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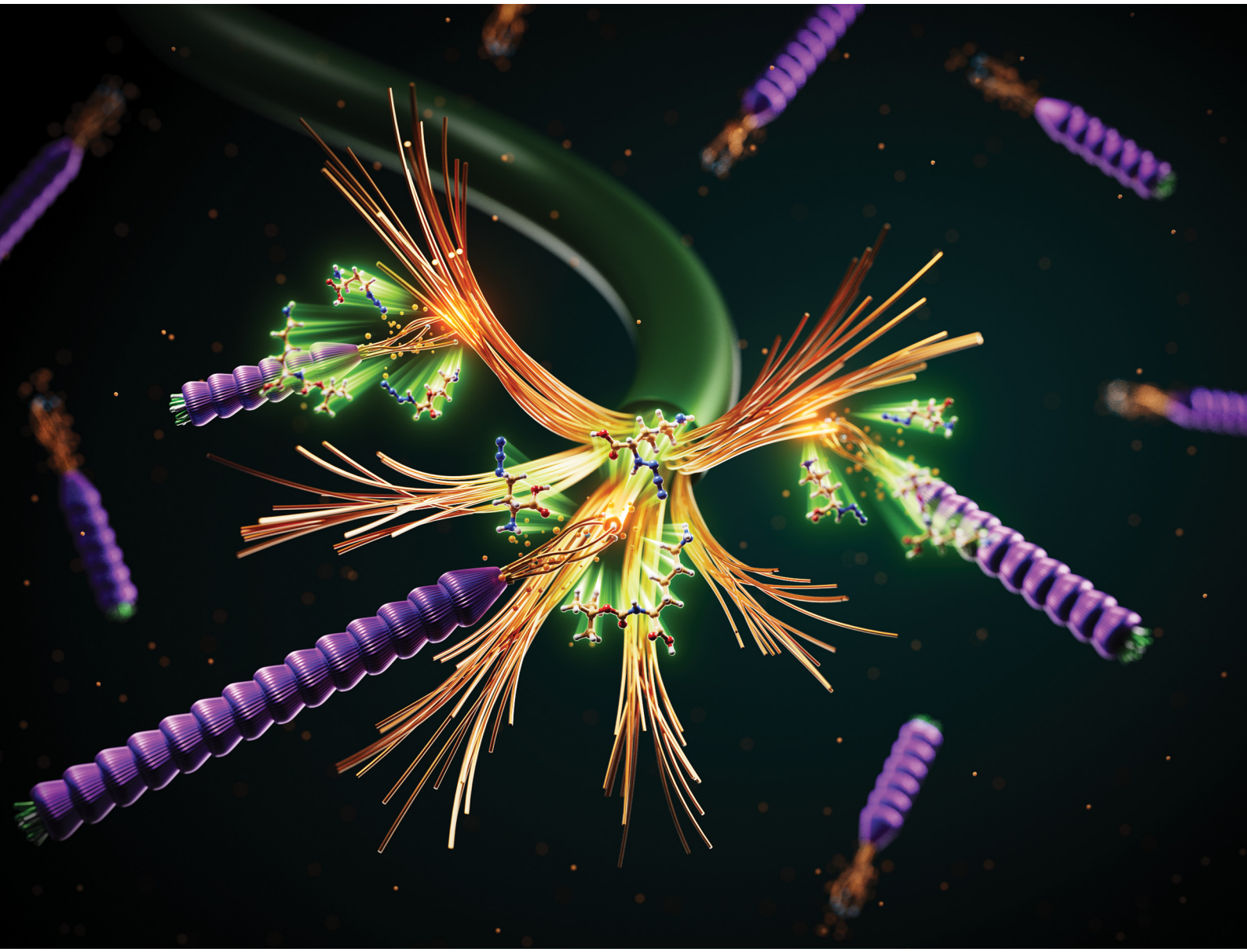

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PAPER
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Copper-catalysed azide–alkyne cycloaddition on live M13 bacteriophage for expanding the molecular diversity of phage-displayed peptide libraries

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Phage display is a powerful platform for ligand evolution, but conventional phage display libraries are confined to the twenty canonical amino acids, greatly limiting the chemical space that these libraries can be used to explore. Here we present an approach to expand the molecular diversity of phage-displayed peptides that combines unnatural amino acid mutagenesis with chemical post-translational modification. By incorporating azide-functionalized unnatural amino acids into phage-displayed peptides and applying optimized conditions for copper-catalysed azide–alkyne cycloaddition, we achieve quantitative and selective peptide modification with a series of alkyne-functionalized small molecules. This approach provides a general platform for constructing chemically augmented phage-displayed libraries with broad utility in ligand discovery.

Introduction

Phage display is a powerful platform for molecular evolution that is widely used to identify peptide ligands with high affinity and specificity for biological targets.[1] However, traditional phage-displayed peptide libraries are restricted to the twenty canonical amino acids, limiting their chemical and structural diversity compared to synthetic libraries or those prepared by *in vitro* translation systems.[2,3] This constraint hinders the discovery of ligands with expanded functions or complex binding profiles, thereby reducing the broader utility of phage display technology in research and drug discovery applications.

One approach to expand the chemical diversity of phage display libraries is through the incorporation of unnatural amino acids (uAAs) by genetic code expansion (GCE).[2,4] This method uses engineered, orthogonal aminoacyl-tRNA synthetase (o-aaRS) and suppressor tRNA pairs to mediate co-translational and site-specific incorporation of uAAs into peptides as they are produced in *Escherichia coli*. GCE has enabled the incorporation of hundreds of structurally distinct uAAs into proteins in living cells, and several uAAs have been deployed to expand the functionality of phage display libraries.[5–17] For example, cysteine-reactive uAAs have been used to produce phage-displayed libraries of macrocyclic peptides, yielding ligands with improved target binding properties.[10,14,15] Libraries bearing genetically encoded post-translational modifications have yielded potent inhibitors of epigenetic reader[11] and eraser enzymes.[9,10,12] In addition, uAAs with reactive chemical warheads have enabled the discovery of antibodies that covalently bind their target antigen.[8] Despite these advances, GCE faces several limitations. Incorporating a new uAA often requires labor-intensive engineering of a novel o-aaRS variant that accepts the uAA as a substrate—a process that is both time-consuming and unpredictable.[18] Furthermore, the uAAs that can be successfully installed by GCE are limited in size and composition due to fundamental limitations of the bacterial translation machinery.

A second strategy to expand the chemical diversity of phage display libraries is through chemical post-translational modification (cPTM).[2,4] In this approach, phage-displayed peptides are modified *in vitro* to produce semi-synthetic libraries of peptide-small molecule conjugates. cPTM has enabled a wide range of applications, including the creation of highly stable mono- and bicyclic peptide libraries,[19,20] the discovery of pathogen-specific peptide antibiotics,[21] as well as the discovery of covalent inhibitors of enzymes[22] and protein–protein interactions.[23] One particularly promising embodiment of cPTM is genetically encoded fragment-based discovery (GE-FBD).[24] Analogous to traditional fragment-based drug discovery, GE-FBD involves conjugating known small-molecule ligands onto phage-displayed peptide libraries. This enables the discovery of hybrid ligands in which

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the peptide and small molecule components synergistically enhance target binding properties.[25,26] Because cPTM does not rely on the protein synthesis machinery of cells to introduce chemical diversity, it permits the incorporation of a wider range of functionalities than GCE. However, cPTM requires the selective modification of natural amino acids—most commonly cysteine. Because library peptides can have multiple cysteine residues, controlling the regiospecificity and stoichiometry of the modification can be challenging.[4] Additionally, off-target modification of native phage coat proteins can compromise infectivity and selection fidelity.[19,27] Several strategies to address these challenges have been developed, including methods for regioselective cysteine modification,[20] and bioconjugation chemistries that exploit the unique reactivity of N-terminal residues.[26,28–30] Despite these innovations, technical challenges remain—particularly when applying cPTM to complex or very large peptide libraries.

Here, we present a hybrid strategy that integrates the site-specific precision of GCE with the modularity of cPTM. By genetically encoding azide-functionalized uAAs into phage-displayed peptides and applying optimized conditions for copper-catalysed azide–alkyne cycloaddition (CuAAC), we achieve efficient and selective attachment of diverse chemical fragments onto phage-displayed peptides. This approach offers a generalizable platform for constructing chemically augmented phage display libraries with broad utility in ligand discovery.

Results and discussion

Genetically encoding azide-functionalized unnatural amino acids into phage-displayed peptides

Our strategy for combining GCE with cPTM was to incorporate azide- or alkyne-functionalized uAAs into phage-displayed peptides for subsequent modification by CuAAC. Numerous uAAs bearing azides and alkynes have been incorporated into proteins by GCE.[18,31] In this study, we focused on azide-functionalized uAAs because, in addition to CuAAC, azides undergo strain-promoted azide–alkyne cycloaddition (SPAAC) with strained alkynes, such as dibenzocyclooctyne (DBCO).[35] This additional reactivity provides a convenient means to assess uAA incorporation and reactivity using DBCO-based probes (*e.g.* biotin-DBCO; *vide infra*).

To evaluate site-specific modification of phage-displayed peptides *via* cPTM, we selected a panel of four uAAs (uAAs **1–4**, Fig. 1A). uAAs **2–4** contain azide functional groups in their side chains which serve as bioorthogonal handles for modification. In contrast, uAA **1** lacks an azide and was included as a negative control to confirm that phage modification is dependent on the azide reactivity. Each of these uAAs has previously been incorporated into proteins in *E. coli* by GCE using archaeal aminoacyl-tRNA synthetase and suppressor tRNA pairs. uAAs **1** and **2** are substrates of the wild-type pyrrolysyl-tRNA synthetase (PylRS) from *Methanosarcina mazei*.[31] uAA **3** is a substrate of an engineered variant of the *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase.[32] uAA **4** is recognized by an engineered variant of the *Methanomethylophilus alvus* PylRS.[33]

To install uAAs **1–4** into phage-displayed peptides, we cloned a model, disulfide-constrained cyclic peptide (^{N}CQLXWMC^{C}) as a genetic fusion to the N-terminus of the phage coat protein pIII. X corresponds to the position of the uAA which is encoded by an amber (UAG) codon. The peptide-pIII fusion protein was expressed from a phagemid vector in *E. coli* that were co-transformed with a second plasmid encoding the desired o-aaRS and suppressor tRNA pair (Fig. S1, SI). Phagemid vectors require complementation with a helper phage that encodes the additional proteins needed for progeny phage assembly.[1]

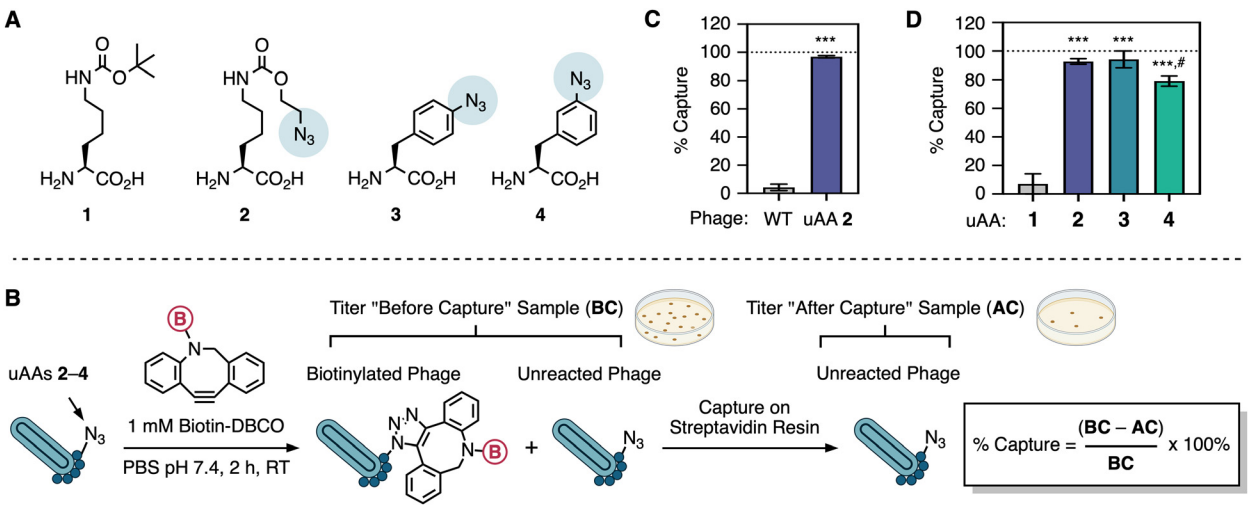


Fig. 1 Incorporation of azide-functionalized unnatural amino acids into phage-displayed peptides. (A) The chemical structures of uAAs **1–4**. (B) Schematic of the streptavidin pulldown assay used to monitor reaction between biotin-DBCO and phage-displayed peptides. (C) A mixture of wild-type M13 (WT) and M13 displaying a peptide with uAA **2** were incubated for 2 h with 1 mM biotin-DBCO and then captured on streptavidin-coated magnetic beads. Only phage containing uAA **2** were captured, demonstrating that the biotin-DBCO reaction is selective for the uAA (***$p < 0.005$, Student's *t*-test). (D) Streptavidin capture of phage displaying a peptide containing one of the uAAs **1–4** (Significant difference from uAA **1**: ***$p < 0.005$; Significant difference from uAA **2**: $^{\#}p < 0.05$, unpaired Student's *t*-test). All data are presented as mean ± SEM, $n \geq 3$.

We selected a helper phage (Hyperphage) with a deletion of the gene encoding pIII, which was previously used for phage display of peptides and scFvs containing uAAs.[6,15] This system produces phage with five copies of the displayed peptide at the tip of the viral particle.[1] Phage were expressed overnight in 2 × YT media supplemented with 1 mM of one of the uAAs **1**–**4** and then purified by two rounds of precipitation with polyethylene glycol.

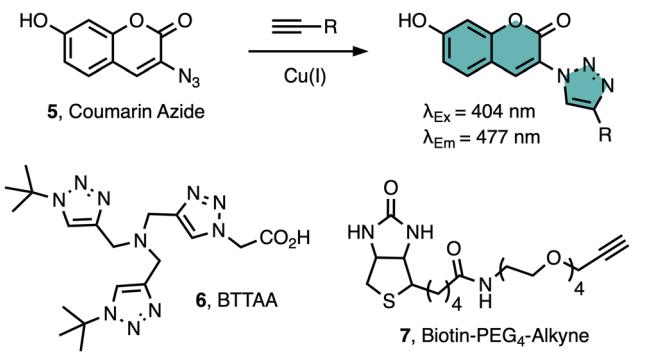
To confirm the incorporation of uAAs **2**–**4** into phage-displayed peptides, we employed a streptavidin pulldown assay inspired by previously reported methods for monitoring reactions on the M13 phage.[10,21,25,28,34] In this assay, purified phage were incubated with biotin-PEG$_4$-dibenzocyclooctyne (biotin-DBCO), which reacts rapidly with azides *via* SPAAC (Fig. 1B).[35] The resulting biotin-conjugated phage were then captured on streptavidin-coated magnetic beads, and the fraction of phage captured was quantified using a colony formation assay. Dibenzocyclooctynes can also react, to some degree, with cysteine residues *via* thiol–yne addition.[36] To evaluate the extent to which biotin-DBCO reacts with native cysteines in wild-type M13, we quantified biotinylation of wild-type phage mixed in the same reaction as uAA-containing phage. To do this, we generated wild-type M13 with a phagemid carrying the *lacZα* reporter gene (M13-*lacZα*). These phages produce blue colonies on X-gal agar plates—distinct from phage containing uAAs, which produce white colonies (Fig. S2, SI). These distinct phenotypes allowed us to monitor modification of uAA-containing and wild-type phage in the same reaction (Fig. S2, SI), analogous to a plaque formation assay developed by Derda and colleagues.[28] To quantify biotinylation, phage containing uAA **2** were mixed with M13-*lacZα* phage in phosphate-buffered saline (PBS, pH 7.4) and incubated with 1 mM biotin-DBCO for 2 h at room temperature. Following incubation, the mixture was subjected to streptavidin pulldown and colony formation assays were used to quantify capture efficiency. More than 96% of phage containing uAA **2** were captured on streptavidin beads after reaction with biotin-DBCO (Fig. 1C). In contrast, less than 5% of wild-type phage were captured after the 2 h reaction with biotin-DBCO. Longer incubations resulted in increased capture of wild-type phage—up to ∼28% capture after 24 h—indicating that extended reaction times can result in significant off-target reactivity of biotin-DBCO with wild-type phage (Fig. S2, SI).

We repeated the streptavidin pulldown assay with phage containing uAAs **3** or **4**, or the control uAA lacking an azide (uAA **1**). With uAAs **3** and **4**, 94% and 79% of phage were captured on streptavidin beads after reaction with biotin-DBCO, respectively (Fig. 1D). Only ∼7% of phage containing uAA **1** were captured, further demonstrating the azide-dependence of phage capture (Fig. 1D). Collectively, these results demonstrate minimal off-target reactivity of biotin-DBCO with native phage cysteines and confirm the successful incorporation of uAAs **2**–**4** into the phage-displayed peptides. It should be noted that this direct-capture pulldown assay does not report on the yield of peptide modification. This is because, as noted above, our phagemid system produces phage displaying five copies of the peptide per virion. Modification of even a single peptide is sufficient for capture on streptavidin beads.[28]

M13 phage are unstable under standard CuAAC conditions

SPAAC provides a convenient method for modifying phage-displayed peptides; however, it requires a bulky dibenzo-cyclooctyne moiety, which is not ideal for modifying peptide libraries. Therefore, we sought to modify phage-displayed peptides using CuAAC, which will produce peptide-small molecule conjugates with a more compact triazole linkage. Additionally, CuAAC is compatible with a myriad of commercially available alkynes and has been widely applied in fragment-based drug discovery applications.[37] We envisioned that applying CuAAC to phage-displayed libraries would provide a convenient and general method to rapidly diversify libraries for GE-FBD applications.

Although M13 phage have previously been used as synthetic scaffolds for CuAAC reactions to produce nanomaterials and bioimaging probes,[38,39] for these applications it is not necessary that the phage retain biological activity. However, to harness the power of iterative selection, reactions for modifying phage display libraries must be sufficiently mild to preserve the phage's ability to replicate in *E. coli*. Several studies have reported that M13 phage are highly sensitive to standard CuAAC conditions, and prior attempts to modify live M13 phage using CuAAC were unsuccessful.[5,40,41] To confirm these findings, phage containing uAA **2** were incubated in PBS (pH 7.4) with $CuSO_4$, the Cu^I-stabilizing ligand **6** (BTTAA),[42] and the alkyne **7** (biotin-PEG$_4$-alkyne; Scheme 1), under reaction conditions previously optimized for protein bioconjugation.[43,44] Reactions were initiated by the addition of sodium ascorbate, which produces the active Cu^I catalyst from Cu^{II} *in situ*.[43] After incubation, all reactions were quenched with ethylenediaminetetraacetic acid (EDTA), serially diluted in 2 × YT medium, and used to infect *E. coli* for quantification *via* a colony formation assay. Consistent with prior reports, we found that standard CuAAC conditions were highly detrimental to phage viability. After just one hour of incubation, phage titers were reduced by more than 1350-fold (Fig. 2A), and no viable phage were detected following overnight incubation. Although all samples were quenched with EDTA and diluted (typically $\geq 10^4$-fold) before infecting *E. coli*, we considered the possibility that residual Cu^{II} might be toxic to *E. coli* and thereby interfere with our tittering assay. However, control experiments showed that $CuSO_4$ alone did not



Scheme 1 Schematic of the fluorogenic assay to monitor product formation under different CuAAC conditions. Also shown are the structures of the Cu^I-stabilizing ligand BTTAA (**6**) and biotin-PEG$_4$-alkyne (**7**).

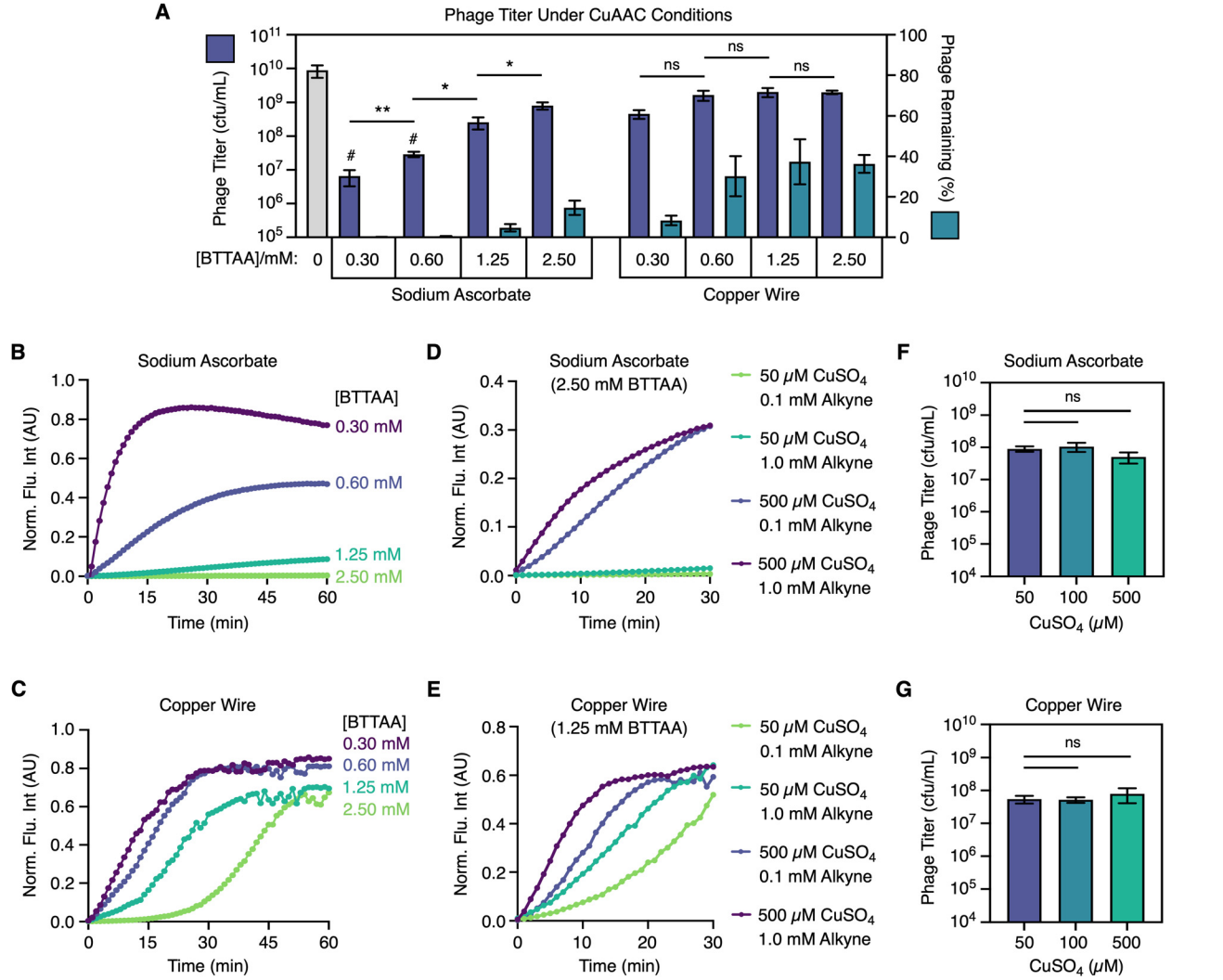


Fig. 2 Optimizing CuAAC to identify phage-compatible reaction conditions. (A) Phage titers and percentage of phage remaining following a 1 h reaction containing 100 μM biotin-PEG_4-alkyne, 50 μM $CuSO_4$, and varying concentrations of BTTAA ligand, using either sodium ascorbate or copper wire to generate Cu^I *in situ*. The gray bar represents the phage titer following incubation in PBS (pH 7.4) only for 1 h. (*$p < 0.05$, **$p < 0.005$, ns = not significant; Significant difference from control sample: $^{\#}p < 0.05$, unpaired Student's *t*-test) (B) and (C) Kinetic traces of product formation in ascorbate- and copper wire-mediated reactions with varying BTTAA ($CuSO_4$ = 50 μM; biotin-PEG_4-alkyne = 100 μM). (D) and (E) Kinetic traces of product formation for ascorbate- and copper wire-mediated reactions with varying concentrations of $CuSO_4$ and biotin-PEG_4-alkyne. The BTTAA concentration is indicated. (F) and (G) Phage titers following ascorbate- and copper wire-mediated reactions with varying concentrations of $CuSO_4$. All data are presented as the mean values; error bars (where shown) represent SEM, $n \geq 3$ for all panels except panel G ($n \geq 2$).

significantly affect assay results, indicating that the observed loss of phage viability is a direct consequence of the CuAAC reaction (Fig. S3; SI).

Identifying phage-compatible CuAAC reaction conditions

Next, we sought to identify reaction conditions that would enable efficient peptide modification while minimizing the loss of phage viability. As noted above, ascorbate is commonly used as a reducing agent in CuAAC to generate the active Cu^I catalyst from Cu^{II} salts.[43] However, the reduction of Cu^{II} to Cu^I by ascorbate, in the presence of molecular oxygen, produces reactive oxygen species (ROS) including hydrogen peroxide, superoxide, and hydroxyl radical.[45,46] These ROS can damage phage coat proteins and induce single- and double-stranded breaks in phage DNA.[45,47,48] It was previously shown that Cu^I ligands, such as BTTAA, can act as sacrificial reductants that intercept ROS, to protect biomolecules from oxidative damage.[43,44] Therefore, we first investigated whether increasing the ligand concentration would protect M13 phage from loss of infectivity during CuAAC reactions.

We observed that increasing the ligand concentration from 0.3 mM up to 2.5 mM significantly protected the phage in a concentration-dependent manner (Fig. 2A). However, because the catalyst-to-ligand ratio influences the rate of CuAAC,[43,49] we considered the possibility that this protective effect could be attributed to a slower reaction rate, which would likely limit the production of ROS. To assess this, we used a fluorogenic assay (Scheme 1) to monitor the reaction between **5** (coumarin azide)

and biotin-PEG_4-alkyne under conditions identical to those used to modify phage.[50] At our standard ligand concentration of 0.3 mM, the reaction proceeded rapidly, whereas higher ligand concentrations significantly slowed the reaction (Fig. 2B). At 2.5 mM BTTAA, the highest concentration tested, minimal product formation was observed (Fig. 2B, Fig. S4, SI). For these initial experiments, we used 50 μM $CuSO_4$, the concentration we routinely use for bioconjugations based on previously optimized protocols;[44,51,52] however, increasing the concentration of $CuSO_4$ led to much faster product formation at elevated ligand concentrations (Fig. 2D). Importantly, although the reaction progressed more rapidly with higher concentrations of copper, phage viability was not compromised up to 500 μM $CuSO_4$ (Fig. 2F). We also found that increasing the concentration of biotin-PEG_4-alkyne from 100 μM to 1 mM enhanced the reaction rate, though less effectively than increasing $CuSO_4$ (Fig. 2D). Based on these findings, we selected 500 μM $CuSO_4$, 2.5 mM BTTAA, and 1 mM biotin-PEG_4-alkyne as optimized conditions to test ascorbate-mediated CuAAC reactions on phage.

While ascorbate is widely used to reduce Cu^{II} to Cu^{I}, its oxidation product, dehydroascorbate, and associated byproducts can react with amino acid residues damaging phage coat proteins.[53,54] To address this issue, alternative methods to generate Cu^{I} have been developed for use in sensitive applications.[44] One such approach employs metallic copper, such as Cu wire, which generates Cu^{I} *via* comproportionation in solutions of Cu^{II}.[55] This method was successfully applied to modify cowpea mosaic virus by CuAAC,[55] but an earlier attempt to modify live M13 phage using Cu wire was not successful.[5] However, this earlier study required very long reaction times (16 h) as it was published prior to the advent of modern ligands—such as BTTAA—that greatly accelerate CuAAC reactions.[42] Therefore, we revisited the possibility of using Cu wire, together with rate-accelerating ligands, for M13 phage modification. We first evaluated the effect of Cu wire-mediated reactions on phage viability with varying concentrations of BTTAA. Phage containing uAA **2** were incubated in PBS (pH 7.4) with 50 μM $CuSO_4$, 0.1 mM biotin-PEG_4-alkyne, and varying concentrations of BTTAA. Reactions were then initiated by adding a small piece 14-gauge copper wire (50 ± 5 mg). Across all ligand concentrations tested, reactions using Cu wire preserved phage infectivity better than those using sodium ascorbate (Fig. 2A).

To determine whether the enhanced phage survival in Cu wire-mediated reactions was simply a result of slower reaction kinetics, we monitored product formation using the same fluorogenic assay used for sodium ascorbate reactions.† Reactions mediated by Cu wire exhibited sigmoidal product formation curves (Fig. 2C), with a pronounced lag phase likely reflecting delayed Cu^{I} formation.[56] As observed with sodium ascorbate, increasing BTTAA concentrations slowed the reaction mediated by Cu wire; however, after the initial lag phase, product formation proceeded more rapidly than in ascorbate-mediated reactions (compare Fig. 2B and C). Interestingly, in Cu wire-mediated reactions, BTTAA primarily affected the duration of the lag phase, with minimal impact on the maximum reaction rate. The lag phase duration increased linearly with BTTAA concentration, from ~0.7 min at 0.3 mM BTTAA to ~28 min at 2.5 mM (a 40-fold increase), while the maximum rate decreased by only 1.4-fold over the same range (Fig. S5, SI). We further optimized reaction conditions by testing increased concentrations of $CuSO_4$ and alkyne substrate. Higher $CuSO_4$ concentrations accelerated the reaction (Fig. 2E) and phage viability was maintained up to 500 μM (Fig. 2G). Based on these data, we selected 500 μM $CuSO_4$, 1.25 mM BTTAA, and 1 mM biotin-PEG_4-alkyne as optimized conditions to test Cu wire-mediated reactions on phage.

We also evaluated hydroxylamine as a mild alternative to sodium ascorbate for producing Cu^{I} *in situ*.[44] However, initial kinetic studies revealed that the reaction proceeded very slowly (Fig. S6, SI). Therefore, this approach was not pursued further.

In early experiments, we found that phage viability during CuAAC could be improved through specific sample handling practices. Although reactions were not conducted under strict anaerobic conditions, we took measures to minimize oxygen exposure and thereby limit Cu^{I}-mediated ROS formation. For example, phage infectivity was approximately 2-fold higher in reactions mixed gently by pipetting compared to those subjected to vortex mixing (Fig. S7, SI). This effect was specific to the CuAAC reaction, as vortex mixing did not reduce phage titer in PBS alone (Fig. S8, SI). These findings are consistent with previous observations by Finn and colleagues, who reported increased DNA damage in CuAAC reactions subjected to vortex mixing, attributed to ROS generation.[44]

We also tested a range of reaction additives to determine whether they could further improve phage stability in ascorbate-mediated reactions. As previously noted, dehydroascorbate—a product of ascorbate oxidation—can form covalent adducts with amino acid side chains, potentially damaging phage coat proteins. Aminoguanidine (AG) has been reported to scavenge dehydroascorbate, thereby preventing unwanted protein modifications during CuAAC.[43,53] AG was shown to prevent aggregation of cowpea mosaic virus under CuAAC conditions[43] and is routinely used in live-cell labeling of *Saccharomyces cerevisiae*.[57] However, the addition of AG did not significantly improve the viability of M13 phage in CuAAC reactions (Fig. S7, SI).

We further evaluated several ROS scavengers, including D-mannitol.[58] *N*,*N*′-dimethylthiourea (DMTU),[59] Trolox,[60] and sodium pyruvate.[61] We first investigated the influence of these additives on reaction kinetics. While most scavengers showed minimal impact at their effective concentrations, DMTU significantly inhibited the reaction and was therefore excluded from further testing (Fig. S9, SI). Among the remaining scavengers, only D-mannitol and pyruvate produced a significant, albeit modest, increase in phage survival (Fig. S7, SI). Finally, we tested an enzymatic oxygen scavenging system, composed of glucose oxidase and catalase (ClickOx), that was previously

† In the fluorogenic plate reader assay, data from reactions with copper wire were consistently noisier than reactions with sodium ascorbate. We suspect that this is due to light scattering by the small piece of metallic copper in the reaction vessel.

shown to mitigate ROS damage in cell culture.[62] However, this system also failed to improve survival of M13 phage (Fig. S7, SI). Based on these results, none of the tested scavengers were used in subsequent experiments.

Modifying phage-displayed peptides by phage-compatible CuAAC

The preceding experiments identified reaction conditions that support efficient coupling between model substrates while preserving phage viability. We next sought to determine whether these conditions could be applied to modify phage-displayed peptides containing azide-functionalized uAAs. To test this, phage containing uAA **2** were incubated in PBS (pH 7.4) with 500 μM $CuSO_4$, 1 mM biotin-PEG_4-alkyne, and either 1.25 mM BTTAA (for Cu wire-mediated reactions) or 2.5 mM BTTAA (for ascorbate-mediated reactions) (Fig. 3A). A lower concentration of BTTAA was used for wire-mediated reactions because it supported faster reactions (Fig. 2C) and, unlike the ascorbate-mediated reaction, higher ligand concentrations did not significantly improve phage survival in reactions mediated by Cu wire (Fig. 2A). Phage modification was evaluated by quantifying the number of phages captured on streptavidin-coated magnetic particles using a colony formation assay similar to that depicted in Fig. 1B. For these experiments, we used phage at a concentration of $\sim 1 \times 10^9$ cfu mL^{-1}, with each phage displaying five copies of the model peptide. This corresponds to an azide-containing peptide concentration of approximately 8 pM—substantially lower than the azide concentration used in our fluorogenic assay. Accordingly, reaction times were extended to 2 or 4 h in these experiments.

In Cu wire-mediated reactions, 86% and 92% of phage were captured on streptavidin beads after 2 h and 4 h reactions with biotin-PEG_4-alkyne, respectively (Fig. 3C). Control reactions lacking $CuSO_4$ resulted in less than 1% capture, confirming the copper-dependence of phage modification (Fig. 3C). Notably, these high levels of phage modification were achieved with only modest losses in viability: phage titers decreased by 3.5-fold after 2 h and 8-fold after 4 h (Fig. 3D). The ascorbate-mediated reaction exhibited similarly high levels of phage capture, with 91% of phage captured after 2 h and 95% of phage captured after 4 h (Fig. 3E). Under these optimized conditions, phage titer dropped by only 2-fold after 2 h and 9-fold after 4 h in the ascorbate-mediated reaction (Fig. 3F). Although the average percent capture increased slightly between 2

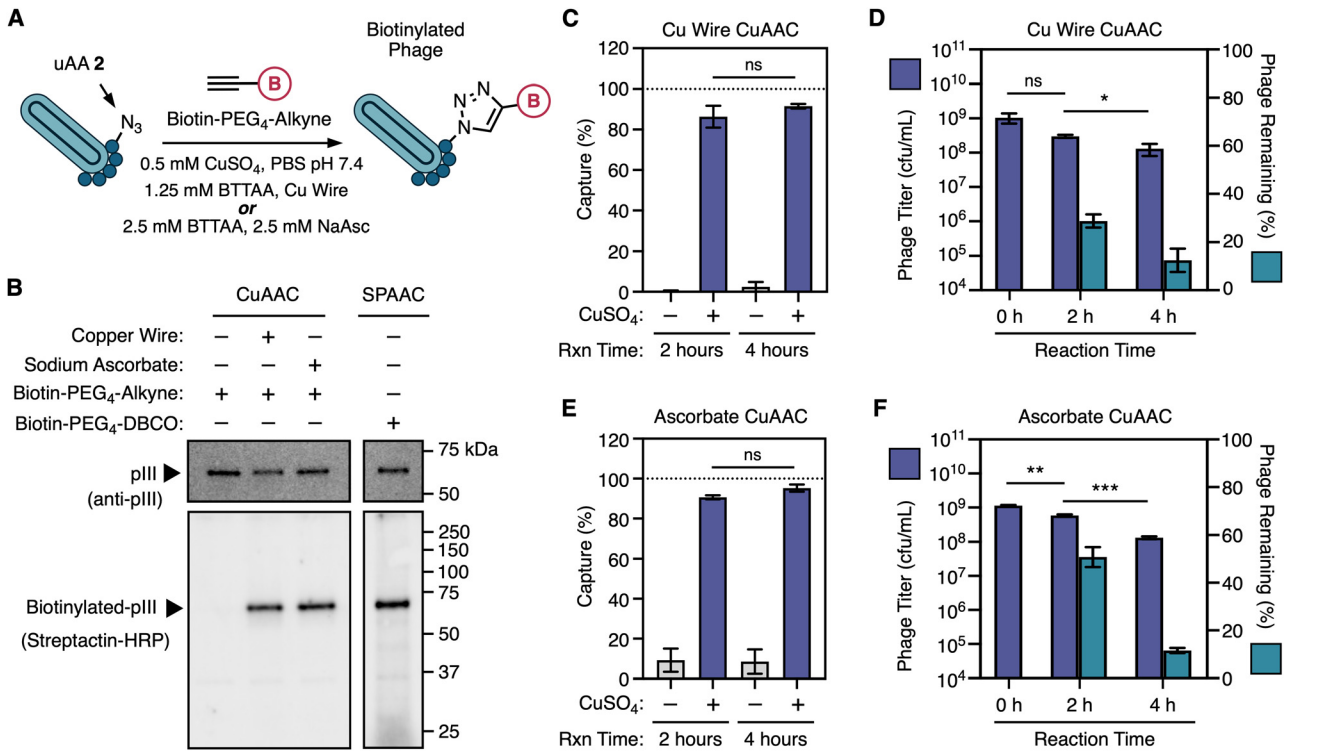


Fig. 3 Modification of live M13 bacteriophage by CuAAC. (A) Phage-displayed peptides containing uAA **2** were modified with biotin-PEG_4-alkyne using either copper wire or sodium ascorbate to generate Cu^I. Both reaction types contained 1 mM biotin-PEG_4-alkyne and 0.5 mM $CuSO_4$. Copper wire-mediated reactions included 1.25 mM BTTAA and a 50 ± 5 mg piece of 14-gauge copper wire, while ascorbate-mediated reactions used 2.5 mM BTTAA and 2.5 mM sodium ascorbate. (B) Western blot analysis demonstrating that biotinylation of phages is specific for the peptide-pIII fusion protein containing uAA **2**. The membrane was probed with anti-pIII antibody, stripped, and re-probed with streptactin-HRP to detect biotinylation. (C) and (D) Phage capture on streptavidin-coated magnetic beads (C) and phage titer and percentage of phage remaining (D) following 2 h and 4 h copper wire-mediated reactions. (E) and (F) Phage capture on streptavidin-coated magnetic beads (E) and phage titer and percentage of phage remaining (F) following 2 h and 4 h ascorbate-mediated reactions. All data are presented as the mean ± SEM, $n \geq 3$. *$p < 0.05$, **$p < 0.005$, *etc.* ns = not significant, unpaired Student's *t*-test.

and 4 h in both the ascorbate- and Cu wire-mediated reactions, this increase was not statistically significant (Fig. 3C and E). In contrast, phage titers were significantly reduced over the same time period (Fig. 3D and F). Thus, for biotin-PEG_4-alkyne, extending reaction times beyond 2 h does not improve modification efficiency and comes at the cost of decreased phage survival. However, we anticipate that less reactive alkynes may benefit from longer reactions, and that optimal reaction times may need to be determined empirically for each alkyne. In any case, the less than 10-fold reduction in phage viability observed in both ascorbate- and Cu wire-mediated reactions is comparable to other cPTM methods commonly used to modify phage-displayed peptide libraries.[19]

We used Western blot analysis to further confirm that the biotin-PEG_4-alkyne probe reacted specifically with the uAA-containing peptide on the phage coat protein pIII. Phage containing uAA **2** were incubated with biotin-PEG_4-alkyne for 4 h under reaction conditions optimized for either Cu wire or ascorbate, resolved by SDS-PAGE, and blotted on a nitrocellulose membrane. The membrane was then probed with an anti-pIII antibody and streptactin-HRP was used to detect biotinylation. Western blotting revealed specific biotinylation of the phage coat protein pIII, with no detectable labeling of other phage proteins (Fig. 3B). As expected, biotinylated pIII was observed in reactions containing Cu wire or sodium ascorbate, and not in a control reaction lacking a reducing agent. The migration and band intensity of biotinylated pIII in both reactions were comparable to that seen in phage modified *via* SPAAC using biotin-DBCO (Fig. 3B). Collectively, these results demonstrate that both Cu wire and sodium ascorbate are effective reductants for modifying peptides on live M13 phage.

Late-stage reshaping of phage-displayed peptides using alkyne-functionalized small molecules

Biotin-PEG_4-alkyne is a convenient probe for optimizing CuAAC reactions, as it enables easy detection of phage modification by streptavidin pulldown or Western blot. However, monitoring reactions with other alkynes is more challenging due to a lack of convenient analytical methods.[63] As a result, most studies rely on indirect "pulse-chase" assays, in which a biotin probe is used to monitor the loss of a reactive functional group on the phage, rather than directly detecting product formation.[10,21,25,28,34] Inspired by these approaches, we developed a pulse-chase assay to track the reaction between azide-functionalized uAAs in phage-displayed peptides and various alkyne-functionalized small molecules. In an initial "pulse" step, phage are incubated with the desired alkyne under the phage-compatible CuAAC reaction conditions described above (Fig. 4A). The reaction is then quenched with EDTA and followed by a "chase" step in which biotin-DBCO is added (Fig. 4B). The biotin-DBCO will biotinylate any phage that contain an unreacted azide following the initial, copper-catalysed reaction (Fig. 4C). The number of biotinylated phage can be quantified using a streptavidin pulldown assay, and the percentage captured used to estimate the yield of the initial CuAAC reaction.[28] Notably, because each phage displays five copies of the pIII protein—each bearing a peptide with an azide-functionalized uAA—all five peptides must be modified for the phage to evade capture on streptavidin beads.

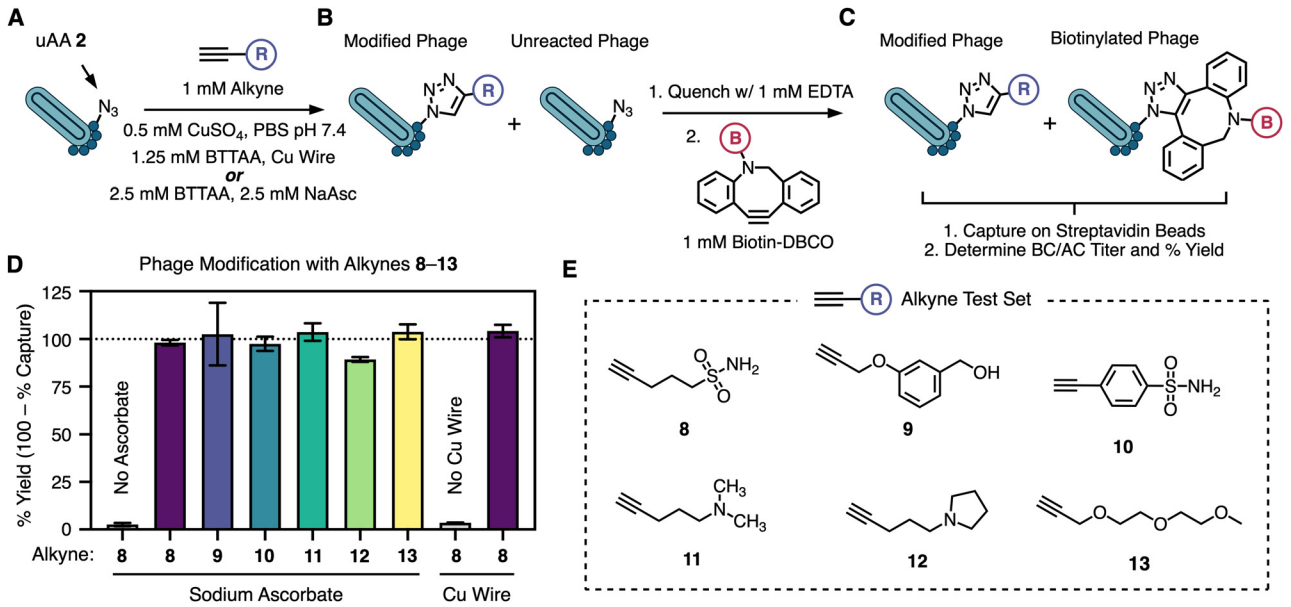


Fig. 4 Late-stage reshaping of phage-displayed peptides using alkyne-functionalized small molecules. (A–C) Schematic of the pulse-chase assay used to monitor the reaction between phage-displayed peptides containing uAA 2 and various alkyne-functionalized small molecules. (A) Phages were modified with 1 mM of the alkyne using optimized conditions for ascorbate- or copper wire-mediated CuAAC. (B) After 3 h, reactions were quenched with EDTA and chased with 1 mM biotin-DBCO to label any remaining azide. (C) Phages were subjected to streptavidin pulldown to quantify biotinylation which reflects the fraction of unreacted azide (uAA 2). (D) Percent yield of CuAAC reactions with alkyne-functionalized small molecules 8–13, as determined by the pulse-chase assay. Data are presented as mean ± SEM, $n \geq 2$. (E) The chemical structures of the alkyne test set evaluated in this study.

Table 1 Modification of phage-displayed peptides containing uAA **2** with alkynes **8–13** using ascorbate-mediated CuAAC reaction conditions

Entry	Alkyne	Yield (%)	Rem.[a] (%)
1	**8**	98 ± 2	31
2	**9**	103 ± 33	18
3	**10**	98 ± 5	19
4	**11**	104 ± 7	14
5	**12**	89 ± 2	33
6	**13**	104 ± 6	48
7	—	3 ± 1	—

[a] Percentage of phage remaining following a 3-hour reaction.

Initial attempts to use this pulse-chase assay to monitor CuAAC reactions on phage were successful but suffered from low precision. This was because small differences in plating efficiency or sample handling led to disproportionately large differences in the calculated yields. To address this, we introduced M13-*lacZα* phage as a spike-in internal control, allowing us to correct for experimental variability in colony counts. We first attempted to modify phage with a small sulfonamide alkyne (**8**, Fig. 4E). Phage displaying a model peptide with uAA **2** were spiked with M13-M13-*lacZα* phage and incubated in PBS (pH 7.4) with 1 mM of alkyne **8** under either Cu wire- or ascorbate-mediated CuAAC conditions. After 3 h at room temperature, the reaction was quenched with EDTA and chased with 1 mM biotin-DBCO for 2 h. This 2 h chase step was sufficient to capture 97% of unreacted uAA **2**-containing phage in control experiments (Fig. S10, SI). Following the chase step, phage capture on streptavidin beads was quantified using a colony formation assay as described in the SI. Using this method, we found that 98% of phage-displayed peptides were successfully modified with alkyne **8** under ascorbate-mediated conditions (Fig. 4D and Table 1). The Cu wire-mediated reaction gave similarly high levels of modification within the same timeframe (Fig. 4D). In control reactions containing all components except ascorbate or Cu wire, the extent of peptide modification measured with this assay was less than 4% (Fig. 4D). These results demonstrate that both the ascorbate and Cu wire serve as efficient reductants for achieving near-quantitative modification of phage-displayed peptides under phage-compatible conditions.

Building on our initial proof-of-concept results with alkyne **8**, we next evaluated modification of phage-displayed peptides using a series of small alkynes (*i.e.*, **9–13**; Fig. 4E) under ascorbate-mediated, phage-compatible CuAAC conditions. All tested alkynes yielded high levels of peptide modification with most achieving quantitative conversion within the 3 h reaction (Fig. 4D and Table 1). To assess the impact of these reactions on phage viability, we also measured phage titers before and after the reaction. In all reactions, the loss of infective phage was modest—less than 7-fold in every case, with an average decrease of 4.4-fold (Table 1). This corresponds to an average of 27% of phage surviving the 3-hour reaction. These results demonstrate that the optimized CuAAC conditions identified here support efficient, high-yielding modification of phage-displayed peptides with a broad range of alkynes, while maintaining high phage viability.

Conclusions

In summary, our work establishes a versatile and efficient strategy to chemically diversify phage-displayed peptide libraries by integrating GCE with cPTM. This hybrid approach leverages the precision of GCE for site-specific installation of bioorthogonal handles and the modularity of cPTM for late-stage reshaping with diverse chemical functionalities. Here, we focused on installing azide-functionalized uAAs into phage-displayed peptides. Through reaction optimization, we identified CuAAC reaction conditions that enable quantitative peptide modification while preserving phage viability. Additionally, we developed a streptavidin pulldown assay to monitor the reaction between phage-displayed peptides and a range of alkyne-functionalized small molecules.

In addition to azide, GCE methodology has been used to install an ever-expanding repertoire of bioorthogonal reactive handles, including alkynes, aldehydes, ketones, cyclopropene, and tetrazines[64]—offering exciting opportunities for further library diversification. Advances in GCE that enable the incorporation of multiple distinct reactive groups opens the door to next-generation phage libraries bearing multivalent or multifunctional chemical modifications.[13,33,65] Together, these innovations lay the groundwork for creating more complex peptide libraries and greatly expanding the functional capabilities of phage display technology.

Author contributions

J. M. T. designed the study. O. D., C. A. W., C. S., and J. M. T. performed experiments. O. D., C. A. W., C. S., A. T., and J. M. T. analysed the data. J. M. T. wrote the manuscript. All authors approved of the final manuscript before submission.

Conflicts of interest

There are no conflicts to declare.

Data availability

Raw data are available from the corresponding author upon request.

Materials and methods, plasmid sequences, recommended general protocol for phage modification by CuAAC, and supplementary figures. See DOI: **https://doi.org/10.1039/d5cb00140d**

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