Bioconjugate Chemistry

Communication

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Conjugation of a Reactive Thiol at the Nucleotide Binding Site for Site-Specific Antibody Functionalization

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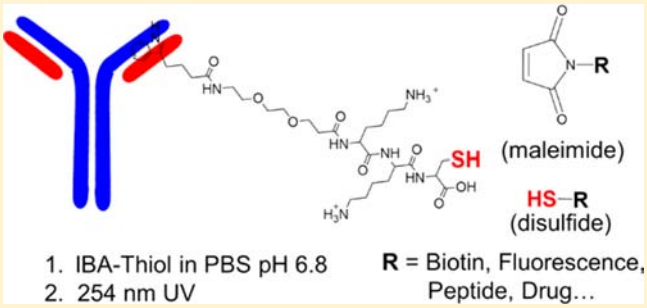
Supporting Information

ABSTRACT: Described here is a UV photo-cross-linking method that utilizes the NBS (nucleotide binding site) for site-specific covalent functionalization of antibodies with reactive thiol moieties (UV-NBS^{Thiol}), while preserving antibody activity. By synthesizing an indole-3-butyric acid (IBA) conjugated version of cysteine we site-specifically photo-cross-linked a reactive thiol moiety to antibodies at the NBS. This thiol moiety can then be used as an orthogonally reactive location to conjugate various types of functional ligands that possess a thiol reactive group through disulfide bond formation or reaction with a maleimide functionalized ligand. Our results demonstrate the utility of the UV-NBS^{Thiol} method by successfully functionalizing a prostate specific antigen antibody (IgG^{PSA}) with IBA-Thiol and subsequent reaction with maleimide-fluorescein. An optimal UV energy of 0.5−1.5 J/cm^2 was determined to yield the most efficient photo-cross-linking and resulted in 1−1.5 conjugations per antibody while preserving antibody/antigen binding activity and Fc recognition. Utilizing the IBA-Thiol ligand allows for an efficient means of site-specifically conjugating UV sensitive functionalities to antibody NBS that would otherwise not have been amenable by the previously described UV-NBS photo-cross-linking method. The UV-NBS^{Thiol} conjugation strategy can be utilized in various diagnostic and therapeutic applications with nearly limitless potential for the preparation of site-specific covalent conjugation of affinity tags, fluorescent molecules, peptides, and chemotherapeutics to antibodies.



INTRODUCTION

Described here is a method for site-specific conjugation of reactive thiol ligands to antibodies at the nucleotide binding site (NBS). Found within the variable region of the Fab arm, the highly conserved NBS has been previously implicated in diverse site-specific conjugation strategies allowing for conjugation of various functional ligands as well as for oriented antibody immobilization for use in medical diagnostics.[1−3] Antibodies are implemented in numerous diagnostic and therapeutic applications, taking advantage of their ability to selectively target antigens with minimal cross reactivity.[4−7] It is often necessary to conjugate functional ligands to antibodies to endow them with unique capabilities depending upon each specific application.[8−11] Common antibody conjugates include affinity tags, fluorescent probes, and peptides.[1,12] It is also becoming increasingly more prevalent for pharmaceutical antibodies to be conjugated with cytotoxic drugs making antibody drug conjugates (ADC) in an effort to enhance cancer treatments.[13−15] In some ADC constructs a disulfide bond is used as an intermediary to conjugate pH sensitive hydrolyzable linkers that facilitate the release of chemotherapeutics upon internalization of the antibody within cancer cell lysosomes.[16,17] To be used as viable clinical tools these complex antibody conjugates necessitate the use of conjugation strategies that have a high coupling efficiency that is not detrimental to antigen binding activity or Fc recognition.

Chemical ligation to the abundant lysine side chains (Lys-ε-NH_3^+) throughout the surface of antibodies utilizing *N*-hydroxysuccinimide (NHS) ester functionalized ligands is the most common method for functionalizing antibodies.[8,10] It is not, however, possible to accurately control the number or sites of conjugation with this method resulting in a heterogeneous functionalized antibody population. Non-site-specific conjugation techniques, such as Lys side chain functionalization, can reduce antibody activity as a result of conjugations to the complementarity determining region (CDR), by impairing antigen binding. Furthermore, conjugations to the Fc domain may interfere with Fc detection via a secondary antibody as well as inhibit antibody-dependent cellular cytotoxicity (ADCC) pathways.[12,18,19] Site-specific covalent conjugation methods, such as partial reduction of disulfides for sulfur chemistry and oxidation of antibody glycosylation sites for carbohydrate chemistry, have also been developed.[20−28] These methods, however, require complex chemical procedures and result in highly variable outcomes from antibody to antibody.[12,29,30] The

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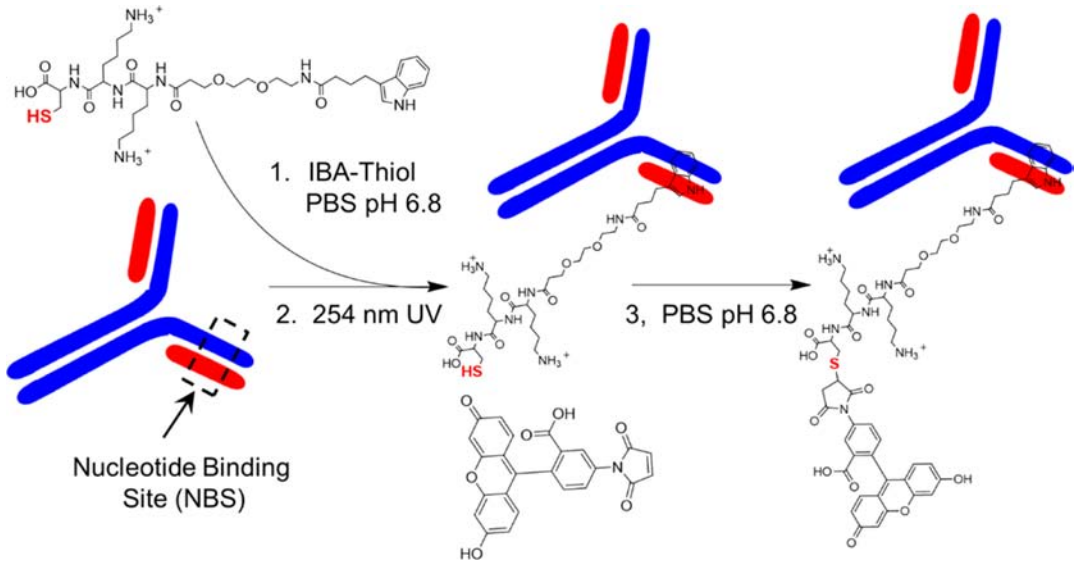

Figure 1. Schematic representation of the method for UV photo-cross-linking of reactive thiol ligands to antibodies at the NBS. IBA-Thiol first associates with the antibody at the NBS, and upon UV exposure, a covalent bond forms between IBA and the antibody. The site-specific IBA-Thiol functionalized antibody can then be reacted to a maleimide bearing molecule such as maleimide−fluorescein.

lack of reproducible site-specific antibody modification techniques demonstrates a clear scientific area in need of improvement.

We previously characterized the NBS by overlaying >260 antibody crystal structures from the RCSB Protein Data Bank, across diverse antibody isotypes and species of origin, and determined four conserved residues that comprise the NBS: Y/F42 and Y/F103 residues in the variable region of the light chain (V_L); and Y103 and W118 in the variable region of the heavy chain, using the IMGT numbering system.[1,31−33] We also demonstrated NBS targeting and covalent conjugation by indole-3-butyric acid (IBA, K_d = 1−8 μM) functionalized ligands.[2,3,31,34,35] By taking advantage of the unique high density of aromatic residues within the NBS, a reactive radical driven cross-linking event between IBA and the Y/F42 residue in the antibody light chain can be initiated through exposure to UV light at a wavelength of 254 nm (Figure S1 in Supporting Information).[1,36,37] The UV-NBS cross-linked antibodies were analyzed by mass spectrometry MS/MS fragmentation, in silico docking minimizations, and Western blot analysis to verify covalent bond formation and determine a mechanism of photo-cross-linking.[1] We previously showed that IBA-conjugated versions of functional ligands such as affinity tags (biotin), chemotherapeutics (paclitaxel), cell penetrating peptides (cyclic iRGD peptide), and imaging molecules (fluorescein) can be photo-cross-linked to antibodies at the NBS.[1] This UV-NBS conjugation technique takes advantage of all of the benefits of photo-cross-linking in that a very defined amount of energy can be delivered to the sample, a high cross-linking yield can be achieved through highly efficient radical activation, and it is a very simple process to reproduce. Nevertheless, certain functional ligands with inherent UV sensitivity may not be suitable for conjugation using the UV-NBS photo-cross-linking technique, which may cause structural damage and result in loss of function.

In this study, we expand the capabilities of the UV-NBS antibody functionalization strategy by implementing a two-step conjugation method utilizing site-specific photo-cross-linking of a reactive thiol ligand at the NBS. This thiol group then functions as a moiety for site-specific conjugation of any thiol reactive ligand, including those that would not be amendable to conjugation through direct UV exposure. By synthesizing an IBA conjugated version of cysteine (IBA-Thiol) a reactive thiol group can be site-specifically photo-cross-linked to antibodies at the NBS (Figure S2 in Supporting Information). This thiol group can then be used as an orthogonally reactive site to conjugate any UV sensitive functional ligand that possesses either a thiol (for disulfide bond formation) or a maleimide moiety (Figure 1). The results detailed here provide a universal technique for the site-specific conjugation of any functional ligand, including UV sensitive molecules, to antibodies at the NBS, while preserving antibody activity.

RESULTS AND DISCUSSION

We first evaluated the effect of UV energy exposure to IBA-Thiol ligand used in the photo-cross-linking reaction, in the presence and absence of antibody. Both IBA and thiol moieties are UV reactive and can function as sensitizers upon UV exposure.[37−41] Therefore, it is important to demonstrate that the thiol remains active post UV exposure and that the IBA/NBS interaction still results in efficient photo-cross-linking. A fixed concentration of IBA-Thiol was exposed to a range of UV energies (0−3 J/cm^2) and reactive thiol groups were quantified utilizing Ellman's Reagent, a chromogenic substrate that absorbs light at 412 nm (ε = 13 600) upon binding to thiols (Figure S3 in Supporting Information).[42,43] In the absence of antibody, 3 J/cm^2 of UV energy resulted in 74% reactive thiol moieties remaining in solution (Figure S4 in Supporting Information). When IBA-Thiol (300 μM) and antibody (20 μM) are both present in solution, the same UV energy exposure results in no appreciable reduction in thiol reactivity (Figure S5 in Supporting Information). In the presence of antibody, thiol reactivity is preserved for two reasons: (i) the reactive species that is generated target the NBS and form the intended IBA/antibody conjugate instead of reacting with the sulfur, and (ii) antibody molecules adsorb part of the UV energy preventing full exposure to the unbound IBA-Thiol ligands.

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It was also necessary to verify that cross-linking IBA-Thiol ligand to the antibody did not have a negative effect on the antibody antigen binding activity at the UV energies necessary to facilitate cross-linking. To simultaneously test for successful cross-linking of IBA-Thiol and conservation of thiol reactivity post UV exposure, maleimide−fluorescein was allowed to conjugate with the introduced thiol and used as a tag in measurements. An anti-prostate specific antigen IgG (IgG^{PSA}) was used in our studies to validate site-specific conjugation. IgG^{PSA} was exposed to increasing UV energies (0−5 J/cm^2) in the presence of a saturating concentration of IBA-Thiol (300 μM) in PBS pH 6.8 to allow for covalent photo-cross-linking between the IBA and NBS. PBS pH 6.8 was selected due to the reduced rate of disulfide bond formation providing for 97% free thiols remaining after 6 h at room temperature and 85% remaining after 24 h (Figure S6 in Supporting Information). This pH also provides for efficient IBA/NBS binding and maintains a high level of site-specific photo-cross-linking efficiency.[1]

The excess IBA-Thiol was removed via membrane filtration and the UV-exposed antibodies were then incubated with a 5-fold excess of maleimide−fluorescein to react to all conjugated reactive thiols. The conjugated antibodies were then allowed to bind to surface immobilized prostate specific antigen (PSA). Total antibody activity was determined using a polyclonal Fc-specific HRP conjugated secondary antibody to allow probing of the entire Fc region for modifications, with 100% antibody activity signified by no reduction in antigen binding or anti-Fc signal intensity at increasing UV energies (Figure 2A). The IBA-Thiol photo-cross-linking efficiency was determined using an anti-fluorescein HRP conjugated secondary antibody (Figure 2B). We compared the experimental results for IBA-Thiol photo-cross-linking to IBA-Biotin photo-cross-linking results that we had extensively studied and reported in detail in an earlier publication.[3] IBA-Thiol ligand provides some UV shielding that protects the antibody from damage at high UV energies similarly to IBA-Biotin with an antibody activity level of 95% at 1.5 J/cm^2 UV (Figure 2A and Figure S7 in Supporting Information). The photo-cross-linking efficiency and thiol reactivity post UV exposure follows a similar trend as IBA-Biotin with an increased UV sensitivity above 1.5 J/cm^2. These results demonstrate that IBA-Thiol was successfully photo-cross-linked to IgG^{PSA} with the thiol moiety and antigen binding remaining active post conjugation.

The effect of UV energy on the average number of IBA conjugations per antibody was then evaluated. IBA-FITC at 300 μM (Figure S8 in Supporting Information) was incubated with IgG^{PSA}, providing for complete noncovalent association of IBA-FITC to all NBS, and was then exposed to the indicated UV energies (Figure S9 in Supporting Information). The IBA-FITC conjugated antibody was then injected in a size exclusion chromatography (SEC) column where nonconjugated IBA-FITC ligand eluted separately from the antibody conjugate. Utilizing a fluorescein calibration curve, based on SEC elution peak integrations of known amounts of fluorescein (494 nm), the average number of IBA-FITC conjugations per antibody was calculated (Figure 3).[1] Increasing UV energy resulted in an increase in the number of conjugations, reaching a maximum of 1.71 conjugations per antibody at 1.5 J/cm^2 UV exposure. Since the IgG^{PSA} antibody activity was greatly reduced above 1.5 J/cm^2, higher UV energies were not investigated. No cross-linking was observed in the absence of IBA or in the absence of UV energy (Figure 3).[1]

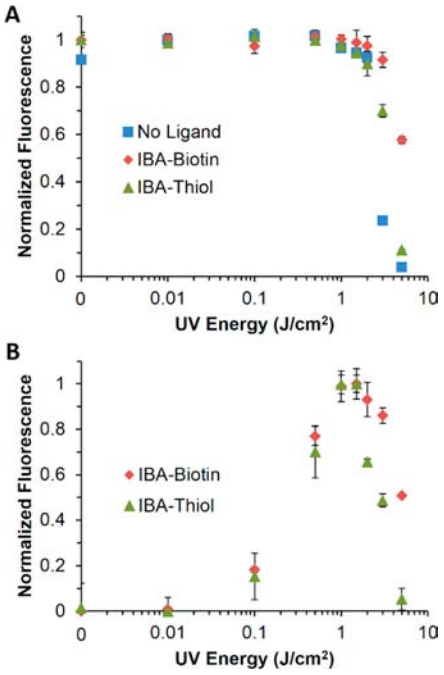

Figure 2. (A) Effects of UV energy exposure on antigen binding activity combined with Fc recognition as determined by an ELISA assay. UV exposed IgG^{PSA}, in the presence or absence of 300 μM IBA-Biotin or IBA-Thiol, via subsequent maleimide-fluorescein reaction, was allowed to bind to surface immobilized PSA. The total amount of bound antibody was quantified using an Fc-specific, HRP conjugated antibody. (B) Photo-cross-linking efficiency of IBA-Biotin or IBA-Thiol to IgG^{PSA} at the NBS was determined by an indirect ELISA assay, where the total biotinylation or thiolation, via reacted maleimide−fluorescein, was detected post binding to surface immobilized PSA by streptavidin-HRP or an anti-fluorescein HRP conjugated antibody. All data represents means (±SD) of triplicate experiments.

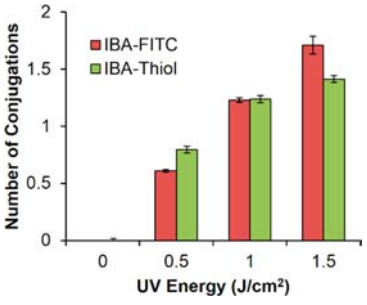

Figure 3. Effect of UV energy on the average number of conjugations per antibody of IBA-FITC and IBA-Thiol, via maleimide−fluorescein detection. The number of conjugations was determined from absorbance at 494 nm SEC peak integrations at fixed ligand and antibody concentrations of 300 μM and 20 μM, respectively. All data represents means (±SD) of triplicate experiments.

A similar analysis of the average number of active IBA-Thiol conjugations per antibody at increasing UV energies was also investigated. Antibody was incubated with a saturating concentration of IBA-Thiol and then exposed to the indicated UV energies. Maleimide−fluorescein was again employed to react to all reactive thiol groups and the mixture was analyzed via SEC injection. Utilizing the same peak integration method and fluorescein calibration curve, the number of reactive IBA-Thiol conjugations was quantified, reaching a maximum of 1.41

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conjugations at 1.5 J/cm^2 UV (Figure 3). The average number of IBA-FITC and IBA-Thiol conjugations per antibody followed a very similar UV energy dependence with very comparable conjugation efficiencies. These results demonstrate that the UV energies necessary to provide for IBA-Thiol photo-cross-linking have no effect on the conjugated thiol reactivity. To further demonstrate the UV energy dependent photo-cross-linking of IBA-Thiol selectively to the antibody NBS a Western blot was performed under reducing conditions displaying site-specific photo-cross-linking to the antibody light chain consistent with previously reported IBA-Biotin photo-cross-linking results (Figure S10 in Supporting Information).[1,3] Taken together, IBA-Thiol can be efficiently photo-cross-linked to the antibody NBS providing for the site-specific incorporation of an orthogonally reactive thiol moiety to the antibody.

CONCLUSION

In this study we demonstrated the site-specific functionalization of IgG^{PSA} with biotin (IBA-Biotin), fluorescein (IBA-FITC), and reactive thiol ligand (IBA-Thiol) using the UV-NBS photo-cross-linking method. Through the coupling of maleimide−fluorescein the proof of concept for site-specific photo-cross-linking of reactive thiol groups to the antibody NBS via an IBA moiety possessing ligand has been established. Utilizing IBA-Thiol ligand allows for an efficient means for site-specifically conjugating a UV sensitive functionality via subsequent maleimide or disulfide bond formation that would otherwise not have been amenable by the UV-NBS photo-cross-linking method.

ASSOCIATED CONTENT

Supporting Information

Detailed materials, experimental methods, and instrumentation. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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