Bioconjugate Chemistry

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Enhanced Binding and Reduced Immunogenicity of Glycoconjugates Prepared via Solid-State Photoactivation of Aliphatic Diazirine Carbohydrates

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Cite This: https://dx.doi.org/10.1021/acs.bioconjchem.0c00555

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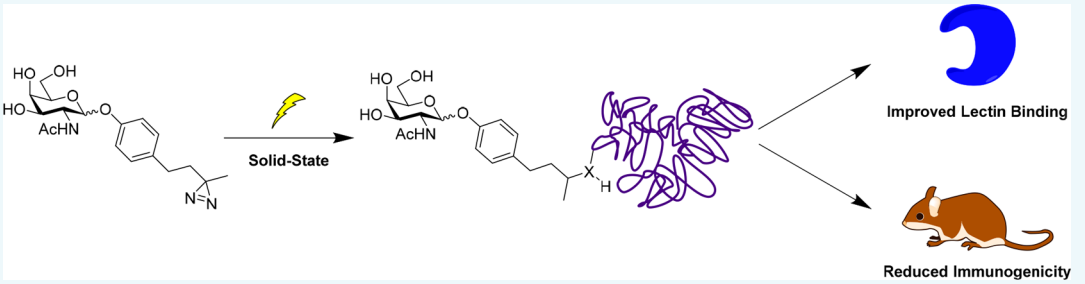

ABSTRACT: Biological conjugation is an important tool employed for many basic research and clinical applications. While useful, common methods of biological conjugation suffer from a variety of limitations, such as (a) requiring the presence of specific surface-exposed residues, such as lysines or cysteines, (b) reducing protein activity, and/or (c) reducing protein stability and solubility. Use of photoreactive moieties including diazirines, azides, and benzophenones provide an alternative, mild approach to conjugation. Upon irradiation with UV and visible light, these functionalities generate highly reactive carbenes, nitrenes, and radical intermediates. Many of these will couple to proteins in a non-amino-acid-specific manner. The main hurdle for photoactivated biological conjugation is very low yield. In this study, we developed a solid-state method to increase conjugation efficiency of diazirine-containing carbohydrates to proteins. Using this methodology, we produced multivalent carbohydrate−protein conjugates with unaltered protein charge and secondary structure. Compared to carbohydrate conjugates prepared with amide linkages to lysine residues using standard NHS conjugation, the photoreactive prepared conjugates displayed up to 100-fold improved binding to lectins and diminished immunogenicity in mice. These results indicate that photoreactive bioconjugation could be especially useful for *in vivo* applications, such as lectin targeting, where high binding affinity and low immunogenicity are desired.

INTRODUCTION

Biological conjugation, the process of covalently linking a molecule of interest and a biomolecule, is a vital tool employed in many basic and clinical applications.[1−3] Bioconjugation reactions are frequently used to attach a wide range of molecules, such as affinity tags and fluorophores, to proteins, DNA, and other biopolymers. In the field of glycoscience, bioconjugations are used to produce multivalent glycoconjugates as probes/inhibitors of endogenous lectins, immunogens for inducing antibody responses, and as reagents for various assays.[4−10] Bioconjugates are also valuable agents for a variety of clinical applications.[11,12] Some prime examples are antibody−drug conjugates such as Gemtuzumab ozogamicin (acute myelogenous leukemia), Trastuzumab emtansine (HER-2 positive metastatic breast cancer), and Sacituzumab govitecan (triple-negative breast cancer). Additionally, carbohydrate−protein conjugate vaccines such as Prevnar (*Streptococcus pneumoniae*) as well as Menactra and Menveo (*Neisseria meningitides*) have been administered to millions of people and have significantly improved human health.[13]

The preparation of biological conjugates is challenging for several reasons. First, most biomolecules require aqueous media, mild temperatures, and a narrow pH range, which significantly limits the spectrum of reaction conditions that are acceptable.[1,14,15] Additionally, it is often difficult to obtain large amounts of biomolecules, so reactions are typically conducted at lower molar concentrations and/or on small scales. Furthermore, they must be compatible with the many functional groups found on proteins and other biomolecules as well as a high molar excess of water.

Bioconjugation reactions are especially challenging when producing conjugates with higher valency, i.e., the number of units linked to the biomolecule. High valency can be critical for

Received: October 6, 2020
Revised: November 25, 2020

bioactivity. For example, carbohydrate−protein conjugates with multivalent displays are more immunogenic[6,16−18] and display higher avidity to lectins due to the ability to simultaneously engage multiple binding sites on a protein.[8,19] To achieve high valency with a residue specific conjugation method, however, the target protein must have a sufficient number of reacting residues on its surface. In addition, driving a conjugation to favor a higher valency can result in protein destabilization and denaturation and can increase the likelihood that one will modify residues in a protein active sites, or generate conjugates that possess diminished activities.[20,21] Additionally, increased valency can substantially alter the protein's isoelectric point (pI) and net charge. For example, coupling molecules with carboxylic acids to the side chain amines of lysine residues produces an amide bond and a charge change at the conjugation site. As multiple sites are modified, the net charge of a protein can be substantially perturbed leading to destabilization, reduced activity, and increased nonspecific adsorption/adherence.

Despite these challenges, many effective methods have been developed for bioconjugations.[1,10,15,22−24] Some commonly employed reactions include reductive amination, amide formation, Michael addition with maleimide-modified biomolecules, use of isothiocyanates, and azide−alkyne click chemistry.[1,14,25] While powerful, there are some important limitations for these methods. The majority of the reactions require surface-exposed lysine and/or cysteine residues as nucleophilic sites.[14,15,22,25] Some target proteins lack the necessary residue(s), while others have a competing nucleophile in a critical location, such as the active site of an enzyme. Modification can be deleterious to biophysical features of a protein/biomolecule too, such as solubility, net charge, stability, and overall structure.

Photochemical methodologies offer a complementary approach. They are used extensively for photoinduced cross-linking and photoaffinity labeling, which have become valuable tools to identify molecular binding sites, protein binding partners, and identify protein−protein interactions within heterogeneous systems.[26−29] With this approach, light is used to convert photoreactive functional moieties such as azides, benzophenones, and diazirines, into highly reactive intermediates (e.g., carbenes, nitrenes, or radicals) that can covalently link with the biological target through N−H, O−H, and C−H insertions or cycloaddition mechanisms.[26,30−35] Some photophores have high residue specificity, such as benzophenone, which displays a strong preference for methionine residues,[36] while others are not amino-acid-specific. The incorporation of a nonspecific photophore into a molecule of interest can theoretically modify a protein at any surface-exposed area without modifying the protein charge. As a result, this approach is not dependent on any specific residue and can theoretically modify a protein at any surface-exposed area without modifying the protein charge. Additionally, photoreactions are typically compatible with common buffers near physiological pH and are often completed within minutes to a few hours.[34,37,38] While offering several beneficial features, one serious disadvantage of photoconjugation is extremely low yield. This problem severely limits the use of photoactivation for producing bioconjugates, especially in applications that require high valency conjugates such as carbohydrate−protein conjugates. We note that elegant approaches have been developed for carbohydrate-based photoaffinity labeling[33,38−42] and attachment of glycans to surfaces,[33,43−46] but this approach is not typically used to prepare glycoconjugates.

In this study, we developed a solid-state approach to improve the efficiency of photoactivated conjugation of aliphatic diazirine-linked carbohydrates to proteins and then used it to prepare a set of glycoconjugates. This solid-state methodology significantly improves efficiency compared to standard solution-state protocols. The high-valency conjugates prepared with the solid-state photoreactive conditions displayed little or no alteration to protein structure, protein charge, and pI. When the carbohydrate conjugates were employed in biological assays, they exhibited up to 100-fold increased binding by mammalian and plant lectins and displayed reduced immunogenicity in mice.

RESULTS AND DISCUSSION

Our goal was to compare the biophysical properties and biological activities of glycoconjugates prepared via photoconjugation to analogous glycoconjugates prepared by traditional amide bond formation. The first phase of the project involved the design and synthesis of carbohydrate derivatives, and optimization of the photoconjugation reaction, while the second phase focused on the evaluation of the binding properties and immunogenicity of the glycoconjugates.

Design and Synthesis of Conjugates. To test the effects of photoreactive conjugation, we designed a matched pair of haptens (compounds **8** and **9**), each incorporating a single GalNAc residue attached to a phenol. These structures mimic a newly discovered form of glycosylation wherein GalNAc is attached to the side chain oxygen of tyrosine residues of proteins.[47,48] GalNAc-tyrosine has been found on a variety of proteins, is known to have altered expression in Alzheimer's patients, and has been reported to interact with lectins of the immune system (i.e., the macrophage galactose type lectin; MGL).[49] While potentially very important, little is known about its biosynthesis, expression, or biological effects.[47,48,50] Access to defined glycopeptides and structurally related haptens could be useful for studying this form of glycosylation.

For the reactive functional groups and coupling methodology, we compared the photoactivation of a diazirine to an amide coupling with a carboxylic acid. Aliphatic diazirines have been used extensively for photoaffinity labeling and have been shown to interact with a wide range of amino acids including Ala, Arg, Asp, Cys, His, Leu, Lys, Gln, Glu, Met, and Tyr residues.[35] This broad reactivity should assist in the generation of multivalent conjugates. In addition, diazirines are small, provide minimal perturbation of the glycan/hapten structure, and are likely to have low immunogenicity, as opposed to larger photoreactive groups like benzophenones. For photoreactive hapten **8**, a secondary diazirine was used for synthetic ease. The diazirine moiety was incorporated three carbons away from the phenyl ring on a butyl chain to retain structural similarity to the control hapten **9**, such that the overall length of the GalNAc haptens would be identical after conjugation. For control hapten **9**, we employed a 3-(4-hydroxyphenyl)-propanoic acid linker that we have previously used to generate a GalNAc-Tyr selective monoclonal antibody (manuscript in preparation). Conjugates prepared with hapten **8** are designated GalNAc-α/β-DZ-valency#-protein, while conjugates prepared with hapten **9** are designated GalNAc-α/β-PA-valency#-protein. DZ indicates conjugation via photoreactive diazirine and PA indicates a phenyl amide linkage to the protein.

https://dx.doi.org/10.1021/acs.bioconjchem.0c00555
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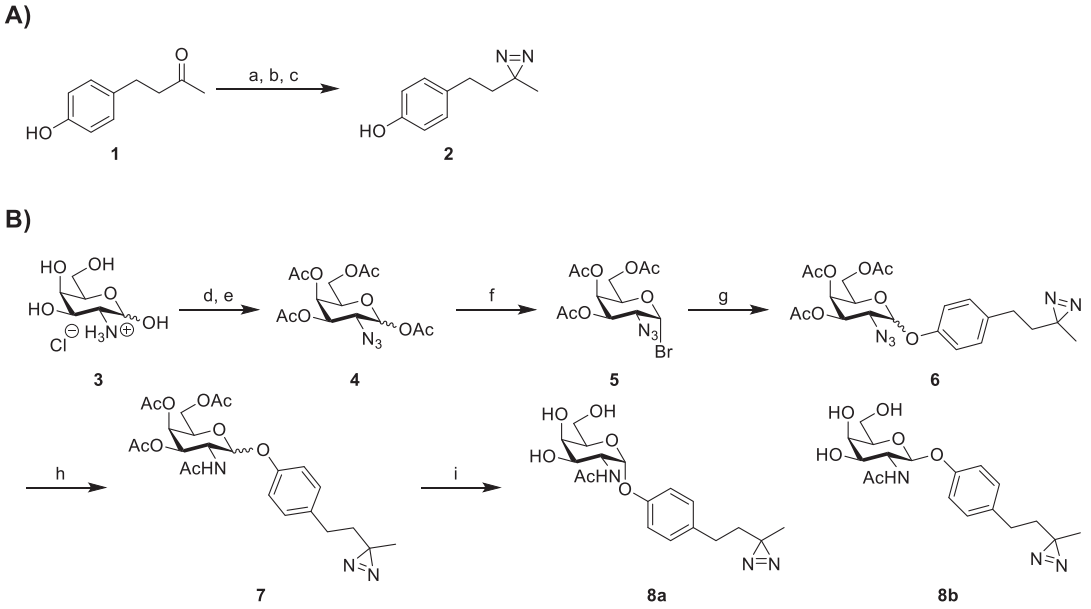
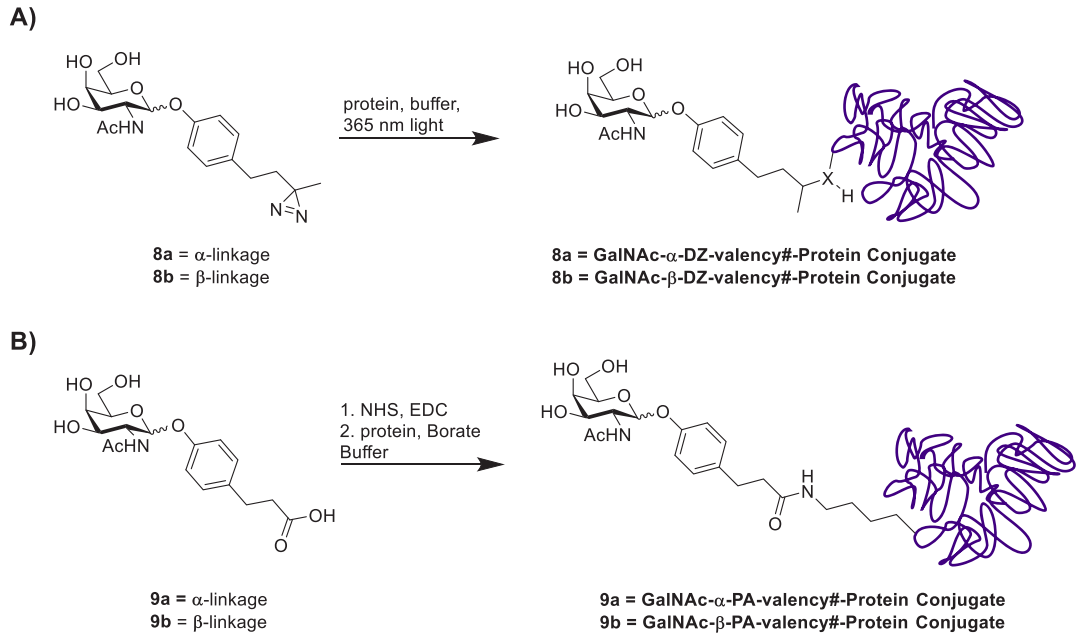
Scheme 1. Synthesis of Aliphatic Diazirine and GalNAc-Diazirine Compounds[a]



[a](a) 7N NH_3 in MeOH, 0 °C, 3 h; (b) Hydroxylamine-*O*-sulfonic acid, rt, overnight; (c) TEA, MeOH, 0 °C, 5 min, then I_2 (55%); (d) Imidazole-1-sulfonyl azide hydrochloride, K_2CO_3, $CuSO_4{\cdot}5H_2O$, MeOH, rt, 2 h; (e) Ac_2O, pyridine, rt, 16 h, 75%; (f) $BiBr_3$, TMSiBr, DCM, rt, overnight 84%; (g) 2, DTBMP, AgOTf, 4 Å MS, DCM:Tol. (1.5, 1 v:v) −78 °C − rt, overnight, 86%, (1:1 α:β); (h) AcSH, pyridine, (1:1 v:v). 0 °C − rt, overnight, 90% (1:1 α:β); (i) 0.5 MeOMe, MeOH, rt, 1 h, 97% (1:1 α:β).

Scheme 2. General Protein Conjugation Reactions of Haptens[a]



[a](A) GalNAc-DZ-protein conjugation conditions and (B) GalNAc-PA-protein conjugation conditions.

For this study, both the α- and β-anomers of aliphatic diazirine **8** were prepared as shown in Scheme 1. In short, the phenol diazirine (**2**) was prepared using standard literature conditions for installing the diazirine functionality.[51] Azido-bromide **5** was prepared from D-galactosamine hydrochloric acid (**3**) using standard conditions.[52] Glycosylation of **5** with **2** gave the desired protected sugar in good yield as a 1:1 mixture of α:β-anomers.[49] Conversion of the azide to the acetylated amine was achieved with thioacetic acid and pyridine to produce **7** in good yield.[53,54] Finally, selective deacetylation with 0.5 M sodium methoxide in methanol produced the desired GalNAc derivative (**8**) in excellent yield.[53] The α- and β-anomers of **8** (**8a** and **8b**, respectively) were separated and purified by preparative HPLC.

Optimization of Photoreactive Conjugation Conditions. One of the largest hurdles for photoactivated reactions is efficiency. With the desired aliphatic diazirine containing GalNAc haptens in hand, we attempted to prepare the photoreactive protein conjugates (Scheme 2A). To determine how much optimization of the reaction conditions were

https://dx.doi.org/10.1021/acs.bioconjchem.0c00555
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Table 1. Select Photoreactive Conjugation Optimization Conditions[a]

entry	protein	buffer	pH	equiv 8	state	365 nm irradiation time (min.)	ave. number of 8 added
1	BSA	1× PBS	7.4	40	solution	10	1.0
2	BSA	1× PBS	7.4	80	solution	40	1.3
3	BSA	1× PBS	7.4	80	solid	40	7.9
4	BSA	1× PBS	5.9	80	solid	40	4.1
5	BSA	1× PBS	8.3	80	solid	40	5.4
6	BSA	Borate	8.2	80	solid	40	9.7
7	BSA	Borate	9.2	80	solid	40	8.9
8	BSA	Borate	10.2	80	solid	40	8.7
9	BSA	Na_2CO_3	8.0	80	solid	40	9.6
10	BSA	Na_2CO_3	9.1	80	solid	40	8.5
11	BSA	Na_2CO_3	10.1	80	solid	40	9.5
12	HSA	1× PBS	7.4	80	solid	40	9.0
13	HSA	Borate	8	80	solid	40	3.5
14	HSA	Na_2CO_3	9.1	80	solid	40	11.3

[a]All reactions were protected from light exposure and incubated for 30 min at room temperature before irradiation or snap freezing and lyophilization.

required, our initial conjugation attempts employed commonly used diazirine photoaffinity labeling conditions with 40 equiv of GalNAc−hapten per mole of protein.[35,38,41,51] Upon conjugation, the loading levels of the resulting glycoconjugates were determined using MALDI-TOF analysis. Successful conjugation of the aliphatic diazirine hapten was also verified by ELISA with *Helix pomatia Agglutinin* (HPA) and *Vicia Villosa* (VVL) lectins which recognize the GalNAc residue. Unfortunately, the standard photoreaction conditions yielded very low loading levels of only ∼1 unit per molecule of BSA (Table 1, entry 1). Doubling the number of equivalents to 80 provided very little improvement (∼1.3 units per molecule of BSA, entry 2). To increase the yield, numerous parameters were varied, such as buffer, temperature, and irradiation time, but no improvement was observed. Based on these results, we concluded that it would be extremely difficult or impossible to prepare a multivalent conjugate using this approach.

Carbenes and other highly reactive intermediates are rapidly quenched by reaction with solvent, intramolecular rearrangements, and intermolecular reactions. We hypothesized that one could minimize these side reactions by removing the water and conducting the reaction in the solid state. In principle, the carbene would be generated in close proximity to the protein, improving the probability that it would react with the protein and decreasing the time that it has to undergo intramolecular rearrangements. In addition, only minimal solvent would be present, minimizing side reactions with water. To test this hypothesis, the reactants were combined, snap frozen, and then lyophilized. Irradiation of the resulting powder displayed a 6-fold increase in loading efficiency (Table 1, entry 3). Thus, the solid-state approach provided significant improvements over the solution-phase reaction.

To further improve the yield, we evaluated a variety of parameters using the solid-state approach. The effects of PBS buffer concentration (0.1−10× PBS at pH 7.4), incubation temperature, and incubation time were found to have minimal effects on the efficiency of the reaction (SI Table S1). Moreover, increasing or decreasing the pH in PBS led to lower yields (Table 1, entries 4 and 5). Fortunately, improvements could be obtained by altering the buffer. While ammonium carbonate provided yields similar to PBS (SI Table S1), the use of sodium borate or sodium carbonate buffers for the initial reaction mixture both provided improved efficiency. With these buffers, we could obtain 8−10 units per molecule of BSA using 80 equiv of diazirine-linked carbohydrate (Table 1, entries 6−11). In addition to improved efficiency, sodium carbonate also provided a consistent yield of ∼10% over a wide range of equivalents (SI Table S2). At the highest ratio tested, 120 equiv, we obtained ∼14 units/BSA. Based on these results, we concluded that the optimal conditions used sodium carbonate between pH 8−10 for the initial reaction mixture followed by solid-state photoconjugation at room temperature.

Having successfully identified conditions that could produce a multivalent carbohydrate−protein conjugate with a valency of at least 10, we next examined the generality of the method by coupling to other proteins (Table 1, entries 12−14). Using sodium carbonate buffer and the solid-state approach with 80 equiv of diazirine-linked GalNAc, we were able to successfully couple to HSA (pH 9.1; 11.3 units/BSA) and CRM197 (pH 8.0; 17.5 units/CRM197, SI Table S3). These conditions were then employed to prepare the various HSA and BSA glycoconjugates used in the mice immunizations and biological assays, including a GalNAc-α-DZ-10-HSA conjugate (average of 10 units/HSA) for immunological studies and high and low valency BSA conjugates for use in characterization studies and biological assays.

The control GalNAc-phenyl amide linked glycoconjugates were prepared via NHS−ester coupling (Scheme 2B) of carboxylic acids **9a** or **9b** with BSA or HSA. For immunological evaluation, a GalNAc-α-PA-10-HSA conjugate was prepared. High and low valency BSA conjugates were prepared for characterization studies and use in biological assays.

Characterization of the Conjugates. To verify that the photoconjugation approach was not disrupting the protein structure or causing degradation, we evaluated the biophysical properties of the conjugates using several methods. Both native and SDS-PAGE gels revealed that there was no noticeable degradation of BSA as a result of the solid-state conjugation conditions (SI Figures S2−S3) or CRM197 (SI Figure S4). The conjugates were also examined by circular dichroism (CD) to evaluate potential effects on overall structure (SI Figure S5). CD spectra of both the low and high loaded diazirine and NHS-ester prepared conjugates retain the indicative "W-shape" due to the hydrogen bonding α-helix structure of BSA.

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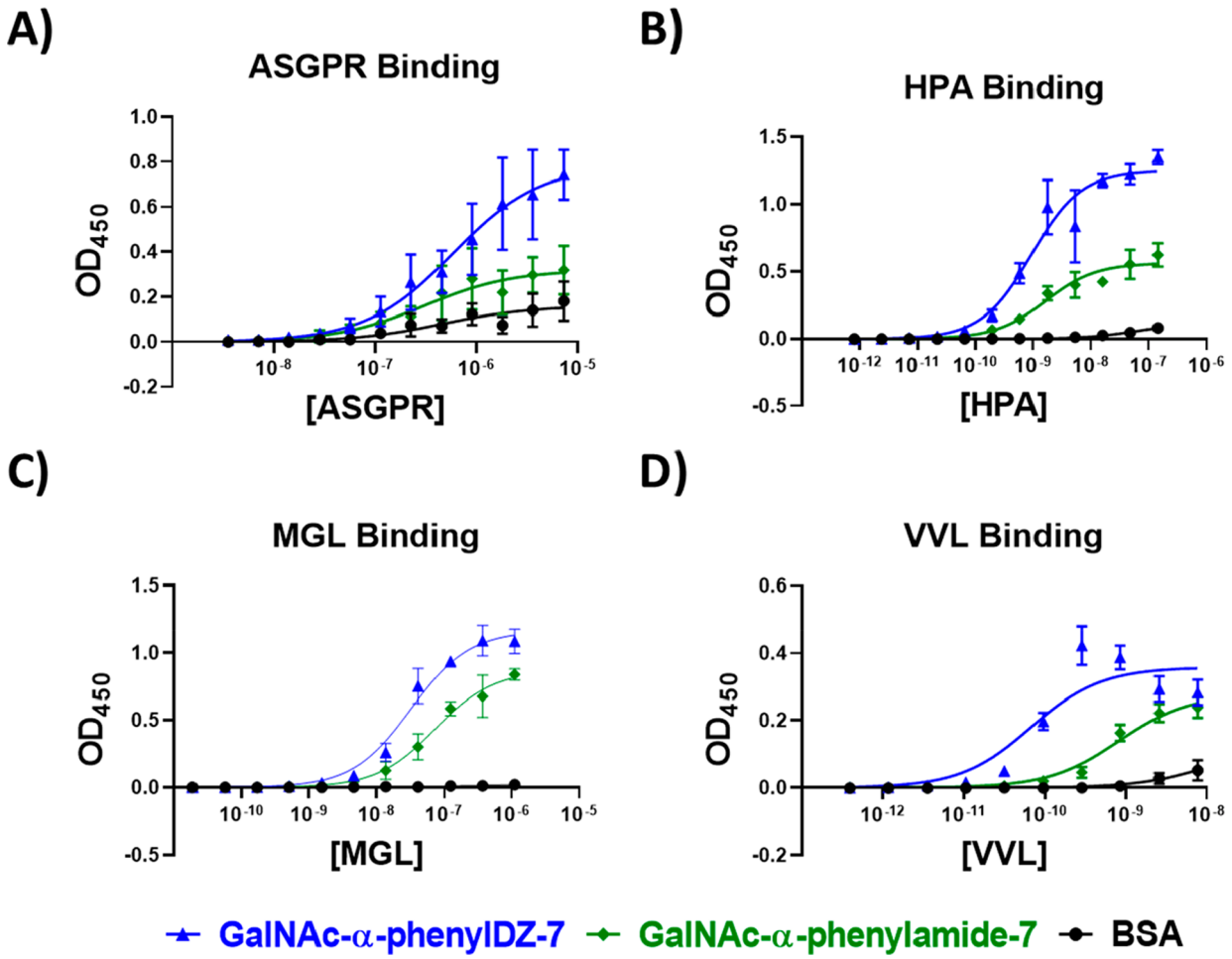

Figure 1. Lectin binding affinity to conjugates. (A) ASGPR, (B) HPA, (C) MGL, (D) VVL. Samples: Blue Triangles − GalNAc-α-DZ-7-BSA, Green Diamonds − GalNAc-α-PA-7-BSA, Black Circles − BSA. Plots display the mean with ± SD of triplicate runs for each lectin.

Based on differences in the chemistry of the two conjugation methods, we anticipated there would be differential effects on overall charge and pI. The carbene generated from the diazirine would likely insert into a wide variety of bonds on the protein surface. As such, it is unlikely to significantly alter the overall charge or pI of the carrier protein. In contrast, amide formation converts a positively charged lysine side chain into a neutral functional group; therefore, we expected substantial changes to protein charge and pI. High levels of charge on a protein can have significant effects on a variety of biophysical properties, including nonspecific adsorption due to modified electrostatic interactions.[55] A change of as few as ±3 charge units can alter the physiochemical properties of the peptide polymer chains and increase aggregation.[56,57] Studies have shown that more extensive modification of protein charge can significantly alter other properties, such as resistance to proteolysis and cell permeability.[58]

The effects of the solid-state and NHS-ester conjugation methods on overall conjugate charge and size were examined by native gel and isolelectric focusing (IEF; SI Figure S2A−C), as well as size exclusion chromatography (SEC, SI Figure S5). We observed a noticeable charge decrease with the GalNAc-β-PA-11-BSA conjugate, verifying the neutralization caused by formation of amide bonds. The size of the charge decrease was correlated with valency and, for high loaded conjugates, was easily distinguishable compared to the BSA standard. In contrast, the diazirine GalNAc-β-DZ-12-BSA conjugate (SI Figure S2A/B, lane 4) had a similar charge to BSA, and both high and low valency conjugates had the same mobility on the gel. These results were recapitulated in the IEF gel (SI Figure S2C). The diazirine-prepared GalNAc-β-DZ-12-BSA conjugate displays a pI of ∼4.7 (SI Figure S2C, lane 4) which is similar to that of BSA (pI ∼4.7). As expected, the isoelectric points of GalNAc-β-PA-11-BSA (pI ∼4.3, SI Figure S2C lane 2) displayed a greater shift as a result of amide bond formation and reduction of charged lysine residues. Despite the observed shifts in pI as a result of the conjugation methods, GalNAc-β-DZ-7-BSA and GalNAc-β-PA-11-BSA conjugates had similar profiles via SEC (SI Figure S6), indicating no significant differences in aggregation state.

Binding Affinity Evaluation of Conjugates. The most important factor when considering bioconjugation methods is bioactivity. To evaluate the effects of our solid-state conjugation methodology on binding affinity, we evaluated binding of a variety of terminal GalNAc-recognizing plant (HPA and VVL) and mammalian [Asialoglycoprotein Receptor (ASGPR) and MGL] lectins using ELISA. ASGPR and MGL are C-type binding lectins found on liver hepatocytes and dendritic cells, respectively. The ability to selectively target these lectins could be beneficial avenues for site-specific drug delivery and activating immune response.[59−61] To make comparisons between the diazirine

https://dx.doi.org/10.1021/acs.bioconjchem.0c00555
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and amide-linked conjugates, we measured binding at a series of concentrations and then determined the apparent binding constants (app K_D).

In most cases, the photoconjugation method provided significant benefits for binding over the amide-coupled glycoconjugate (Figure 1A–D and Table 2). The biggest effects were observed for VVL, which displayed 100-fold stronger binding to the diazirine conjugate (apparent K_D of 0.0067 nM for GalNAc-α-DZ-7-BSA and 0.81 nM for GalNAc-α-PA-7-BSA). HPA and MGL also had improved binding to the diazirine conjugate, although it was a smaller effect. In addition to affinity, all 4 lectins displayed at least some increase in total binding to the diazirine conjugate, with the largest effects on the maximum signal observed for HPA and ASGPR. Thus, in addition to affinity, the method of conjugation also affects the maximum number of lectin molecules that will bind to the glycoconjugate-modified surface. This improvement could be beneficial for the development of diagnostics where increased signal-to-noise is needed.

Table 2. Apparent K_D Values of Select Lectins to Conjugates

protein	lectin apparent K_D			
	HPA	VVL	MGL	ASGPR
BSA	N.D.	N.D.	N.D.	467 nM
GalNAc-α-phenylDZ-7	0.93 nM	0.0067 nM	32.3 nM	585 nM
GalNAc-α-phenylamide-7	1.6 nM	0.81 nM	74.1 nM	304 nM

Although we do not yet know the mechanistic basis for the improved binding to the diazirine conjugate, differences in electrostatic interactions could be a key contributor. BSA is a negatively charged protein at physiological pH, with an estimated net charge of −18 at pH 7.4. While the net charge does not change for the diazirine conjugate, the estimated net charge for the GalNAc-α-PA-7-BSA conjugate at pH 7.4 decreases to −25. This difference could manifest itself in several ways. First, it could influence the total number of glycoconjugate molecules that will stick to the surface of the ELISA plate. The surface may be able to accommodate more molecules of diazirine than amide conjugate due to less charge−charge repulsion. Second, the difference in net charge could influence overall binding via interactions between protein surfaces. Three of the four lectins we studied are also negatively charged at pH 7.4, with MGL at −10, VVL at −10, and ASGPR at −13.6; HPA is close to neutral with a net charge of +1.6. This effect could involve localized charge−charge interactions near the binding pockets or secondary interactions that influence the total number of lectins and molecules that can be in close proximity to each other. In addition to charge, the spacing and orientation of glycans on the carrier protein could also be affected. Additional studies will be needed to more fully understand these effects.

Immunological Evaluation of Conjugates. Immunogenicity can be beneficial for the development of vaccines and antibodies, but it can be detrimental in other cases such as targeting endogenous lectins. To evaluate the effects of the conjugation method on immunogenicity, HSA conjugates were prepared with **8a** and **9a** (conjugates GalNAc-α-DZ-10-HSA and GalNAc-α-PA-10-HSA, respectively). Two groups of 5 BALB/c mice were immunized with either GalNAc-α-DZ-10-HSA or GalNAc-α-PA-10-HSA. Sera were collected from each mouse pre-immunization and on days 24, 38, and 52 after

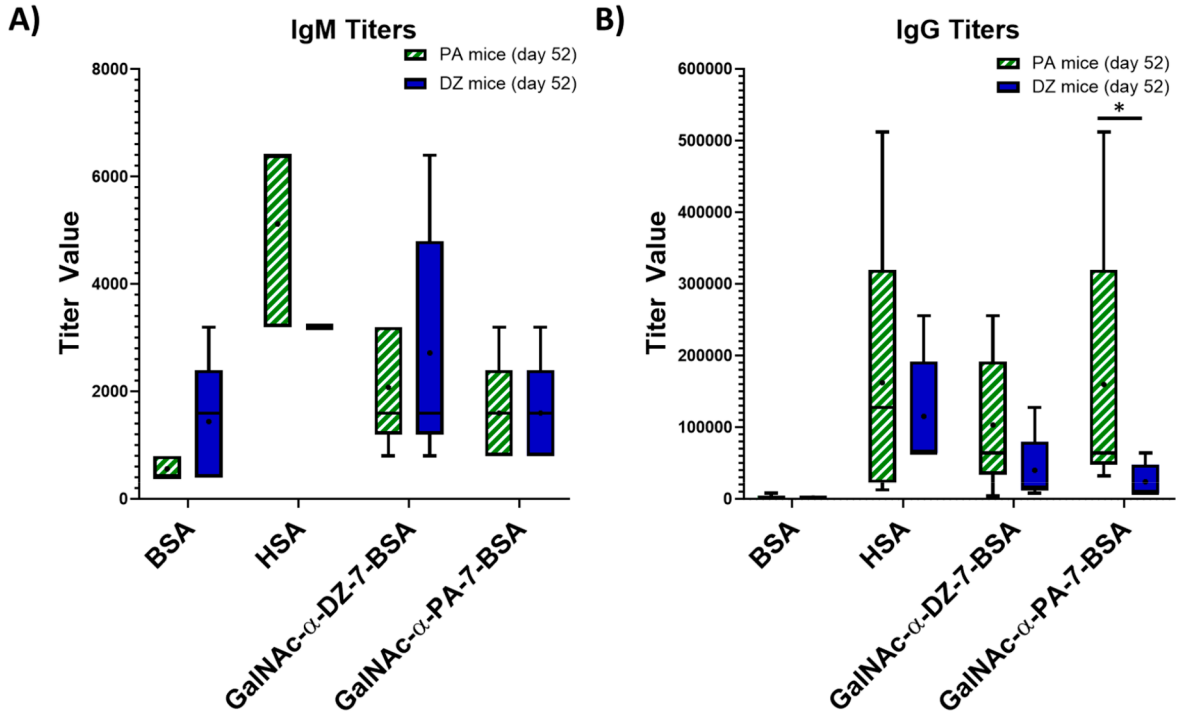

Figure 2. IgM and IgG titers for conjugates. (A) IgM titers, (B) IgG titers. Samples: green striped bars − GalNAc-α-PA-10-HSA final bleed sera (day 52), dark blue bars − GalNAc-α-DZ-10-HSA final bleed sera (day 52). Boxes display the mean titer values (horizontal line) of each group of 5 mice ± SD. The whiskers correspond to ±2SD. IgM and IgG titers on day 52 were performed in triplicate for each mouse. *P*-values were determined using the Mann−Whitney Test. *($p < 0.05$) shows a statistically significant difference in IgG titers to the GalNAc-α-PA-7-BSA conjugate between the serum antibodies on day 52 from the two groups of mice.

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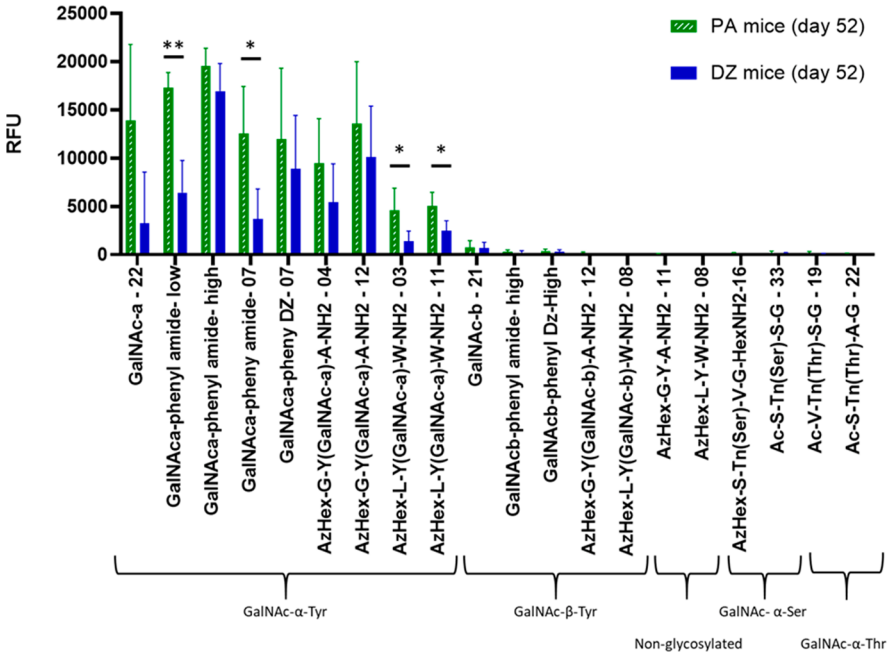

Figure 3. IgG selectivity to GalNAc-α and GalNAc-β and GalNAc-a-S/T/Y peptides. Final bleed sera GalNAc-α-PA-10-HSA mice − dark green striped bars; Final bleed sera GalNAc-α-DZ-10-HSA mice − dark blue bars. The final bleed (day 52) IgG selectivity for each mouse was evaluated on the glycan microarray once at a 1:400 serum dilution. Plots display the mean with ± SD based on RFU of the 5 mice in each group. *P*-values were determined using the Mann−Whitney Test. **($p < 0.01$) and *($p < 0.05$) show statistically significant differences in IgG response of day 52 serum antibodies between the two groups of mice to various array components..

immunization and subsequent boosters as shown in SI Figure S7. Once the study was completed, serum antibody titers, as well as serum antibody specificity, were evaluated.

Preimmunization, first draw (day 24), and post-immunization (day 52) sera IgM and IgG titers from each mouse were evaluated against the protein carrier (HSA), GalNAc-α-DZ-7-BSA, GalNAc-α-PA-7-BSA, and BSA (Figure 2A,B, SI Figures S8−S9). IgM responses were only evaluated in sera preimmunization and on day 52, the final day of the study. As expected, all mice possessed minimal IgM responses to the various components before immunization. IgM responses increased by the end of the immunization schedule but were substantially lower than IgG responses in both groups of mice. This response was anticipated due to general duration of IgM immune response and class switching during the course of the study. After 24 days, all mice developed IgG responses to both GalNAc conjugates and proteins, and the titers increased substantially after 52 days. Interestingly, the amide-linked conjugates produced higher titers than the diazirine at both time points (SI Figure S9). On day 24, the mice immunized with GalNAc-α-PA-10-HSA possessed 2.4-fold and 2.8-fold stronger responses for the GalNAc-α-DZ-7-BSA and GalNAc-α-PA-7-BSAconjugates, respectively. On day 52, the IgG responses from the mice immunized with GalNAc-α-PA-10-HSA were 2.6-fold and 6.7-fold stronger for the GalNAc-α-DZ-7-BSA and GalNAc-α-PA-7-BSA conjugates, respectively. IgG titers for the carrier protein, HSA, were not significantly different indicating that the effects discussed above were due to the hapten/linker/conjugation method and not an unexpected difference in immune capacity for the groups of mice. The mechanistic basis for the difference in immunogenicity is not yet clear. Prior studies have shown that certain glycopeptide fragments can be presented on major histocompatibility class II (MHCII), initiating a carbohydrate specific CD4+ T cell and cytokine response.[62] Glycans linked to lysine residues may provide a higher proportion of suitable glycopeptides relative to a more random conjugation strategy.

In addition to appraising the immunological strength of the diazirene and NHS-ester conjugates, we evaluated the selectivity and breadth of the IgM and IgG serum antibodies pre-immunization and on day 52 using a 740-component carbohydrate microarray (Supporting Information). Our glycan microarray contains a diverse assortment of N-linked and O-linked glycans, glycolipid glycans, glycopeptides, and glycoproteins.[7,63,64] In particular, it contained 154 components with a terminal GalNAc residue.

Both haptens induced antibody responses with narrow selectivity (Figure 3). As anticipated, both groups of mice did not possess substantial IgM or IgG antibodies to GalNAc-α and GalNAc-β-glycoconjugates or GalNAc-glycopeptides before immunization with the glycoconjugates (SI Figures S10−S11, SI Table S4). After 52 days, both groups displayed robust IgG responses that were highly selective for GalNAc-α-Tyr glycoconjugates. Modest IgM responses to GalNAc-α-glycoconjugates and GalNAc-α-Ser/Thr/Tyr peptides were also observed. Aside from the antibodies that are naturally present in a mouse,[18,65] little or no signal was observed for any other glycans on the array, including GalNAc-α-Ser/Thr glycopeptides, blood group A [GalNAcα1−3(Fucα1−2)Gal], GalNAcα1−6Gal, and GalNAcα1−3Gal. Within the subset of GalNAc-Tyr related glycoconjugates and glycopeptides, induced antibodies displayed selectivity for α- over β-linked GalNAc for both conjugates. Depending on the array component, IgG antibodies were 3−15 times more selective for GalNAc-α than the corresponding GalNAc-β construct. The IgG response to the diazirine conjugated immunogen was more selective for the hapten and GalNAc-Tyr peptides than the response to the amide-linked immunogen or GalNAc

https://dx.doi.org/10.1021/acs.bioconjchem.0c00555
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residues linked through a non-phenyl-containing mercaptoethylaminoglutarate linkage (GalNAc-α-22, GalNAc-β-21) to the protein carrier.

CONCLUSIONS

Photoconjugation reactions offer numerous potential advantages for the preparation of bioconjugates. The main disadvantage has been extremely low yields, making it nearly impossible to produce conjugates with high valency. By devising a solid-state approach, we were able to reduce side reactions and significantly improve the yield. While additional improvements in efficiency would be beneficial, a 10−12% yield is sufficient to prepare multivalent conjugates with a valency of 10 units/protein while also using practical amounts of carbohydrate hapten (80 equiv). The approach is compatible with different proteins as well as multiple buffers and pHs.

The conjugates produced via the photoreaction have several beneficial features. First, they have minimal alteration to protein charge, which may help reduce nonspecific adsorption to surfaces, improve protein stability and solubility, and minimize changes to other biophysical properties of the protein. Second, the glycoconjugates exhibit up to 100-fold enhanced binding to plant and mammalian lectins when compared to a similar GalNAc-containing glycoconjugates constructed with amide linkages to lysine residues. Third, the photoreactive glycoconjugates were significantly less immunogenic in mice as compared to the corresponding amide-linked glycoconjugate. The unique combination of high affinity and low immunogenicity make glycoconjugates prepared via photoconjugation especially useful for *in vivo* targeting of endogenous lectins. They may also be useful as capture ligands for various *in vitro* assays, such as glycan microarrays.

MATERIALS AND METHODS

Materials and experimental procedures are included in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00555.

Additional synthetic methods, characterization of compounds, characterization of protein conjugates, and supporting tables and figures (PDF)

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All authors contributed to the writing of the manuscript and have given approval of the final version. All experimental work was carried out by MDC.

Funding

This work was supported by the intramural research program of the National Institutes of Health.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the Consortium for Functional Glycomics (GM62116; The Scripps Research Institute), X. Huang (Michigan State University), T. Tolbert (University of Kansas), Lai-Xi Wang (University of Maryland), J. Barchi (National Cancer Institute), T. Lowary (University of Alberta), Omicron Biochemicals Inc., GlycoHub, and Glycan Therapeutics and for generously contributing glycans for the array. This work was supported by the Intramural Research Program of the National Cancer Institute, NIH.

ABBREVIATIONS

GalNAc, N-Acetylgalactosamine; ASGPR, Asialoglycoprotein Receptor; HPA, *Helix pomatia Agglutinin*; MGL, Macrophage Galactose Lectin; VVL, *Vicia Villosa* Lectin

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