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Site-Specific Photoconjugation of Antibodies Using Chemically Synthesized IgG-Binding Domains

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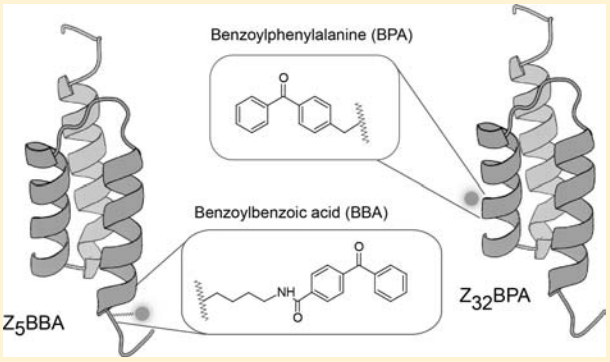
ABSTRACT: Site-specific labeling of antibodies can be performed using the immunoglobulin-binding Z domain, derived from staphylococcal protein A (SpA), which has a well-characterized binding site in the Fc region of antibodies. By introducing a photoactivable probe in the Z domain, a covalent bond can be formed between the Z domain and the antibody by irradiation with UV light. The aim of this study was to improve the conjugation yield for labeling of different subclasses of IgG having different sequence composition, using a photoactivated Z domain variant. Four different variants of the Z domain (Z5BPA, Z5BBA, Z32BPA, and Z32BBA) were synthesized to investigate the influence of the position of the photoactivable probe and the presence of a flexible linker between the probe and the protein. For two of the variants, the photoreactive benzophenone group was introduced as part of an amino acid side chain by incorporation of the unnatural amino acid benzoylphenylalanine (BPA) during peptide synthesis. For the other two variants, the photoreactive benzophenone group was attached via a flexible linker by coupling of benzoylbenzoic acid (BBA) to the ε-amino group of a selectively deprotected lysine residue. Photoconjugation experiments using human IgG1, mouse IgG1, and mouse IgG2A demonstrated efficient conjugation for all antibodies. It was shown that differences in linker length had a large impact on the conjugation efficiency for labeling of mouse IgG1, whereas the positioning of the photoactivable probe in the sequence of the protein had a larger effect for mouse IgG2A. Conjugation to human IgG1 was only to a minor extent affected by position or linker length. For each subclass of antibody, the best variant tested using a standard conjugation protocol resulted in conjugation efficiencies of 41−66%, which corresponds to on average approximately one Z domain attached to each antibody. As a combination of the two best performing variants, Z5BBA and Z32BPA, a Z domain variant with two photoactivable probes (Z5BBA32BPA) was also synthesized with the aim of targeting a wider panel of antibody subclasses and species. This new reagent could efficiently couple to all antibody subclasses that were targeted by the single benzophenone-labeled Z domain variants, with conjugation efficiencies of 26−41%.



INTRODUCTION

Antibodies are extensively used in both clinical applications, such as therapy, and *in vitro* assays as research reagents or diagnostic tools. For many applications the antibody needs to be conjugated to a reporter group, such as a cytotoxic molecule for use in therapy or a detection probe when used in diagnostics. With the increased use of conjugated antibodies in different applications, the labeling of antibodies has become more important. However, the methods for antibody conjugation most commonly used today all have drawbacks. Conjugation to the N-terminal and side chain amino groups often results in a heterogeneous product, due to the large numbers of available lysine residues at the surface of the antibody. There is also a risk that the antigen binding affinity is affected by conjugation to amino acids located in or near the complementarity-determining regions of the antibody. A more controlled way of labeling is to instead introduce a cysteine residue, which could offer a selective handle for conjugation of maleimide-based thiol-reactive probes. This could either be accomplished by genetically introducing an extra cysteine residue, such as in THIOMABs,[1] or by reduction of existing disulfide bridges.[2] Any unpaired cysteine could however be expected to have significant impact on antibody production yield or on antibody folding.

Antibody heterogeneity can be expected to be a serious problem when the antibody is labeled for use in *in vivo* applications. Antibody−drug conjugates (ADC) are currently extensively explored as therapeutic agents, e.g., for cancer therapy, and the selection of conjugation chemistry and conjugation site can be crucial for the performance of the ADC.[3] Since several lysine residues are found in the antibody constant regions,[4] it is evident that using standard amine coupling will result in a pool of antibodies with different drug-to-antibody ratio (DAR). It has further been shown that the different number of drugs on each antibody might lead to differences in the pharmacokinetic properties and could

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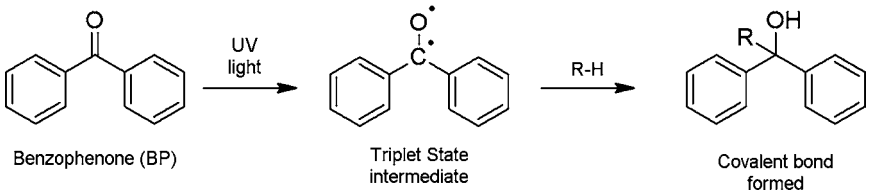

Figure 1. Proposed reaction mechanism for benzophenone photoconjugation via a triplet state intermediate.

decrease the therapeutic window, compared to methods giving site-specific and stoichiometric labeling, e.g., transglutaminase-mediated conjugation to specific glutamine residues in the antibody framework.[5] Also for other applications, a reproducible and site-specific labeling method can be desirable. For example, in the evaluation of a panel of candidate antibodies, it can be an advantage to use a site-specific labeling technology, since the effect that the labeling may have on the properties of the antibody (e.g., denaturation or loss of binding activity) will be comparable within a panel of different antibodies of the same subclass. Also for certain *in vitro* assays, such as fluorescence correlation spectroscopy (FCS) and fluorescence lifetime imaging microscopy (FLIM), the degree of labeling has a large impact on the performance of the assay and it is preferred to employ a controllable labeling method giving minimal batch-to-batch variation.[6]

An ideal labeling technique should thus be rapid, efficient, and give rise to a homogeneously labeled product. By using an immunoglobulin-binding protein with known interaction with the antibody, such as protein A, protein G, or protein L, it is possible to target a specific binding site in the antibody for labeling or surface immobilization. The five homologous immunoglobulin-binding domains E, D, A, B, and C from protein A, which is expressed on the surface of *Staphylococcus aureus*,[7] have a well-characterized binding site in the fragment crystallizable (Fc) of the antibody, located to the region between CH2 and CH3.[8,9] The protein A-derived domains also bind to the fragment antigen binding (Fab) region of the antibody; however, the B domain has been further engineered for alkaline stability, which resulted in an obligate Fc binder, the Z domain.[10]

The interactions between the above-mentioned immunoglobulin-binding proteins and antibodies are of high affinity, but they are reversible and may not be sufficiently stable for certain applications. To covalently link the antibody to the protein domain, a photoactivable probe can be introduced in the protein. Upon irradiation, the photoactivable probe is activated and can form a covalent bond to an amino acid situated in close proximity, a strategy widely used to map interaction sites in receptor−ligand pairs.[11] Benzophenones (see Figure 1), in particular, the commercially available amino acid analogue *p*-benzoyl-L-phenylalanine, are commonly used as photoactivable probes thanks to their favorable biochemical properties. Benzophenones are more stable than other photo-cross-linking groups, e.g., diazido esters, aryl azides, and diazarine, and they insert relatively nonspecifically into unreactive C−H bonds, even in the presence of nucleophiles such as water. Furthermore, benzophenones efficiently return to the ground state in the absence of photoreaction, thus allowing for repetitive photoactivation.[12,13] Recently, the same photochemistry has been investigated for covalent conjugation of immunoglobulin-binding domains to antibodies,[14−16] as a means to generate site-specifically labeled antibody conjugates. Since the immunoglobulin-binding domain is small in size and has a simple structure not requiring any disulfide bridges for correct folding, different reporter groups can readily be incorporated in the protein domain by chemical synthesis or standard bioconjugation techniques, prior to photolabeling of the antibody.

Based on the earlier work performed on protein A-derived immunoglobulin-binding domains for antibody conjugation,[15,16] the aim of the current study was to further investigate the photoconjugation efficiency to antibodies of different species and subclasses. The study was focused on the antibodies most commonly used in therapy and diagnostics, i.e., antibodies of human and mouse origin, in order to develop suitable reagents for labeling these important subclasses of antibodies. While the position of the photoactivable probe in the protein has earlier been demonstrated to have large impact on the binding affinity and/or conjugation efficiency, it could also be anticipated that the length and flexibility of the linker between the probe and the peptide backbone would have an effect. Four different variants with the photoactivable probe incorporated at different positions and with different distance to the backbone, Z5BPA, Z5BBA, Z32BPA, and Z32BBA, were therefore synthesized and tested (see Figure 2), and based on the results a general reagent for photoconjugation to the Fc-portion of a panel of different antibodies subclasses was developed.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies from different sources were used in the conjugation experiments. Human IgG1 and mouse IgG2A (targeting FITC-BSA), human IgG1 (B1), human IgG2 (B1), human IgG4 (B1), and human IgG1 (PA-1) were kind gifts from Bioinvent International AB, Lund, Sweden. Mouse IgG1 (LDL-11) and mouse IgG2A (LDL-20) targeting Apo lipoprotein B (ApoB) were kind gifts from Mabtech AB, Nacka strand, Sweden. Mouse IgG1 targeting 14−3−3 sigma and mouse IgG1 targeting carcinoembryogenic antigen-related cell adhesion molecule 5 (CEACAM5) were obtained from Abnova, Tapei, Taiwan.

Synthesis of IgG-Binding Proteins Using Solid Phase Peptide Synthesis. The variants Z5BPA, Z5BBA, Z32BPA, Z32BPA, and Z5BBA32BPA were chemically synthesized using an automated peptide synthesizer with an integrated microwave owen (Liberty, CEM Corporation Matthews, NC, USA) and Fmoc chemistry in 0.05 mmol scale. The proteins were assembled on Fmoc Rink amide resin with a substitution of 0.27 mmol/g as the solid support (Merck, Darmstadt, Germany) and *N*-methyl-2-pyrrolidone (NMP) was used as the main solvent throughout the synthesis. The temporary protecting group was removed with 20% (v/v) piperazine in NMP before conjugation of the subsequent amino acid. The amino acids were preactivated with *N*-hydroxybenzotriazole (HOBt) (AK Scientific, Union City, CA, USA) (5 equiv), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Apollo Scientific, Cheshire, United Kingdom) (5 equiv), and *N*,*N*-diisopropylethylamine (DIEA)

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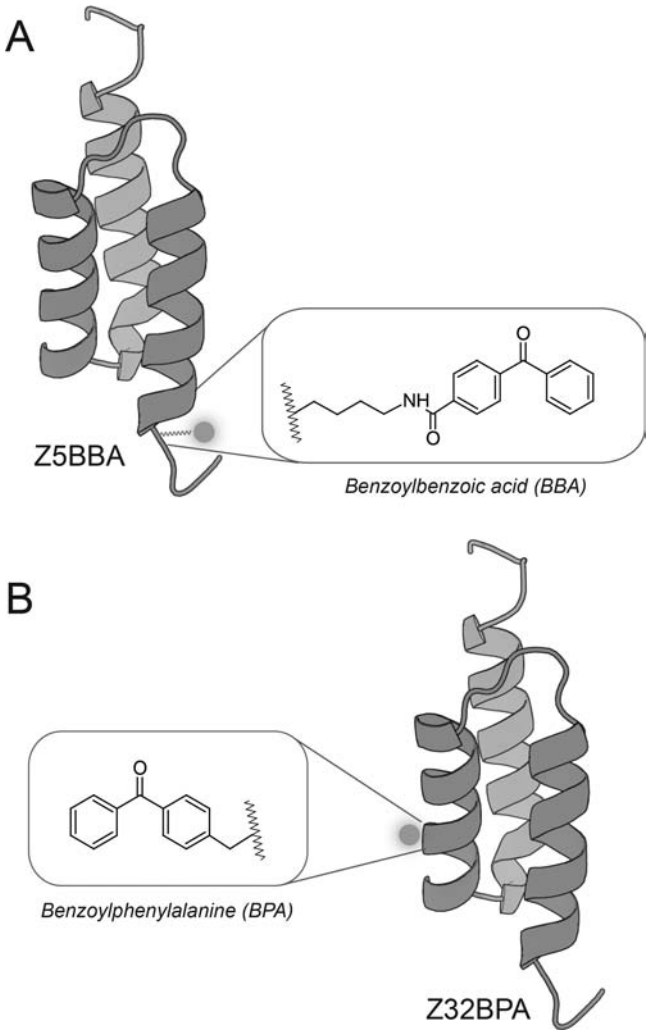

Figure 2. Schematic figure of the benzophenone-labeled Z variants. (A) Z5BBA in ribbon illustration, with BBA conjugated to the ε-amino group of lysine in position 5. (B) Z32BPA in ribbon illustration, highlighting the chemical structure of BPA in position 32.

(10 equiv) and coupled in 5-fold excess with microwave irradiation. After each conjugation, any remaining nonreacted amino groups were capped with 0.5 M acetic acid. The C-terminal Lys58 was incorporated as Fmoc-Lys(Alloc)-OH for the possibility of further functionalization and an Asp^2Glu substitution was introduced in all variants to avoid aspartimide formation.

For preparation of Z5BPA and Z32BPA, the commercially available amino acid derivative Fmoc-BPA-OH (Chem-Impex, Wood Dale, IL, USA) was incorporated at the selected position during synthesis as a regular amino acid. For Z5BBA and Z32BBA, the protein was synthesized with the orthogonally side chain-protected Fmoc-Lys(Mtt)-OH derivative at the selected position. After completed synthesis of $Z\text{-}Lys^5(Mtt)$ and $Z\text{-}Lys^{32}(Mtt)$, the resin was treated with 1% trifluoroacetic acid (TFA) and 5% triisopropylsilane (TIS) in dichloromethane (DCM) for 10 × 2 min, selectively cleaving off the acid-labile protecting group Mtt. 4-Benzoylbenzoic acid (BBA) (Sigma Aldrich) was manually conjugated to the deprotected ε-amino group using 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (5 equiv) and *N,N*-diisopropylethylamine (DIEA) (5 equiv) in NMP at RT for 2 h. The reaction was monitored by ninhydrin test to ensure complete coupling resulting in Z5BBA and Z32BBA. As a combination of two of the previously synthesized variants, a Z-variant was synthesized with BBA conjugated to a selectively deprotected Lys(Mtt) residue in position 5, and Fmoc-BPA-OH incorporated in position 32, resulting in Z5BBA32BPA. All proteins were treated with TFA:TIS:H_2O (95:2.5:2.5) for 2 h in RT for single step cleavage of the side-chain protecting groups and the linker to the solid support. Proteins were extracted in *tert*-butyl methyl ether:H_2O (50:50) three times before the water phase was filtered and lyophilized.

Protein Purification and Analysis. All proteins were purified by reversed phase HPLC (1200 series, Agilent) on a Zorbax SB300-C18 3.5 μm (4.6 × 150 mm) column (Agilent) with a gradient ranging from 20% to 50% B over 25 min (Buffer A: 0.1% TFA:H_2O and Buffer B: 0.1%TFA:CH_3CN). The molecular weight was determined by an electrospray ionization quadrupole time-of-flight mass spectrometer (6540 ESI-Q-TOF-MS) (Agilent).

Antibody Photoconjugation. Photoconjugation was performed in solution using a panel of human IgG1, human IgG2, human IgG4, mouse IgG1, mouse IgG2A, and mouse IgG2B antibodies for determination of the conjugation efficiency. For each conjugation, 3 μg monoclonal antibody was incubated with a 10-fold molar excess of Z variant at a final antibody concentration of 0.06 mg/mL in 1×PBS in a 48-well non-tissue prepared flat bottom plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The reaction was incubated for 2 h at RT with gentle shaking before exposure to UV light at 365 nm (Spectronics Corporation) using a UVC 500 Ultraviolet Cross-linker (Amersham Bioscience) equipped with 5 × 8 W UV-A tubes. An exposure time of 120 min was used for the cross-linking experiments. All conjugations were performed in 48-well plates with a surface of 0.75 cm^2 on ice.

Analysis of Photoconjugation Efficiency. To analyze the photoconjugation efficiency, aliquots of the reaction samples were run on 4−12% Bis-Tris SDS-PAGE gels (Invitrogen) at 175 V for 1 h at 4 °C under reducing conditions. Gels were stained with SimplyBlue SafeStain (Invitrogen) according to the manufacturer's instructions and scanned before quantification of the band intensities with the GelQuant.NET software, provided by biochemlabsolutions.com. The antibody heavy chain covalently conjugated to the Z domain was well separated from the unconjugated heavy chain and the conjugation efficiency could be calculated from the fraction of heavy chain with covalently attached Z domain. The contribution from the Z protein to the staining of the conjugated heavy chain was regarded as negligible when determining the conjugation efficiency. The determination was based on two separate SDS-PAGE gels where each Z variant was conjugated to three different antibodies each for human IgG1 and mouse IgG1, and to two different antibodies each for mouse IgG2A and IgG2B.

RESULTS

Z5BPA and Z32BPA were successfully synthesized using automated solid phase peptide synthesis with phenylalanine in position 5 or glutamine in position 32 substituted with benzoylphenylalanine. For the Z5BBA and Z32BBA variants, selective deprotection of $Lys^5(Mtt)$ and $Lys^{32}(Mtt)$ was required before coupling of benzoylbenzoic acid yielding Z5BBA and Z32BBA. After TFA cleavage, the Z variants were separated using RP-HPLC and the correct masses were

determined using ESI-Q-TOF MS (see Table 1). Based on the photoconjugation results, a double mutant containing two photoreactive probes was later successfully synthesized, using the same synthetic strategy.

Table 1. Molecular Weights of Synthesized Proteins

Z variant	Theor. M_w	Exp. M_w
Z5BPA	6758 Da	6757 Da
Z5BBA	6926 Da	6927 Da
Z32BPA	6860 Da	6861 Da
Z32BBA	6945 Da	6944 Da
Z5BPA32BBA	7048 Da	7047 Da

A standard photoconjugation protocol was applied to determine the difference in conjugation efficiency for the different combinations of Z variant and antibody subclass. The photoconjugation experiments were analyzed by SDS-PAGE and the conjugation efficiency was determined by quantification of the band intensities and calculating the fraction of antibody heavy chain with attached Z protein compared to unconjugated heavy chain (see Figure 3). It can be noted that 50% conjugation yield corresponds to on average one Z domain attached to each antibody, whereas 100% yield would correspond to two Z molecules conjugated to the symmetrical antibody. The antibodies were conjugated with 10× excess of Z domain and no purification step removing Z domain after the reaction was included, as the purpose of the experiment was only to determine the conjugation efficiency. The SDS-PAGE gels thus also show an additional band, corresponding to the unconjugated, excess Z domain. The results were shown to be reproducible for experiments performed on different days for all antibodies.

The different Z variants showed large differences in conjugation efficiency for the different tested antibody species and subclasses. For human IgG1, the conjugation efficiency ranged from 9% for Z5BPA to 41% for Z32BPA (see Table 2). Z32BPA was also shown to conjugate efficiently to human IgG2 and IgG4, although with somewhat lower efficiency to IgG4 (data not shown). For mouse IgG1, there was a dramatic difference in conjugation efficiency between the Z variant with the photoactivable probe in position 5 and a short linker between the probe and the backbone, for which the conjugation efficiency was barely detectable, and Z5BBA with the longer flexible linker where the conjugation efficiency was 66%. The other two tested Z variants ranged in conjugation efficiency between 38% and 46%. For mouse IgG2A, the linker length did not seem to have a large impact on the conjugation efficiency; however, the selected position in the protein had a large influence. Both Z5BPA and Z5BBA showed high conjugation efficiencies to mouse IgG2A, 44% and 50%, respectively, compared to Z32BPA and Z32BBA, which only showed conjugation efficiencies of 6% and 19%, respectively. Mouse IgG2B was also tested in photoconjugation experiments with the four Z variants, but no conjugation could be detected by SDS-PAGE for any of the combinations (data not shown).

In order to produce a conjugation reagent that would be generally applicable to a wider range of antibody species and subclasses, the two best performing variants with regard to linker length and probe position, Z5BBA and Z32BPA, were combined in a double benzophenone-labeled Z domain: Z5BBA32BPA. The resulting double-labeled variant could successfully be photoconjugated to all antibody subclasses targeted by the earlier tested variants with similar conjugation efficiency for human IgG1, but with somewhat lower conjugation efficiency than the best single benzophenone-

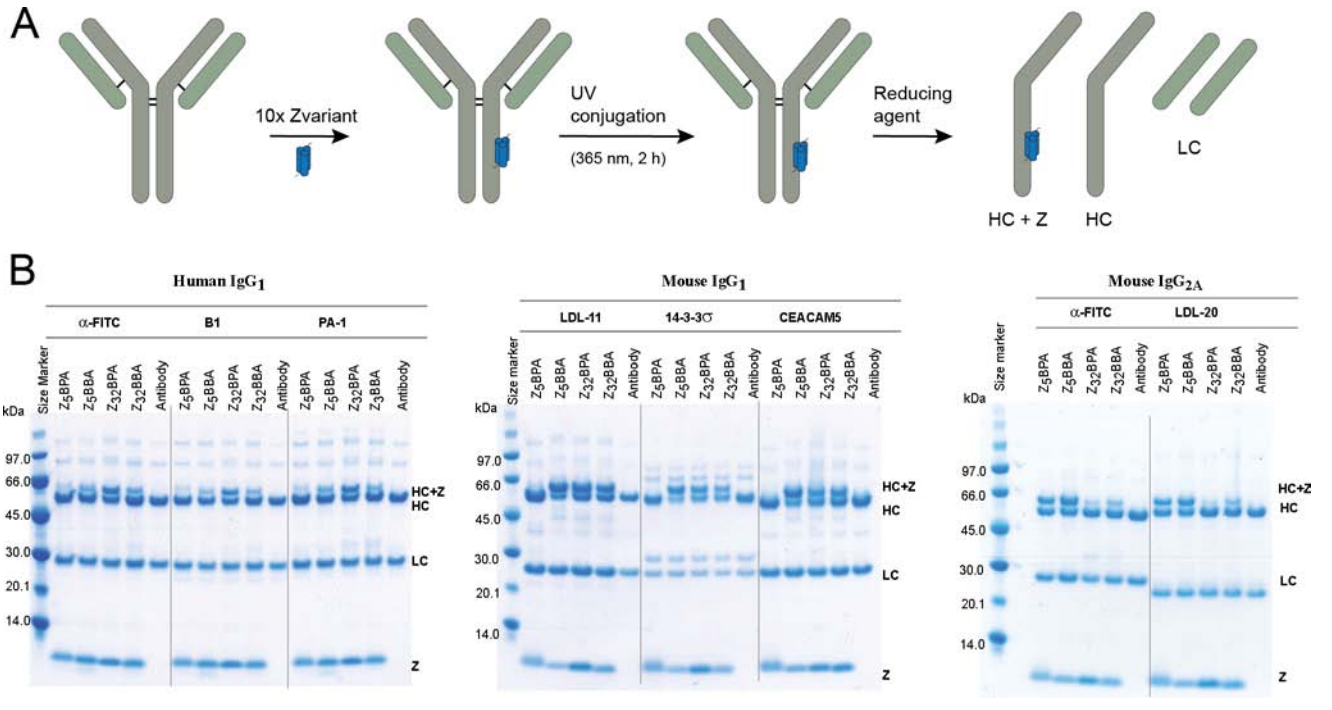

Figure 3. (A) Schematic representation of the antibody photoconjugation followed by reduction. The conjugated heavy chain is separated from the free heavy chain when analyzed by SDS-PAGE. (B) SDS-PAGE analysis of photoconjugated antibodies. The four different benzophenone-labeled Z-variants Z5BPA, Z5BBA, Z32BPA, and Z32BBA were conjugated to a panel of antibodies of different species and subclasses. The intensities of the bands corresponding to unconjugated heavy chain (HC) and conjugated heavy chain (HC+Z) were used to determine the conjugation efficiency.

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Table 2. Conjugation Efficiencies for the Different Antibody Subclasses and Subtypes[a]

	Human IgG1				Mouse IgG1				Mouse IgG2A		
Variant	α-FITC	B1	PA-1	Avg.	LDL-11	14−3−3σ	CEACAM5	Avg.	α-FITC	LDL-20	Avg.
Z5BPA	13%	8%	6%	9%	2%	1%	0%	1%	45%	44%	44%
Z5BBA	27%	17%	22%	21%	60%	69%	70%	66%	53%	48%	50%
Z32BPA	40%	44%	40%	41%	45%	51%	42%	46%	8%	4%	6%
Z32BBA	19%	16%	17%	17%	40%	37%	38%	38%	20%	19%	19%
Z5BBA32BPA	42%	40%	40%	41%	21%	36%	37%	31%	28%	25%	26%

[a]The conjugation efficiency is presented as a mean from two separate SDS-PAGE gels for each antibody.

labeled Z domain variant for mouse IgG1 and mouse IgG2A (see Figure 4).

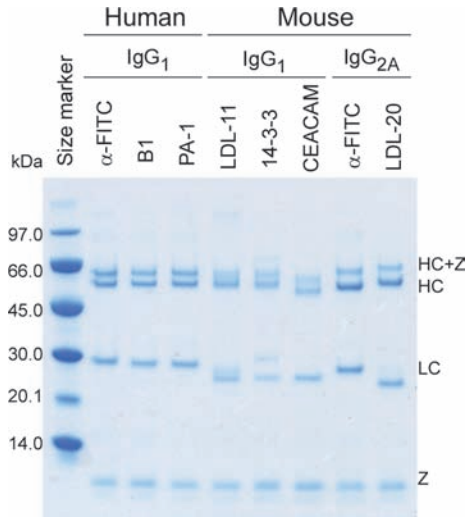

Figure 4. SDS-PAGE analysis of the double benzophenone-labeled Z5BBA32BPA photoconjugated to different antibodies.

DISCUSSION

Site-specific antibody labeling can be performed using the immunoglobulin-binding Z domain, which has a well-characterized binding site in the Fc region. By introducing a photoactivable probe in the Z domain, it is possible to create a covalent bond between the Z domain and the antibody by irradiation with UV light, and this strategy can be used as an alternative to standard bioconjugation methods for labeling of antibodies, which typically lead to a heterogeneous product. However, the binding affinity of the Z domain to different species and subclasses of IgG varies significantly and the aim of the present study was to explore the possibilities for photolabeling different antibodies and to develop a generally applicable reagent for antibody labeling. The antibody subclass predominantly used today for antibody therapy is human IgG1[17] whereas mouse IgG1 is still the most employed antibody subclass for diagnostic assays and other *in vitro* biotechnological applications. The site-specific labeling of these selected antibody subclasses and isotypes are thus of particular importance.

It has earlier been shown that the positioning of the photoactivable probe in the Z domain influences its binding and conjugation efficiency to antibodies. In one recent study, phenylalanine in position 5 of the Z domain was substituted with the photoactivable amino acid benzoylphenylalanine to generate an antibody labeling reagent. The selected position was chosen as it is in close proximity to the binding surface and considered to be involved in the interaction with the Fc part of the antibody.[18] SPR experiments showed that high affinity binding was retained and the proteins could be photoconjugated to human IgG1, mouse IgG2A, and rabbit polyclonal IgG.[15] In the same study, histidine in position 18 was also substituted with benzoylphenylalanine. However, this variant did not show any binding to IgG and there was no detectable photolabeling. In a different study, efforts were made to increase the photoconjugation efficiency of the Z domain to mouse IgG1, and several mutants were produced and evaluated. The results showed that a high conjugation yield could be observed when the photoactivable probe was introduced in position 32. However, in these Z variants the photoactivable benzophenone probe was conjugated to the side chain of a cysteine residue, via a maleimido-benzophenone reagent, instead of incorporation as part of the amino acid benzoylphenylalanine, as in the first study. Since the maleimido reagent provides a longer and more flexible linker between the benzophenone moiety and the peptide backbone, it could not be ruled out that this difference also influences the photoconjugation efficiency.

Based on these earlier results, positions 5 and 32 in the Z domain were in the present study selected for incorporation of the photoreactive probe. To investigate the influence of the linker length, the direct incorporation of benzoylphenylalanine was compared to benzoylbenzoic acid coupled to the side chain of a lysine residue. In contrast to the previously reported strategy,[16] which employed a thiol-reactive maleimide reagent for introduction of the photoactivable probe, the chemical synthesis approach based on an orthogonally protected lysine derivative preserved the option of later introducing a unique cysteine residue in the protein for conjugation to a reporter group, e.g., a fluorophore, as an alternative to incorporating the probe during peptide synthesis.

SDS-PAGE analysis demonstrated that the synthesized Z variants could be site-specifically and covalently conjugated to the antibody heavy chain and appeared in the gel analysis as an extra band with higher molecular weight above the non-conjugated heavy chain. Importantly, no conjugation to the light chain could be detected, confirming the selectivity of the reaction. The conjugation efficiency to three different monoclonal human IgG1 antibodies was determined and the average conjugation efficiency was 41% for Z32BPA, which was found to be the best variant for human IgG1 compared to the variants with the benzophenone probe located at a larger distance from the backbone, or with the probe in the position 5. The small differences in conjugation efficiency for the three tested monoclonal antibodies can possibly be explained by minor heterogeneities in the antibody preparations, introduced during production, purification, or storage.

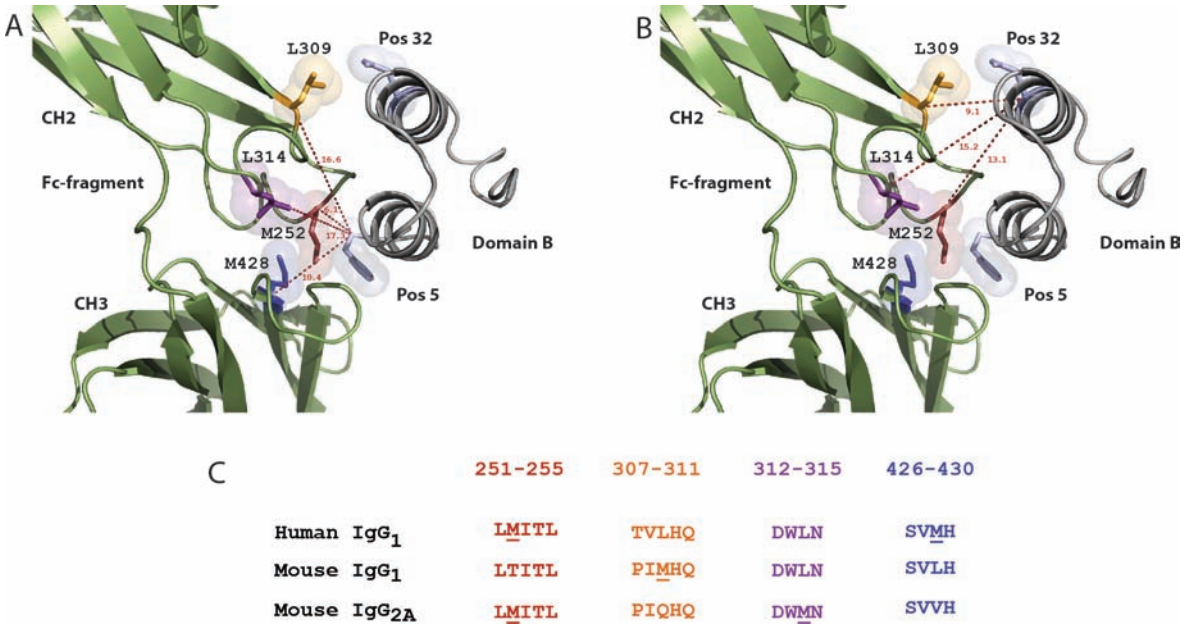

Figure 5. Ribbon representation of part of the complex between the B domain, from which the Z domain is derived, and human IgG1 Fc (pdb entry 1FC2). The distances (in Å) between selected positions in the Fc fragment and (A) position 5 of the B domain, and (B) position 32 of the B domain, are shown. (C) Sequence alignment of loop regions in CH2 and CH3 having different Met residues in human IgG1, mouse IgG1, and mouse IgG2A.

The influence of the linker length was surprisingly large when Z5BPA was compared to Z5BBA in the conjugation experiment with mouse IgG1. The protein with the short linker between the probe and the backbone showed little or no detectable photoconjugation to the mouse IgG1, whereas the longer linker resulted in the highest conjugation efficiency of all tested variants, resulting in more than one Z5BBA molecule per antibody on average. The linker effect was however marginal for conjugation to mouse IgG2A, where Z5BBA only showed slightly higher conjugation efficiency compared to Z5BPA. When BBA is conjugated to the lysine side chain, it provides flexibility that is not available for BPA, in which the benzophenone moiety is separated from the peptide backbone only by a methylene bridge. Increased photolabeling yield with a more conformationally flexible photoactivable ligand has been seen in other photoconjugation studies, and it has been suggested that the conformational flexibility is more important than the binding affinity to achieve efficient photo-cross-linking.[19] However, in the present study there is no simple correlation between probe flexibility and photoconjugation efficiency. The large differences in conjugation efficiency observed for the tested antibody subclasses can probably be explained by not only the differences in the heavy chain sequences at or near the binding site for the Z domain, which may affect the binding affinity to the Z variants, but also the availability of properly positioned amino acid side chains in the antibody that can react with the activated benzophenone probe during the lifetime of the activated state, and avoid competing intramolecular reactions within the Z domain.

UV-activated benzophenone in a triplet state can react with C−H bonds (see Figure 1) located either in the backbone or in the amino acid side chains of proteins, but it has been reported to have a preference for the carbon next to the heteroatom in the side chains of Met, Arg, or Lys, and the tertiary carbon in the side chains of Leu and Val.[12] Several studies of photoconjugation to peptides and proteins have shown that benzophenones have a particular preference for Met and are conjugated to this residue to a larger extent than to other residues.[20,21] It has even been suggested that Met acts as a magnet for benzophenone and is preferentially modified even when other amino acids are present at a shorter distance from the photoactivable probe.[22]

The crystal structure of the B domain, from which the Z domain is derived, in complex with a human Fc fragment, shows that the B domain binds to a region of the antibody involving contact residues in both the CH2 and CH3 domains.[18] Based on this crystal structure, the distance from selected positions in the analogous Z domain to different regions in the Fc part of the antibody can be estimated (see Figure 5). Interestingly, the antibodies primarily used in this study, i.e., human IgG1, mouse IgG1, and mouse IgG2A, have different Met residues in the Fc region within approximately 10−15 Å from the benzophenone probes incorporated in the Z domain, when the distance is measured from backbone to backbone of the selection positions, and this could possibly explain the different conjugation efficiencies. Position 32, shown to be important for efficient conjugation to human IgG1 as demonstrated by the highest labeling yield for the Z32BPA variant, is located within 15 Å from Met252 (using the Kabat numbering scheme[23]), making it a possible candidate for the photoconjugation reaction. This Met residue is present also in the sequence of mouse IgG2A, but not in mouse IgG1, which instead has a Thr residue in this position. The fact that mouse IgG1 still is efficiently labeled by Z32BPA could possibly be explained by Met309, which is present in mouse IgG1, but not in human IgG1 or mouse IgG2A. Met309 is located in a different loop of the CH2 domain than Met252, but it can be estimated to be within 10 Å from position 32 in the Z domain. Another sequence variation in this loop region is position 314, which is a Met residue in mouse IgG2A, but a Leu residue in human IgG1 or mouse IgG1. This residue appears to be accessible to position 32, but possibly also to position 5, which

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is the other position in the Z domain used for incorporation of the benzophenone probe. The latter position in the Z domain can based on the crystal structure be estimated to be approximately 6 Å from the Met252 residue, but it is also close to residues in the CH3 domain. Similarly to the CH2 domain, the CH3 loop regions have different sequence composition and show differences with respect to the presence of Met residues. In human IgG1, Met428 is located approximately 10 Å from position 5 in the Z domain and could be a candidate for the photoconjugation reaction, but in mouse IgG1 and mouse IgG2A, this Met residue is replaced with Leu and Val, respectively. Estimated from the crystal structure, position 5 in the Z domain is close to Met residues in both human IgG1 and mouse IgG2A, whereas in mouse IgG1 the closest residue, Met309, is found at 17 Å. This could possibly explain why a long, flexible linker between the benzophenone group and the backbone is needed for efficient photoconjugation to mouse IgG1 when the photoactivable probe is located in position 5, as demonstrated by the striking difference in conjugation yield between Z5BPA and Z5BBA. In summary, it can be speculated that the different sets of Met residues present in the antibody CH2 and CH3 domains at least partly explain the different conjugation efficiencies observed for the different Z variants, but the sequence variation between the antibody subclasses could also have other effects on the conformation of the antibodies, which could make individual amino acids more or less accessible to the photoconjugation reaction.

As an attempt to produce a more versatile IgG-labeling reagent, which could be used for conjugation to a panel of different antibody subclasses, the two most promising variants, Z5BBA and Z32BPA, were combined in the double benzophenone-labeled Z5BBA32BPA protein variant. Although lower conjugation efficiency to mouse IgG1 and mouse IgG2A was observed for the double-labeled variant compared to the best Z variant-antibody combinations, the protein could efficiently be conjugated to all the tested antibodies (except mouse IgG2B) and it gave the same conjugation efficiency to human IgG1 as the best performing single-labeled variant. Although it would probably not be the reagent of choice for labeling an antibody for a clinical application, such a general reagent could be expected to be of value in a research laboratory, as the preparation of different reagents for different antibody subclasses would not be required.

In conclusion, with the new benzophenone-labeled Z domain variants, high conjugation efficiency can be obtained for the therapeutically important human IgG1 subclass, as well as for mouse IgG1 and mouse IgG2A, which are two of the most important antibody subclasses in diagnostic and biotechnological applications. A general reagent giving lower conjugation yield, but having wider applicability, has also been generated in the project. It is clear from the results that the differences in conformational flexibility of the photoactivable probe and the location in the immunoglobulin-binding protein have profound effects on the efficiency of photo-cross-linking to different antibody subclasses.

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Notes

The authors declare no competing financial interest.

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