

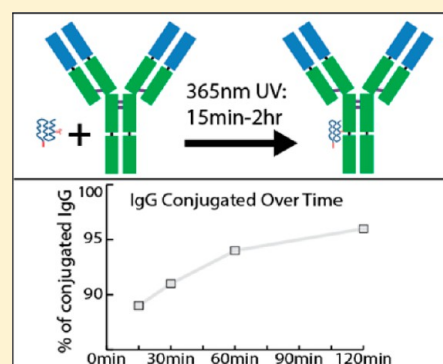
Optimization of Photoactive Protein Z for Fast and Efficient Site-Specific Conjugation of Native IgG

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Supporting Information

ABSTRACT: Antibody conjugates have been used in a variety of applications from immunoassays to drug conjugates. However, it is becoming increasingly clear that in order to maximize an antibody's antigen binding ability and to produce homogeneous antibody-conjugates, the conjugated molecule should be attached onto IgG site-specifically. We previously developed a facile method for the site-specific modification of full length, native IgGs by engineering a recombinant Protein Z that forms a covalent link to the Fc domain of IgG upon exposure to long wavelength UV light. To further improve the efficiency of Protein Z production and IgG conjugation, we constructed a panel of 13 different Protein Z variants with the UV-active amino acid benzoylphenylalanine (BPA) in different locations. By using this panel of Protein Z to cross-link a range of IgGs from different hosts, including human, mouse, and rat, we discovered two previously unknown Protein Z variants, L17BPA and K35BPA, that are capable of cross-linking many commonly used IgG isotypes with efficiencies ranging from 60% to 95% after only 1 h of UV exposure. When compared to existing site-specific methods, which often require cloning or enzymatic reactions, the Protein Z-based method described here, utilizing the L17BPA, K35BPA, and the previously described Q32BPA variants, represents a vastly more accessible and efficient approach that is compatible with nearly all native IgGs, thus making site-specific conjugation more accessible to the general research community.



INTRODUCTION

Antibody conjugates, which include antibody–drug, –enzyme, –hapten, and so forth, have been used for a wide variety of applications in the biomedical sciences, from detecting antigens in immunoassays to acting as vehicles for targeted drug delivery. Antibodies remain the targeting agent of choice for these diverse biological studies due to their wide availability, broad range of validated targets, and proven clinical efficacy.^{1–4} Traditionally, antibody conjugates have been prepared using inefficient conjugation methods, such as those based on carbodiimide (e.g., EDC) and/or *N*-hydroxysuccinimide (NHS) chemistries.⁵ While these methodologies have proven sufficient in a variety of settings, e.g., the preparation of the antibody–drug conjugate herceptin-emtansine, they nonetheless suffer from various shortcomings such as heterogeneities with respect to the number and location of antibody modifications.^{6–9} This lack of control limits the efficacy of the resulting antibody conjugates and can hamper their wider adoption. What's more, many novel applications of antibodies, such as fluorescent resonance energy transfer (FRET) probes or some antibody–drug conjugates (ADC), necessitate, not merely benefit from, precise control over antibody labeling.¹⁰ For example, it is increasingly apparent that substantial subpopulations of randomly conjugated ADCs show little, if any, therapeutic activity, yet account for most of the toxicity.^{11–13} As such, obtaining the next generation of ADCs with maximal therapeutic index, not to mention new immuno-

assays with higher sensitivity, hinges on developing more precise conjugation chemistries that enable site-specific attachment onto antibodies.^{14–17}

While numerous site-specific conjugation methods have been developed, most are technically complex, requiring genetic engineering of the parental antibody and subsequent hybridoma preparation.^{14,15} Genetic engineering approaches have included the incorporation of unnatural amino acids, peptidyl substrates for modifying enzymes, and cysteine handles to direct downstream site-specific modifications.^{18–20} Similarly, antibody fragments such as scFV and antibody alternatives such as affibodies have also been cloned and expressed in part to simplify site-specific attachment.^{21,22} However, these agents often suffer from decreased stability, short serum half-life, and weak antigen binding. Alternative conjugation strategies for full-length antibodies, such as those that attach conjugates onto glycosylated residues, avoid the need for cloning, and can accelerate the time needed to produce a functional conjugate. However, consecutive chemical or enzymatic reactions are still required, which can take days.^{23,24} To ensure speedy production and wider adoption, it would be ideal to develop site-specific conjugation methods that can be used with native, full-length IgGs. Given the wide

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availability of IgGs against a variety of targets, such a method would lower the technical and economical barriers to conjugation and potentially allow the identification and development of novel antibody conjugates to be “crowd-sourced” to the research community at large.

One versatile and promising approach for conjugating native antibodies that has emerged involves utilizing a small engineered antibody-binding protein, Protein Z, containing both a UV-active benzophenone cross-linker within the Fc-binding domain and one or more functional moieties such as a biotin, fluorophore, or azide at its C-terminus.^{25–28} Derived from the IgG-binding B domain of Protein A, Protein Z exhibits nanomolar affinity for a binding site in the CH2-CH3 region of most IgG isotypes. Upon binding to IgG and exposure to long wavelength UV (365 nm), the engineered Protein Z constructs can be site-specifically and covalently conjugated to the Fc region of the IgGs. In addition to being compatible with native IgGs, this approach is also attractive because of the fast kinetics of conjugation, since photo-cross-linking only takes hours, and the employment of long wavelength UV light, which does not damage IgG.

Despite the advantages described above, broader adoption of this approach can be hampered by the difficulty of producing Protein Z with multiple modifications. Neither peptide synthesis nor post-translational modification of recombinant proteins, both of which have been used before, can efficiently produce photoactive Protein Z with high yields.²⁸ Adding to the woes, even after these Protein Z constructs have been produced, they may exhibit low cross-linking efficiencies. The location of the photo-cross-linker greatly influences both the cross-linking extent and the isotypes that can be cross-linked, in often unpredictable ways (Table 1).^{25–28} Previous studies have found that only those variants with benzophenone at the F5 (Phe5), F13 (Phe13), and Q32 (Gln32) positions can appreciably cross-link to human IgG1, IgG2 (Q32), mouse IgG1 (Q32), IgG2a (F5, F13), and rabbit polyclonal IgG (F5), all with only around 40–60% of all heavy chains cross-linked after 2 h of UV exposure.²⁷ As such, it is important to further characterize additional Protein Z constructs with the photo-cross-linker at different locations, so we can both better understand the mechanism of cross-linking and engineer improved Protein Z variants that can more efficiently cross-link a greater range of antibodies.

Recently, we developed an efficient bacterial expression system that allows for the recombinant production of photoactive Protein Z with a diverse range of C-terminal modifications (e.g., azides, haptens, fluorophores, etc.).²⁸ This system combined a well-validated unnatural amino acid incorporation technique using an engineered Amber codon suppressor pair with intein mediated expressed protein ligation (EPL), which allows functional moieties to be added to the C-terminus of Protein Z.^{22,29} To further improve the efficiency of EPL, we have now replaced intein-mediated EPL with sortase-tag expressed protein ligation (STEPL) (Figure 1).³⁰ Using this robust and high-yield expression system, we incorporated the unnatural amino acid benzoylphenylalanine (BPA) into various sites in Protein Z and screened for optimal cross-linking to a range of IgG isotypes. We were able to identify three Protein Z variants that, taken together, can effectively, or in some cases completely, cross-link to almost all commonly used IgG isotypes with less than 1 h of UV exposure, thereby representing a vastly faster and more effective labeling method.

Table 1. Variants of Protein Z-Based IgG Conjugation Reagents

sites/mutations	cross-linker ^a	reference
D2	Maleimide-BP	Yu ²⁶
N3	Maleimide-BP	Yu ²⁶
K4	Maleimide-BP	Yu ²⁶
F5 ^b	BPA/BBA	Konrad, ²⁵ Perol, ²⁷ Hui ²⁸
Q9	Maleimide-BP	Yu ²⁶
N11	Maleimide-BP	Yu ²⁶
F13 ^b	BPA	Hui ²⁸
E15	Maleimide-BP	Yu ²⁶
L17 ^b	BPA	
H18	BPA	Konrad ²⁵
N23 ^b	BPA	
E24 ^{b,c}	Maleimide-BP/BPA	Yu ²⁶
E25 ^{b,c}	BPA	
N28 ^{b,c}	BPA	
A29 ^b	BPA	
I31 ^b	BPA	
Q32 ^b	Maleimide-BP/BPA	Yu, ²⁶ Perol ²⁷
Q32 F5I ^b	BPA	Yu, ²⁶ Perol ²⁷
K35 ^b	BPA	
K35 F5I ^b	BPA	
D36 ^b	BPA	
L17 Q32 ^b	Two BPAs	
L17 K35 ^b	Two BPAs	

^aCross-linker abbreviations: BP, Benzophenone; Maleimide-BP, Maleimide-benzophenone; BBA, benzoylbenzoic acid; BPA, benzoylphenylalanine. ^bMutations explored in this work. ^cLittle to no expression—excluded from study.

RESULTS

Selecting Sites of Cross-Linker Placement. Previous works have studied Protein Z variants with benzophenone-based cross-linkers placed at several different locations (Table 1).^{25–28} In addition it has been reported that a F5I (Phe5Ile) background mutation could confer increased binding and cross-linking between the Q32 Protein Z variant and human and mouse IgG1 subtypes.²⁶ Based on these studies and the crystallographic model of Protein A binding to human IgG, we tested the three previously reported sites that demonstrated the best cross-linking (F5, F13, Q32) and nine additional sites in Protein Z for BPA incorporation (Figure 2, Table 1). These sites cover all remaining Fc-facing residues on helix 1 and 2 of Protein Z that have yet to be explored in previous studies. Additionally, residues in the loop connecting the first two helices (E24, E25 in PBD model 1FC2) were also investigated. The Q32BPA and K35BPA variants were also combined with the background F5I mutation to investigate whether this substitution consistently provides improved cross-linking performance. Additionally, based on the results of the three best performing single-BPA-containing Protein Z variants, constructs with two BPA moieties were also investigated for potentially synergistic effects in cross-linking.

Construction of Mutant. The investigation of a broad array of mutations is made tractable due to the facile recombinant protein production technique used here (Figure 1), which does not require any post-translational modifications. As was done previously, selected codons in the Protein Z sequence were mutated to Amber codons (TAG) for BPA incorporation during expression.²⁸ The resulting fusion proteins, which contain a histidine tag, can be efficiently purified using metal affinity columns. To elute the bound protein from the column, calcium

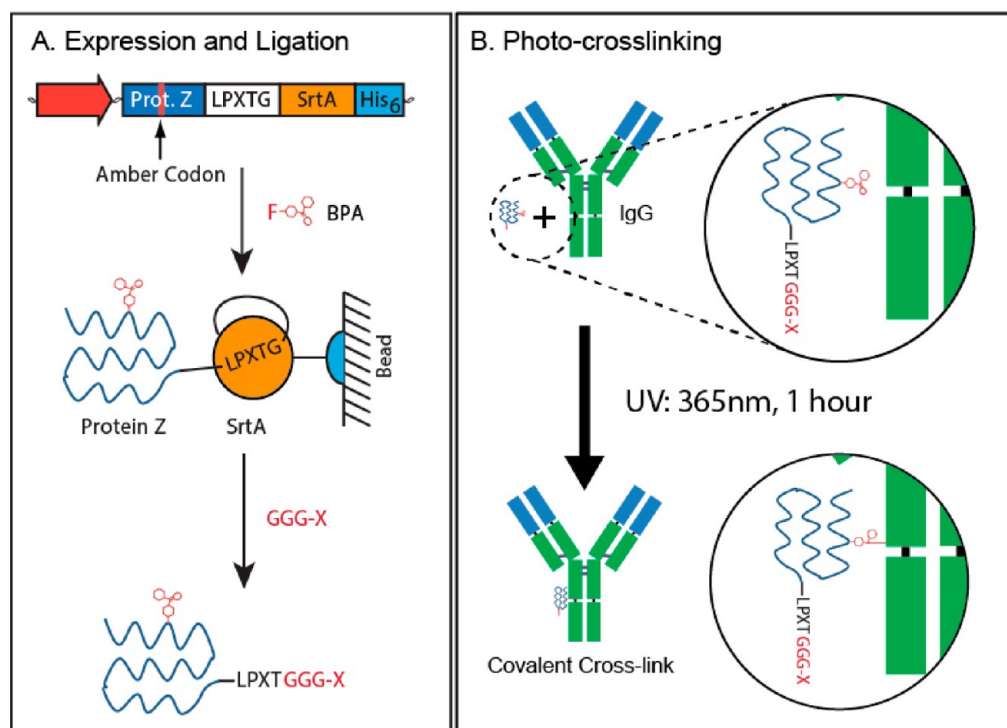


Figure 1. Schematics of Protein Z production and IgG cross-linking. (A) The Protein Z sequence is engineered to contain an Amber codon that encodes BPA and is cloned into the STEPL vector pSrtA. Protein Z is expressed as a fusion protein containing a self-cleaving sortase domain and polyhistidine tag, to be subsequently purified using metal affinity column. Simultaneous sortase cleavage and ligation are triggered by the addition of calcium and triglycine (GGG) or any peptide with a triglycine at the N-terminus (GGG-X, where X represents peptides carrying functional moieties). Only ligated Protein Z is released from the column. (B) After Protein Z is mixed with IgG, it first binds to the Fc region noncovalently. Upon irradiation with long wavelength UV (365 nm), BPA is activated and a covalent bond is formed between the BPA on the Protein Z and the IgG.

along with either triglycine or any synthetic peptides with N-terminal triglycine are added into the column. This triggers sortase catalyzed fusion protein self-cleavage and the simultaneous ligation of either triglycine or a triglycine-containing peptide to the C-terminus of the Protein Z (Figure 1A). By combining the ligation and purification steps, the STEPL system can efficiently generate native Protein Z, as was done in this work, or Protein Z-conjugates with synthetic peptides containing functional moieties such as FITC or azide ligated to the C-terminus.^{22,28,30} Unlike intein based EPL systems, STEPL does not release unligated Protein Z from the column. Hence there is no need for an additional purification step between Protein Z elution from the column and its use in antibody labeling. Instead, after UV cross-linking of IgG, labeled antibodies can be separated from both un-cross-linked Protein Z and the excess peptides used during the ligation step. For characterization, however, pure Protein Z can be obtained by high performance liquid chromatography (RP-HPLC), as was done here.

All variants, except E24BPA, E25BPA, and N28BPA, were successfully expressed and purified. Very low protein expression level was seen for N28BPA and no expression was seen for E24BPA and E25BPA. These variants were therefore excluded from further analysis.

Cross-Linking of Human IgGs. The panel of Protein Z variants was screened for the ability to cross-link all four subtypes of human IgG (hIgG). Five molar excess of Protein Z variant was mixed with human IgG samples and exposed to long wavelength UV on ice for 1 h. The reaction products were then analyzed on a reducing SDS-PAGE gel (Figure 3).

Most mutations, including the previously reported F5BPA and F13BPA variants, demonstrated poor cross-linking with 1 h of

UV exposure. The Q32BPA variant, however, was able to significantly cross-link human IgG1 at a level (37% of heavy chains cross-linked) consistent with the previously reported estimate of near 40%.²⁷ The best cross-linking performance for hIgG1, however, was demonstrated by the K35BPA variant (47%). The presence of the F5I mutation in the background did not significantly improve cross-linking for either the Q32BPA or K35BPA variants.

Similarly for hIgG2, the Q32BPA and K35BPA variants were found to exhibit the greatest extent of cross-linking, albeit with a much lower efficiency, at 12% for Q32BPA and 17% for K35BPA. No cross-linking of hIgG3 was seen with any Protein Z, likely because Protein A, the parental molecule of Protein Z, is known to have little to no affinity for hIgG3.³¹ Finally, hIgG4 was cross-linked by several variants including F5BPA, as reported by Perol et al., as well as F13BPA, Q32BPA, and K35BPA.²⁷ As was the case for hIgG1, K35BPA was found to perform best, cross-linking approximately 50% of the heavy chains. The F5I mutation in the background did not significantly improve cross-linking for either of the variants tested.

In all, three out of four human IgG isotypes can be cross-linked. The K35BPA variant displayed the best performance, with nearly 50% of all hIgG1 and hIgG4 heavy chains cross-linked, exceeding that of the previously reported Q32BPA variants.²⁷

Notably, different antibodies of the same isotype were cross-linked with similar efficiencies (Supporting Information Figure S1), supporting the idea that the difference among isotypes, rather than individual antibody variations, accounts for the observed differences in cross-linking efficiency. This is consistent with previous reports.^{25–27}

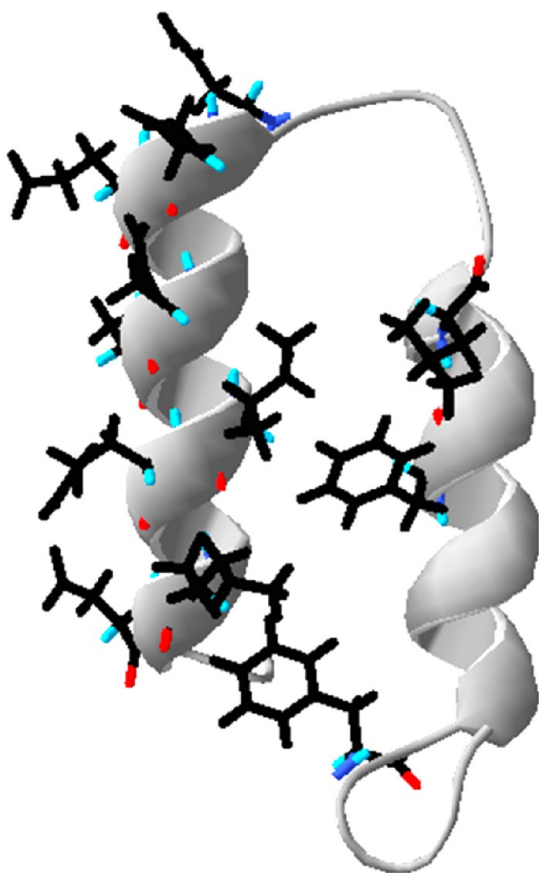


Figure 2. Model of Protein Z showing sites of BPA cross-linker placement. Twelve residues within Protein Z were selected for BPA incorporation. These sites include nearly all Fc facing residues that were not previously investigated in previous works. Helix one (right) and two (left) of the Protein Z are shown along with the modeled side chains of the original residues. Helix three is not shown for clarity. All mutations were successfully expressed except for E24BPA, E25BPA, and N28BPA variants. Models used include: 1Q2N (shown), 1FC2 (not shown).

Cross-Linking of Mouse IgGs. The cross-linking of various isotypes of mouse IgGs (mIgG) was also screened using the 11 Protein Z variants. As with human IgGs, mIgG samples were mixed with a five times molar excess of Protein Z and exposed to long wavelength UV for 1 h. Reducing SDS-PAGE gel of the cross-linking reactions showed dramatically different cross-linking profiles between mIgG1, mIgG2a, mIgG2b, and mIgG3 (Figure 4).

Consistent with previous reports, the Q32BPA variant gave the maximum cross-linking of mIgG1, with 50% of heavy chains cross-linked in 1 h.^{26,27} However, mIgG2a, mIgG2b, or mIgG3 isotypes were not appreciably cross-linked by Q32BPA. Instead they were all extensively cross-linked by L17BPA, with efficiencies of 60% (mIgG2a), 71% (mIgG2b), and 80% (mIgG3). The F5BPA, F13BPA, and K35BPA variants were able to modestly cross-link mIgG2a, mIgG2b (F5BPA and F13BPA only), and mIgG3 isotypes, but to a lesser extent than L17BPA.

Encouragingly, all mouse IgG isotypes were able to be significantly cross-linked with Protein Z. With 80% of mIgG3 heavy chains labeled after only 1 h of UV exposure, the L17BPA variant displayed the best cross-linking performance ever reported of any Protein Z or Protein G based conjugation technique.^{27,32}

Cross-Linking of Rat IgGs and Other IgGs. Protein Z variants were also screened for their ability to cross-link rat IgGs. As with human and mouse IgGs, representative isotypes were cross-linked with five molar excess Protein Z and exposed to UV light for 1 h. The results were analyzed by reducing SDS-PAGE gel (Figure 5). While it is generally recognized that most rat IgGs do not bind Protein A or Protein Z, all but rat 2a isotype were able to be cross-linked, albeit with lower efficiencies than seen with human and mouse IgG. The Q32BPA variant cross-linked the rat IgG1 subtype with 10% efficiency, while the rat 2b subtype was cross-linked by the L17BPA variant with a slightly higher 20% efficiency. In contrast to other rat isotypes, the rat 2c isotype was substantially cross-linked by the L17BPA variant, with an efficiency of ~60%. This enhanced cross-linking can perhaps be attributed to Protein A and Protein Z's natural affinity toward the rat IgG 2c isotype.³³

While the association constants between Protein Z and rat IgG1, 2a and 2b, are low, the formation of a covalent bond by BPA shifts the equilibrium such that even the low level of binding can result in detectable cross-linking.^{31,33} The specificity of cross-linking is still maintained in this case, as demonstrated by cross-linking that is specific to the heavy chain. While in this work only 1 h of UV exposure and five times molar excess of Protein Z were used, both of these parameters can be increased to achieve a higher cross-linking extent if so desired. This will be especially important when conjugating precious antibody samples.

In addition to the above-mentioned IgG isotypes, Protein Z variants were also used to cross-link hamster and rabbit IgGs (Figure 6). Cross-linking was seen with both hamster IgG1 and rabbit polyclonal antibody. Hamster IgG1 displayed a similar cross-linking pattern to those of human IgG1 and IgG2, where only Q32BPA and K35BPA showed appreciable cross-linking, with K35BPA (30%) outperforming Q32BPA (20%). Interestingly, the additional background mutation of F5I significantly improved the cross-linking of hamster IgG (37% vs 30%). Rabbit IgG, on the other hand, was equally well cross-linked by both the L17BPA (30%) and K35BPA (34%) variants, with little difference seen upon the addition of the F5I mutation.

Kinetics of Cross-Linking. To assess the kinetics of the cross-linking reaction, three pairs of Protein Z-IgG that showed the best cross-link formation: L17BPA with mIgG3, Q32BPA with mIgG1, and K35BPA with hIgG1 were mixed at a 5:1 Protein Z to IgG molar ratio and UV exposed for various durations of time from 15 min to 2 h.

As shown in Figure 7A, the cross-linking between L17BPA and mIgG3 demonstrated the fastest kinetics with more than 70% of all heavy chains already covalently cross-linked within 15 min of UV exposure. After 2 h, 90% of all mIgG3 heavy chains were cross-linked. To assess whether IgG needs to be incubated with Protein Z prior to UV exposure to achieve optimal cross-linking, each Protein Z and IgG pair was also preincubated for 1 h at 37 °C followed by UV exposure for 2 h (Figure 7A, Incub.). No increased cross-linking was observed, demonstrating that preincubation is not necessary to achieve sufficient Protein Z binding to IgG during the subsequent UV exposure.

To analyze the cross-linking extent of intact IgG, rather than reduced heavy chains, the samples were analyzed using nonreducing conditions (Figure 7B). Protein Z was able to cross-link onto one or both of the heavy chains in an intact mIgG3. Within 15 min, the gel revealed that 90% of mIgG3 had at least one Protein Z covalently attached, while more than 95% and 96% of IgG were cross-linked to Protein Z at 1 and 2 h, respectively.

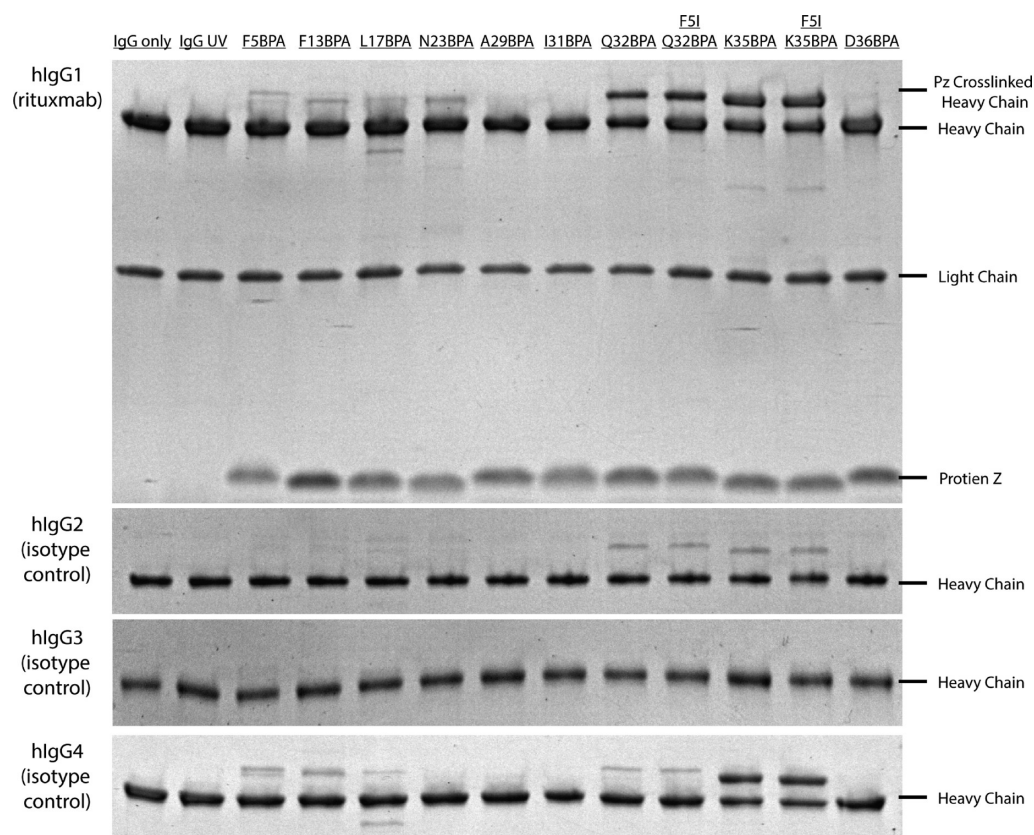


Figure 3. Cross-linking of human IgGs with photoactive Protein Z variants. Human IgG isotypes (hIgG1, hIgG2, hIgG3, hIgG4) were each cross-linked using 11 Protein Z variants, each containing BPA in different locations as indicated. Five molar excess of Protein Z was mixed with IgG and exposed to 365 nm UV light for 1 h. The product was analyzed on a reducing SDS-PAGE gel.

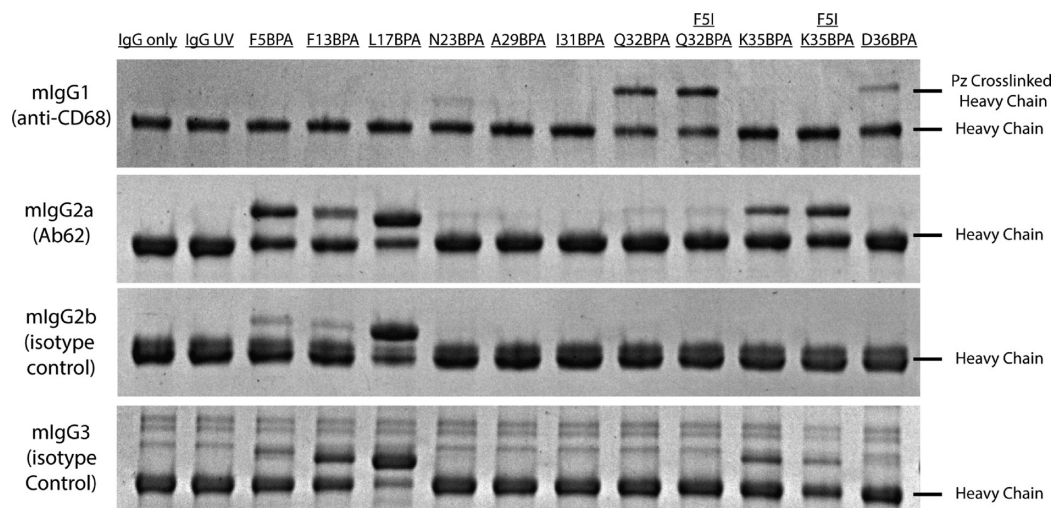


Figure 4. Cross-linking of mouse IgGs with photoactive Protein Z variants. Mouse IgG isotypes (mIgG1, mIgG2a, mIgG2b, mIgG3) were each cross-linked using 11 Protein Z variants with BPA placed in different locations. Five molar excess of Protein Z was mixed with IgG and exposed to 365 nm UV light for 1 h. The product was analyzed on a reducing SDS-PAGE gel.

The cross-linking of both Q32BPA and K35BPA with their respective IgG also displayed fast reaction kinetics, albeit slower than that demonstrated by L17BPA (Supporting Information Figure S2). In both cases, approximately 50% of heavy chains were cross-linked within 1 h of UV exposure and more than 55% were cross-linked by 2 h. When assessed on nonreducing gel, it was found that at 1 h approximately 60% of intact IgG were cross-

linked by at least one Q32BPA (mIgG1) or K35BPA (hIgG1), while the percentages reach more than 70% by 2 h.

The fast cross-linking kinetics of the examined Protein Z variants make them ideal tools for convenient and efficient antibody labeling. The ability to bypass the preincubation step also speaks to the fast binding kinetics between Protein Z and IgG, such that binding equilibrium can occur either near-instantaneously or over the duration of UV cross-linking. For the

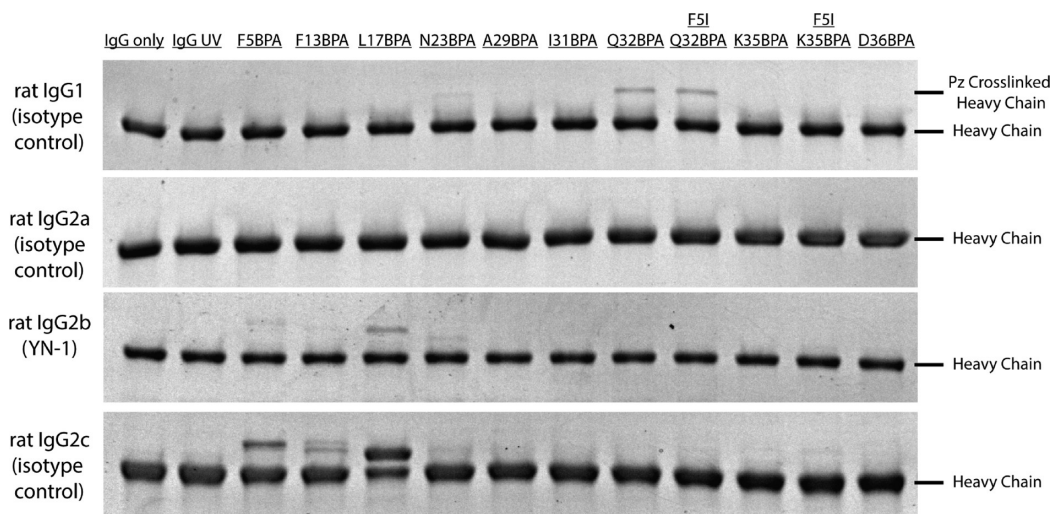


Figure 5. Cross-linking of rat IgGs with photoactive Protein Z variants. Rat IgG isotypes (rIgG1, rIgG2a, rIgG2b, rIgG2c) were each cross-linked using 11 Protein Z variants with BPA placed in different locations. Five molar excess of Protein Z was mixed with IgG and exposed to 365 nm UV light for 1 h. The product was analyzed on a reducing SDS-PAGE gel.

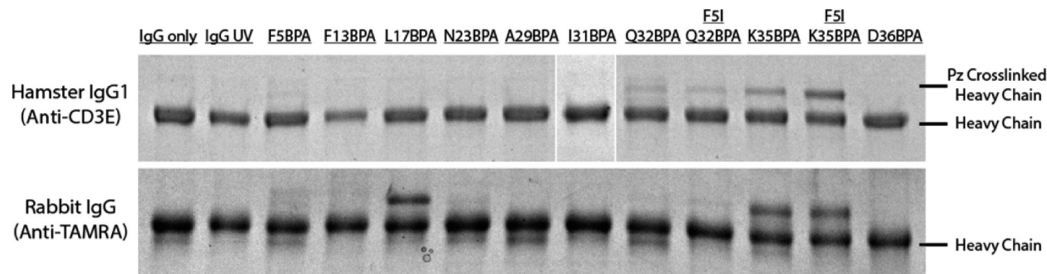


Figure 6. Cross-linking of hamster and rabbit IgGs with photoactive Protein Z variants. Hamster IgG1 and Rabbit polyclonal IgG were cross-linked using 11 Protein Z variants with BPA placed in different locations. Five molar excess of Protein Z was mixed with IgG and exposed to 365 nm UV light for 1 h. The product was analyzed on a reducing SDS-PAGE gel.

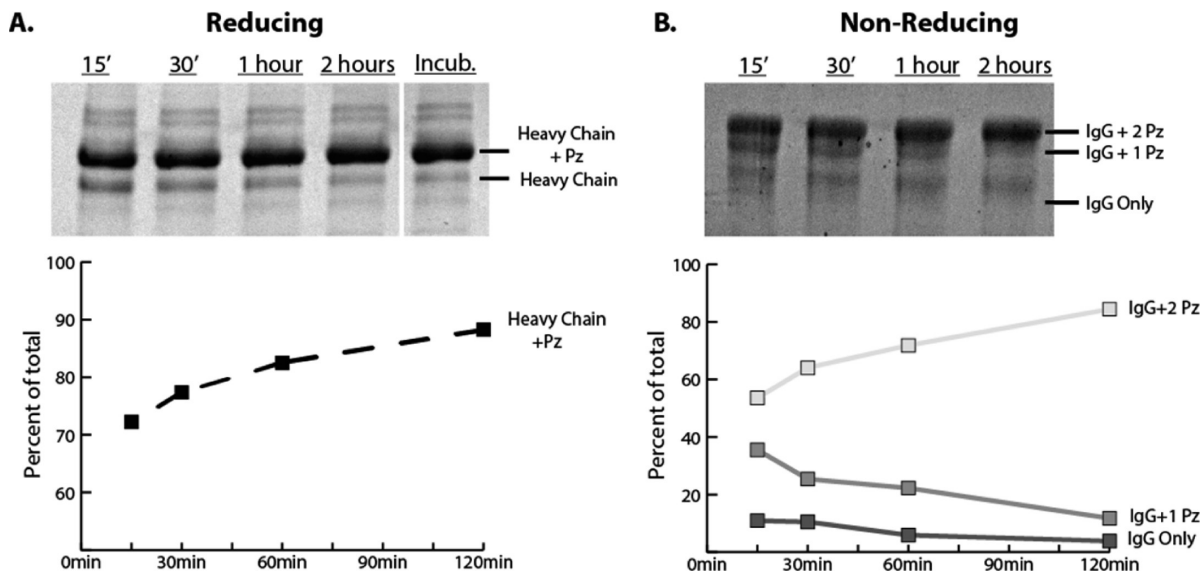


Figure 7. Reducing and nonreducing gel showing cross-linking kinetics between mIgG3 and photoactive Protein Z. To examine the kinetics of cross-linking, L17BPA Protein Z and mIgG3 were subjected to UV exposure for 15 min to 2 h. The results were analyzed using (A) reducing (showing percent of heavy chain cross-linked) and (B) nonreducing (showing percent of intact IgG cross-linked) SDS-PAGE gels stained with Coomassie blue. The gel images were analyzed using ImageJ software.

majority of the isotypes tested (mIgG2a, mIgG2b, mIgG3, rat 2c), more than 70% of intact IgGs are conjugated in 15 min and nearly all are conjugated by 1 h. For human IgG1 and mouse

IgG1 isotypes, more than 60% conjugation is obtained in 1 h while a higher level can be achieved with longer exposure. This makes the Protein Z based approach not only much faster than

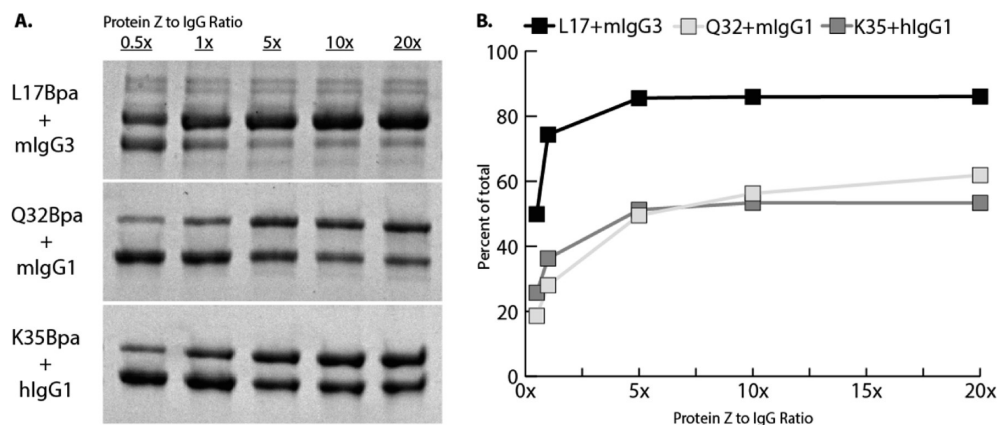


Figure 8. Influence of Protein Z to IgG ratio on cross-linking kinetics. Three photoactive Protein Z and IgG pairs (L17BPA + mIgG3, Q32BPA + mIgG1, K35BPA + hIgG1) were cross-linked at varying ratios of Protein Z to IgG (0.5, 1, 5, 10, and 20 times). (A) The products were analyzed using Coomassie stained reducing SDS-PAGE gel. (B) The gel images were analyzed using ImageJ software.

existing site-specific conjugation methods that may take 1–2 days, but comparable to many general conjugation techniques such as those using EDC/NHS, which often take 30 min or more.^{5,23,24}

Effects of Protein Z to IgG Ratio on Cross-Linking. To better optimize the parameters of conjugation, the effect of altering the ratio of Protein Z to IgG on cross-linking was also examined. The L17BPA, Q32BPA, and K35BPA variants of Protein Z were each cross-linked for 1 h with their compatible isotypes at Protein Z to IgG molar ratios of 0.5×, 1×, 5×, 10×, and 20× (Figure 8). It was found that the cross-linking extent improved with increasing Protein Z amount when less than 5 molar excess was used. Exceeding this ratio provided little, as was the case of Q32BPA variant, to no improvement in cross-linking.

The inability to further improve cross-linking when more than five molar excess of Protein Z was used suggested that conjugation in this case is limited by the formation of the covalent bond rather than Protein Z binding onto IgG. In practice, these findings suggest that only five molar excess of these Protein Z variants, as compared to 10–20× reported for other Protein Z based reagents, is sufficient to saturate the IgG and achieve maximum cross-linking.^{25–27} This reduced reagent requirement, when combined with the short UV duration and the ease of recombinant production, makes site-conjugation of IgG using Protein Z more accessible for researchers at large.

Construction of Additional Protein Z Format. To further enhance cross-linking, additional Protein Z variants containing two BPAs, L17BPA-Q32BPA and L17BPA-K35BPA, were constructed. The recombinant nature of production allowed double mutants to be easily produced using two site-directed mutagenesis reactions followed by the normal expression protocol. All mutants were successfully expressed and purified without any noticeable decrease in yields. However, no improvement in cross-linking efficiency was seen with any of the double mutants (Supporting Information Figure S3). In fact, in most cases there was a loss in cross-linking efficiency and some Protein Z variants lost the ability to cross-link IgG altogether. It is hypothesized that the addition of a second BPA at these locations may significantly interfere with Protein Z binding to IgGs, thereby resulting in poor cross-linking efficiency.

While no promising double mutation variants were identified in our preliminary screen, further work is needed to better identify optimal placements of BPAs in multimutation variants.

The recombinant approach as used here nonetheless demonstrated the ease of constructing and producing such species.

DISCUSSION

While the utility of photoactive Protein Z has been previously demonstrated, it is very challenging to produce such a recombinant protein with multiple modifications. For example, Protein Z has previously been produced by peptide synthesis, but standard synthetic approaches give less than 35% theoretical yield for peptides longer than 50 amino acid (Protein Z has 56 amino acids), and much worse actual yield after purification.³⁴ Alternatively, photoactive protein Z constructs have been made by post-translationally modifying a recombinant protein, but the need for additional reaction and purification steps complicates production and limits the final yield.²⁶ Moreover, it is very challenging to introduce multiple post-translational modifications onto a single recombinant protein. We previously utilized an intein-based EPL technique to make BPA-containing Protein Z that is site-specifically modified at the C-terminus with functional moieties (e.g., fluorescent dyes, click chemistry compounds, etc.).²⁸ While this approach was promising, it had a number of downsides, including the requirement for an additional purification step to get rid of those Protein Z that are not ligated with moieties of interest. In contrast, by incorporating the recently developed sortase-tag expressed protein ligation (STEPL) into our system,³⁰ this additional purification step is avoided in the method described in this current work. Further, STEPL also has the advantage of been able to utilize nearly all of the peptides carrying the functional moieties during ligation. This is economically advantageous since these synthetic peptides can be cost-limiting for Protein Z production. Notably, in this study unlabeled peptides (i.e., triglycine) were used since the objective was to optimize the placement of BPA.

Using this facile production approach, we were able to quickly construct a panel of Protein Z with BPA placed into different locations throughout the first and second IgG binding helices. By screening for the ability of these variants to cross-link a range of IgG isotypes, several variants were discovered which taken together can cross-link nearly all isotypes to a significant extent. This greatly expands the repertoire of native antibodies that can be conjugated. What's more, Protein Z, in particular, the L17BPA variant, displays extremely fast cross-linking kinetics that enables conjugation to be completed in less than 1 h (<15 min in the case of L17BPA). This is significantly faster and easier than alternative

site-specific conjugation methods such as those requiring hybridoma construction or those using engineered β -galactosidases to modify glycosylated residues.^{11,19,20,23} The Protein Z based method is also more advantageous than another recently developed site-specific conjugation approach where a UV-active nucleotide analogue was used to covalently cross-link a conserved nucleotide binding site (NBS) within the Fab region. The proximity of the NBS to the Fab region poses the risk of sterically hindering antigen binding.³⁵ Additionally, the NBS method requires cross-linking with short wavelength UV light (250 nm), which has the potential to damage proteins. Taken together, the recombinant nature of Protein Z production combined with the ease of cross-linking will greatly expand the accessibility and utility of this conjugation method.

Conceptually, the cross-linking reaction can be viewed as a two-step reaction composed first of Protein Z binding non-covalently to IgG followed by the UV-activated covalent bond formation between the BPA and IgG. Definitely understanding the contribution of these two steps toward cross-linking will require additional experiments such as using surface plasmon resonance (SPR) to measure binding affinity, which is the subject of ongoing work. Nonetheless, the different isotype cross-linking profiles and kinetics seen with different Protein Z variants can give mechanistic insights into the cross-linking reaction. For example, the inability to further improve cross-linking when more than five molar excess of Protein Z was used suggests that the rate limiting step in these cases is the formation of a covalent bond between the Protein Z and IgG, rather than slow or poor binding between the two. The observed plateauing of cross-linking extent, despite prolonged UV exposure, at around 55% for the Q32BPA and K35BPA variants also supports this conclusion. These two findings raise the possibility of competing reactions that prevent some proportion of the IgG-bound Protein Z from forming the proper covalent link with IgG. Preliminary results in our lab suggest against intramolecular cross-linking within Protein Z, since pre-UV exposure does not reduce the efficiency of antibody cross-linking and non-cross-linked Protein Z from one experiment can still cross-link fresh batches of antibody (data not shown). The L17BPA variant's ability to cross-link a variety of antibodies in a shorter time frame than other variants suggests that any competing reactions that do exist are sensitive to the local environment and can be avoided if more ideal BPA location can be chosen. It is well-known that benzophenones preferentially cross-link some amino acids, particularly methionine.^{36,37} While this effect may explain the outstanding performance of the L17BPA variant toward mIgG2, mIgG3, and rat IgG2c, since these isotypes all share a Met252 that lies closely to L17BPA, it cannot account for all of the observed variations in cross-linking extent. Further, this theory does not explain the plateauing of cross-linking extent for Q32BPA and K35BPA variants, since inefficient cross-linking should be able to be overcome by prolonged UV exposure.

While adequate cross-linking extent can already be obtained for most of the isotypes using the above-reported Protein Z variants, better insight into the cross-linking mechanism will be important for understanding the properties of the resulting conjugates and for further improving Protein Z variants. Recent work by Perol et al. showed that the linkage length of the benzophenone group greatly influences cross-linking when placed in some, but not other, sites in Protein Z.²⁷ It would be valuable to investigate whether this is also true for the K35 and L17 sites discovered in this work. Additionally, in this work it was found that while the background FSI mutation did not

significantly improve cross-linking for most mutations and isotypes, it did increase K35BPA cross-linking to hamster IgG1. This opens up the possibility that other background mutations can interact with BPA placement to improve cross-linking. What's more, while those variants containing two BPAs tested in this work did not outperform single BPA variants, the possibility that the right combinations of BPA placements can improve cross-linking cannot be ruled out, as previously suggested.²⁷

An ideal site-specific conjugation method should demonstrate several properties. First, the antigen-binding domain should be unaltered, thereby maximizing the antibody function. Second, the conjugation method should be sufficiently versatile to allow a variety of moieties to be conjugated to a broad range of antibodies. Lastly, the method should be broadly accessible to a large number of researchers. We approached our work with these goals in mind and created a versatile Protein Z based method that can be used to efficiently and effectively conjugate a range of native IgGs site-specifically.

■ MATERIALS AND METHODS

Materials. Clinical grade anti-CD20 antibody, rituximab, was kindly provided by Dr. Eline Luning Prak at the University of Pennsylvania. Clinical grade anti-EGFR antibody, cetuximab, was kindly provided by Dr. Daqing Li at the University of Pennsylvania. Human IgG2 isotype control antibody (HCA108A), human IgG3 isotype control antibody (HCA178), and human IgG4 isotype control antibody (HCA050A) were all purchased from AbD Serotec (Kidlington, UK). Mouse IgG1 anti-Dextran (10730) antibody was purchased from StemCell Technologies (Vancouver, Canada). Mouse IgG1 anti-Rat CD68 antibody (MCA341R) was purchased from AbD Serotec Inc. (Kidlington, UK). Mouse IgG1 anti-Fas (DX2) antibody was purchased from R&D Systems (Minneapolis, MN). Mouse IgG2a anti-PECAM (AB62) antibody was kindly provided by Dr. Vladmir Muzykantov at the University of Pennsylvania. Mouse IgG2a anti-CD3 antibody (OKT3) was purchased from Bio-X-Cell (West Lebanon, NH). Mouse IgG2b isotype control antibody (14-4732) was purchased from eBioscience (San Diego, CA). Mouse IgG3 anti-prostate lipid antibody (F77) was kindly provided by Dr. Mark Greene at the University of Pennsylvania. Mouse IgG3 isotype control antibody (14-4742) was purchased from eBioscience (San Diego, CA). Rat IgG1 isotype control antibody (14-4301) was purchased from eBioscience (San Diego, CA). Rat IgG2a isotype control antibody (14-4321) was purchased from eBioscience (San Diego, CA). Rat IgG2b anti-ICAM1 antibody (YN-1) was kindly provided by Dr. Ann-Marie Chako at the University of Pennsylvania. Rat IgG2c isotype control antibody (400701) was purchased from Biolegend (San Diego, CA). Hamster IgG1 anti-CD3E antibody (145-2C11) was purchased from Thermo-Fisher (Waltham, MA). Polyclonal Rabbit anti-tetramethylrhodamine (A-6397) antibody was purchased from Invitrogen (Carlsbad, CA). Triglycine was purchased from Sigma-Aldrich. (St. Louis, MO) All other reagents were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise noted.

Cloning of Protein Z Fusion Protein into pSrtA Vector.

Two complementary oligonucleotides encoding the Protein Z amino acid sequence and flanked at both ends by 15 base sequences homologous to the desired NdeI and AgeI restriction sites of the destination vector pSrtA were ordered from Integrated DNA Technologies (Coralville, IA). The full amino acid sequence for the Protein Z can be found in the Supporting Information.³⁸ Oligonucleotides were incubated together at a

final concentration of 5 μM and hybridized at room temperature for 30 min. The resulting Protein Z sequence was then digested with NdeI and AgeI restriction enzymes (New England Biolabs, Ipswich, MA) and gel purified. The corresponding sortase pSrtA vector was also double digested with NdeI and AgeI and gel-purified. The pSrtA vector was previously described.³⁰ Ligation was done overnight using T4 ligase (New England Biolabs, Ipswich, MA) at 16 °C. Insertion of the Protein Z sequence was verified by Sanger DNA sequencing using the T7 promoter as the sequencing primer. Site-directed mutagenesis of selected codons into an amber codon (TAG) was done using Quikchange Mutagenesis Kit (Agilent, Santa Clara, CA). For double mutations, two consecutive Quikchange mutageneses were performed.

Expression and Purification of Protein Z Fusion Protein. The pSrtA plasmid containing the cloned Protein Z sequence and the pEVOL-pBpF plasmid (Addgene.org) were cotransformed into the T7 Expression Crystal Competent Cells (New England Biolabs, Ipswich, MA). Bacterial cell cultures were initially grown overnight in an air shaker (225 rpm) at 37 °C in 3 mL of lysogeny broth (LB) media. Cultures were scaled up to 50 mL of LB media and grown overnight under the same conditions, and then inoculated into 1 L LB media containing 50 mg/L of ampicillin and 25 mg/L of chloramphenicol.

For BPA incorporation, L-benzoylphenylalanine (Bachem, King of Prussia, PA) was added into the culture for a final concentration of 300 μM and the culture was left to grow for 30 min. Next, at optical density (OD) 600 nm = 0.6, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and arabinose to a final concentration of 0.1% to begin the inductions of the pSrtA and pEVOL plasmids, respectively.

Cultures were allowed to express for 2 h at 37 °C. Bacterial cultures were centrifugally pelleted at 10 000 g for 5 min, resuspended in 10 mL of B-PER lysis buffer (Pierce, Rockford, IL) containing 0.75 g/L lysozyme, 1 $\mu\text{g}/\text{mL}$ DNase, and 50 mM phenylmethylsulfonyl fluoride. Cells were lysed by incubation for 1 h in room temperature and then pulse sonicated on ice. Cell lysates were centrifuged at 15 000 g for 30 min at 4 °C. Supernatant was collected and stored at -20 °C. For the following purification steps, all procedures were run at 25 °C. The supernatant (9 mL) was incubated for 1 h in a 10 mL Poly-Prep chromatography column (Bio-Rad, Hercules, CA) packed with 1 mL of Talon metal affinity resin (Clontech, Mountain View, CA). Supernatant was then allowed to pass through the column and resin beads were washed with 50 mL of column buffer (0.1 M Tris-HCl, pH 8.5) at a flow rate of approximately 2 mL/min and then drained. The stopper was placed back onto the column.

Expressed Protein Ligation. Triglycine (30 μL of 150 mM solution in column buffer) and calcium chloride (2.4 μL of 50 mM solution in column buffer) was added into 1 mL of column buffer and then applied to the column. The resin was vortexed to ensure uniform distribution of the triglycine solution and then incubated at 37 °C for 4 h. Afterward, the column was eluted using 2 mL column buffer.

Purification and concentration of the final product can be performed using a 3 kDa molecular weight cutoff (MWCO) filter (Amicon Ultra, Milipore, Temecula, CA) or size-exclusion chromatography (Zeba 7kD columns, Pierce, Rockford, IL).

Alternatively, Protein Z can also be purified with RP-HPLC (Varian Prostar) as was done here. A C8 300 \AA 5 μm column (Agilent) was used. Protein Z was eluted at 1 mL/min using a

mixture of water and acetonitrile, both containing 0.1% TFA. The solvent gradient used was: 95–75% water over the first 10 min, then 75–69% over the next 60 min. Absorbance was monitored at 215 nm. The collected fractions were then dried using vacuum centrifuge concentrator (Labconco, Kansas City, MO) and reconstituted in column buffer. Protein concentration was determined using BCA assay (Pierce, Rockford, IL).

Cross-Linking. Unless otherwise stated, Protein Z were cross-linked with IgGs by first mixing the IgG (final concentration 0.4 μM) and Protein Z (final concentration 2 μM) in 0.1 M Tris-HCl buffer at molar ratio of 1 to 5 in a clear 1.5 mL centrifuge tube. Next, the mixture was immediately placed on an ice bath and irradiated for 1 h with 365 nm UV light using a UVP CL-1000L UV cross-linker (Upland, CA). Samples were then analyzed using SDS-PAGE gel as described below.

To assess the effect of irradiation length on cross-linking, the samples were prepared as above, but irradiated for 15 min, 30 min, 1 h, and 2 h. To test whether preincubation of IgG with Protein Z was necessary, samples were first incubated in 37 °C for 1 h after mixing and then UV irradiated for 2 h.

To assess the effect of IgG to Protein Z ratio on cross-linking, the IgG (final concentration 0.4 μM) were mixed with Protein Z at final concentrations of 0.2 μM (0.5 \times), 0.4 μM (1 \times), 2 μM (5 \times), and 4 μM (10 \times) and 8 μM (20 \times) and UV irradiated for 1 h as above.

Analysis of Cross-Linking. Cross-linked products were analyzed directly using SDS-PAGE electrophoresis. For reducing SDS-PAGE, cross-linked samples were boiled for 3 min with equal volume of SDS-PAGE loading buffer (Biorad, Hercules, CA) containing 1:20 dilution of β -mercaptoethanol (Biorad). The samples were then loaded onto AnyKd gradient PAGE gels (Biorad) and ran for 21 min at constant 250 V. For nonreducing gel, the samples were boiled as above, but using SDS-PAGE loading buffer without any β -mercaptoethanol, and then also loaded onto a AnyKD gradient gel and ran for 1.5 h at 250 V. The gels were stained for protein using SimplyBlue Coomassie stain (Invitrogen).

Images of the gel were taken using a Kodak Gel Logic 100 system (Rochester, NY) with background illumination correction. The images were then analyzed using ImageJ software (<http://imagej.nih.gov>). Specifically, images were background corrected using the background subtraction function with a rolling ball radius of 50 pixels. Next, the lanes were selected using the rectangle tool and band intensity profiles plotted using the gel analysis functions. For reducing gels, the intensity of the cross-linked heavy chain as a percentage of total heavy chain was taken to reflect cross-linking extent. For nonreducing gels, the percentages of double cross-linked IgG, single cross-linked IgG, and non-cross-linked IgG were all calculated as percentages of total IgG.

■ ASSOCIATED CONTENT

📄 Supporting Information

Protein Z sequence, Cross-linking of additional IgG isotypes by Protein Z, Additional cross-linking kinetics. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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