Cu(II)/THPTA-Mediated Thiazolidine Deprotection for Living Phages and Cell Surfaces Labeling

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Abstract

Incorporating unnatural bioorthogonal groups into peptides and proteins offers an excellent opportunity to endow them with new properties in a precise and controlled manner. Among these, the α-oxo aldehyde group is particularly suitable for the post-functionalization of peptides and proteins due to its versatility and stability in aqueous buffers. However, the facile and site-specific incorporation of α-oxo aldehyde into proteins, especially in living systems, remains a long-lasting challenge. Here, we describe a novel Cu(II)/THPTA-Mediated Thiazolidine Deprotection (CUT-METHOD) strategy for post-installation of a

highly-active α-oxo aldehyde moiety, which is released from a thiazolidine ring borne by a genetically encoded unnatural amino acid ThzK. This reaction is performed under physiological conditions, thereby enabling the chemoselective and site-specific modification of proteins via oxime ligation without compromising their integrity and function. To validate its versatility, we successfully performed site-specific incorporation of α-oxo aldehyde into recombinant proteins and those displayed on M13 filamentous bacteriophage particles and bacterial cell surfaces. In addition, by leveraging Spycatcher/Spytag chemistry and oxime ligation, the bacterial cells bearing aldehyde generated via the CUT-METHOD could be simultaneously decorated with two distinct functional molecules, providing a novel one-pot dual labeling platform for the construction of living bacterial cell-based cancer targeting systems. Put together, we have demonstrated that the CUT-METHOD strategy is a significant addition to the current bioorthogonal chemistry toolbox with broad applications anticipated in the near future.

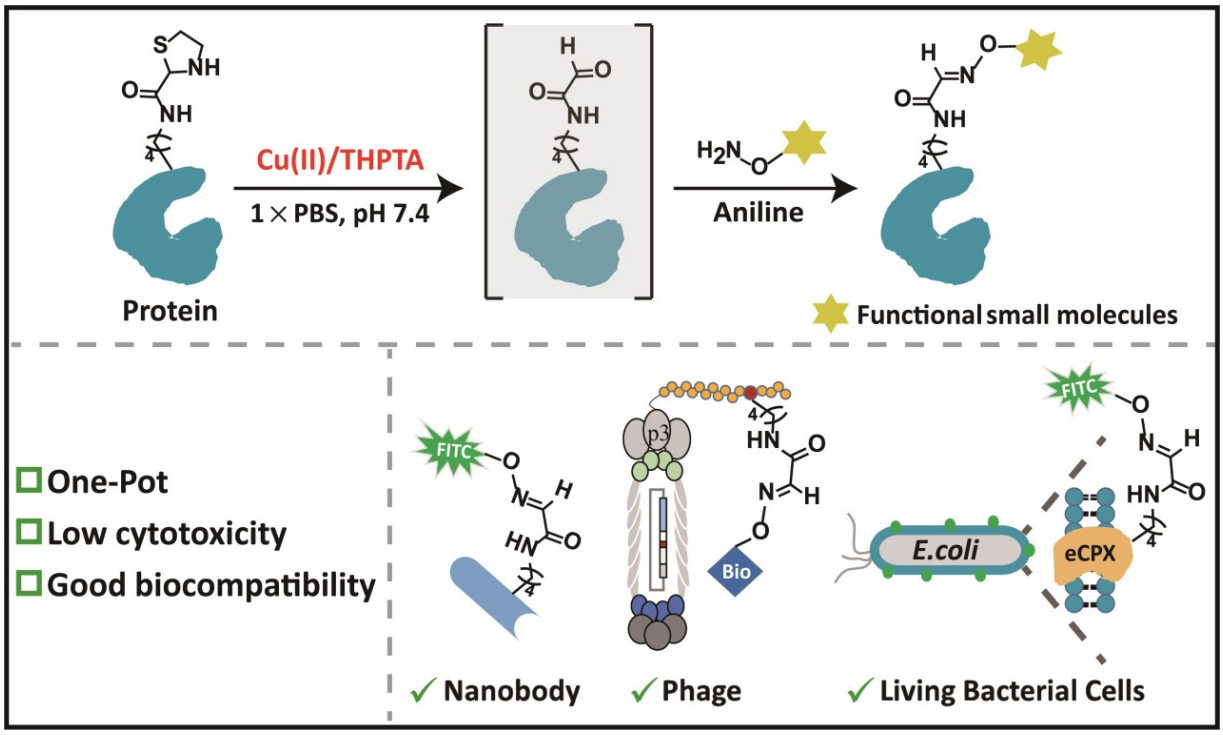
Introduction

Chemical modification of proteins has emerged as a pivotal technique for bestowing novel functionalities upon them for diverse applications.[1,2] However, chemoselectivity and site-specificity remains a daunting task. Directly modifying the side chain functional groups of canonical amino acid residues presents a straightforward approach,[3,4] but it is often besieged by issues such as indiscriminate labeling sites and the difficulty in controlling the extent of the reaction due to the presence of multiple identical residues.[5] In contrast, the incorporation of non-natural bioorthogonal groups into proteins offers advantages in chemoselectivity and site-selectivity over conventional methods.[6] Considerable efforts have been made to develop chemical and biological strategies to incorporate the unique chemical handle at either the two termini or internal sites of proteins.[7-13] A notable example is genetic code expansion (GCE) technology, wherein orthogonal aminoacyl-tRNA synthetase and tRNA pairs facilitate the site-specific incorporation of a non-canonical amino acid equipped with a specific bioorthogonal group into a predetermined location within the protein,[14,15] thereby providing an excellent opportunity to precisely modify proteins at a single-residue-resolution without affecting other residues. With the increasingly

expanded repertoire of genetically encodable unnatural amino acids (UAA),[16] this technology has already become an indispensable tool to manipulate proteins with tailor-made functionalities for diverse applications, both in vitro and in vivo, such as the development of antibody-drug conjugates, the study of protein post-translational modifications, and the *in situ* capture of unknown protein-protein interactions in living cells.[17-19]

Thiazolidine chemistry serves as a pivotal "click" type of bioorthogonal reaction, wherein a thiazolidine ring is formed via the specific condensation of an aldehyde and 1,2-aminothiol in aqueous buffers. The exceptional chemoselectivity of this reaction has rendered it indispensable in various peptide and protein chemical modification applications, including peptide cyclization, protein immobilization, construction of ubiquitin dimers, and modification of living cell surfaces.[20-23] While it has been found that benzylaldehydes with boronic acid substitutions can react with 1,2-aminothiols to yield stable thiazolidine rings or derivatives under physiological conditions,[24-27] this chemistry is generally considered a reversible process.[28] The deprotection of the thiazolidine ring can be triggered by various stimuli, both in vitro and in vivo, such as basic pH, small molecules, and transition metals.[29-34] This property has made the thiazolidine ring useful for temporarily caging the activity of aminothiol or aldehyde functionalities, with many applications such as the deprotection of caged-Cys for native chemical ligation, traceless release of cytotoxic aldehyde-containing drugs from antibodies in vivo, and control of the ubiquitin aldehyde probe for profiling deubiquitinase in living cells.[31,35,36,37-39] In spired by these studies, herein a novel Cu(II)/THPTA-Mediated Thiazolidine Deprotection (CUT-METHOD) strategy was successfully developed. This method facilitates the release of highly active α-oxo aldehyde from the thiazolidine ring of the genetically encoded thiazolidine-lysine (ThzK) in proteins under physiological conditions. Consequently, it enables chemoselective and site-specific protein modification via oxime ligation without compromising protein integrity and function. Furthermore, using the CUT-METHOD, for the first time this chemical moiety was incorporated site-specifically into peptides displayed on phages and bacterial

cell surfaces without compromising their viability (Scheme 1). This study significantly broadens the toolkit for precise protein engineering and cellular manipulation.



Scheme 1. Schematic presentation of the incorporation of α-oxo aldehyde into proteins, phage particles, and live bacterial cell surfaces achieved by the biocompatible Cu(II)/THPTA-mediated thiazolidine deprotection. The liberation of the aldehyde and its chemoselective conjugation with hydroxylamine functional reagents could be carried out in a one-pot manner, with applications in nanobody, phage and bacteria cell surface chemical modifications.

Results

Proof of concept study on small model peptide

To validate our hypothesis on Cu(II)-induced decaging of thiazolidine, we firstly demonstrate it on a short model peptide Thz-FYG-NH_2 **1** due to its accessibility and ease of characterization (Figure 1a). The Boc-protected thiazolidine (Thz) acid compound was synthesized conveniently following our established protocols.[37] It was then incorporated into the N-terminus of a growing peptide on MBHA resin using Fmoc chemistry via solid-phase peptide synthesis (SPPS), resulting in the expected Thz-bearing peptide **1**.

The purity of peptide **1** was confirmed by HPLC and MS analysis. Notably, due to the presence of mixed diastereomers in the Thz compound, **1** exhibited two peaks on the HPLC profile despite having identical mass values (Figure 1b, panel I). 1 mM **1** was treated with 2 mM $CuSO_4$ for 2 h in the reaction buffer (6 M guanidine, pH 6.0). HPLC analysis of the reaction mixture revealed a distinct peak, and prolonged incubation for 12 h led to complete conversion of **1** into this new product. MS analysis of the new peak showed a mass matching the expected hydrated form of aldehyde **3** (Figure 1b, panel II and III). To further corroborate the reactivity of the released α-oxo-aldehyde group on peptide **2**, reaction was performed again along with 3 mM aminooxy-biotin **4** at room temperature. HPLC analysis of the reaction mixture showed the quantitative conversion of **1** into the biotinylated product **5** after 12 h, as confirmed by MS analysis (Figure 1c). This data indicates that once the aldehyde was released from thiazoldine by the Cu(II)-mediated ring rupture reaction, an oxime ligation with **4** happened spontaneously in a one-pot manner. As 6 M guanidine is not compatible with proteins and living systems, we next attempted to perform this thiazolidine deprotection reaction in PBS at neutral pH without denature reagents. However, $CuSO_4$ exhibited poor solubility in PBS buffer at neutral and slightly basic pH, leading to the failure of this test. To address this issue, we decided to test whether a water-soluble Cu(II)/THPTA complex could serve as a biocompatible thiazolidine decaging reagent, considering that it has been widely used in copper-catalyzed azide-alkyne cycloaddition (CuAAC) for various bioconjugation applications, including protein chemical modifications and living cells labeling.[40-42] Remarkably, after 2 h treatment of **1** with Cu(II)/THPTA (2 equivalents) in PBS at pH 7.4, HPLC and MS analysis revealed a complete conversion of **1** into **2** (Figure S1a). Moreover, Cu(II)/THPTA mediated thiazolidine deprotection is well-compatible with oxime ligation to be conducted in a one-pot manner to yield the expected biotinylated product **5** in PBS at pH 7.2-7.4 (Figure S1b). Collectively, these findings indicate that Cu(II)/THPTA-mediated thiazolidine deprotection (CUT-METHOD) is efficient on liberating a highly active α-oxo-aldehyde on peptides by rupturing the thiazolidine ring under physiological pH. This inherent biocompatibility presents great potential for the chemical modification of proteins and living cell systems.

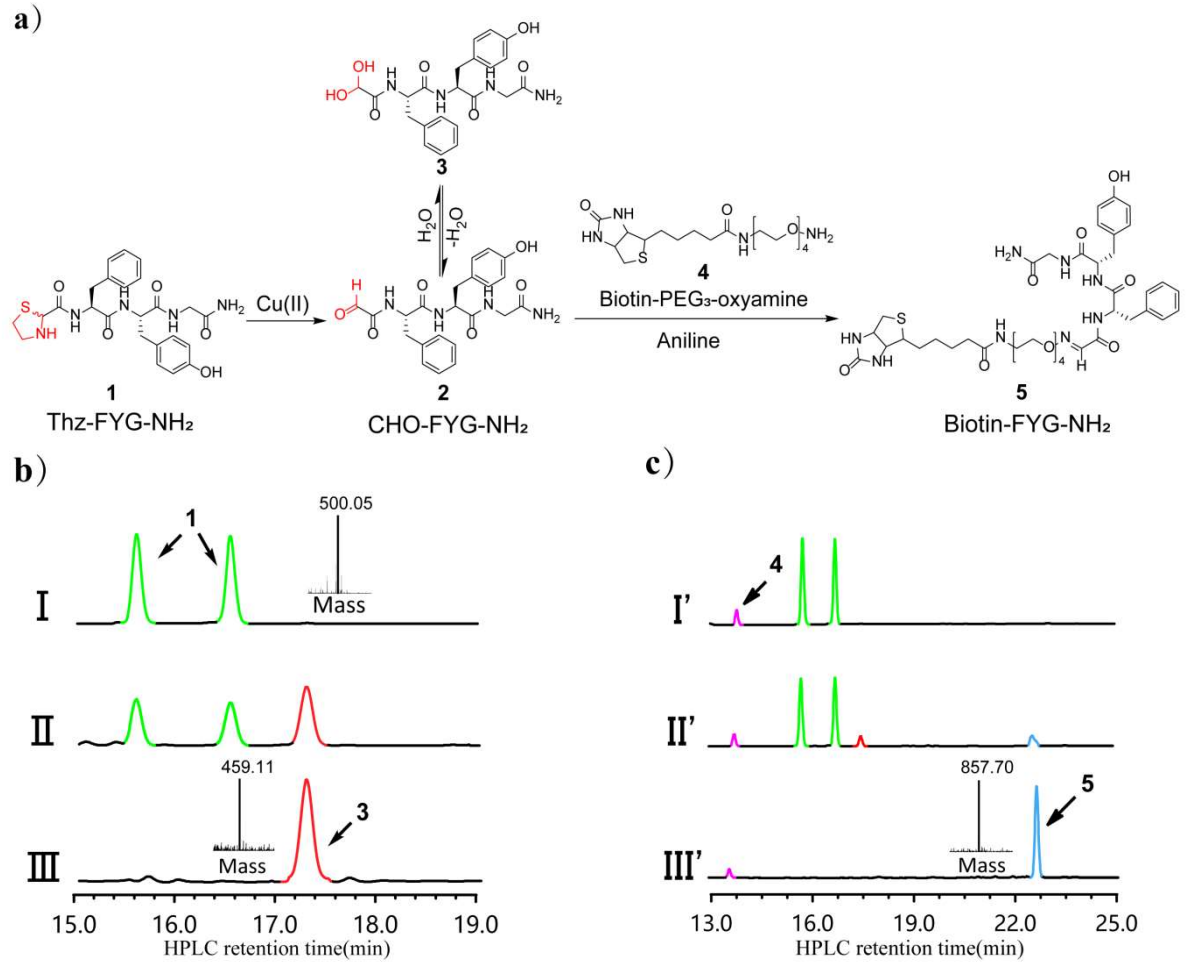
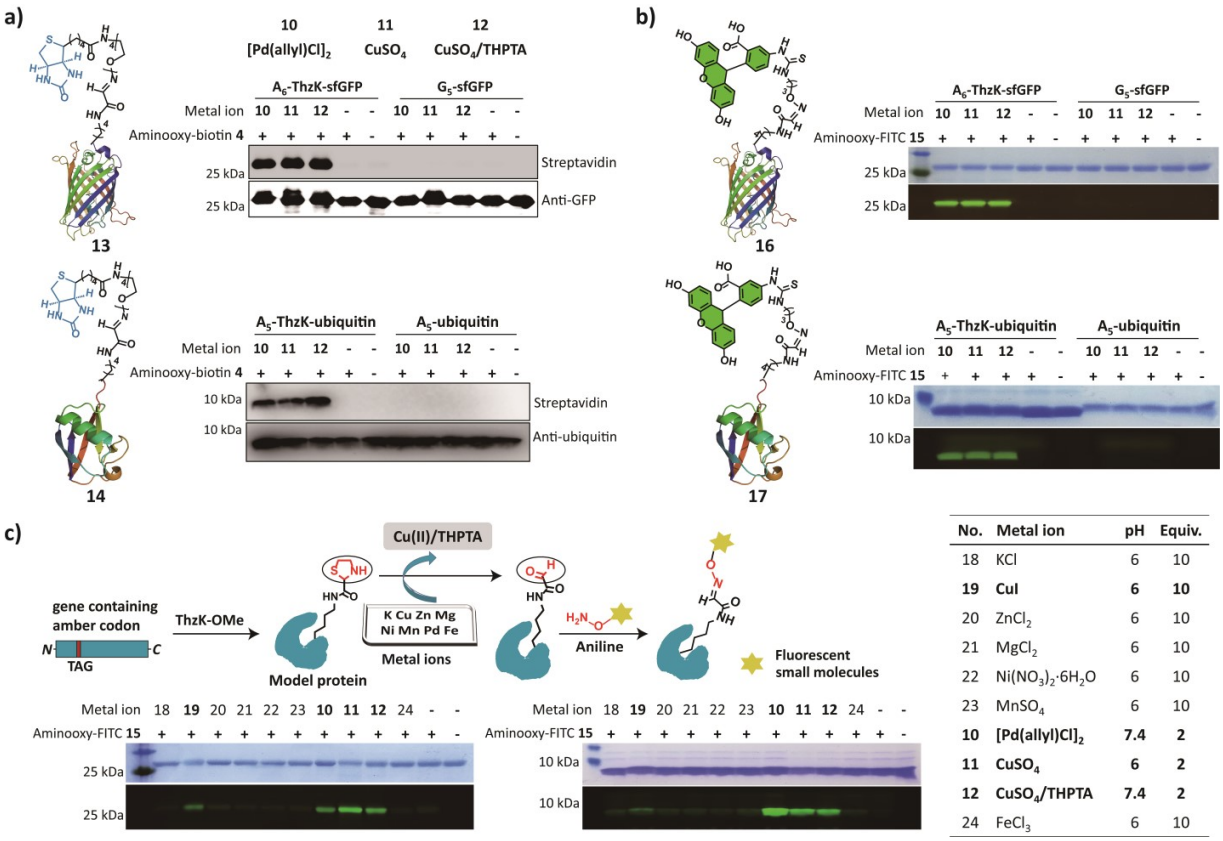


Figure 1. a) Scheme for the Cu(II)-mediated thiazolidine deprotection and oxime ligation for peptide modification. b) Representative C18 analytical HPLC profile and MS analysis of the starting material peptide **1** and the thiazolidine deprotection reaction treated with $CuSO_4$ in 6 M guanidine at pH 6.5. (I): the starting material **1** with two green peaks; the observed mass is 500.05, calcd 499.19. (II) and (III): the thiazolidine deprotection reaction for 2 h and 12 h, respectively; the red peak represents the expected aldehyde **3** with an observed mass of 459.11 (hydrated form), calcd 458.18; c) Representative C18 analytical HPLC profile and MS analysis of the reaction of peptide **1** with $CuSO_4$ and a biotin probe **4** in 6 M guanidine at pH 6.5. (I'): reaction at 0 time point, the purple peak is **4**. (II') and (III'): reaction for 1 h and 12 h, respectively; the blue peak represents the conjugated product with an observed mass of 857.70, calcd 856.99.

Site-specific modification of sfGFP and ubiquitin incorporating with ThzK

To validate the biocompatibility of CUT-METHOD, we introduced Thz functionality carried by an UAA (ThzK-OMe) into two model proteins, sfGFP and ubiquitin, using our previously established procedure.[37] Briefly, the pETDuet plasmid encoding sfGFP or ubiquitin with a single TAG mutation after

a multiple Ala sequence at the N-terminus was transformed into *E. coli* BL21(DE3) strain, along with the pEVOL-MbPylRS plasmid encoding the wild type MbPylRS/tRNA pair capable of assigning ThzK-OMe to the amber codon during protein translation. In the presence of 2 mM ThzK-OMe, both full-length proteins were obtained with a yield of 1-2 mg/L after immobilized metal affinity chromatography (IMAC) purification, yielding A_6-ThzK-sfGFP **6** and A_5-ThzK-ubiquitin **7**, respectively. In the absence of ThzK-OMe, no desired proteins were harvested (Figure S2). A_6-ThzK-sfGFP **6** (30 uM) was then treated with 2 equivalents of Cu(II)/THPTA, and 5 equivalents of aminooxy-biotin **4** in PBS pH 7.4 at room temperature for 12-18 h. In parallel, this protein was also treated with Pd(II) **10** and Cu(II) **11** for comparison. For the Cu(II)-treated group, the labeling reaction was conducted in acidic PBS buffer (pH ~6) as $CuSO_4$ could be solubilized to a moderate extent at low concentration. Meanwhile, wild type G_5-sfGFP **8** lacking ThzK was treated at the same condition to serve as a negative control. Western Blot (WB) analysis revealed successful biotinylation of **6** treated with Cu(II), Pd(II) and Cu(II)/THPTA **12**, whereas no bands were detected in **6** in the absence of any metal ions or ThzK-installation (Figure 2a, up panel). Similarly, A_5-ThzK-ubiquitin **7** and WT-A_5-ubiquitin **9** were subjected to the same treatment, also showing that only in the presence of ThzK-OMe and three decaging reagents, the biotinylated band could be detected (Figure 2a, down panel). Besides, we found that if aminooxy-biotin **4** reaching 100 equivalents, **6** could also be labeled even in the absence of the decaging reagents (Figure S3), indicating that the aminooxy group itself can lead to the deprotection of thiazolidine at high concentrations and the addition of decaging reagents could significantly accelerate the thiazolidine ring opening. To further demonstrate the versatility of the CUT-METHOD, a fluorescent aminooxy-FITC **15** was prepared (Figure S4) and used to label the aforementioned proteins under the same conditions. After the one-pot labelling reaction, fluorescent gel analysis again showed that both ThzK-bearing proteins (**6** and **7**) were successfully labeled, while the control protein (**8** and **9**) lacking Thz functionality and the group without metal ion treatments exhibited no signal (Figure 2b). Inspired by these results, we envisaged that other transition metals may also rupture the thiazolidine ring to release the aldehyde. **6** and **7** were then treated with different metal ions and **15** in one-pot manner according to the conditions shown in Figure 2c, right panel. It was found that proteins



No.	Metal ion	pH	Equiv.
18	KCl	6	10
19	**CuI**	**6**	**10**
20	$ZnCl_2$	6	10
21	$MgCl_2$	6	10
22	$Ni(NO_3)_2{\cdot}6H_2O$	6	10
23	$MnSO_4$	6	10
10	**$[Pd(allyl)Cl]_2$**	**7.4**	**2**
11	**$CuSO_4$**	**6**	**2**
12	**$CuSO_4$/THPTA**	**7.4**	**2**
24	$FeCl_3$	6	10

Figure 2. a) Western blot analysis of A_6-ThzK-sfGFP/WT-G_5-sfGFP (upper panel) and A_5-ThzK-Ubiquitin/WT-G_5-ubiquitin (lower panel); b) SDS-PAGE and fluorescent gel analysis of A_6-ThzK-sfGFP/WT-G_5-sfGFP (upper panel) and A_5-ThzK-Ubiquitin/WT-G_5-ubiquitin (lower panel) treated with Cu(II), Pd(II), and Cu(II)/THPTA, and labeled with FITC-ONH_2 **15** under identical conditions. Proteins were detected and visualized by Coomassie Blue staining and fluorescent emission under blue light; c) General scheme, reaction conditions, and SDS-PAGE/fluorescent gel analysis for screening metal ions capable of deprotecting the thiazolidine ring on proteins. A_6-ThzK-sfGFP/WT-G_5-sfGFP (left panel) and A_5-ThzK-Ubiquitin/WT-G_5-Ubiquitin (right panel) were labeled with FITC, and the decaging ability of individual metal ions was measured by detecting green fluorescent signals on the gel.

treated by treated with **10**, **11**, **12** and **19** were successfully labeled, as confirmed by the green fluorescent bands shown on the gel, indicating that these metal ions can mediate the thiazolidine ring deprotection to release the aldehyde. Compared to Pd(II), Cu(II), Cu(II)/THPTA, Cu(I) was less efficient based on the weaker green fluorescent signal on the gel. No such effects were observed in other metal ions (Figure 2c). These findings suggest that our CUT-METHOD is a reliable approach to release the masked aldehyde from the thiazolidine ring in intact proteins under physiological conditions using low concentrations of Cu(II)/THPTA.

One-pot labeling of nanobody 7D12

Nanobodies, which are single-domain antibody fragments derived from the distinctive heavy-chain antibodies found in camelids, have garnered considerable attention in recent years due to their notable attributes, including small size, high stability, and facile production.[43] As nanobodies continue to emerge as potent tools for diagnostics and therapeutics, there is a growing interest in enhancing their functionality through precise site-specific modifications.[44] Therefore, we aimed to test the compatibility of our CUT-METHOD with nanobodies and determine whether their functions remain intact after chemical transformation. The nanobody 7D12, well-known for its targeting of the Epidermal Growth Factor Receptor (EGFR) on cells, was selected for this demonstration.[45] Previous studies have shown that Gln13 is a permissive site allowing for the encoding of UAAs without abolishing its binding capability.[46] Thus, a pETDuet plasmid encoding the 7D12 nanobody harbouring a Q13TAG mutation was constructed. This plasmid was then co-transformed into *E. coli* BL21(DE3) with the pEVOL-MbPylRS plasmid to produce the 7D12-Q13ThzK **25** protein in the presence of 2 mM of ThzK-OMe. The expression yield reached approximately 1 mg/L after IMAC purification (Figure S5). The integrity of 7D12-Q13ThzK **25** and 7D12-Q13CHO **26** treated by Cu(II)/THPTA was confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis (Figure 3a). After a 12-18 h one-pot labeling reaction, the labeled 7D12 was characterized by WB and fluorescent gel analysis, respectively, revealing that only in the presence of Cu(II)/THPTA and ThzK-OMe could the biotinylated and fluorescent bands be detected while the WT 7D12 **27** lacking ThzK and 7D12-Q13ThzK without metal ions treatment exhibited no signal under the same conditions (Figure 3b), which is consistent with the above results obtained with sfGFP and ubiquitin labeling. To assess whether the FITC-conjugated 7D12 still retained its ability to target EGFR, two cell lines, A549 (EGFR-positive) and SW620 (EGFR-negative), were tested. Remarkably, an evident green rim was observed only in the A549 cell line, while no obvious signal detected in SW620, confirming the preserved targeting capability and selectivity of the labeled 7D12-Q13ThzK (Figure 3c). This observation was further confirmed by flow cytometry analysis (Figure 3d). These findings indicate the excellent

biocompatibility of our CUT-METHOD with intact functional proteins, highlighting its potential utility in the development of targeted protein molecules for diagnostic and therapeutic applications.

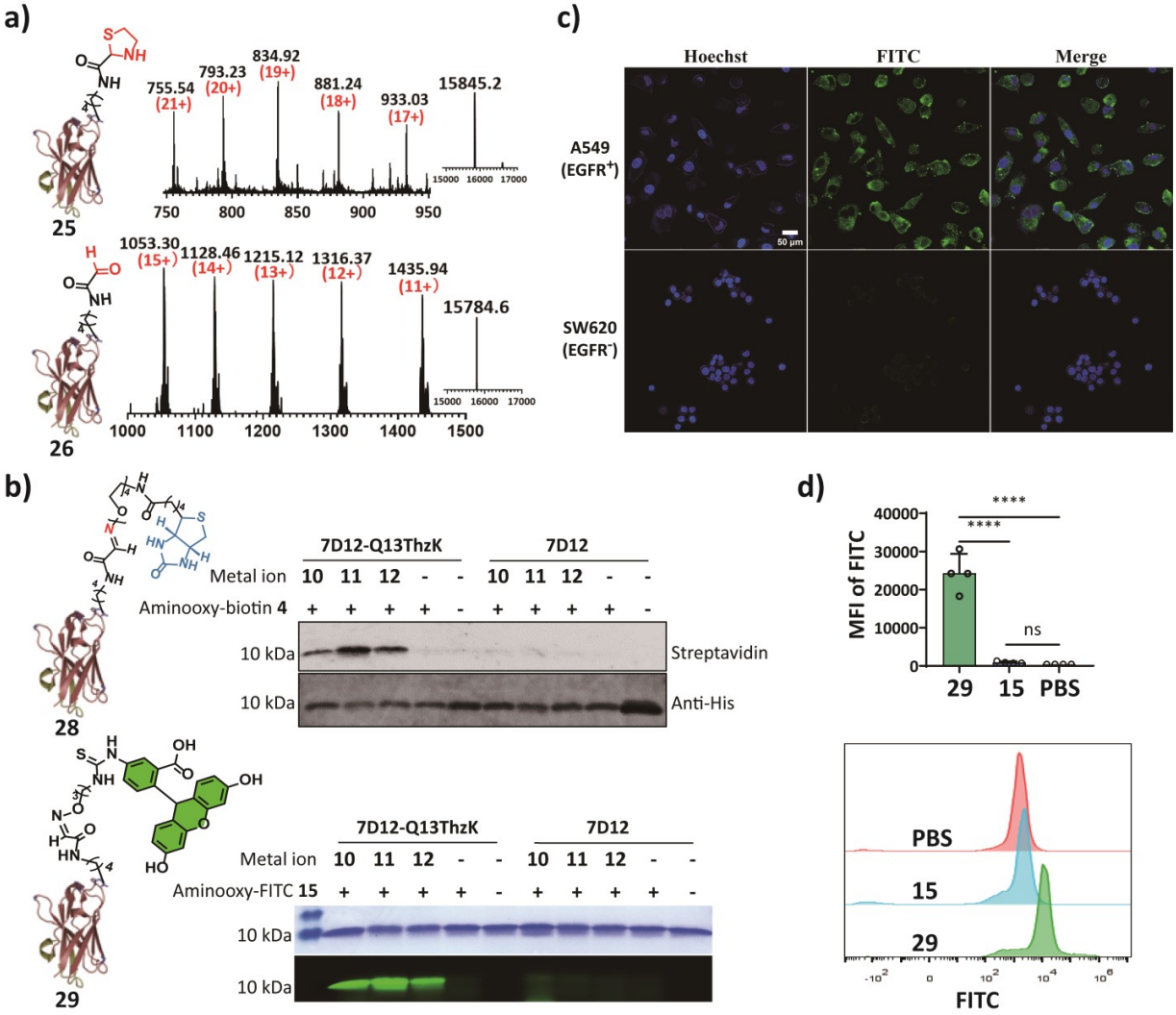
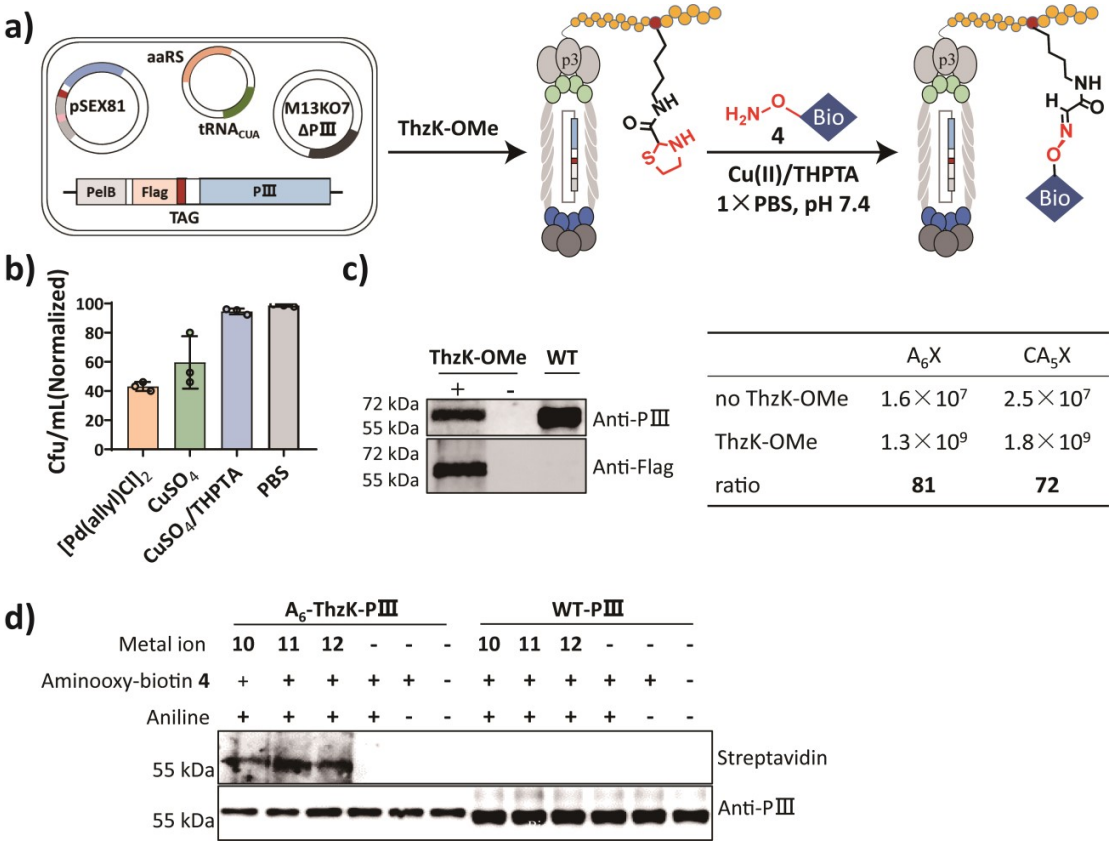


Figure 3. Site-directed chemical modification of nanobody 7D12 incorporating ThzK using CUT-METHOD and oxime ligation and its characterization. a) ESI-MS analysis of 7D12-Q13ThzK **25** with the observed mass 15845.2 Da, calcd 15845.4 Da (up panel) and7D12-Q13aldehyde **26** with the observed mass 15784.6 Da, calcd 15786.3 Da (down panel); b) Western blot analysis of products generated by reacting 7D12 **25** with biotin-PEG_3-oxyamine **4**; SDS-PAGE/fluorescent gelanalysis of products generated by reacting 7D12-Q13ThzK 25 with FITC-ONH_2 **15**; c) Confocal microscopy images of A549/SW620 cells incubated with 100 nM 7D12 **29** (green) for 30 min in 37°C. The nuclei were stained with Hoechst (blue). Scale bars = 50 µm; d) Mean fluorescence intensity of A549 cells incubated with 3 different samples. Statistical significance was calculated by one-way ANOVA with Tukey's multiple comparisons test (**** < 0.0001); Histogram showing fluorescence intensity distribution of A549 cells incubated with 3 different samples.

Site-specific incorporation of α-oxo-Aldehyde into bacteriophage particles

Phage display is a widely used technique in molecular biology and biotechnology, serving as a valuable tool for exploring protein-protein interactions, protein engineering, and the discovery of antibodies.[47,48] Traditional phage display techniques can only present peptides and proteins made from the 20 canonical amino acids with limited chemical functionalities. To address this limitation, GCE technology has been recognized as a powerful approach to enable the accurate insertion of a single or even multiple UAAs into phage, expanding the chemical space of functionalities encoded by the phage for various applications.[49-53] Up to date, incorporating aldehyde moiety into phage particles has been achieved by serine oxidation and pyridoxal 5'-phosphate (PLP)-mediated transformation.[54,55] However, the generated aldehyde is only confined to the N-terminus of peptide/proteins displayed on the phage.[56] Moreover, such chemical transformations might also inadvertently abolish other amino acids such as Cys and Met, potentially compromising the phage's activity and the function of the peptides and proteins it presents. A reliable technique for the site-specific incorporation of α-oxo-aldehyde into live phage particles is still in need. Hence, our subsequent focus was directed to incorporate ThzK-OMe into phage particles and assess the compatibility of our CUT-METHOD with live phages (Figure 4a). First, to test the effects of Cu(II), Pd(II) and Cu(II)/THPTA on the viability and infectivity of phages, wild phage lacking ThzK were used, as they can be very easily prepared due to rapid propagation and high yield. Results show that Cu(II)/THPTA was fully compatible without any obvious adverse effects on phage viability compared to PBS treatment. In contrast, long-term exposure of phages to Pd(II) would significantly decrease their infectivity as shown in Figure 4b. Based on this data, we determined to use Cu(II)/THPTA as the best decaging reagent due to its excellent biocompatibility. Next, to site-specifically incorporate ThzK-OMe into bacteriophages, a tri-plasmids system was constructed according to previous reports.[51,57] In this system, the pSEX81 plasmid encodes either a FLAG tag-A_6X-pIII or FLAG tag-CA_5X-pIII fusion protein, where 'X' denotes the incorporated ThzK. The pIII encoding sequence in the M13KO7 helper phage has been deleted, resulting in the inability to generate viable phage without the



	A$_6$X	CA$_5$X
no ThzK-OMe	1.6×10^7	2.5×10^7
ThzK-OMe	1.3×10^9	1.8×10^9
ratio	**81**	**72**

Figure 4. a) Schematic diagram illustrating a tri-plasmid system employed to generate a phage containing ThzK at the N-terminus of pIII protein, and its labeling with biotin using CUT-METHOD and oxime ligation; b) Assessment of effect of metal ions on bacteriophage activity. Each group was repeated 3 times for titer testing and the average value was taken (retaining 3 significant figures); c). Western blot analysis depicting phage expression (FLAG-A$_6$X-pIII and WT) in the absence or presence of ThzK-OMe (left panel); The table presents two phage derivatives, one featuring an N-terminal CA$_5$X peptide and the other with a N-terminal A$_6$X peptide, where X denotes ThzK, along with their corresponding phage yields (CFU/mL) in the presence and absence of ThzK-OMe (left panel); d) Western blot analysis of A$_6$-ThzK-PIII and WT phage labelled with biotin-PEG$_3$-oxyamine 4.

supplementation of full-length pIII (Figure S6). Therefore, only after ThzK-OMe was successfully incorporated, could a FLAG tag-pIII fusion peptide be expressed, and intact phage particles formed with the pentavalent display of peptides-ThzK-pIII fusion protein. WB analysis shows that only in the presence of ThzK-OMe, the FLAG band could be detected using Anti-FLAG antibody, with no bands observed otherwise, indicating that ThzK-OMe have been incorporated into the phages (Figure 4c, left panel). Moreover, A$_6$X-phage and CA$_5$X-phage have a 81 and 72 fold increase in infectivity in the presence of ThzK-OMe than that of phage without ThzK-OMe supplement (Figure 4c, right panel), which is

consistent with previous observation that phage incorporating with UAA has higher infectivity.[53] To further verify the successful installation of ThzK-OMe on phage, ThzK-incorporating phages were treated with Cu(II)/THPTA, Cu(II), Pd(II) and biotin-aminooxyl **4** at room temperature for 2 h in PBS. Wild-type phages served as a negative control. WB analysis again showed that biotinylated labeled pIII was only detectable in ThzK-incorporating phages, with no bands observed in the wild-type phages (Figure 4d). Overall, this proof-of-concept study suggest that the combined utilization of our CUT-METHOD and GCE technology enables the site-specific incorporation of α-oxo-aldehyde into phage particles, expanding the chemical space of functionalities genetically encoded by the phage.

One-pot dual labeling platform for bacterial surfaces decoration

To further demonstrate the broad applicability of our approach, we attempted to expand it to more complicated biological systems. Similar to phage display, bacterial cell surface display is also a powerful biotechnology with widespread applications among others, including antibody epitope mapping and bacterial cancer therapy.[58] Several chemoenzymatic strategies have been innovatively developed for externally decorating bacterial cells with a variety of functional molecules.[59,60] including those using UAAs.[61,62] Despite these advances, currently, there remains a scarcity of methods enabling one-pot, bioorthogonal dual labeling of bacterial cell surfaces. To this end, we made endeavours to develop a new strategy to achieve simultaneous dual labeling of bacterial cells in a one-pot fashion by combined use of Spycatcher/SpyTag chemistry and our CUT-METHOD. The process involved the simultaneous incorporation of ThzK and SpyTag003 (a SpyTag derivative with higher reaction kinetics in low concentration)[63] into the peptide displayed on bacteria cell surface using eCPX system.[64] These elements served as two orthogonal ligation handles for subsequent attachment of two functionalities onto cell surface in a one-pot manner,[65] endowing them with fluorescent property and cancer cell-selective targeting ability, as depicted in Figure 5a. As eCPX can display two different peptides at its both ends, a model peptide GGGGGX followed by a FLAG tag, where X represented ThzK, and Spytag003 were

introduced at the N and C terminus, respectively. This ensured that only when ThzK-OMe was incorporated successfully could the full length of eCPX be detected via WB analysis using anti-FLAG antibody (Figure S7). To verify whether our CUT-METHOD is compatible with living bacterial cells, we first assessed the cytotoxic effects of the metal ions at different concentrations on *E. coli* over a 6 h incubation period. Results demonstrated that even at the highest concentrations (100 μM), Cu(II)/THPTA exhibited minimal toxicity, contrasting with Pd(II), which resulted in the complete eradication of bacterial cells at the same concentration (Figure 5b). This assay again highlights the excellent compatibility of Cu(II)/THPTA with biological systems. To minimize the potential effect of metal ion treatments on bacterial activity, we adopted two-step process to label cells. Firstly, bacterial cells were treated with Cu(II)/THPTA for 2 h to liberate the aldehyde. Then, the Cu(II)/THPTA were removed by extensive PBS washing, ensuring there is no toxic effect on their activity. To determine whether these bacterial cells bearing aldehyde and SpyTag003 could be simultaneously labeled, FITC-aminooxyl 15 and mCherry-SpyCatcher003 were co-incubated with cells in PBS for 4 h at 37°C. Confocal microscopy analysis revealed colocalized green and red fluorescent signals, observable only in the presence of ThzK-OMe (Figure 5c, upper panel), which was attributed to bacteria surface-conjugated FITC and mCherry, respectively. This data suggests the successful attachment of two functional molecules concurrently via oxime ligation and isopeptide bond formation. In contrast, in the absence of ThzK-OMe, no fluorescent signals were detected on the bacterial cell surface (Figure 5c, down panel).

Bacterial cancer therapy is increasingly recognized as a promising method for cancer treatment, leveraging bacteria to target and eliminate cancer cells through direct destruction or by eliciting an immune response against the tumor.[66,67] To explore the potential of our method in generating fluorescently labeled bacterial cells with targeted tumor-binding ability, a trifunctional affibody-mCherry-SpyCatcher003 fusion protein was designed (Figure S8), in which the affibody can target EGFR over expressed on cancer cells, mCherry was used for red fluorescent detection and SpyCatcher003 mediated the installation of this protein onto the bacteria cells by reacting with the surface displayed SpyTag003.

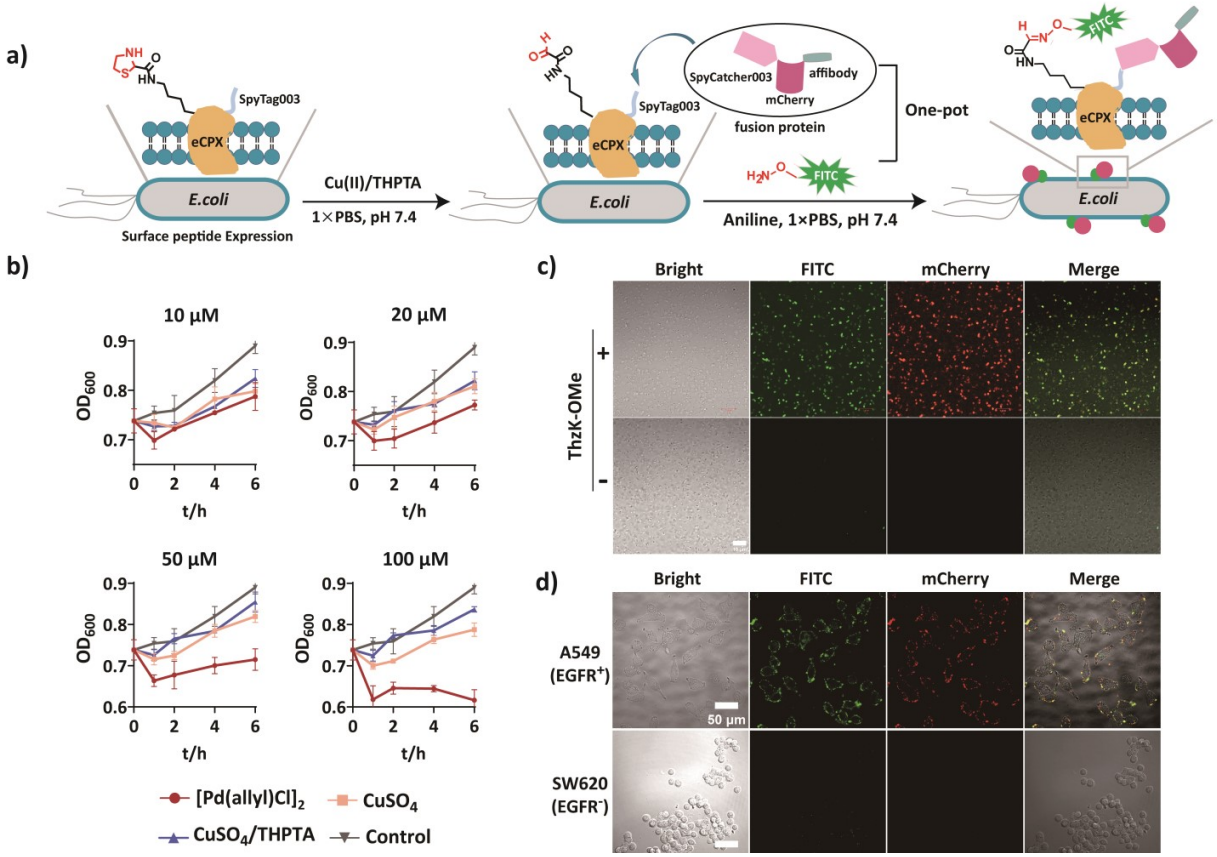


Figure 5. a) Schematic representation illustrating the dual labeling of live bacterial cell surfaces in a one-pot fashion using oxime ligation and SpyCatcher/SpyTag chemistry, along with their tumor cell targeting applications. ThzK and SpyTag003 were incorpo rated into the N- and C-terminal ends of the surface-localized eCPX on *E. coli*, respectively, and the aldehyde was released using the CUT-METHOD; b) Effects of different metal ions on the growth activity of *E. coli* at four different concentrations during a 6 h period; c) Confocal microscopy images of FITC and mcherry-labeled *E. coli*. Scale bar = 10 μm; d) Representative confocal microscopy images of A549/SW620 cells labeled with FITC and mcherry-labeled *E. coli* samples at 37°C for 1 h. Rinse cells with PBS before observation. Scale bar = 50 μm.

Then, the bacterial cells underwent the same one-pot labeling procedure as previously described. Evaluation of efficacy in targeting tumors was tested using A549 and SW620 cells. Confocal microscopy analysis revealed green and red spots exclusively on the surface of A549 cells, indicating the adherence of dual-color labeled bacteria. By contrast, no signal was observed in EGFR-negative SW620 cells, which is attributed to the lack of specific interaction between affibody and EGFR (Figure 5d and S9-10). Collectively, these findings suggest that oxime ligation is bioorthogonal to Spycatcher/Spytag chemistry, and the combination of these two reactions along with our CUT-METHOD provides a reliable and

biocompatible approach to decorate living cells with two distinct functional molecules. This innovative one-pot labeling platform holds potential for diverse applications in the future, including the development of new live cell drugs for tumor therapy.

Discussion

In summary, In this study, we present a novel method for liberating the highly active α-oxo-aldehyde from a thiazolidine ring under physiological conditions, leveraging a stable and biocompatible Cu(II)/THPTA complex. Coupled with genetic code expansion and oxime ligation, this work provides a robust and biocompatible tool for site-specific and chemoselective modifications of peptides, proteins, phage particles, and living bacterial cells without compromizing their normal functions. Despite the widespread use of Cu(II)/THPTA in click chemistry,[41] our study represents the first exploration of its utility in generating aldehydes by rupturing the thiazolidine ring under physiological buffer systems. Its excellent biocompatibility allows for seamless integration of the aldehyde into proteins and biological systems. Moreover, it could be envisaged that by combined with click chemistry, this shared reagent would also be also useful for synthesizing the dual-modified peptide/protein conjugates in a one-pot manner. Additionally, our work presents other innovative aspects. Through genetic code expansion, we achieved the site-specific incorporation of α-oxo-aldehyde into peptides displayed on phage particles, expanding the chemical space of functionality that can be genetically encoded in phages. This breakthrough enables precise modification of phages for potential drug delivery and opens avenues for selecting novel affinity peptides carrying the aldehyde or its derivatives. We also present a novel one-pot, bioorthogonal dual labeling platform by the combined use of SpyCatcher/SpyTag chemistry with oxime ligation,[68] facilitating the facile and fast generation of dual-functionalized bacteria with tailor-made properties for various applications. Overall, our CUT-METHOD offers a versatile approach for selectively modifying biomolecules and living cells, with vast potential in chemical biology and biotechnology, such as on-demand manipulation of protein activity in living cells.

Supporting Information

Detailed methods and experimental procedures could be found in the Supporting Information file, including recombinant protein expression, phage preparation, dual-labeling of bacterial cell surface, HPLC, ESI-MS, SDS-PAGE, Western blot, et al.

Contributions

X. B. conceived, designed and supervised the project. C. M. performed the experiments, collected and analyzed data. H. X., B. Y and X. B. wrote the manuscript. J. Y. collected the confocal data of labeled bacterial cells. G. L. completed the model peptide study. J. S. and D. Y. conducted some cell labeling experiments. D. L. and W. H. completed the synthesis of FITC-ONH_2, and S. P. assisted in the ESI-MS analysis of the protein. All authors discussed the results and commented on the manuscript.

Data Availability

All other relevant data that support the findings of this study are available from the corresponding authors on reasonable request.

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Acknowledgements

This study was supported by the National Key Research and Development Program of China (2018YFA0900404), the National Natural Science Foundation of China (22134003), Zhejiang Provincial Natural Science Foundation of China under Grant No. LY24B020008 and Zhejiang University of Technology High-Level Talents Startup Funds (2021414800229, X. B. Bi).

Ethics declarations

Competing interests

The authors declare no conflict of interests.