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Journal
Chemical Communications, 56(74)

ISSN
1359-7345

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Publication Date
2020-09-17

DOI
10.1039/d0cc04600k

Peer reviewed

HHS Public Access

Author manuscript

Chem Commun (Camb). Author manuscript; available in PMC 2021 September 17.

Published in final edited form as:

Chem Commun (Camb). 2020 September 17; 56(74): 10883–10886. doi:10.1039/d0cc04600k.

Chemically triggered crosslinking with bioorthogonal cyclopropenones

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Abstract

We report a proximity-driven crosslinking strategy featuring bioorthogonal cyclopropenones. These motifs react with phosphines to form electrophilic ketene-ylides. Such intermediates can be trapped by neighboring proteins to form covalent adducts. Successful crosslinking was achieved using a model split reporter, and the rate of crosslinking could be tuned using different phosphine triggers. We further demonstrated that the reaction can be performed in cell lysate. Based on these features, we anticipate that cyclopropenones will enable unique studies of protein-protein and other biomolecule interactions.

Covalent crosslinkers are valuable tools for examining biomolecule interactions in physiologically relevant environments. Such tools typically fall into two categories: photocrosslinkers[1] and chemical crosslinkers. Popular photocrosslinkers include diazirines,[2] aryl azides,[3] benzophenones,[4] and aryl carboxytetrazoles.[5] These groups are routinely used in vitro to monitor interactions between proteins and other biomolecules.[6] When exposed to intense UV light, the motifs photolyze to provide high-energy intermediates; these species can be trapped by neighboring biomolecules to forge covalent adducts. UV light can be delivered to samples on demand, enabling both spatially and temporally controlled crosslinking. Furthermore, the small size of most photocrosslinkers ensures that they are compatible with a variety of cellular targets and pathways for installation.[6a,6c,6d,7]

While widely employed, photocrosslinkers also have limitations. Many suffer from high levels of background signal due to off-target labeling.[8] Additionally, the requisite UV light precludes applications in thick tissues and live organisms–environments that are either refractory to light delivery or sensitive to irradiation.[9] These issues can be avoided using chemical crosslinkers, such as α-haloacetamides or other chemical warheads for covalent

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†Electronic Supplementary Information (ESI) available: Experimental data, spectra, and experimental details. See DOI: https://dx.doi.org/10.1039/d0cc04600k

Conflicts of interest

There are no conflicts to declare.

trapping.[10] However, such electrophiles are permanently “on” and not responsive to external stimuli, contributing to non-specific labeling. Efforts to tune the specificity of these probes have come at the expense of versatility, as less potent electrophiles react with a more limited set of nucleophiles.[10e,11]

To develop more general and triggerable crosslinkers, we investigated cyclopropenones (CpOs) as *chemically* activated motifs. These scaffolds undergo bioorthogonal reactions with functionalized phosphines.[12] The reaction proceeds through a ketene-ylide intermediate, which can be trapped by a variety of nucleophiles to produce covalent adducts (Fig. 1). Nucleophile trapping is typically accomplished in an *intra*molecular fashion using ortho-substituted phosphines. We hypothesized that *inter*molecular trapping would afford biomolecule crosslinks. Upon phosphine treatment, CpOs positioned near targets of interest would be activated for covalent adduct formation.

We previously showed that ketene-ylides form upon phosphine incubation with CpOs, and that such intermediates can be trapped with alkylamines.[12a] Large concentrations (>10 mM) of nucleophile were required to outcompete ketene hydrolysis (Fig. 1). We hypothesized that such features would be ideal for biomolecule crosslinking. High local concentrations of trapping residues would only be achieved with interacting biomolecules. In all other cases, hydrolysis would dominate, generating an innocuous byproduct and minimizing non-specific crosslinking. Most protein binding interfaces also comprise residues amenable to trapping electrophiles and thus affording stable crosslinks.[13]

To test whether a broad range of residues could suffice for *inter*molecular trapping, we incubated a model cyclopropenone (CpO **S1**, Table S1, ESI) with a panel of amino acids. CpO **S1** was activated for trapping via treatment with a water-soluble triarylphosphine (TPPTS, **1**), and the reactions were monitored by LCMS. Crosslinked products were observed in the presence of Ser, Cys, Lys, and Tyr (Fig. S1–S4, ESI), but only when excess amino acid was used (Table S1, ESI). The hydrolyzed product was the major species formed in each case. These results suggested that CpO-mediated crosslinks could be forged at protein interfaces, where local concentrations of amino acids can exceed 100 mM.[14]

We proceeded to examine CpO crosslinking using a model protein interaction: split Nanoluciferase (Nluc, Fig. 2A).[15] Split Nluc comprises a short C-terminal peptide (SmBiT) and a larger engineered fragment (LgBiT). LgBiT and SmBiT bind readily and can reconstitute the full-length, light-emitting enzyme in aqueous buffers. Examining the Nluc crystal structure, we hypothesized that CpO appended to the N-terminus of SmBiT could access a handful of potential trapping residues when bound to LgBiT (Fig. 2B).[16] To prepare the functionalized peptide, we first synthesized CpO amino acid **2**. This Fmoc-protected variant could be prepared in gram-scale quantities, and was amenable to solid-phase peptide synthesis. CpO **2** was installed at the N-terminal position of SmBiT to provide the desired conjugate (SmBiT-CpO **3**, Fig. 2C and Fig. S5, ESI). Importantly, SmBiT-CpO **3** was still capable of binding LgBiT to produce a light-emitting enzyme (Fig. S6, ESI).[15]

We next examined whether the functionalized peptide could form a covalent crosslink with LgBiT. LgBiT and peptide **3** were incubated in phosphate buffered saline (PBS, pH 7.4) and

treated with phosphine **1**. SDS-PAGE analysis revealed a higher molecular weight band, indicating that crosslinked Nluc was formed. The identity of the product was verified via mass spectrometry (Fig. 2D). Hydrolysis products were also observed after 3 h, along with cyclized SmBiT (due to *intra*molecular trapping). Unreacted SmBiT-CpO was still present at this point, though, and available for crosslinking.

To test whether crosslinking was specific to interacting fragments, we performed the reaction in the presence of a non-target protein, hen egg-white lysozyme (HEWL). HEWL is similar in size to LgBiT. Both proteins also comprise >20 surface-exposed nucleophiles that could potentially trap the activated cyclopropenone.[17] When SmBiT-CpO **3** was incubated with HEWL, though, no crosslinked products were observed upon phosphine treatment (Fig. 2E). These data suggest that the crosslinking strategy can provide a selective readout on productive interactions.

We further examined whether the approach could distinguish among interactions of variable affinity. We synthesized two additional SmBiT-CpO peptides (**4** and **S9**, Fig. S5, ESI) comprising sequences known to exhibit different degrees of binding to LgBiT.[15] We hypothesized that the range of binding affinities would manifest in variable degrees of crosslinking. The SmBiT peptides were mixed with LgBiT, and the CpO residues were activated with phosphine **1**. SDS-PAGE analysis revealed a clear trend in crosslinking efficiency (Fig. S7, ESI). The highest affinity peptide (**4**) provided the largest number of LgBiT crosslinks, while no adducts were observed with the weakest binder (**S9**). Importantly, SmBiT-CpO **4** (i.e., the highest affinity peptide) also did not form covalent crosslinks with off-target proteins (Fig. S8, ESI).

While split luciferase adducts were captured upon CpO activation, maximal crosslinking was not achieved until 4 h post-phosphine addition (Fig. S9, ESI). We hypothesized that crosslinking speed could be improved by tuning the phosphine trigger. Alkyl substituents are well known to increase the nucleophilicity of phosphines, but can render the probes susceptible to oxidation.[18] However, monocyclohexyl diaryl phosphines are bench stable and have been used in biological environments.[12b] Thus, we reasoned that phosphine **5** would strike a balance between increased reactivity and stability toward oxidation, and also be water-soluble (Fig. 3A).[19] Phosphine **5** was easily accessed from commercially available starting materials using standard sulfonation chemistries. When **5** was used to activate SmBiT-CpO **4** in the presence of LgBiT, crosslinked products were observed in as little as 10 minutes (Fig. 3B). The probe could also activate CpO motifs for crosslinking in a dose-dependent manner (Fig. S10, ESI). No adducts were observed with triarylphosphine **1** over the same time period, and prolonged reaction times were required for robust crosslinking signal (Fig. S11, ESI).

The crosslinking approach was further examined in bacterial cell lysate. In this complex environment, many proteins could potentially outcompete LgBiT for trapping activated CpOs. LgBiT and SmBiT-CpO **4** were added to freshly prepared lysate, then treated with phosphine **5**. The expected crosslinked product was observed after 30 min of incubation at 37 °C (Fig. 3C). Interestingly, SDS-PAGE analysis suggested that crosslinking was more efficient in lysate than in buffer alone. A similar result was observed with the less

nucleophilic phosphine **1** (Fig. S12, ESI). Macromolecular crowding likely facilitates biomolecular association—and thus more effective crosslinking—in heterogeneous environments.[20]

Having demonstrated successful crosslinking with split luciferase probes, we were curious about the nature of the covalent linkage. We initially suspected LgBiT residues K124 or K136 were involved in CpO trapping (Fig. 2B). However, when these sites were mutated to alanine, no decrease in crosslinking efficiency was observed (Fig. S13, ESI). Mass spectrometry analyses of tryptic digests revealed LgBiT fragment (residues 16–64) involved in the trap (Fig. S14, ESI). Further attempts to localize the crosslink (via MS/MS analysis) were inconclusive, though, possibly due to non-specific cleavage of the crosslink. Within the LgBiT fragment, Tyr16, Ser28, and Ser29 were the most likely traps based on predicted proximity to bound SmBiT (Fig. 2B). We prepared LgBiT mutants Y16F, S28A, and S29A to probe their participation in CpO trapping. Only mutant Y16F exhibited reduced crosslinking efficiency not attributed to impaired LgBiT binding (Fig. S15 and Fig. S16, ESI). Few chemical crosslinkers target tyrosine or other less potent nucleophiles, highlighting the versatility of our approach.

The CpO crosslinking strategy was also compared to a common photocrosslinking approach with diazirine (Dz) motifs. Upon UV irradiation, diazirines form carbenes that can react with a variety of protein residues to afford covalent adducts. A diazirine-functionalized SmBiT peptide (SmBiT-Dz, **S10**, Fig. S19, ESI) was synthesized. The Dz linker was one carbon shorter than the corresponding CpO probe, but was pursued based on the synthetic tractability of a precursor amino acid (**S13,** Synthetic Procedures, ESI). SmBiT-Dz (**S10**) was incubated with LgBiT, and the sample was irradiated with UV light (312 nm). Crosslinked adducts were observed via SDS-PAGE analysis (Fig. S17, ESI). Prolonged irradiation times resulted in more crosslinked adducts, but also promoted LgBiT degradation. The crosslinking efficiency of SmBiT-Dz was also examined alongside SmBiT-CpO (**4**). Each peptide was incubated with LgBiT. Samples containing SmBiT-CpO were exposed to phosphine **5** for chemical crosslinking, while samples containing SmBiT-Dz were irradiated with UV light for photocrosslinking. Crosslinked products were observed in both cases (Fig. S18, ESI), with the chemically triggered reaction providing more adducts.

While quantitative comparisons cannot be made due to differences in probe structure, SmBiT-Dz and SmBiT-CpO exhibited similar affinities for LgBiT (Fig. S19, ESI), suggesting that the observed differences in crosslinking were not simply due to altered binding interactions. They could instead be due to differences in probe reactivity. Carbenes are formed irreversibly upon diazirine photolysis. These high-energy intermediates exhibit relatively short half-lives (ns–μs) and can insert into a variety of different bonds.[21] As a consequence, carbenes can trap even weakly associated off-target molecules, leading to false positives. Ketene-ylides, by contrast, are formed reversibly from cyclopropenones and phosphines. They have relatively long half-lives and react with a smaller subset of biological functional groups.[12a,22] Such differences likely influence the degree and specificity of crosslinking observed.

In conclusion, we demonstrated that cyclopropenones can be chemically activated by phosphines to forge biomolecule crosslinks. Both the phosphine and cyclopropenone reagents are readily accessible, biocompatible, and easy to handle. Using a model split reporter, we showed that the triggered crosslinking reaction is specific and proceeds readily in physiological buffers and in the presence of cell lysate. CpO probes are complementary to photocrosslinkers and add to the growing arsenal of tools for capturing protein contacts. Phosphine accessibility to CpO probes will dictate the full scope of targets, and is the subject of ongoing work. The ability to robustly and site-specifically encode CpO motifs will also provide additional opportunities for interrogating protein binding in cells,[23] and these experiments are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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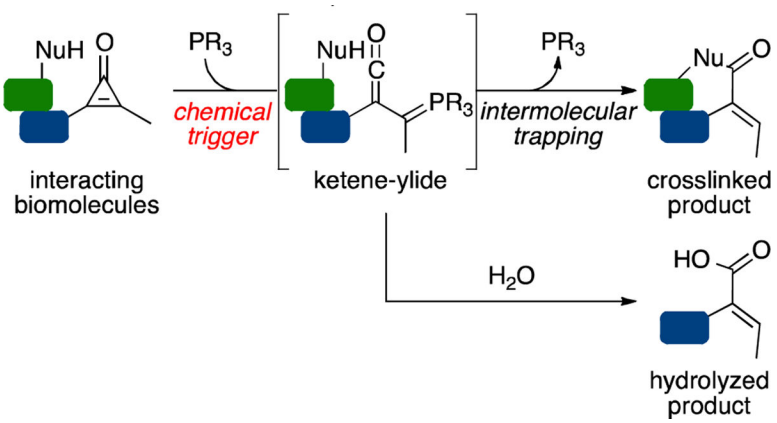

Fig. 1.

Cyclopropenones as chemically triggered crosslinkers. Cyclopropenones generate ketene-ylides upon treatment with bioorthogonal phosphines. These intermediates can be trapped by neighboring nucleophiles to form covalent crosslinks or hydrolyze to give innocuous byproducts.

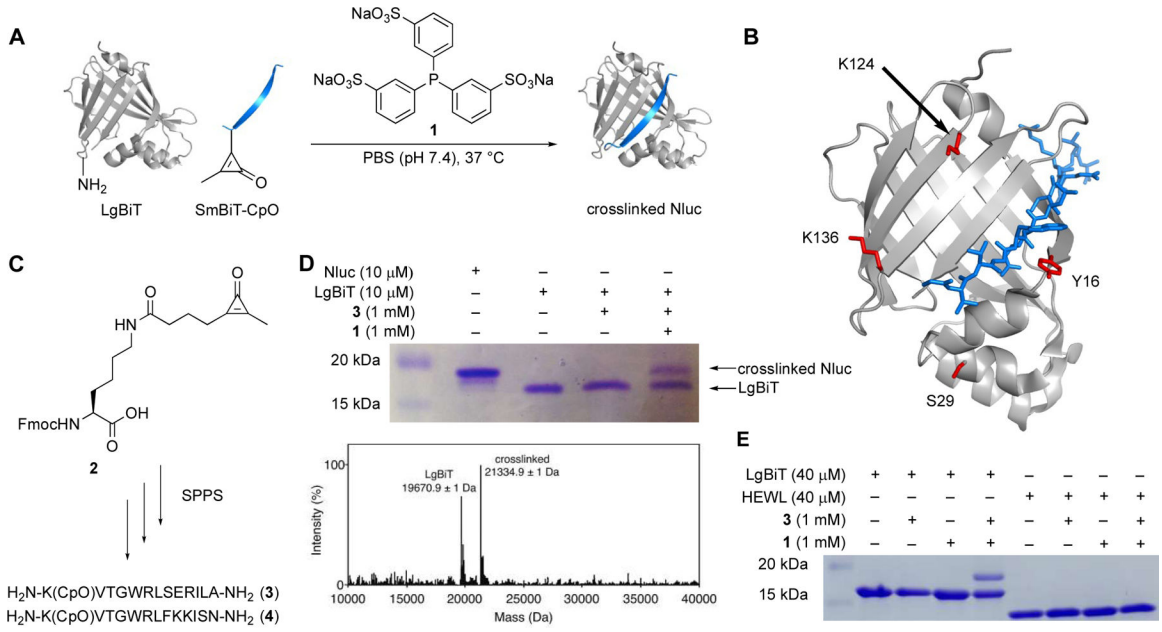

Fig. 2.

Chemically triggered crosslinking of split luciferase probes. (A) Crosslinking of LgBiT and SmBiT to form Nanoluciferase (Nluc). (B) Crystal structure of Nluc (PDB 5IBO), with SmBiT highlighted in blue. Potential trapping residues are shown in red. (C) CpO-functionalized SmBiT-CpO **3** and **4** were prepared via solid-phase peptide synthesis. (D) Covalent crosslinking of LgBiT and SmBiT-CpO **3** with phosphine **1**. Crosslinked Nluc was observed by SDS-PAGE (top) and mass spectrometry (bottom). The crosslinking yield (33%) was determined by ImageJ analysis. (E) SmBiT-CpO **3** does not form covalent adducts with off-target proteins. LgBiT (40 μM) or HEWL (40 μM) and SmBiT-CpO **3** (1 mM) were incubated in PBS (pH 7.4) in the presence of phosphine **1** (1 mM) at 37 °C for 10 h. Samples were analyzed by SDS-PAGE. The crosslinking yield (31%) was determined by ImageJ analysis.

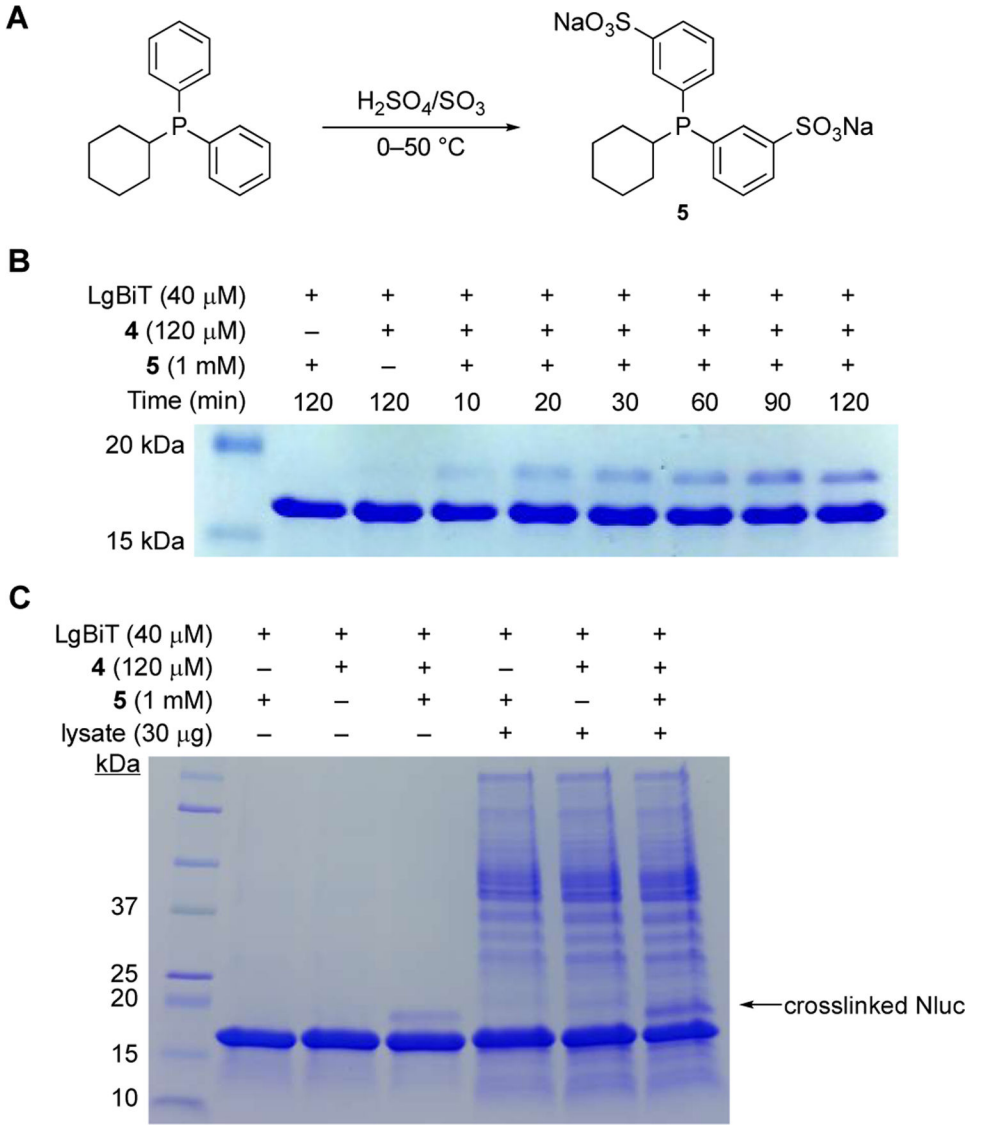

Fig. 3.

Improved crosslinking with an optimized trigger. (A) Phosphine **5** was designed as a more nucleophilic, water-soluble probe. (B) Rapid crosslinking was observed with optimized phosphine. LgBiT (40 μM) and SmBiT-CpO **4** (120 μM) were incubated with phosphine **5** (1 mM). Samples were analyzed by SDS-PAGE. The crosslinking yields (5–16%) were determined by ImageJ analysis. (C) Crosslinking in cell lysate. LgBiT (40 μM) and SmBiT-CpO **4** (120 μM) were incubated in PBS (pH 7.4) containing bacterial lysate (30 μg) and phosphine **5** (1 mM). Samples were incubated at 37 °C for 30 min, then analyzed by SDS-PAGE. The crosslinking yields in the presence (13%) and absence (5%) of lysate were determined by ImageJ analysis.