Equimolar Cross-Coupling Using Reactive Coiled Coils for Stable Covalent Protein Assemblies

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ABSTRACT: Biocompatible cross-coupling reactions enable the efficient covalent attachment of large biomolecules at near-stoichiometric ratios, ensuring the stability and integrity of the resulting products. We present an affinity-based peptide platform utilizing coiled coils containing reactive side chains for proximity-driven protein cross-coupling in the presence of a cross-linking agent. This platform supports both chemical synthesis and recombinant expression, using canonical amino acids to generate reactive affinity tags. Employing the E3/K3 coiled coil pair as a scaffold, we design four complementary coils with cysteine residues as cross-linking sites, achieving >90% conversion to stable, covalent heterodimeric coupling products using 3,4-dibromomaleimide. Equimolar mixtures of proteins with reactive coils at their termini yield near-quantitative heterodimeric cross-coupling products. The strategic selection of complementary coiled coil pairs and cross-linking agents enables orthogonal assembly of macromolecules with diverse architectures. This method offers a versatile approach for creating covalent, stable fusion proteins, enhancing their stability and functionality for applications in chemical biology, biotechnology, and medicine.

Fusion proteins are a versatile class of molecules with a range of applications in chemical biology, biotechnology, and medicine. Recombinant fusion proteins gained significant importance in the field of biopharmaceuticals due to diverse functionalities arising from each individual component.[1] Fusion proteins have been used for drug targeting, wherein bispecific fusion proteins, including those containing antibodies, can specifically bind to specific cell types.[2,3] Another application of fusion proteins involves extending the half-life of protein drugs in the bloodstream by incorporating the fragment crystallizable (Fc) region of immunoglobulin G (IgG), or carrier proteins like human serum albumin (HSA) and transferrin (Tf).[4,5] While genetic techniques offer a straightforward approach for synthesizing fusion proteins with multiple functions, there are obstacles related to the translation and folding processes, particularly in the case of large fusion proteins, as well as requirements for N-to-C terminal ligation.[1]

Conversely, post-translational bioconjugation methods such as chemical, enzymatic, and bioorthogonal conjugation by incorporation of unnatural amino acid residues enable the assembly of fusion proteins with more complex structures including connections at both C-termini and N-termini or branched molecules, which cannot be achieved with biological expression methods.[6] Efficient protein-protein cross-coupling reactions mediate the coupling of large molecules with near-stoichiometric amounts of reaction partners and achieve desired hetero/homoselectivity.[7] Because protein solutions are typically prepared in the micromolar concentration range, attaining a high conversion rate necessitates the selection of reactions with rapid kinetics.[8]

Peptides have been used in bioconjugation methodologies toward affinity-induced site-specific modifications of proteins with small molecules.[9] We investigated the use of affinity-based peptide components of coiled coils for proximity-driven cross-coupling reactions between proteins. Coiled coils are often found in proteins in which two or more adjacent α-helices wrap around each other.[10] Coiled coil systems have been widely utilized for sequence-selective labeling of proteins with small molecules[11–14] and programmable macromolecular assembly.[15,16] To enhance the stability of fusion proteins, there is a demand for the development of molecular assembly systems that employ covalent bonds. However, prior heterodimerization reactions utilizing coiled coils have relied

on the formation of reversible disulfide bonds.[17] We hypothesized that incorporation of complementary coil-based reactive affinity peptides into proteins could enable rapid and specific cross-coupling under equimolar conditions.

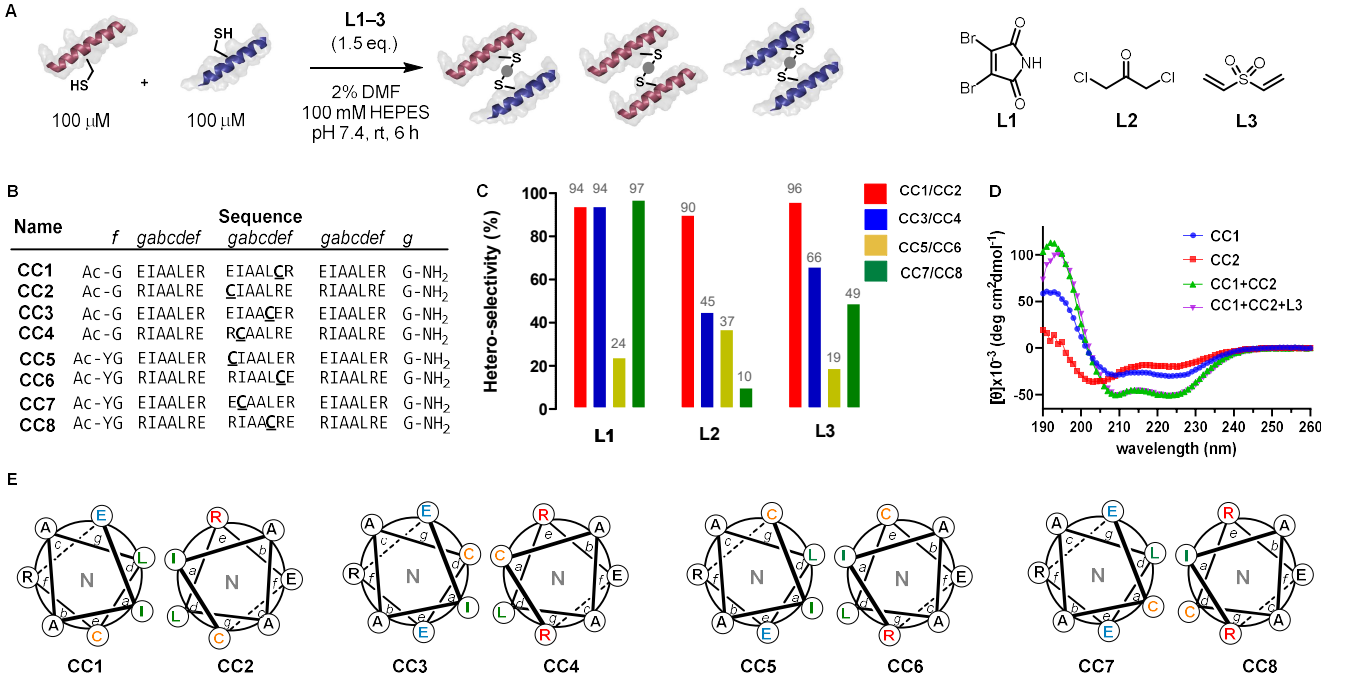

Figure 1. Appropriate choice of reactive coiled coil pair and cross-linker enables hetero-selective dimerization. (A) Reaction conditions and cross-linkers examined. (B) Sequences of reactive coil pairs. All peptides were N-terminally acetylated and C-terminally amidated. The sequence modifications with cysteine residues are highlighted in bold and underlined. (C) Heteroselectivity of reactive coiled coil pairs cross-coupling with different cross-linkers (1.5 equivalents) in 2% v/v DMF 100 mM HEPES (pH 7.4) at 100 μM. The conversion to the corresponding heterodimers was estimated by integration of TIC chromatogram acquired by LC-MS. (D) CD spectra of the **CC1** (blue), **CC2** (red), the equimolar mixture of **CC1** + **CC2** (green) and cross-coupled heterodimer (purple) measured in 10 mM HEPES buffer at pH 7.4. Peptide concentration of all samples was 100 μM. (E) Helical wheel diagram depicting the second heptads of four reactive coiled coil pairs designed in this study. Reactive cysteine residues are highlighted in rectangles.

We report the development of an affinity peptide-based cross-coupling platform that is amenable to recombinant expression of the coupling partners, as the reactive affinity tags are composed of canonical amino acids. Furthermore, the judicious selection of the complementary matched coiled coil pairs potentially enables orthogonal assembly of macromolecules with varied architectures.

We investigated the sequences of the E3/K3 pair of coils which were originally designed by Hodges et al.[18] because they are relatively short in length (3 heptads, 21-residues) and show strong affinity to each other (dissociation constant, K_D = 70 nM) at pH 7.[19] We focused on four pairs of complementary coiled coil sequences labeled as **CC1**–**8** (Figure 1B). The reactive affinity peptides were synthesized with cysteine introduced as a reaction site (Figure 1B and 1E). We replaced all lysine residues in these sequences with arginine (R) to reduce the chance of undesired non-selective cross-coupling at amine-containing side chains. We tested three crosslinkers: 3,4-dibromomaleimide (**L1**),[20] 1,3-dichloroacetone (**L2**),[21] and divinyl sulfone (**L3**).[22] The reactions were carried out at room temperature in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer at pH 7.4 (Figure 1A). For the substrate pair **CC1**/**CC2**, all crosslinkers exhibited high heteroselectivity with conversions to the desired coiled coil heterodimer between 90–96%, estimated by integrating the peak areas in the total ion current (TIC) chromatograms using the following equation: % heteroselectivity = $A_{desired\ product}/A_{all\ relevant\ species}$ where $A_{desired\ product}$ is the peak area of the desired product, and $A_{all\ relevant\ species}$ is the sum of the peak areas of all relevant peptidic species (Figure 1C and S6–S17). However, for pairs **CC3**/**CC4** and **CC7**/**CC8**, only **L1** demonstrated substantial heteroselectivity (94% and 97% conversion to the heterodimer, respectively), whereas the pair **CC5**/**CC6** resulted in poor heteroselectivity across all linkers investigated (<40% conversion to the heterodimer). The structures of the helical tags and their corresponding heterodimers were analyzed using circular dichroism (CD) spectroscopy (Figure 1D and S42). At pH 7.4, both **CC1** and **CC2** display typical

random coil-like structures. However, the CD spectrum of an equimolar mixture of **CC1** and **CC2** shows a strong signal corresponding to an α-helix secondary structure with two minima at 208 and 222 nm.

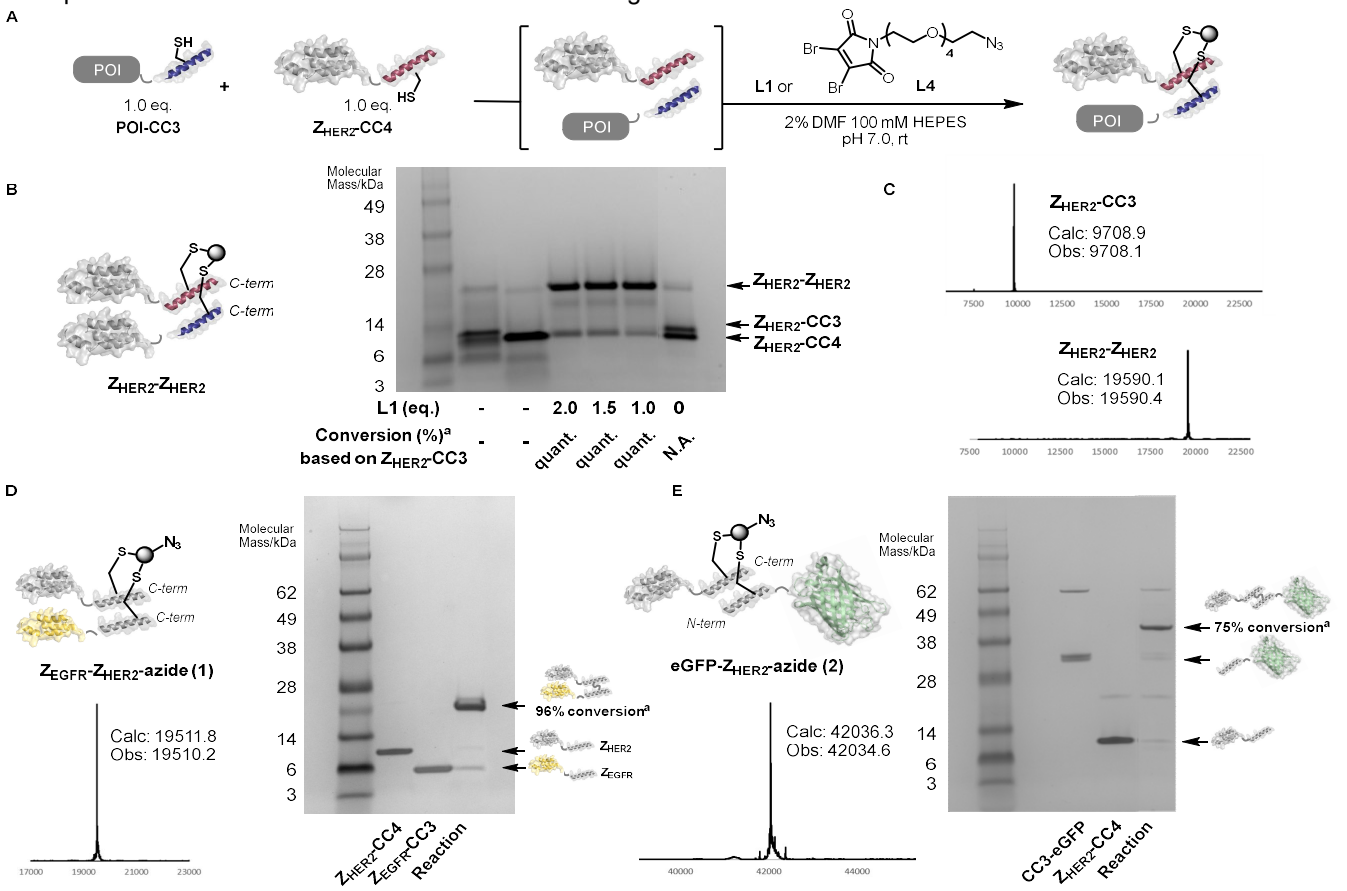

Figure 2. Stoichiometric amounts of cross-linkers enable equimolar cross-coupling of proteins with appended coil pairs. (A) Affinity peptide-based cross-coupling reaction of proteins. (B) Synthesis of Z_{HER2} affibody dimer. Yield estimation by non-reducing SDS-PAGE analysis allows for optimization of cross-coupling conditions. (C) Deconvoluted mass spectra are depicted for Z_{HER2}-CC3 (top), and the conjugate Z_{HER2}- Z_{HER2} (bottom). (D) Cross-coupling of Z_{EGFR}-CC3 and Z_{HER2}-CC4 in the presence of **L4**. (E) Cross-coupling of eGFP-CC3 and Z_{HER2}-CC4. [a]Conversion was determined by SDS-PAGE followed by densitometry analysis.

The CD spectra of covalent heterodimer (**CC1** + **CC2** + **L3**) also shows an α-helix secondary structure almost identical to their non-covalent complex. It is notable that **L1** enables the release of each coupling partner through a cysteine exchange reaction under high glutathione conditions.[23] On the other hand, **L2** and **L3** form non-cleavable irreversible conjugates.

Our group implemented automated fast-flow peptide synthesis technology (AFPS) that enables high-fidelity stepwise production of sequences approaching the length of single domain proteins.[24,25] Using AFPS instruments, we successfully synthesized affibody molecules Z_{HER2}[26] containing appended C-terminal tags **CC3** and **CC4**, respectively (Figure S23 and S24), which enable subsequent protein cross-coupling reactions. Both polypeptide chains are 88 residues long. We examined the amount of cross-linking reagent **L1** required for coupling of partners Z_{HER2}-CC3 and Z_{HER2}-CC4 (Figure 2B). Each affibody containing the complementary tags was incubated at 5 μM, and reaction conversion was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that using 1.0 equivalent or more of **L1** yielded affibody-affibody in quantitative yield. Heteroselectivity was confirmed by LC-MS analysis of the crude products in the presence of 1.0 equivalent of cross-linker **L1** (Figure 2C). Next, we investigated the protein substrate scope of our cross-coupling methodology (Figure 2D and 2E). Z_{EGFR} affibody[27] with helical tag **CC3** at the C-terminus was synthesized using AFPS (Figure S25), while eGFP[28] was expressed in *E. coli* (Figure S1). We incorporated **CC3** at the N-terminus of eGFP through a sortase-mediated reaction using the appropriate sortags on each partner (Figure S2). Treatment of an equimolar mixture of Z_{EGFR}-CC3 and Z_{HER2}-CC4 (final concentration of 5 μM) with 1.0 equivalent of the 3,4-dibromomaleimide-PEG4-azido **L4** achieved 96% conversion after 15 hours, as determined by SDS-PAGE (see Figure S30). For this purpose, linker **L4** was designed by modifying linker **L1**

with an azido moiety to allow further functionalization of the resulting conjugates by click chemistry, if desired. After size-exclusion chromatography, the protein-protein conjugate **1** was isolated in 39% yield (736 µg). We further performed the cross-coupling reaction between eGFP-CC3 and Z_{HER2}-CC4, obtaining the eGFP-Z_{HER2} conjugate (**2**) (SDS-PAGE, 75% conversion, also see Figure S32).

The covalent bispecific Z_{EGFR}-Z_{HER2} protein dimer displayed affinity toward the two respective antigens. We introduced a biotin molecule and a fluorescent probe to **1** through a click reaction, resulting in the formation of compounds **3** and **4**, respectively (Figure 3A). We determined the apparent dissociation constant (K_D) of **3** to epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2) using bio-layer interferometry (BLI) (Figure 3B). The K_D values determined were 23 nM against EGFR and 1.2 nM against HER2, respectively. To investigate the binding of compound **4** to HER2-positive breast cancer cells (SK-BR-3), the cells were incubated with **4** (5.1 µM) at 37 °C for 24 h. Subsequently, the cells were imaged using a 559 nm laser (Figure 3C). The bispecific Z_{EGFR}-Z_{HER2} retains its affinity for HER2-positive breast cancer cells.

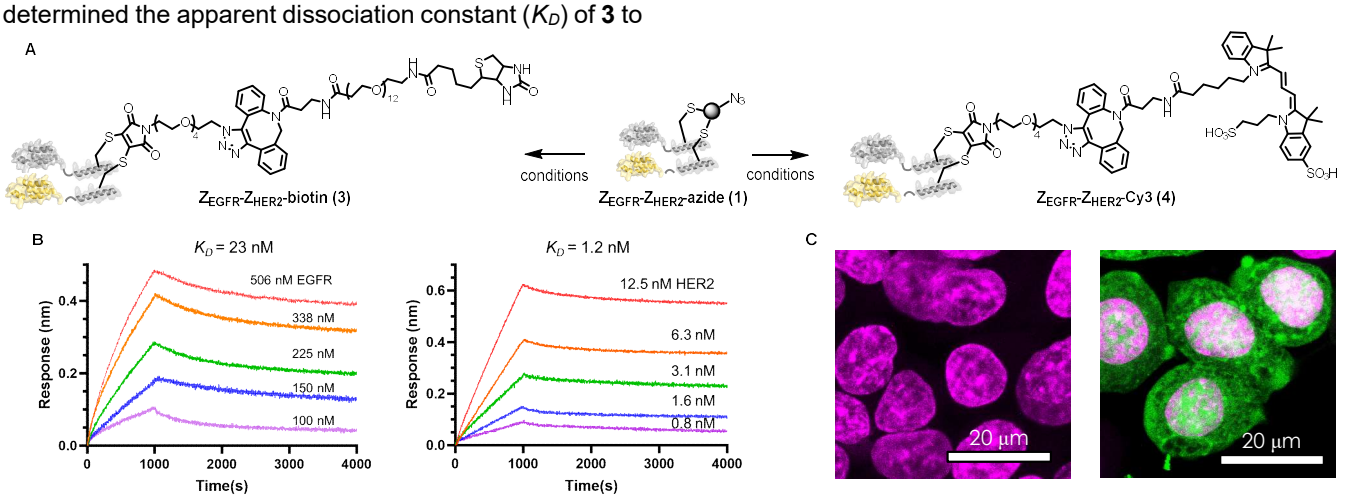

Figure 3. Bispecific affibodies demonstrate dual binding affinity towards two distinct antigens. (A) Derivatization of **1** with biotin and Cy3 fluorophore, respectively. Conditions: 10 equivalents of DBCO-PEG4-Biotin or Sulfo-Cy3-DBCO in 100 mM HEPES buffer (pH 7.4) at room temperature for 1 h. (B) BLI sensorgram of **3** immobilized on the sensor tip sampled against solutions of EGFR Fc chimera or HER2 Fc chimera proteins, respectively, at decreasing concentrations. (C) Left: negative control (PBS). Nuclei were stained with Hoechst 33342 (20 µM). Right: fluorescence imaging of SK-BR-3 cells incubated with **4** (5.1 µM) at 37 °C and 5% CO_2 for 24 h. Cells were imaged using an Olympus FV1200 Laser Scanning Confocal Microscope, using 405 nm and 559 nm lasers.

Recently, Baker et al. reported the thiol-amine coupling via **L1** using excess amount of aniline type nucleophile and found that the resulting conjugate exhibited greater stability compared to its cysteine analogues.[29] We hypothesized that proximity effect of helical tags enables thiol and primary amine coupling using **L1**. We synthesized **CC9**, a lysine-containing mutant of **CC1** (C14K) (Figure 4A). After screening thiol and amine-reactive crosslinkers, **L1** gave the moderate cross-coupling conversion of **CC9** and **CC2** (77% conversion, see Figure S19–S22). To extend thiol-amine coupling to protein-protein cross-coupling reactions, we introduced lysine-containing **CC11** onto an albumin-binding domain (ABD094)[30] through AFPS to give ABD094-CC11 (**5**). Subsequently, we investigated the cross-coupling reaction between **5** and **CC12**. **CC11** and **CC12** are single point mutants of **CC9** (I2N) and **CC2** (I2N), respectively.[31] The reaction of **5** with 2 equivalents of **CC12** at pH 8.7 resulted in substantial heteroselectivity (98%) (Figure 4B). It is notable that even when an excess amount of **CC12** (10 equivalents) was employed, almost no reaction at other lysine moieties on ABD094 was observed. Finally, we synthesized the conjugate between GLP-1-CC12 (**7**) and **5** through affinity-based amine and thiol coupling. 3.0 equivalents of **5** were required to react with the GLP-1 analogue probably due to steric hindrance caused by the protein cargo (90%) (Figure 4C and S36).

We developed a coiled coil-based cross-coupling method to access fusion proteins using double thiol conjugation of a preorganized complex. This nocel approach allows for the preparation of heterodimeric proteins under near-equimolar conditions. The requisite complementary reactive coils are conveniently installed onto the target proteins by biological expression. We also demonstrated the feasibility of mixed thiol-amine cross-coupling reactions with the same approach. Taking advantage of the plug-and-play nature of the coiled coil system and its sequence selectivity,[32] we are currently investigating the expansion of our strategy to a programmable affinity-based conjugation platform. We anticipate the unique features of reactive coiled coils will drive development of a versatile, modular, and

customizable platform for the synthesis of complex biomolecular structures.

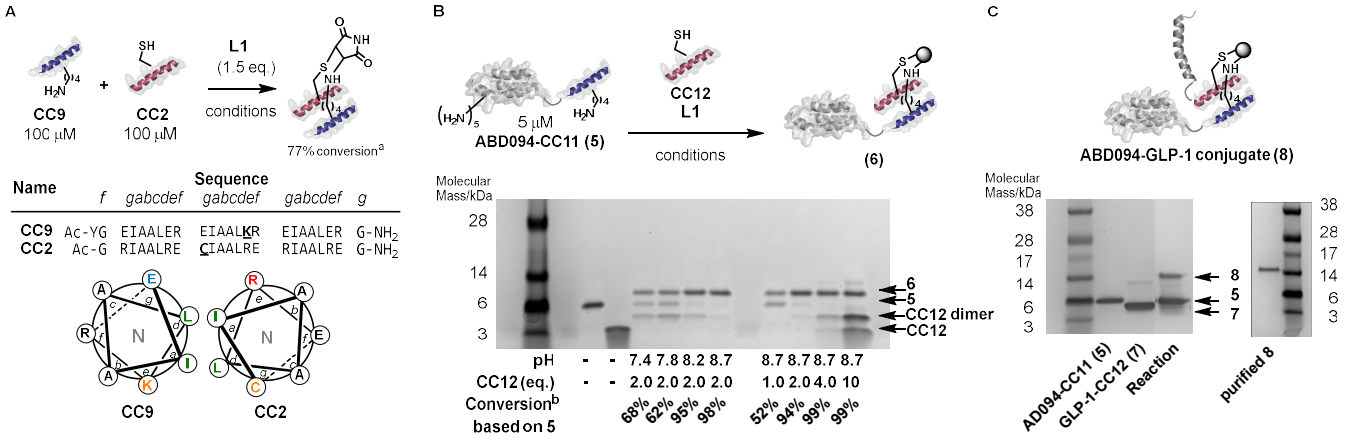

Figure 4. The appropriate combination of reactive coils and cross-linker enables lysine- and cysteine-based cross-couplings. (A) Conditions: reactions were conducted at 100 μM of **CC9** and **CC2** with **L1** (1.5 equivalents) in 2% v/v DMF 100 mM HEPES buffer at pH 8.7 at room temperature for 12 h. All peptides were N-terminally acetylated and C-terminally amidated. The sequence modifications with cysteine or lysine residues, respectively, are highlighted in bold and underlined. (B) Conditions: reactions were conducted at 5 μM of ABD094-CC11 (**5**) in 100 mM HEPES buffer at room temperature for 20 h. (C) Synthesis of ABD094-GLP-1 conjugate. Conditions: rection was conducted at 5 μM of GLP-1-CC12 (**7**) and 3 equivalents of ABD094-CC11 (**5**) with **L1** in 100 mM HEPES buffer at room temperature for 20 h. [a]The conversion to the corresponding heterodimers was estimated by the peak area of chromatogram acquired by HPLC. [b]Conversion was determined by SDS-PAGE followed by densitometry analysis.

ASSOCIATED CONTENT

Supporting Information. Materials and reagents; experimental details on peptide synthesis protocols, cell culture, protein production, coiled coil-based reactions, and additional BLI, CD, LC-MS, microscopy and NMR characterization data (PDF).

Author Contributions

H.T., A.L., and B.L.P. designed research. H.T., E.S., and S.G. performed the experiments. H.T. generated figures. All authors analyzed data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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