Published in final edited form as:

Bioconj Chem. 2017 June 21; 28(6): 1749–1757. doi:10.1021/acs.bioconjchem.7b00220.

Bioorthogonal Protein Conjugation: Application to the Development of a Highly Sensitive Bioluminescent Immunoassay for the Detection of Interferon- γ

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Abstract

Bioorthogonal conjugation eliminates the short-comings of classical conjugation methods. The conjugation of antibodies to reporter proteins, such as bioluminescent protein, can be controlled with orthogonal conjugation methods. Here we report a bioluminescent immunoassay for the sensitive detection of interferon- γ (IFN- γ) that utilizes orthogonal conjugation of bioluminescent protein, *Gaussia* luciferase to anti-IFN- γ antibody. The IFN- γ is produced by the immune system and the detection of the IFN- γ is pivotal for the detection of persistent viral and bacterial infections. A bioorthogonal conjugation approach is used to conjugate an anti-IFN- γ antibody with a GLuc mutant containing the N-terminal tyrosine using formylbenzene diazonium hexafluorophosphate reagent (FBDP) in hydrophilic mild pH environment yielding high conjugation efficiency (60%). This reagent is shown to be specific for tyrosine (Tyr) residues. Therefore, conjugation through Tyr was orthogonal and not detrimental to the bioluminescence activity of GLuc. The immunoassay described in this paper is a sandwich type assay and involves a capture and a detection antibody. The assay was validated for its robustness, precision, accuracy, limit of detection, and recovery.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.7b00220. SDS-PAGE of the GLucY expression; Western blot analysis of the bioconjugation with glutaraldehyde; experimental procedure of the optimization studies (PDF)

INTRODUCTION

Reporter-labeled antibodies are commonly prepared for use in a variety of applications including immunoassays,¹ in situ binding assays,² imaging,³ diagnosis,⁴ targeted therapy,⁵ and drug delivery.⁶ Reporters that are most commonly conjugated to antibodies are fluorescent dyes,⁷ and proteins, such as enzymes,⁸ fluorescent proteins,⁹ and bioluminescent proteins.¹⁰ Although the fluorescent dyes are commonly employed as reporters, they usually lack the required sensitivity. In order to achieve high sensitivity, labels such as enzymes or bioluminescent proteins are commonly employed.¹¹ Bioluminescence-based immunoassays¹² have shown exquisite sensitivity and ease of use in complex matrices since the background associated with the bioluminescent detection is very low. This makes bioluminescent protein¹³ an attractive reporter for conjugating to antibodies.

Thus, far, bioluminescent proteins are conjugated to antibodies using specific and nonspecific chemical methods and genetic fusion, if the gene sequence for the antibody is available.^{14–16} Nonspecific chemical conjugation can result in heterogeneous conjugates that can lead to loss of the functional property of the antibody as well as the bioluminescent reporter. In general, specific conjugation of bioluminescent proteins to antibodies is also a significant challenge due to the unavailability of unique amino acids with appropriate chemical functionality necessary for conjugation, which necessitates protein engineering to introduce unique cysteines if not detrimental to protein activity.^{10,17,18} Further, a few important criteria to be considered while designing protein reporter–antibody conjugation include maintaining the structural and functional integrity of the biomolecules involved, reproducibility of the conjugation, control of biomolecule conjugation ratio, stability of the conjugates, ability to perform reaction in hydrophilic environments, and so forth. Many different orthogonal bioconjugation methods with superior performance over the traditional conjugation method have been developed that involve the use of less common amino acid residues.^{19,20} However, these methods have not been used for conjugating bioluminescent proteins to antibodies. Among the orthogonal conjugation methodologies, modification of the tyrosine (Tyr) residue is gaining popularity among researchers since they appear with intermediate frequency in a protein, and chemical reactivity of the Tyr phenol ring provides for several diverse reactions. The types of reactions performed with the Tyr residue can be varied according to the reaction conditions. For example, under basic conditions, the phenol

ring provides a proton for the O-acylation of the Tyr.²¹ On the other hand, an ene-type reaction at an aromatic carbon can occur under acidic conditions.²¹ Several groups have performed a Mannich-type reaction modifying the *ortho* position of the phenol of the Tyr²² in a peptide for the purpose of the bioconjugation of the peptide to poly(ethylene glycol).²³ The Barbas group developed an orthogonal method for specific labeling of Tyr residues.^{21,24} Additionally, the Barbas group introduced a stable diazonium salt, the formylbenzene diazonium hexafluorophosphate (FBDP) for the modification of the Tyr residues. This diazonium salt provides bioorthogonal aldehyde functionality and reacts specifically with the *ortho* position of the phenol group of the Tyr residue.²⁵ Francis and coworkers also used the diazonium-coupling reactions with the most accessible Tyr residue to modify the interior of a bacteriophage.²⁶ The most outstanding feature for the diazonium-coupling reaction is that it occurs in an aqueous solution, near neutral pH, and at room temperature.²⁵

In this paper, we have employed the orthogonal Tyr labeling strategy described above for the conjugation of a mutant of the bioluminescent protein, *Gaussia luciferase* (GLuc) to an interferon- γ (IFN- γ) antibody and employed this reporter-labeled antibody in the development of a highly sensitive bioluminescent immunoassay. GLuc, originally isolated from copepod, *Gaussia princeps*, has been used by many groups as the bioluminescent reporter of choice.^{27,28} In general, GLuc is one of the smallest known bioluminescent proteins²⁹ consisting of 185 amino acids with a molecular weight of 19.9 kDa. Upon addition of its substrate, coelenterazine, GLuc emits a blue light with an emission maximum at 480 nm³⁰ and is one of the brightest coelenterazine-dependent bioluminescent proteins. Bright bioluminescence emission and high sensitivity that can be achieved using GLuc as a label in antibody-based sensing has not been exploited to the fullest yet. Additionally, the chemical reaction between GLuc and its substrate does not require any cofactors such as ATP or calcium ions. Lastly, this protein has four cysteine residues that improve its thermostability and hence conjugation through the cysteine could be detrimental to its stability.^{30,31} In previous work, we have introduced a Tyr residue on GLuc creating a mutant termed GLucY containing Tyr at an easily accessible location which can be chemically modified to develop bioanalytical applications.³² This GLuc mutant contains only one other Tyr that is embedded inside the protein structure and hence not easily accessible for conjugation. Therefore, this GLuc mutant is an ideal candidate to study bioorthogonal conjugation through Tyr for selective conjugation to an antibody. In order to perform the chemical conjugation through the N-terminal Tyr residue of the GLuc mutant, the stable diazonium salt, FBDP, was synthesized as per the literature reported method.²⁵ FBDP provides aldehyde functionality on the Tyr residue of GLuc, which under slightly acidic conditions reacts with a hydrazine group introduced on the antibody through the sulfo-succinimidyl-6-hydrazino-nicotinamide (sulfo-S-Hynic) and amine reaction.

Here, as a proof of concept for the applicability of the Tyr-based bioconjugation methodology, we have developed a bioluminescent immunoassay to detect the antigen, IFN- γ . The IFN- γ is produced by the immune system as a response to a bacterial and viral infection. A continuous production of the IFN- γ along with other inflammatory cytokines is triggered in order to activate the immune system when challenged by an infecting virus, such as HIV.³³ As such IFN- γ is an important biomarker for monitoring infections.

RESULTS AND DISCUSSION

In this paper, we demonstrate the applicability of the Tyr-based bioconjugation methodology and show that the orthogonal conjugation method provides for a facile conjugation of bioluminescent protein to antibody. The conjugate prepared was employed in the development of a bioluminescent sandwich-type immunoassay to detect the antigen, IFN- γ .

Reporter, GLucY Protein Expression, and Purification

For our work, we use GLucY, a mutant of GLuc, containing a Tyr residue at the N-terminus, which was previously prepared in our laboratory.³² The Tyr residue on GLucY provides a phenol moiety that can be used specifically for bioconjugation. The most important consideration for conjugation through the Tyr residues is that they are much less common than lysine residues. Therefore, if we introduce Tyr at a site away from the active site of the protein its modification is less likely to cause a detrimental effect on the activity of the protein. The protein GLucY was expressed and purified, in high concentration, from the soluble fraction of an *E. coli* culture (Origami 2 strain).³² This method yielded about 15–20 mg purified protein per liter of culture, using immobilized-metal affinity chromatography (IMAC) following cell lysis. The calculated molecular weight for GLucY was ~22.1 kDa when analyzed by SDS-PAGE (Supporting Information, Figure S1).

Conjugation of GLucY to Antibody Using Conventional Method

As a comparison, we performed chemical conjugation of GLucY to antibody using a commonly used glutaraldehyde-based method. For that, we followed a standard published protocol used for antibody conjugation without optimizing it.¹⁵ The GLucY–antibody conjugation was confirmed using Western blot (Supporting Information, Figure S2). However, the conjugate did not retain any bioluminescence activity of GLucY and hence we did not employ this conjugate for immunoassay development. This loss of activity potentially could be due to the overconjugation of GLucY or the steric crowding of the GLucY active sites by the IFN- γ antibody. This could be avoided by further optimizing the protocol; nevertheless, this study points to the difficulty when conjugation is performed through nonspecific methods.

Modification of GLucY Using FBDP

To perform conjugation through Tyr on GLucY, we chose the diazonium salt chemistry because it provides a more controllable conjugation reaction with the biomolecule. The diazonium salt, formylbenzene diazonium hexafluorophosphate (FBDP), has two major advantages; it can be easily synthesized and is stable. FBDP was synthesized according to the literature method²⁵ (Figure 1A) and the product was characterized with ¹H NMR spectroscopy (data not shown). The diazonium salt, FBDP, provides a bioorthogonal aldehyde functionality that is at the *ortho* position to the phenol group on the Tyr residue, since the phenol moiety activates the *ortho* and the *para* positions for the electrophilic substitution reaction (Figure 1B). A secondary reaction can be performed with the aldehyde using the sulfo-S-Hync modified biomolecule. Furthermore, the modification of the Tyr residue with the FBDP can be monitored based on a change in the electronic absorption spectrum. The absorption spectrum between 250 and 450 nm shows a broad band around

350 nm (Figure 1C), which is absent in the spectra of unreacted GLucY and FBBDP. Also, mass spectroscopy data (not shown) indicated a molecular weight increase that is consistent with the molecular weight of FBBDP. After FBBDP labeling, the protein was subjected to tryptic digestion followed by the mass spectroscopy analysis of the resulting peptides. This analysis showed that the diazonium salt reacts only with the Tyr residues and not with the amines of the lysine residues or the amino terminus. The extent of conjugation was calculated using an Aldehyde Quantification Assay Kit (Abcam, Cambridge, Massachusetts) according to the manufacturer's instructions. The results showed that approximately 60% of the GLucY was modified with FBBDP. This is an efficient coupling reaction especially considering its regioselectivity, in which only one of the Tyr residue is preferentially modified with the FBBDP. In order to check if the modification of the protein with FBBDP has any detrimental effect on the bioluminescence activity of GLucY, several aliquots of GLucY were reacted with different mole ratios of FBBDP. The results showed that the protein can be successfully modified with 1 equiv of FBBDP without detriment to its bioluminescence. For convenience, FBBDP-modified GLucY will be referred to as GLucYFBBDP in this manuscript.

Conjugation of Anti-Human Interferon- γ Antibody with GLucYFBBDP

There are many chemicals, such as glutaraldehyde, that can be used to modify antibodies. Sulfo succinimidyl-6-hydrazino-nicotinamide (sulfo-S-Hynic) is a cross-linker that reacts with the amines of lysines present on the antibodies at physiological pH (Figure 2). By optimizing the stoichiometry, the reaction between the mouse anti-human interferon- γ antibody with the sulfo-S-Hynic can be controlled so that about 2–3 lysines would be modified with hydrazinonicotinic acid (Hynic) moieties. This ratio allowed us to preserve the function of the antibody. Also, by controlling the number of Hynic moieties, we can control the number of GLucY that are conjugated to the antibody in order to optimize the sensitivity of the system.

The commercially available mouse anti-human IFN- γ antibody was modified with sulfo-S-Hynic according to the manufacturer's instructions. The stoichiometry of the reaction was adjusted so that the final product would have about 2–3 Hynic moieties per antibody. Then, Hynic-modified antibody and the GLucYFBBDP were mixed in the conjugation buffer and allowed to react overnight at 4 °C to give the GLuc-labeled antibody. This GLucY-anti-IFN- γ antibody conjugate is referred to as the detection antibody (dAb) for the remainder of the text. The final bioconjugation was confirmed by Western Blot analysis (Figure 3). We used two different antibodies to visualize the bands. A primary antibody that targets GLuc and an anti-mouse secondary antibody that binds modified mouse anti-human-IFN- γ primary antibody were employed. Both blots confirmed that the dAb was successfully synthesized, as there is a bright broad band higher than 100 kDa that can be detected with both antibodies, indicating the presence of the GLucY as well as the anti-human-IFN- γ antibody (Figure 3). The presence of a broad smear is an indication of multiple different products of the conjugation of the detection antibody with the bioluminescent protein.

Development of the Immunoassay

The next step was to develop a bioluminescent immunoassay for the detection of IFN- γ . Our immunoassay is a sandwich type immunoassay and involves a capture antibody that binds

human IFN- γ as well as a detection antibody against a different epitope of the IFN- γ . The immunoassay was developed in a Maxisorb polystyrene 96-well black plate. Each assay step was carefully studied under different conditions (Figure 4). Many variables were optimized to achieve the lowest limit of detection (LOD) of the antigen. The parameters that were optimized are the incubation time, incubation temperature, concentration of the capture antibody, concentration of the detection antibody, the incubation time of the antigen, and the effect of several commercially available blocking buffers for an efficient blocking step (Figure 4).

The details of each optimization step are discussed in the Supporting Information. The following time points were selected because they generated the lowest LOD for the assay. Also, the 2 h incubation time of IFN- γ resulted in much higher day-to-day consistency of the results. The optimization studies resulted in the following ideal immunoassay conditions. The wells of the plate were coated with the capture antibody (100 μ L of 1.0 μ g/mL concentration) followed by an overnight blocking step using a commercially available blocking buffer (StartingBlock (PBS) Blocking Buffer, Thermo Scientific Cat#37539, Waltham, MA). Next, several different dilutions of the antigen were incubated for 2 h at room temperature followed by a wash step and the incubation of the detection antibody at a concentration of 2.0 μ g/mL at room temperature for 45 min. Following another wash step, the bioluminescence emission was monitored by adding 2 μ g/mL of the substrate coelenterazine. Using the bioluminescence emission intensity, a calibration curve was generated and a nonlinear dose–response curve was fitted to the data points using GraphPad Prism software (Figure 5). After these optimization parameters we concluded that the lowest limit of detection of the immunoassay was found to be 5.0×10^{-12} M, which is similar to the commercially available IFN- γ immunoassay (Biolegend, Cat#430104, San Diego, CA) with a detection limit of 2.4×10^{-12} M. The limit of detection was interpolated from the GraphPad Prism software using the formula $LOD = S_B + (3 \times SD_B)$ where S_B and SD_B are the mean and the standard deviation of the blank signals, respectively. The range of the calibration curve is 6 orders of magnitude and the total assay time was less than 3 h.

We have also tested the accuracy of the immunoassay by running the assay against known concentrations of IFN- γ (2.5×10^{-8} M) spiked into blocking buffer and measured using the calibration curve and then interpolating the signal to find the concentration. The interpolated concentration was calculated to be 2.27×10^{-8} M. The accuracy of the assay was calculated to be 90.8%. The precision of the assay was tested by running the assay test on different days (Figure 6). The results showed a coefficient of variation of less than 20% for every concentration.

Evaluation of Matrix Effects

Immunoassays are employed to detect antigens in a variety of matrices including blood, serum, saliva, and urine depending upon the type of antigen. The presence of other biologically active molecules can interfere with the assay affecting its analytical performance. For this purpose we have evaluated the possible matrix effects for our test by running the immunoassay in undiluted and 1:10 diluted pooled human serum, which is the sample of choice for IFN- γ detection. Serum samples were spiked with different known

concentrations of IFN- γ and the assay was performed. Using the same plate, we concurrently ran our immunoassay in phosphate buffered saline (PBS) as the control. By adding the substrate coelenterazine and using the bioluminescence emission data, three separate calibration curves were generated and three nonlinear response curves were fitted to the data points using GraphPad Prism software (Figure 7). The results show that the assay can be run in diluted or undiluted serum with similar performance characteristics as in the buffer matrix. We have performed one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism software. The results showed no statistical difference at 95% confidence interval indicating that the mean of each measurement is identical. We have confirmed the lack of matrix effect by performing a recovery study in which a known amount of IFN- γ (2.5×10^{-8} M) was spiked into both serum and diluted serum and the resultant signal was interpolated to the IFN- γ concentration using the calibration curve. The interpolated concentration for the serum was calculated to be 1.78×10^{-8} M and the percent recovery was 71.2%, and for the diluted serum was 2.84×10^{-8} M with the percent recovery equal to 113.6%. The observed percent recoveries are within the accepted range ($\pm 30\%$) for clinical samples.^{34,35} Also, we tested the levels of IFN- γ in three patients with irritable bowel syndrome (IBS), who have significant bacterial infection using our immunoassay. We ran our immunoassay in 1:10 diluted serum and found levels of IFN- γ to be 4.12 nM, 9.51 nM, and 3.63 nM in these samples.

In conclusion, we have developed and validated a very sensitive bioluminescence-based immunoassay for the detection of IFN- γ using a bioorthogonal conjugation strategy between the bioluminescent protein GLucY and an antibody against IFN- γ using a novel reagent FBBDP as the cross-linker. Since the conjugation strategy involves the use of FBBDP, the required reaction conditions are mild and, therefore, do not require pH and temperature extremes. Therefore, the antibody and the bioluminescent protein are not denatured and preserve their functionality. Since our choice of label is a bioluminescent protein attached directly to a primary antibody, we eliminate the secondary antibody incubation, as well as the incubation with the chromogenic substrate. Therefore, our assay is sensitive and requires less incubation time. Our assay demonstrated excellent performance with high precision and accuracy as well as a broad dynamic range and low limit of detection.

MATERIALS AND METHODS

Expression and Purification of Gaussia Luciferase from *Escherichia coli*

The expression of the protein GLucY from Origami 2 *E. coli* was performed according to Hunt et al.³² Briefly, the overnight cultures of *E. coli* containing the pCold-1GLucY plasmid were grown in terrific broth supplemented with 0.1 mg/mL ampicillin. These overnight cultures were then expanded into 300 mL of terrific broth supplemented with 0.1 mg/mL ampicillin. The cultures were grown to an OD₆₀₀ of 1.6. The cells were cold-shocked in an ice bath for 1 h and were induced with IPTG at a final concentration of 1.0 mM following an overnight induction at 15 °C. The cells were collected by centrifugation, resuspended in Lysis buffer³⁶ of 50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 10 mM imidazole, 1% (vol.) nonyl phenoxypolyethoxyethanol (NP-40), 0.2% (vol.) polyoxyethylene (20) sorbitan monolaurate (Tween 20), and 10 mM 2-mercaptoethanol (β -ME). Afterward the 1×

ProBlock Gold Bacterial Protease Inhibitor Cocktail (Gold Biotechnology Inc., St. Louis, MO) was added to the cell suspension followed by sonication (Model 500 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) with a microtip probe for 5 min with a 0.5 s on/off pulse sequence.³⁷ The insoluble material was removed by centrifugation at $10\,000 \times g$ at 4 °C for 30 min and the supernatant was filtered through 0.22 μ m filter. The filtered crude protein was then incubated with Ni-NTA agarose (Qiagen, Valencia, CA) at 4 °C for 45 min, collected on a Pierce Centrifuge Column (Life Technologies, Grand Island, NY) by gravity flow, washed with 10 column volumes of lysis buffer followed by 20 column volumes of a wash buffer of 50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, and 20 mM imidazole. The protein was then eluted with an elution buffer of 50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, and 150 mM imidazole in 1 column volume to each of six elutions. The purity of the protein in each elution fraction was confirmed by SDS-PAGE using a 4–20% Tris-Glycine gel according to manufacturer's instructions.

The elution fractions containing pure protein were pooled and then dialyzed into a phosphate buffered saline (PBS) of 100 mM sodium phosphate pH 7.2 and 150 mM sodium chloride. Slide-A-Lyzer Dialysis Cassettes (Life Technologies, Grand Island, NY) with a molecular weight cutoff (MWCO) of 10 kDa were used. Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) and a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA) for absorbance measurements. The concentration of the purified protein was measured using BCA assay according to manufacturer's instructions.

Synthesis of the Formylbenzene Diazonium Hexafluorophosphate (FBDP)

Formylbenzene diazonium hexafluorophosphate (FBDP) was synthesized according to the protocol described by Gavriluk et al.²⁵ Briefly, the 4-aminobenzaldehyde polymer was suspended in 12 N HCl solution at a concentration of 0.5 M at –15 °C and the cold aqueous solution NaNO₂ (0.7 M) was added dropwise to the mixture. The resulting solution was stirred for 1.5 h. Then, the aqueous 60% HPF₆ (1.70 equiv) was added to the orange-brown solution and the reaction mixture was left to stir for 30 min at –15 °C and for 30 min at room temperature. The white-yellow solid was collected by vacuum filtration and was washed with cold water and ethyl acetate. The yield of the reaction was 40%. The solid was stored in the freezer and under Ar atmosphere.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.39 (d, J = 9.2 Hz, 2H), 8.85 (d, J = 8.4 Hz, 2H), 10.17 (s, 1 H).

A stock solution of 0.01 M FBDP was prepared in PBS. With a total reaction volume of 1 mL reaction, 1 equiv FBDP was added to 5.8×10^{-6} mol of GLucY and incubated at room temperature (~20 °C) for 2 h. The modified variants were then buffer exchanged by dialysis (10 kDa MWCO) into 100 mM sodium citrate pH 6.0 and 150 mM sodium chloride to remove excess FBDP and prepare the modified variants for subsequent reaction with the hydrazine-modified antibody. Specific modification was confirmed by mass spectrometry performed by the Laboratory for Biological Mass Spectrometry at Indiana University (Bloomington, IN). The introduction of an aldehyde functionality by FBDP was also

confirmed using an Aldehyde Quantification Assay Kit (Abcam, Cambridge, MA), and by colorimetric reaction with 2,4-dinitrophenylhydrazine.

Concentration of the Purified Anti-Human IFN- γ Antibody

The purified anti-human IFN- γ antibody (BioLegend, Cat#502502, San Diego, CA) was concentrated using 100 K MWCO Amicon Ultra 0.5 mL Centrifugal filters [Millipore, Cat#UFC510024, Billerica, MA] according to the user's manual to achieve a final concentration of ~2–3 mg/mL. Briefly, the antibody was washed with 500 μ L PBS (100 mM sodium phosphate, 150 mM NaCl, pH = 7.4) by centrifuging the unit at $14\,000 \times g$ for 5 min for a total of four times. The absorbance of each wash was measured using UV–vis spectroscopy at 280 nm (Spectramax 190, Molecular Devices, Sunnyvale, CA) to confirm that there was no loss of the antibody. After collecting the concentrated purified anti-human IFN- γ antibody, the final concentration of the antibody was measured with UV–vis spectroscopy at 280 nm.

Bioconjugation of the Antibody with the GLucYFBDP

A 1.0 mg sulfo-succinimidyl-6-hydrazino-nicotinamide (sulfo-S-Hynic) [Solulink, Cat#S-1011-105, San Diego, CA] was dissolved in 100 μ L PBS. An aliquot of this solution that corresponds to 20.0 equiv was added to the solution of anti-IFN- γ antibody and the mixture was incubated for 2 h at room temperature (~20 °C). Then the unreacted sulfo-S-Hynic was removed from the reaction mixture using microdialysis tubes (MWCO 4K, 20–150 μ L). The buffer was also exchanged to the conjugation buffer (100 mM sodium citrate, 150 mM sodium chloride, pH 6.0) and the concentration of purified antibody was measured with UV–vis spectroscopy at 280 nm. After dialysis the extent of hydrazine modification was determined using UV–vis spectroscopy and measuring the absorbance values at 380 nm. *p*-Nitrobenzaldehyde was used as the standard, which forms a chromogenic product with sulfo-S-Hynic and has absorbance at 380 nm.

Depending on the number of sulfo-S-Hynic molecules on the antibody, an excess of the modified protein was added to the modified antibody solution and incubated overnight at 4 °C.

Bioconjugation of the Antibody via Glutaraldehyde

The GLucY solution was added to 7.5 equiv excess of antibody solution. In a fume hood 10 μ L of 25% glutaraldehyde (Sigma, Cat#G7526, St. Louis, MO) was added per mL antibody/protein. The reaction was left for 2 h at 4 °C. The resultant Schiff bases were reduced using sodium cyanoborohydride at a final concentration of 10 mg/mL and the reduction was performed for 1 h at 4 °C¹⁵.

Purification of Both Conjugated Antibodies

The unreacted protein from the antibody mixture was removed using 100 K MWCO Amicon Ultra 0.5 mL Centrifugal filters [Millipore, Cat#UFC510024, Billerica, MA] according to the user's manual. The concentration of the labeled antibody was measured with UV–vis spectroscopy at 280 nm and the conjugation of the antibody to GLuc was confirmed using Western blot analysis.

Western Blot Analysis

A SDS polyacrylamide gel electrophoresis was performed using a 4–20% Tris-Glycine gel as described previously using 1 μg purified antibody GLuc conjugate in duplicate. The proteins then were transferred onto a polyvinylidene fluoride (PVDF) membrane using a Bio-Rad semidry blotting instrument according to the manufacturer's instructions. After the transfer was complete, the membrane was blocked with Odyssey Blocking Buffer (PBS) [LI-COR, Cat#927-40000] for 2 h at room temperature. Then one of the membranes was incubated with a monoclonal mouse anti-Gaussia luciferase antibody (Nanolight Technology, Cat#401M, Pinetop, AZ) whereas the other membrane was incubated with an anti-mouse secondary antibody labeled with proprietary infrared dye (binds to Fc portion of mouse antibody), overnight at 4 °C. The membranes were washed three times with phosphate buffered saline containing 0.05% Tween-20 (PBST) buffer and three times with phosphate buffered saline (PBS). The first membrane was incubated with an anti-mouse secondary antibody labeled with proprietary infrared dye for 2 h at room temperature. The membrane was washed three times with PBST buffer and three times with PBS. Both membranes were scanned in a LI-COR Odyssey infrared scanner.

Immunoassay Parameter Optimization Studies

See Supporting Information for the description of studies.

Immunoassay

In order to optimize the time of incubation with antigen for a better detection limit, a MaxiSorp 96-well microtiter plate [ThermoFisher Cat#437111, Waltham, MA] was coated with anti-human IFN- γ capture antibody [BD Pharmingen Cat#551221, San Diego, CA]. The capture antibody solution (1.0 $\mu\text{g}/\text{mL}$) was prepared in 100 mM NaHCO_3 , pH 9.6, and an aliquot of 100 μL was dispensed into each well of the plate using a multichannel pipettor. The plate was shaken overnight at room temperature. The next day, capture antibody solution was removed by using the plate washer (MultiWash+, Molecular Devices, Sunnyvale, CA) using Wash Buffer (100 mM sodium phosphate, 150 mM sodium chloride pH 7.2, 0.05% Tween-20, 1% Starting Block buffer). The plate was blocked with 200 μL Starting Block T20 PBS [Thermo Scientific Cat#37539, Waltham, MA] while shaking at 500 rpm overnight at 4 °C. After the overnight blocking was complete, the blocking buffer was removed. Several dilutions of IFN- γ [BioLegend Cat#570206, San Diego, CA] in blocking buffer were prepared. 100 μL aliquots of the IFN- γ at the various concentrations was placed in each well and incubated for 2 h at room temperature. After the incubation, the antigen was removed by using the plate washer with the same settings. Then, 100 μL aliquots of the GLuc conjugated detection antibody at a concentration of 2 $\mu\text{g}/\text{mL}$ was added into each well, and incubated for 45 min at room temperature. After the incubation, the excess detection Ab was removed and the wells were washed with the plate washer. Finally, the bioluminescence intensity of each well was measured in the Clariostar Optima (BMG Labtech, Ortenberg, Germany) by injecting 100 μL of native coelenterazine at a final concentration of 2.0 $\mu\text{g}/\text{mL}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported through NIGMS funding (R01GM047915). S.D. would like to thank NSF CHE-1506740. S.D. thanks the Miller School of Medicine of the University of Miami for the Lucille P. Markey Chair in Biochemistry and Molecular Biology.

ABBREVIATIONS

IFN-γ	Interferon- γ
GLuc	Gaussia luciferase
FBDP	formyl-benzene diazonium hexafluorophosphate
sulfo-S-Hynic	sulfo-succinimidyl-6-hydrazino-nicotinamide
IMAC	immobilized-metal affinity chromatography
Hynic	hydrazinonicotinic acid
dAb	detection antibody
LOD	limit of detection
DTT	dithiothreitol
NP-40	nonyl phenoxyethoxyethanol
β-ME	2-mercaptoethanol
Tween 20	sorbital monolaurate
PBS	phosphate buffered saline
MWCO	molecular weight cutoff
BCA	bicinchoninic acid
PVDF	polyvinylidene fluoride

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Figure 1.

(A) Synthesis of FBDP according to the literature. (B) Schematic of the chemical reaction of modification of the GLucY with FBDP (GLucYFBDP). (C) Absorbance spectra of GLucY (●), GLucYFBDP (red ■), and FBDP (blue ▲).

Figure 2.

(A) Modification of anti-IFN- γ antibody with sulfo succinimidyl-6-hydrazino-nicotinamide (sulfo-S-Hynic). (B) Bioorthogonal conjugation of anti-IFN- γ antibody with GLucY.

Figure 3.

Western blot analysis of the bioconjugation of the anti-human IFN- γ with the GLucYFBDP using two different antibodies. (A) Membrane is probed with primary antibody against GLuc. (B) Membrane is probed with anti-mouse secondary antibody reactive toward anti-human IFN- γ antibody. Lanes (1) Precision Plus Protein Dual Color Standard; (2) commercial anti-human-IFN- γ primary antibody; (3) GLucYFBDP; (4) detection antibody.

Figure 4.

Optimization parameters of the immunoassay. A) Concentration of capture antibody: blue ●, 0.5 $\mu\text{g/mL}$; red ▲, 1.0 $\mu\text{g/mL}$; green ■, 5.0 $\mu\text{g/mL}$; and ▼, 10.0 $\mu\text{g/mL}$. (B) Incubation time of detection antibody: green ■, 45 min; red ◆, 90 min; blue ●, 1 h; gray ▲, 2 h; ▼, 3 h; and purple ●, overnight. (C) Incubation time of IFN- γ : blue ●, 1 h; red ▲, 2 h; and ■, 3 h. (D) Different commercial blocking buffers: blue ●, Starting blocking buffer; red ■, BLOTTO blocking buffer; green ▲, Sea Block buffer; ◆, SuperBlock blocking buffer; and gray ▼, protein-free blocking buffer. All points are the mean of three measurements $\pm$ 1 std dev. Error bars that are not visible are obstructed by the data point.

Figure 5. Dose response curve for IFN- γ . The inset shows the region of calibration curve near the detection limit. The data points are an average of three measurements ± 1 standard error of the mean. Some error bars are obscured by the symbols of the points.

Figure 6. Calibration curve generated using three different plates performed to monitor precision of the assay: 1st day (●), 2nd day (■), 3rd day (▲). The data points are an average of three measurements $\pm$ 1 standard error of the mean. Some error bars are obscured by the symbols of the points.

Figure 7.

Matrix effect with human serum and diluted human serum: red ●, IFN- γ in blocking buffer; blue ▲, IFN- γ in human serum; ■, IFN- γ in 1:10 diluted human serum. The data points are an average of three measurements $\pm$ 1 standard error of the mean. Some error bars are obscured by the symbols of the points.