established that LDM_{C-K} can simultaneously regulate reactivity, chemoselectivity, site-selectivity, and modularity with excellent control.

Molecular dynamics simulation: impact of linchpin formation on the adaptable conformational rigidity of LDM_{C-K} reagent and downstream site-selection. We anticipated that the effectiveness and selectivity of the LDM_{C-K} reagents would be primarily dependent on their geometry and conformation. It is tempting to speculate that these parameters would regulate the effective distance between F_C and F_K. Hence, the BLGA modification must happen if the distance between the linchpin (Cys121) and the target site (Lys) matches the spacer's effective length. However, the dynamics of the protein and the reagent could make the linear correlation unreliable. Hence, we resorted to MD simulation to provide microscopic insights into the structure and dynamics of this complex molecular system and investigate our assumptions' validity.

At first, we analyzed the conformational space of the LDM_{C-K} reagent (capped) in an aqueous medium in the absence and presence of the protein. Figure 5d shows the distance distribution between the atoms C1 and C11, which forms covalent bonds with the S atom of Cys121 and side-chain N atom of target Lys, respectively. Hence, it would be an appropriate parameter to capture the conformational space explored by the LDM_{C-K} reagent and check whether it is consistent with the linchpin-target distance in the protein. It shows the comparison of the distance distribution in both systems. In absence of protein (black line), there is a broad distribution with two predominant peaks around 0.85 nm and 1.0 nm. The representative structures of

these conformations in water are highlighted (Fig. 5d). There is a minor peak above 1.0 nm, which is not analyzed in detail. This data clearly indicates that the LDM_{C-K} reagent can't be treated as a rigid entity. Instead, it exists in multiple possible conformations in an aqueous medium that is characterized by a significant difference in its effective length. Interestingly, this distribution landscape changes drastically in the presence of the protein (BLGA), and there is a major peak at around 0.8 nm (red line). Although there are signatures of other conformations, their population is substantially low. The shift in the conformational ensemble state is induced by the local environment and interactions with the protein. Consequently, a subset of this population is stabilized preferentially. This attribute of adaptable rigidity of otherwise flexible LDM_{C-K} reagent enables it to adjust as per the surface landscape of the protein. These detailed investigations also confirm that the design principle of the bioconjugation reagent merely based on a static length of the extended configuration would not be successful in general.

With these insights, we turned our attention to the LDM_{C-K} reagent in the presence of protein. Being anchored to Cys121, this large and flexible molecule has considerable degrees of freedom to access and interact with different parts of the protein. But the key question is whether it can come into close contact with any of the target Lys residues, particularly the Lys101 residue, which gets selectively modified by LDM_{C-K} reagent **9d**. Further, we investigated the distance distribution between the S atom of the Cys121 and N atom of various Lys residues in the neighborhood (Supplementary Fig. 91). The peaks for both Lys101 and Lys135 appear in proximity around 1 nm, making them tough competitors to be modified selectively by the linchpin.

In order to investigate the propensity of the linchpin to approach various Lys residues in a selective manner, we looked into the probability distribution of the distance between the C11 atom and the N atoms of multiple nearby Lys residues (Lys100, Lys101, and Lys135, Fig. 5e). The comparison of these distance distributions clearly establishes that the C11 atom has the highest propensity to approach the Lys101 residue compared to others. A representative structure showing the proximal interaction is shown in Fig. 5f. Results obtained from all three independent trajectories are consistent with this trend (Supplementary Fig. 92). Thus, our simulation results validate the experimental findings and provide a mechanistic basis of action of the LDM_{C-K} reagent. It also highlights how the conformational flexibility of the reagent coupled with protein-induced rigidity could enable the site selection in protein bioconjugation.

Late-stage modification and dual-probe installation

At this point, we decided to validate the potential of re-engineered linchpin (F_X) for the installation of probes. In this perspective, we treated BLGA with the LDM_{C-K} reagent (**9d**, Fig. 6a). After bioconjugation rendered cyclic protein (**10d**, >99% conv.), we subjected it to one-pot C-S/C-C bond dissociation rendering F_X (**17**). Finally, we distributed the precursor to three reaction vials and subjected them to the parallel installation of probes through oxime formation. Here, the treatment of hydroxylamine derivative (**21–23**) of the probes delivered installation of ¹⁹F NMR tag (**24**, >99% conv. over two steps), biotin-based affinity probe (**25**, 86% overall conv.), and fluorophore (**26**, 91% overall conv.). The remarkable overall efficiency of the protocol is noteworthy. The independent sequencing of the products by MS, proteolytic digestion, peptide mapping (labeled KKY, residues 100–102; m/z 941.9, m/z 927.9, and m/z 875.3 [M + H]⁺), and MS-MS confirms the site-of-conjugation (K101, Supplementary Figs. 71–73). Next, we selected single-site engineered BLGA bioconjugates (**24–26**) for dual-probe installation. In a user-friendly chemoselective protocol, the CPM-maleimide derivative rendered dual-probe conjugates (**28–30**, 71–78%, Supplementary Figs. 75–77) and established its utility for applications in this perspective.

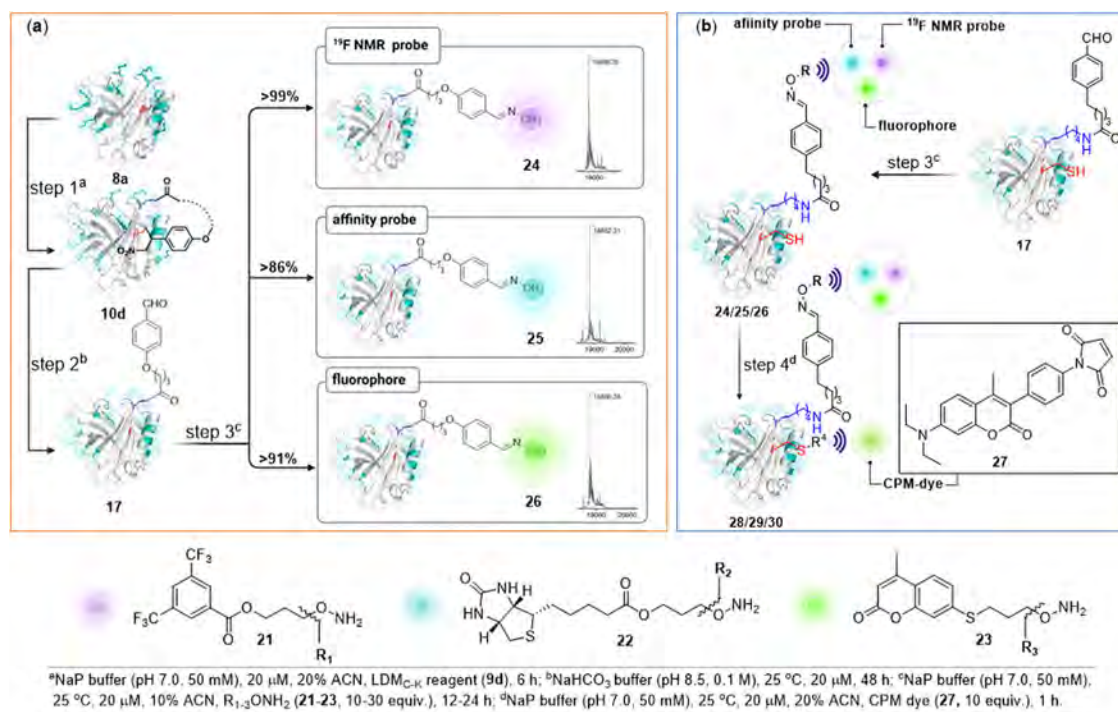


Fig. 6 | Late-stage installation of various tags on protein. a Single-site parallel installation of ¹⁹F NMR probe **21**, affinity probe **22**, and fluorophore **23**. **b** Precise second probe installation through the chemoselective installation of CPM dye (**27**) on protein bioconjugates (**24–26**) to render dual-probe conjugates (**28–30**).

Challenges of serum albumins

At this stage, we decided to display the potential of LDM_{C-K} with serum albumins. These proteins present additional complexity levels due to the display of a large number of solvent-accessible lysine residues and heterogeneous post-translational modifications (Supplementary Figs. 78 and 81). Besides, the solvent accessibility of Cys is much lower in comparison to BLGA. Interestingly, LDM_{C-K} reagent **9d** resulted in single-site modification of bovine serum albumin (BSA **8j**, Supplementary Fig. 79). The proteolytic degradation, peptide mapping (labeled KVPQVSTPTLVEVSR, residues 413–427, m/z 645.69 [$M + 3H$]³⁺), and MS-MS confirmed the site-of-modification as K413 (Supplementary Fig. 79). Further, the single-site labeling of BSA at K431 with LDM_{C-K} reagent **9g** confirmed the modularity of the method (labeled SLGKVGTR, residues 428–435, m/z 570.82 [$M + 2H$]²⁺, Fig. S80). The level of precision becomes even more noteworthy in the backdrop of fifty-nine lysine residues in BSA, where more than twenty are in the proximity of cysteine. With these results in hand, we examined the potential of LDM_{C-K} with human serum albumin (HSA, **8k**). It contains fifty-seven lysine residues, with more than half of them in the proximity of cysteine. Gratifyingly, the LDM_{C-K} reagent **9d** delivered single-site modification of HSA (Supplementary Fig. 82). Further, sequencing confirmed the labeled peptide (LKASLQK, residues 198–205, m/z 621.83 [$M + 2H$]²⁺) and the site-of-modification (K199, Supplementary Fig. 82). The observation of labeled LKASLQK (residues 198–205) with LDM_{C-K} reagents **9e** (m/z 414.89 [$M + 3H$]³⁺) and **9f** (m/z 621.83 [$M + 2H$]²⁺) and subsequent MS-MS re-validated the results (Supplementary Figs. 83–84). The esterase-like activity of HSA remains unperturbed after bioconjugation (Supplementary Fig. 109).

Protein-selectivity empowered by residue-pair selectivity coupled with a purification protocol

We selected a mixture of proteins to establish whether the method can sustain the biomolecular crowding and deliver simultaneous regulation of protein selectivity along with chemoselectivity and site-selectivity (Fig. 7a). At first, a structurally and functionally diverse mixture of myoglobin, lysozyme C, cytochrome C, α-lactalbumin,

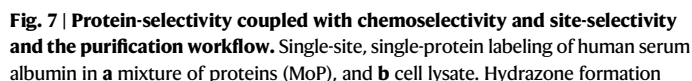
RNase A, ubiquitin, insulin, and HSA was treated with LDM_{C-K} reagent (**9d**). The MS data confirmed the exclusive modification of HSA (**8k**, Supplementary Fig. 85). Next, we coupled the method with a purification protocol. In the process, we used acyl hydrazide functionalized beads to capture and enrich the HSA bioconjugate. After recovering the unreacted proteins, the immobilized HSA bioconjugate on-resin was released through transoximization using hydroxylamine derivative of coumarin (**33**). While the SDS-PAGE (see Supplementary Fig. 86) validated the purification protocol, the peptide mapping, and MS-MS (see Supplementary Fig. 86) of the isolated HSA bioconjugate confirmed the conjugation site (K199). It is noteworthy that the chemoselectivity and site-selectivity remained unaffected by the crowding of other proteins.

Modification of a protein in cell lysate

Later, we challenged the performance of the LDM_{C-K} method in a cell lysate derived from *E. coli* BL21 cells (Fig. 7b). The cell lysate was spiked with HSA (**8k**) and treated with LDM_{C-K} reagent **9d**. We were delighted to note protein-specific modification of HSA. We utilized the purification protocol involving hydrazone-oxime chemistry to enrich the HSA bioconjugate from the cell lysate and installed a coumarin tag in the process. After confirming the purity by SDS-PAGE (Supplementary Fig. 87), the proteolytic digestion, peptide mapping, and MS-MS confirmed the conservation of chemoselectivity and site-selectivity (K199, Supplementary Fig. 87) in combination with protein selectivity. It further revalidates that LDM_{C-K} can negate the biomolecular crowding effect making it a suitable method for diverse applications.

Antibody-drug conjugates

The emergence of platforms for precision engineering of proteins has promised to meet protein-based therapeutics' requirements. One of the biggest beneficiaries in this perspective has been the ADCs. These constructs provide a state-of-the-art platform for directed cancer chemotherapeutics. However, many efforts failed at the pre-clinical stages due to the lack of methods to access homogeneous ADCs. In this regard, the LDM platform offers promise. The ADC involves a



captures and enriches the bioconjugate by ordered on-resin immobilization allowing recovery of unlabeled proteins. Next, the transoximization and centrifugal spin concentration render analytically pure single-site tagged protein bioconjugate.

efficacy and specificity of LDM_{C-K}-ADC towards antiproliferation of HER-2 positive breast cancer cells.

The precision engineering of proteins creates a hinge to connect chemistry, biology, and medicine. Hence, it is not surprising that it has drawn attention from diverse segments of science, specifically the rapidly growing biologics for precision therapeutics. However, the inherent challenges kept the principles for regulating precision elusive. As a result, the technological demand for specifically engineered proteins remained unmet, thrusting the use of heterogeneous bioconjugates. The efforts of our group and a few others helped gain insight into principles for rendering chemoselectivity and site-selectivity. However, simultaneous regulation of multiple selectivity attributes beyond protein-defined reactivity order remains challenging.

This manuscript presents an LDM that establishes the principles for creating a cysteine-based linchpin and directs single-site modification of a proximal lysine residue (LDM_{C-K}). The method renders simultaneous regulation of reactivity, chemoselectivity, site-selectivity, modularity, and protein selectivity within the spectrum of optimized reaction parameters. The selection of low-frequency cysteine was critical to directly modifying a single lysine of a single protein within a complex mixture of proteins. However, finding an appropriate handle to serve multiple attributes around C-S bond formation and

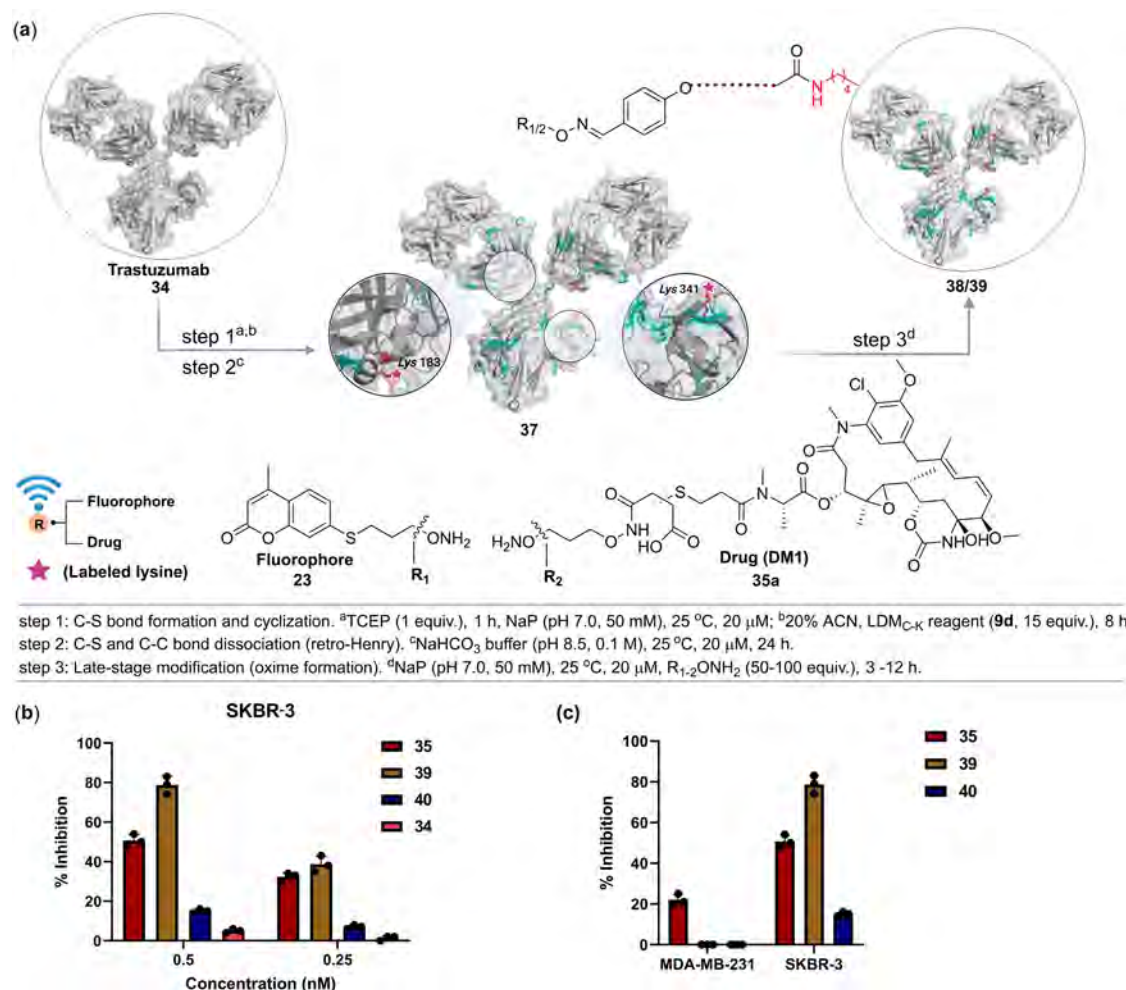


Fig. 8 | LDMC-K for homogeneous antibody-fluorophore and drug conjugate (AFC and ADC). **a** LDMC-K reagent **9d** renders homogeneous trastuzumab conjugates with specific modifications of K183 and K341. Subsequently, AFC (**38**) and ADC (**39**) were prepared by late-stage installation of hydroxylamine derivative of fluorophore (coumarin, **23**) and drug (emtansine, DM1, **35a**). **b** Inhibition of cell proliferation by LDMC-K-ADC (**39**) as compared to trastuzumab (**34**), DM1 (**35**), and

Kadcyla (**40**) in SKBR-3 (HER-2 positive) cancer cell line. The percentage inhibition was calculated using untreated cells as control. **c** Inhibition of cell proliferation by DM1 (**35**), ADC (**39**), and Kadcyla (**40**) at 0.5 nM concentration in HER-2 positive SKBR-3 as compared to HER-2 negative MDA-MB-231 cells. Data are presented as mean values (±SD), *n* = 3 biologically independent experiments.

chemically orthogonal bond dissociation under mild physiological conditions was non-trivial. Hence, the major credit for the success of LDMC-K goes to the unique F_C or nitroolefin chemistry that enabled the cysteine-linchpin by rendering an array of synchronized bond formation and dissociation processes. Besides, its successful transformation to aldehyde under mild conditions enables the integration of orthogonal late-stage transformations. Importantly, it drives a highly efficient purification protocol to deliver ordered protein immobilization and analytically pure single-site probe-tagged bioconjugates. Notably, the seamless operation extends to a complex biomolecular mixture to deliver protein selectivity coupled with single-site precision. Another critical attribute of the LDMC-K technology emerges from the adaptable rigidity of spacer that facilitates the placement of lysine selective group and irreversible bioconjugation. Notably, the overall protocol is user-friendly for non-experts. The platform proved highly efficient for synthesizing homogeneous dual-probe protein conjugates, antibody-fluorophore conjugate (AFC), and ADC. The LDMC-K-ADC exhibited excellent antiproliferative activity toward HER-2-positive breast cancer cells without negatively impacting the HER-2-negative cells. Further, the LDM technology offers principles that promise to bridge the gap between precision engineering of isolated proteins versus when they reside in a complex ecosystem.

Methods

General procedure of peptide labeling

The peptide stock solution was freshly prepared using acetonitrile with two drops of HPLC-grade methanol to enhance the solubility.

Michael addition to the peptide

Peptide containing free cysteine **12a** (68 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent **11** (28 µg, 50 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the Michael addition was monitored by LC-ESI-MS for up to 1 h.

Acylation with the peptide

Peptide containing protected cysteine and free lysine **12c** (80 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent **9d** (40 µg, 100 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the reaction was monitored by LC-ESI-MS for up to 1 h.

Peptide macrocyclization

Peptide containing free cysteine and lysine **12b** (75 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent **9d** (40 µg, 100 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the Michael addition followed by macrocyclization was monitored by LC-ESI-MS up to 1 h. This protocol was also used for peptides **12e–12g** for examining the competition from Tyr, Ser, and His. The peptide **12h** requires a few changes in the method (ESI) due to improved solubility in water.

Site-selective peptide modification

Peptide containing one free cysteine and two lysine residues **12d** (116 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent **9d** (40 µg, 100 nmol) in ACN (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the reaction was monitored by LC-ESI-MS up to 1 h. The site-of-modification was confirmed by MS-MS.

General procedure for site-selective modification of native proteins

Protein **8a** (2 nmol) in phosphate buffer (70–80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, LDM_{C-K} reagent (**9d–9h**, 2.4–20 nmol, each separately) in acetonitrile (20–30 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and LDM_{C-K} reagent was 20 µM and 24–200 µM, respectively. After 1–24 h, the reaction mixture was diluted with water (500 µl), followed by centrifugation for 1 min at 9600×g to precipitate the unreacted LDM_{C-K} reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2 × 0.4 ml). The sample was analyzed by LC-ESI-MS or MALDI-ToF-MS. The sample was then buffer exchanged using NaHCO₃ (100 µl, 0.1 M, pH 8.5) and incubated for 48 h. It resulted in the C-S bond dissociation, and a free aldehyde handle on the protein (**17**) was generated via the retro-Henry reaction. The sample was then buffer exchanged using phosphate buffer (100 µl, 50 mM, pH 7.0). To this solution, hydroxylamine hydrochloride **18** or *O*-benzylhydroxylamine hydrochloride **19** (2 µmol) in water (10 µl) from a freshly prepared stock solution was added for late-stage modification (oxime formation) and incubated for 3 h. The excess hydroxylamine and salts were removed by a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO) and the sample was collected in an aqueous medium. The modification of protein was analyzed by LC-ESI-MS. The aqueous sample was concentrated by lyophilization before subjecting it to digestion, peptide mapping, and sequencing by MS-MS. [Note: The same protocol was followed for the single-site modification of BSA (**8j**) and HSA (**8k**)].

General procedure for molecular dynamics simulations

The atomistic classical molecular dynamics (MD) simulation studies were performed to understand LDM chemistry. The structures were solvated in TIP3P water box, extending 12 Å from the solute in all three directions using the LEAP module in AMBER20. Appropriate numbers of Na⁺ and Cl[−] counterions were added to neutralize the charges and to maintain 150 mM physiological salt concentration. AMBER ff14 force field was used to describe the interaction involving protein and Joung and Cheatham parameters for ions. The resulting solvated structures were subjected to minimization. Then MD was performed under constant pressure-constant temperature conditions (NPT) for 500 ps using a harmonic restraint on the solute with a force constant of 10 kcal/mol/Å². This was followed by a production run of 1000 ns. Three independent trajectories were generated for the production runs. The LDM_{C-K}

reagent and the protein were simulated separately in water using similar protocols as reference systems.

The MD simulations were carried out using the PMEMD module of AMBER20 package with imposed 3D periodic boundary conditions. TIP3P water model was used to solvate the systems. A time step of 2 fs was used to integrate the equation of motion. The temperatures were maintained for the simulations using Langevin dynamics, while pressure was kept constant at 1 atm using Berendsen weak coupling method with anisotropic pressure scaling. The particle mesh Ewald method was used to calculate long-range electrostatic calculations with a real space cut-off distance of 10 Å where the vdW and direct electrostatic interactions were truncated. All bond lengths involving hydrogen atoms were held fixed using the SHAKE algorithm. All analyses were done using CPPTRAJ module of AMBER20 tools. Snapshot generations were done using (Visual Molecular Dynamics).

General procedure for single-site installation of tags

After the installation of free aldehyde handle on the protein (**17**) via retro-Henry process, various derivatives of hydroxylamine (**21–23**, 20–60 nmol) in H₂O:ACN (1:1) from a freshly prepared stock solution were added for the late-stage modification (oxime formation) and incubated for 12–24 h. The excess hydroxylamine and salts were removed by a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO), and the sample was collected in an aqueous medium. The modification of protein was analyzed by LC-ESI-MS. The aqueous sample was concentrated by lyophilization before subjecting it to digestion, peptide mapping, and sequencing by MS-MS.

General procedure for dual-probe installation

Protein **8a** (2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, LDM_{C-K} reagent (**9d**, 20 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and LDM_{C-K} reagent was 20 µM and 200 µM, respectively. After 6 h, the reaction mixture was diluted with water (500 µl), followed by centrifugation for 1 min at 9600×g to precipitate the unreacted LDM_{C-K} reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2 × 0.4 ml). The sample was analyzed by LC-ESI-MS. The sample was then buffer exchanged using NaHCO₃ (100 µl, 0.1 M, pH 8.5) and incubated for 48 h. The C-S bond dissociation and retro-Henry reaction render the free aldehyde handle on the protein (**17**). The sample was then buffer exchanged using phosphate buffer (100 µl, 50 mM, pH 7.0). To this solution, various derivatives of hydroxylamine (**21–23**, 20–60 nmol) in ACN (10 µl) from the freshly prepared stock solution were added. The incubation for 12–24 h rendered the late-stage modification through oxime formation. Later to this solution, the CPM dye (**27**, 20 nmol) in acetonitrile (20 µl) from freshly prepared stock solution was added and incubated for 1 h. The excess hydroxylamine derivative, CPM dye, and salts were removed by a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO), and the sample was collected in an aqueous medium. The modification of protein was analyzed by LC-ESI-MS.

Protein-selectivity coupled chemoselective and site-selective protein modification, enrichment, and late-stage tagging in a representative mixture of eight proteins. Ubiquitin (17 µg, 2 nmol), cytochrome C (24 µg, 2 nmol), RNase A (27 µg, 2 nmol), insulin (23 µg, 4 nmol), α-lactalbumin (28 µg, 2 nmol), lysozyme C (28 µg, 2 nmol), myoglobin (34 µg, 2 nmol), and HSA (25 µg, 0.4 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) were mixed in a 1.5 ml microcentrifuge tube. Here, the HSA concentration was kept five times lower than the other proteins to present a substantial challenge to the method. To this solution, LDM_{C-K} reagent **9d** (1.6 µg, 4 nmol) in ACN (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C.

After 3 h, the reaction mixture was diluted with grade I water (500 μ l). The unreacted LDM_{C-K} reagent **9d** and salts were removed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO) and the protein mixture was collected in the NaHCO₃ buffer (100 μ l, 0.1 M, pH 8.5). Next, the incubation for 48 h generates a free aldehyde handle on protein (**32**) via the retro-Henry reaction. Subsequently, the reaction mixture was diluted with phosphate buffer (500 μ l, 50 mM, pH 7.0). The buffer exchange was performed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO). Subsequently, this reaction mixture was utilized for the enrichment of HSA.

In a 5 ml fritted polypropylene chromatography column with end tip closures, hydrazide beads (400 μ l, hydrazide resin loading: 16 μ mol/ml) were taken. The beads were washed with phosphate buffer (0.1 M, pH 7.0, 5 \times 1 ml) and re-suspended (phosphate buffer, 100 μ l, 50 mM, pH 7.0). The reaction mixture containing modified HSA **32** (40 nmol) in phosphate buffer (500 μ l, 50 mM, pH 7.0) was added to the beads. Next, the end-to-end rotation (29 \times g, rotary mixer) was performed at 25 $^{\circ}$ C for 24 h. The supernatant was collected, and the beads were washed with KCl (0.5 M, 6 \times 1 ml) and phosphate buffer (0.1 M, pH 7.0, 4 \times 1 ml) to remove the unreacted and adsorbed proteins from the resin. The beads were further washed with Grade I water (6 \times 1 ml) and re-suspended (phosphate buffer, 450 μ l, 50 mM, pH 6.5). To release the labeled protein from its immobilized derivative, the *O*-hydroxylamine derivative of coumarin **23** (20 μ M) in ACN:H₂O (50 μ l) was added. The subsequent end-to-end rotation at 25 $^{\circ}$ C for 24 h led to transoximization. The supernatant was collected while the salts and **23** were removed using the centrifugal spin concentrator (10 kDa MWCO). The purity of the labeled protein **33** was confirmed by in-gel fluorescence (please see the source data file), peptide mapping, and MS-MS. The protocol results in coumarin tagged labeled protein **33** with excellent purity.

Single-site, single-protein labeling in cell lysate and its enrichment.

E. coli BL21 cell lysate (2 μ g/1 μ l) in phosphate buffer (75 μ l, 50 mM, pH 7.0) spiked with HSA **8k** (25 μ g, 0.4 nmol, in phosphate buffer, 5 μ l, 50 mM, pH 7.0) were taken in a 1.5 ml microcentrifuge tube. To this solution, the LDM_{C-K} reagent **9d** (1.6 μ g, 4 nmol) in ACN (20 μ l) from a freshly prepared stock solution was added and incubated at 25 $^{\circ}$ C. After 3 h, the reaction mixture was diluted with Grade I water (500 μ l). The unreacted LDM_{C-K} reagent **9d** and salts were removed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO). The reaction mixture was collected in NaHCO₃ buffer (100 μ l, 0.1 M, pH 8.5). The subsequent incubation for 48 h renders a free aldehyde handle on protein (**32**) via retro-Henry reaction. Next, the reaction mixture was diluted with phosphate buffer (500 μ l, 50 mM, pH 7.0). The buffer exchange was performed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO). This reaction mixture was utilized for the enrichment of modified HSA in the subsequent step.

In a 5 ml fritted polypropylene chromatography column with end tip closures, hydrazide beads (400 μ l, hydrazide resin loading: 16 μ mol/ml) were taken. The beads were washed with phosphate buffer (0.1 M, pH 7.0, 5 \times 1 ml) and re-suspended (phosphate buffer, 100 μ l, 50 mM, pH 7.0). The cell lysate containing labeled and unlabeled HSA (combined batch: 2.6 mg, 40 nmol) in phosphate buffer (500 μ l, 50 mM, pH 7.0) were added to the beads, followed by end-to-end rotation (29 \times g, rotary mixer) at 25 $^{\circ}$ C for 24 h. The supernatant was collected, and the beads were washed with KCl (0.5 M, 6 \times 1 ml) and phosphate buffer (0.1 M, pH 7.0, 4 \times 1 ml) to remove the unreacted and adsorbed proteins from resin. The beads were further washed with Grade I water (6 \times 1 ml) and re-suspended (phosphate buffer, 450 μ l, 50 mM, pH 6.5). To release the labeled protein from its immobilized derivative, the *O*-hydroxylamine derivative of coumarin **23** (20 μ M) in ACN:H₂O (50 μ l) was added. Next, the transoximization is facilitated by end-to-end rotation at 25 $^{\circ}$ C for 24 h. The supernatant was collected while the salts and **23** were removed using the centrifugal spin concentrator

(10 kDa MWCO). The purity of the labeled protein **33** was confirmed by in-gel fluorescence (please see the source data file), peptide mapping, and MS-MS. The protocol results in coumarin tagged labeled protein **33** with excellent purity.

Site-selective modification of trastuzumab and synthesis of AFC.

Trastuzumab **34** (300 μ g, 2 nmol, Emcure, N7123B01) in phosphate buffer (80 μ l, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. TCEP (5 μ g, 2 nmol) was added to this solution, and the reaction mixture was vortexed for 1 h. It was followed by the addition of reagent **9d** (12 μ g, 30 nmol) in ACN (20 μ l) from a freshly prepared stock solution and vortexed for 8 h at 25 $^{\circ}$ C. The overall concentration of the trastuzumab **34** and LDM_{C-K} reagent **9d** was 20 μ M and 300 μ M, respectively. After 8 h, the reaction mixture was diluted with water (500 μ l), followed by centrifugation for 1 min at 9600 \times g to precipitate the unreacted LDM_{C-K} reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 ml, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2 \times 0.4 ml). The sample was analyzed by LC-ESI-MS. The sample was then buffer exchanged using NaHCO₃ (100 μ l, 0.1 M, pH 8.5) and incubated for 48 h. This step resulted in C-S bond dissociation to generate the free aldehyde handle on the protein (**37**) enabled by the retro-Henry reaction. This trastuzumab conjugate (**37**) can be stored after lyophilization for late-stage installation of desired probes through their hydroxylamine derivative. The labeled trastuzumab (**37**, 2 nmol) in phosphate buffer (80 μ l, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, the hydroxylamine derivative of coumarin **23** (0.2 μ mol) in Grade I water (10 μ l) from a freshly prepared stock solution was added separately for the late-stage installation of probes. The reaction mixture was incubated for 3 h to yield AFC (**38**, please see the source data file). The excess hydroxylamine derivative and salts were removed by centrifugal spin concentrator (0.5 ml, 10 kDa MWCO).

Synthesis of ADC and antiproliferative assay. The hydroxylamine derivative of DM1 (**35a**, 2 μ mol) in DMSO (20 μ l) and 10% HCl (10 μ l) from a freshly prepared stock solution was added to the antibody bioconjugate **37** in phosphate buffer (80 μ l, 50 mM, pH 7.0). Subsequently, the reaction mixture was vortexed at 25 $^{\circ}$ C for 12 h to form the oxime derivative, i.e., ADC (**39**). The reaction mixture was frozen, lyophilized, followed by the addition of 100 μ l of water. Under these conditions, the unreacted DM1 derivative **35a** is insoluble, enabling its precipitation and removal. The centrifugal spin concentration (10 kDa MWCO) and volume reduction to 250 μ l ensured the complete removal of **35a**. The sample was lyophilized and stored for further studies.

SKBR-3 cells (10⁴, source: NCCS Pune) were seeded in a 96-well plate (tissue culture grade, flat bottom) in a final volume of 100 μ l of MacCoy's 5A culture medium. After seeding for 24 h, the cells were treated with various concentrations (0.25–0.5 nM) of ADC (**39**), Kadcyla (T-DM1, **40**, Roche, N1037B18), DM1 (**35**), and trastuzumab (**34**) for the next 48 h and the total volume was kept 200 μ l after addition of compounds. All the treatments were given in triplicate. The inhibition of cell proliferation was assessed using the MTT assay from Sigma Aldrich (Sigma Aldrich, Saint Louis, USA). Briefly, MTT reagent (100 μ l, final concentration 0.5 mg/ml) was added after removing the medium, and the plates were incubated at 37 $^{\circ}$ C. After 1–1.5 h (depending upon the formation of crystals) of incubation, DMSO (100 μ l) was added, and absorbance was taken on an ELISA plate reader (CYTATION 5, BioTek) with a test wavelength of 570 nm and a reference wavelength of 630 nm. Relative growth inhibition rates for the untreated control were calculated and expressed as % inhibition of cell proliferation. In order to check the selectivity of ADC (**39**), we performed the MTT assay in HER-2 negative MDA-MB-231 cells (please see the source data file). MDA-MB-231 cells (source: ATCC, STR profiling authentication) were seeded in the DMEM medium, and the same protocol was followed as mentioned above.

Statistics and reproducibility. All the experiments were independently repeated at least three times. Data are presented as mean values \pm SD (standard deviation) calculated using OriginPro 8.5 and GraphPad Prism 8.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available within the Article and its accompanying Supplementary Information file. The source data for Figs. 5c, 7a, b, 8b, c and Supplementary Figs. 65, 86a, 87b, 90, 98a, b, 98c, 108, 111, 112, is provided in the Source Data file. The raw data generated in this study have been deposited in the Figshare public repository <https://doi.org/10.6084/m9.figshare.21219896>. Source data are provided with this paper.

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Author contributions

V.R. and N.C.R. conceived the research. N.C.R., R.M., and P.N.J. designed and performed the bioconjugation experiments. R.K.M., S.T.K., and J.K. designed and performed cell-based experiments. S.S. and N.K. designed and performed the antiproliferative assay for ADCs. S.C. and I.B. designed and performed the simulations. All the authors wrote the manuscript.

Competing interests

V.R. is the founder of Plabeltech Private Limited. A patent application (US 11,149,058 B2; Applicant: IISER Bhopal and DBT; Inventors: Vishal Rai and Srinivasa Rao Adusumalli) has been granted on the LDM. The remaining authors declare no competing interests.

Additional information

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