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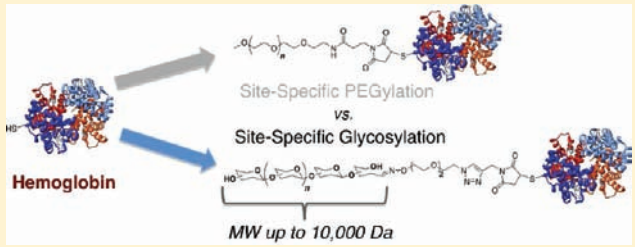
Site-Selective Glycosylation of Hemoglobin with Variable Molecular Weight Oligosaccharides: Potential Alternative to PEGylation

Thomas J. Styslinger,[†] Ning Zhang,[‡,∥] Veer S. Bhatt,[†,§] Nicholas Pettit,[†,‡] Andre F. Palmer,*[,∥,‡] and Peng G. Wang*[,†,‡,§]

[†]Department of Chemistry, [‡]Biochemistry Program, [§]Biophysics Program, and [∥]William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio 43210, United States

Supporting Information

ABSTRACT: Poly(ethylene glycol) (PEG) conjugation (i.e., PEGylation) is a commonly used strategy to increase the circulatory half-life of therapeutic proteins and colloids; however, few viable alternatives exist to replicate its functions. Herein, we report a method for the rapid site-selective glycosylation of proteins with variously sized carbohydrates, up to a molecular weight (MW) of 10 000, thus serving as a potential alternative for PEGylation. More importantly, the method developed has two unique features. First, traditional protecting group strategies that typically accompany the modification of the carbohydrate fragments are circumvented, allowing for the facile site-selective glycosylation of a desired protein with variously sized glycans. Second, the methodology employed is not limited by oligosaccharide size; consequently, glycans of MW similar to that of PEG, used in the PEGylation of therapeutic proteins, can be employed. To demonstrate the usefulness of this technology, hemoglobin (Hb) was site-selectively glycosylated with a series of carbohydrates of increasing MW (from 504 to ∼10 000). Hb was selected on the basis of the vast wealth of biochemical and biophysical knowledge present in the literature and because of its use as a precursor in the synthesis/formulation of artificial red blood cell substitutes. Following the successful site-selective glycosylation of Hb, the impact of increasing the glycan MW on Hb's biophysical properties was investigated in vitro.



INTRODUCTION

The covalent coupling of poly(ethylene glycol) (PEG) to therapeutic proteins and colloids, PEGylation, has long provided an effective means to increase the pharmacological effectiveness of such biopharmaceuticals.[1−4] PEGylation serves to increase the hydrodynamic radius of the protein/colloid, effectively shielding its surface and preventing recognition by the reticuloendothelial system. Benefits of PEGylation include increased circulatory half-life, decreased degradation by metabolic enzymes, and reduced protein immunogenicity. However, despite the numerous benefits of PEGylation, questions regarding its universal application have arisen in the literature.[5,6] In certain cases, PEGylation has been observed to promote decreased biological activity and can accumulate over long periods of treatment, giving rise to macromolecular syndrome.[3]

Currently, various groups are exploring potential alternatives to PEGylation for improving the pharmaceutical effectiveness of therapeutic proteins.[7] In certain cases, inspiration has been taken from nature, in which the glycosylation of proteins, with even small oligosaccharides, functions in much the same way as PEGylation.[8,9] Similar to PEGylation, glycosylation provides a large hydration shell capable of protecting proteins from proteolysis.[10] Interestingly though, glycosylation has the added benefit of being able to stabilize the protein by forming hydrogen bonds with a protein's amino acid residues. Furthermore, glycosylation has been shown to increase thermal stability while decreasing precipitation and aggregation.[11,12] Consequently, due to the importance of glycoproteins in nature, the glycosylation of proteins in the laboratory (glycoprotein synthesis) has long been an area of interest among researchers, and many elegant methods exist in the literature.[13,14]

Unfortunately, two limiting factors are frequently associated with the ability to chemically glycosylate a protein in the laboratory. The first is the rate at which the carbohydrate derivatives, or carbohydrate linkers, used in protein glycosylation can be synthesized—a bottleneck mainly due the tedious protecting group strategies that accommodate carbohydrate modification, as shown in Figure 1A.[13,14] The second limiting factor is that current methods for protein glycosylation are conceivably confined to small oligosaccharides due, in part, to the low solubility of large oligosaccharides and polysaccharides in many organic solvents. This low solubility, or lack thereof, significantly reduces the routes available for glycan modification necessary to employ them in glycoprotein synthesis. Consequently, on the basis of these limitations, we sought to develop a method that would circumvent carbohydrate protecting group chemistry altogether and be

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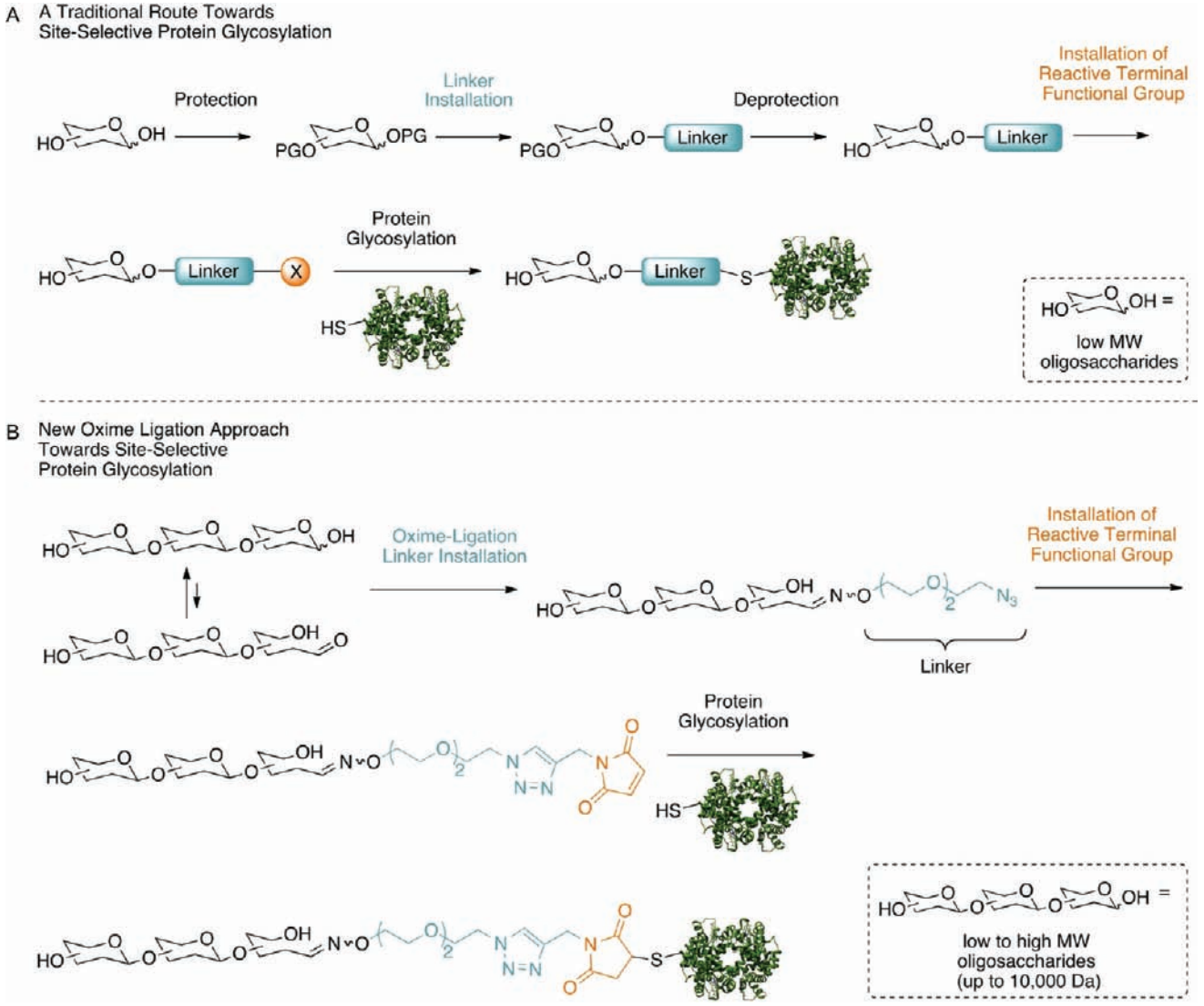

Figure 1. (A) Traditional route toward glycoprotein synthesis involving protecting group manipulation. (B) New oxime-ligation approach avoiding the need for protecting group manipulation.

aqueous-friendly, thus allowing a desired protein to be glycosylated with a wide variety of carbohydrates, regardless of size.

Herein, we report a versatile method for the site-selective glycosylation of proteins that requires no protecting group chemistry on the carbohydrate fragment and, importantly, allows for the use of large molecular weight (MW) oligosaccharides. By taking advantage of the aldehyde available in the open-chain conformation on the reducing end carbohydrate, we were able to employ oxime-ligation chemistry for the installation of a linker fragment (Figure 1B). It is important to note that this method enabled us to employ glycans of MW comparable to that of PEG, commonly used in the PEGylation of therapeutic proteins, to be conjugated site-selectively to proteins. Thus, this method of site-selectively glycosylating proteins with high MW oligosaccharides may provide a new viable alternative to site-selective PEGylation, as carbohydrates are biodegradable, whereas PEG is not, and carry with them further advantageous properties. In addition, the ease by which this method can be employed may allow for the facile fine-tuning of the pharmacokinetic and pharmacodynamic properties of therapeutic proteins, as is done with the PEGylation of similar therapeutics. To the best of our knowledge, this is the first reported method for the site-selective chemical glycosylation of proteins with oligosaccharides of such high MWs.

To demonstrate the usefulness of this methodology, hemoglobin (Hb) was site-selectively glycosylated with three different maltose-type sugars (MWs ranging from 504 to 1153) and a dextran sugar (MW ~10 000). After determination that the glycosylation reactions were successful and indeed site-selective, the biophysical properties of the oligosaccharide-conjugated Hb's were subsequently studied in vitro. Hb was chosen as the test protein for the conjugation chemistry on the basis of several factors, including (1) the significant amount of information present in the literature regarding Hb's biochemistry, (2) the feature of bovine Hb (bHb) containing one cysteine residue on the β chain at position 93 and none on the α chain, making site-selective modification possible, and (3) the potential of Hb to serve as an oxygen therapeutic for use in transfusion medicine to treat moderate to severe blood loss.[15−17] Thus, using thiol-targeting chemistry, we were able to successfully conjugate various MW glycans to the two cysteine residues on the Hb tetramer ($\alpha_2\beta_2$) and, subsequently,

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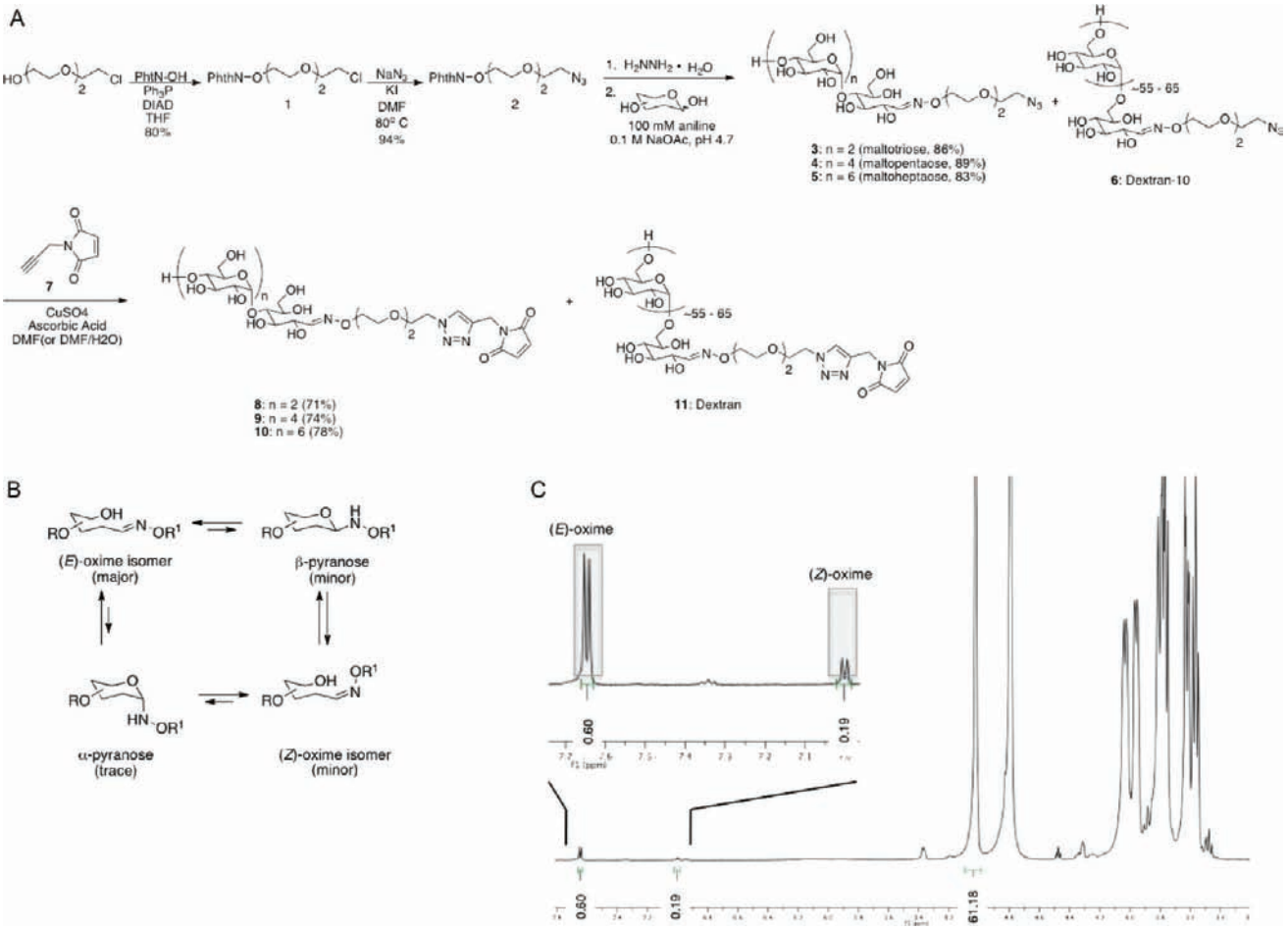

Figure 2. (A) Synthesis of the aminooxy-bearing linker and subsequent synthesis of maltotriose, maltopentaose, maltoheptaose, and dextran linkers. (B) Ring−chain tautonomers of the oximine-ligation products, including (*E*)-oxime, (*Z*)-oxime, *β*-pyranose, and *α*-pyranose.[24,36] (C) ^{1}H NMR of dextran linker **6** showing the presence of the (*E*)-oxime (0.6H) and (*Z*)-oxime (0.19H) H-1, with the integration of the C1−H of the repeating glucose residues being (61.18H).[37]

investigate the biophysical effects of glycosylation. This methodology not only allowed us to explore the potential of glycosylated Hb to serve as an oxygen therapeutic, but also holds the promising ability to be applied to a wide variety of other therapeutic proteins bearing naturally occurring and accessible cysteine residues or to therapeutic proteins that have undergone site-directed mutagenesis to incorporate cysteine residues.

■ RESULTS AND DISCUSSION

Design of the Carbohydrate Linkers. Typically, site-selective protein glycosylation occurs in a fashion similar to that depicted in Figure 1A in which the chosen glycan is chemically modified and then allowed to react with the desired protein.[13,14,18] Chemical modification of the glycan typically involves initial protection of the hydroxyl groups followed by activation at the anomeric position, thus allowing for the subsequent installation of a substituent (referred to as the linker herein) responsible for linking the glycan moiety to the desired protein. Lastly, the carbohydrate must be deprotected and the linker modified to accommodate a functional group capable of reacting site-selectively with the protein. Consequently, when new glycans are chosen for protein glycosylation, an exponentially greater number of synthetic steps are required, slowing the rate at which a protein can be glycosylated with various glycans and subsequent investigation of the biophysical effects of glycosylation.

Thus, in an attempt to abstain from the use of this lengthy process, we searched for a method that would circumvent the requirement of protecting the desired glycan and be aqueous-friendly, allowing for the use of high MW oligosaccharides not soluble in most organic solvents. As a result, we chose to employ an oxime ligation for the installation of a linker and "click" chemistry for its subsequent modification to allow for site-selective glycosylation (Figure 1B). Employing an oxime ligation allowed us to take advantage of the ability of an aminooxy group to react with a ketone or aldehyde—in our case, the aldehyde present in the open-chain conformation on the reducing end carbohydrate of the oligosaccharide—forming an oxime bond. Notably, oxime-ligation conjugates have been shown to be stable under physiological conditions and a wide pH range.[19−21] This type of oxime-ligation chemistry has previously been applied to the development of carbohydrate microarrays,[22,23] glyconanoparticles,[24,25] and solution-phase tagging of glycans.[26−28] Furthermore, at the time of conception, it was our belief that the addition of aniline to the oxime ligation would catalyze the reaction, functioning as a nucleophilic catalyst under slightly acidic conditions, as noted by Dawson and co-workers, but which had yet to be applied to carbohydrate chemistry.[29] Confirming our suspicions, while we

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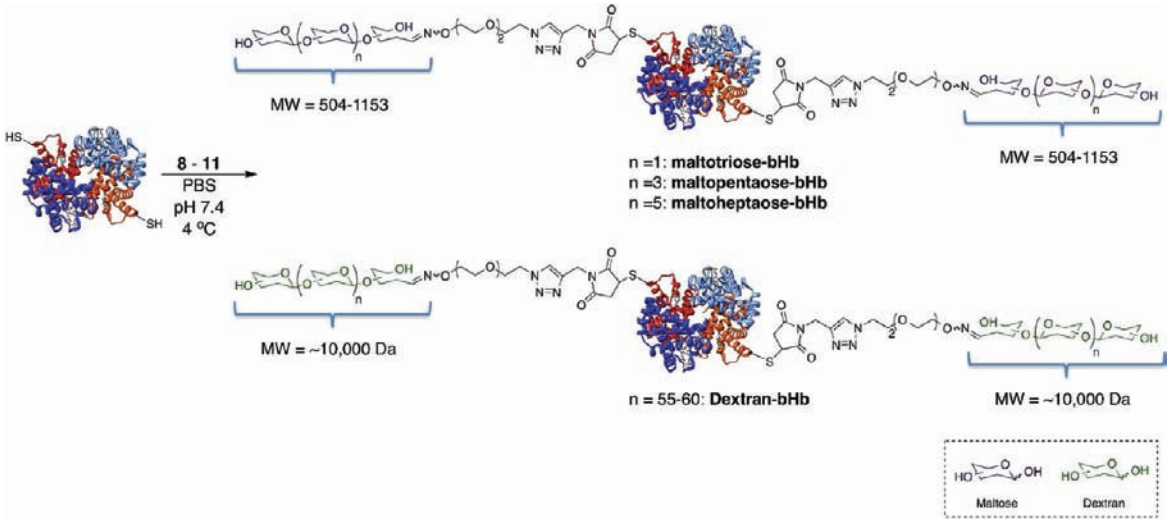

Figure 3. Synthesis of maltotriose−bHb, maltopentaose−bHb, maltoheptaose−bHb, and dextran−bHb.

were carrying out the synthetic portion of this project, Jensen and co-workers published a paper diligently detailing the effects of nucleophilic catalysis on carbohydrate oxime formation by aniline.[30] Accordingly, the linker was designed to incorporate an aminooxy functional group on one end and an azido group on the opposite end, with the two being separated by a short oligoethylene glycol spacer. The aminooxy group was selected to partake in the aforementioned oxime ligation, while the azido group was selected to remain inert during the oxime ligation and to subsequently allow for the installation of a maleimide group—capable of site-selectively reacting with the cysteine residues of a protein—via a CuAAC (copper(I)-catalyzed azide−alkyne cycloaddition) reaction. It is important to note that the overall design of the synthesis was constructed to allow for easy scale-up with a minimal number of synthetic steps.

Four differently sized carbohydrates, spanning a MW range of 504−10 000, were chosen for the glycosylation of bHb. Of the four chosen carbohydrates, three were maltose sugars, maltotriose, maltopentaose, and maltoheptaose, with the other being a dextran sugar with an average MW of 10 000. Both the maltose and dextran sugars are oligomers of glucose with differing linkages, the maltoses being composed of $\alpha(1\rightarrow4)$ linkages and the dextran being composed of $\alpha(1\rightarrow6)$ linkages. Although the maltose carbohydrates have a lower MW range (504−1153), conjugation of smaller oligosaccharides could still be advantageous on the basis of reports that PEGylation with low MW PEG (300−2500) effectively increases the pharmacokinetics and immunogenicity profiles of therapeutic proteins.[31] The dextran glycan, in addition to being of a much higher MW—comparable to that of PEG commonly used in therapeutic protein conjugation—belongs to a family of carbohydrates (dextrans) that are utilized as plasma expanders, coatings for certain biomedical devices, and components of biomedical hydrogels.[32−35]

Synthesis of the Carbohydrate Linkers. Synthesis of the carbohydrate linkers is depicted in Figure 2A and began with 2-(2-[2-chloroethoxy]ethoxy)ethanol to which a protected aminooxy functional group was installed via a Mitsunobu reaction with *N*-hydroxyphthalimide, giving substrate **1**. Subsequent azide displacement of the chloro group gave linker **2**, which was capable of being synthesized on a multigram scale.

Following the removal of the phthalimide protecting group using hydrazine monohydrate, oxime ligations between linker **2** and the desired glycans were carried out under slightly acidic conditions (pH 4.7) in the presence of 100 mM aniline, giving the carbohydrate−azide linkers **3**−**6**. All reactions proceeded with relatively high yields and were purified by size exclusion chromatography and subsequently characterized by LC−MS, ^{1}H NMR, and ^{13}C NMR. The oxime-ligation products **3**−**6** can exist as several ring−chain tautonomeric forms (Figure 2B), of which the (*E*)-oxime and (*Z*)-oxime predominate over the α- and β-pyranoses, with each giving rise to distinctive ^{1}H NMR and ^{13}C NMR peaks for H-1 and C-1 of the conjugated glycan.[24,30] Importantly, the ^{1}H NMR of the dextran linker **6** (Figure 2C) confirmed that the aminooxy linker **2**, once deprotected, was capable of reacting with the high MW dextran oligosaccharide as shown by the presence of the (*E*)-oxime and (*Z*)-oxime H-1 ^{1}H NMR peaks. With the assembly of the four carbohydrate−azide linkers complete, a maleimide functional group was installed via a CuAAC reaction between linkers **3**−**6** and compound **7**, which itself was synthesized by a short one-step procedure (see the Supporting Information), giving the desired carbohydrate maleimide linkers **8**−**11**.

Preparation and Glycosylation of bHb. bHb is a readily available type of Hb capable of being purified on a multikilogram scale, making it an attractive precursor for the synthesis/formulation of oxygen therapeutics.[38,39] Like other forms of Hb, bHb is a tetramer consisting of two identical α chains and two identical β chains. More importantly for our purposes, the two β chains both display one cysteine residue (Cys-93), which has been previously shown to be capable of modification with maleimide-modified compounds.[18,40]

Using a minimal excess of the synthesized glycan linkers **8**−**11**, bHb was successfully site-selectively glycosylated via a maleimide conjugation reaction—introducing a novel chiral center on the resultant succinimide thioether—giving the desired maltotriose−bHb, maltopentaose−bHb, maltoheptaose−bHb, and dextran−bHb glycoconjugates (Figure 3). After purification, the glycosylated bHb samples were submitted for analysis by MALDI, LC−MS, and tryptic digest followed by LC−MS/MS. Initial analysis by MALDI for the linker-treated bHb showed successful glycosylation of the β subunit (Figure 4A). Glycosylation of the β subunit was evident

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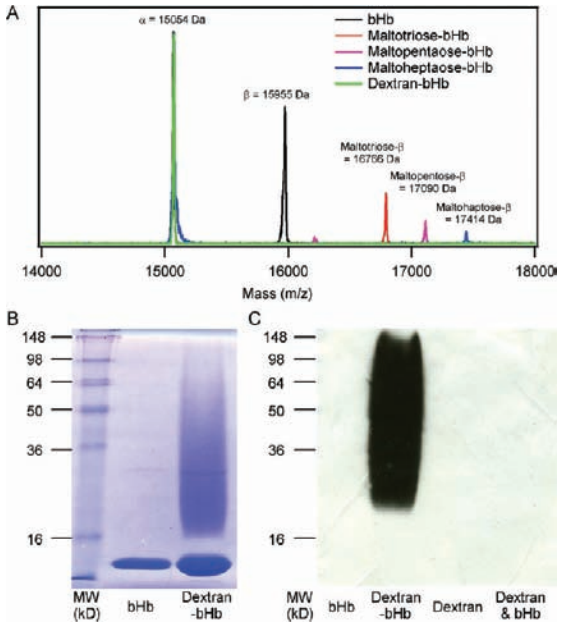

Figure 4. (A) MALDI-ESI of bHb, maltotriose–bHb, maltopentaose–bHb, maltoheptaose–bHb, and dextran–bHb. (B) SDS–PAGE of bHb and dextran–bHb. (C) Western blot of bHb, dextran–bHb, dextran, and dextran–bHb.

through the complete disappearance of the MW peak corresponding to the native β subunit (15 955) and the emergence of a new peak corresponding to that of the carbohydrate-modified β subunit. For instance, when bHb was treated with the maltotriose linker (MW 811), the detected mass shifted from the original mass of 15 955 Da to 16 766 Da (β subunit + linker). For bHb treated with the dextran linker, the disappearance of the native β subunit MW was observed; however, the presence of the dextran-modified β subunit could not be detected by MALDI due to the interference of ionization imposed by the large oligosaccharide dextran, a trait commonly seen with proteins bearing large oligosaccharides.[41] Thus, to further analyze the dextran-modified Hb and confirm glycosylation, SDS–PAGE and Western blot analysis, with an antidextran antibody, were performed (parts B and C, respectively, of Figure 4).[42,43] Both the SDS–PAGE and Western blot analysis showed a large shift in the MW of the β subunit, with the Western blot staining positive for dextran in the region corresponding to the modified β subunit. Further analysis of the glycosylated bHb by LC–MS demonstrated that the glycosylation reaction went to completion, leaving no unmodified β-subunit, and showed an increase in MW corresponding to the linker that the bHb was treated with, as was observed by MALDI. Furthermore, completion of the glycosylation reaction was evident in the RP-HPLC chromatograms of the LC–MS analysis, in which only two peaks were present, corresponding to the α and modified β subunits. Deconvolution of the ESI mass spectra for the peaks corresponding to the modified β subunits yielded molecular ions with masses of 15 955, 16 767, 17 091, and 17 415 Da, correlating to native bHb (Figure S1, Supporting Information), maltotriose–bHb (Figure S2, Supporting Information), maltopentaose–bHb (Figure S3, Supporting Information), and maltoheptaose–bHb (Figure S4, Supporting Information), respectively. Additionally, trypsin digestion, followed by LC–MS/MS analysis, was used to confirm that Cys-93 of the β subunit was indeed the site of glycosylation (Tables S1–S3, Supporting Information), with the results indicating that the maleimide moiety was present in the open maleimic acid form due to the partial hydrolysis of the succinimidyl thioether that occurred during the procedure—a side reaction that has been reported in the literature.[44]

Evaluation of the Biophysical Properties of bHb. After confirming that bHb was indeed glycosylated at the Cys-β93 position, we proceeded to study the effect of glycosylation on the biophysical properties of bHb. In particular, we were interested in examining the effect of glycosylation on the equilibria between oxygen and bHb, the secondary structure and heme environment of bHb, the kinetics of haptoglobin (Hp) binding to bHb, and the 3-dimensional structure of bHb.

Oxygen–bHb/Glycosylated bHb Equilibria. Hb is primarily responsible for gaseous ligand storage and transport in the circulatory system and thus has received significant attention as a precursor for the synthesis/formulation of oxygen therapeutics, i.e., Hb-based oxygen carriers (HBOCs).[15−17] Consequently, in this study, the equilibria between oxygen and bHb/glycosylated bHb was measured to investigate the effects of glycosylation on the oxygen affinity (P_{50}) and cooperativity coefficients (n) of bHb. P_{50} is an indicator of oxygen affinity and refers to the partial pressure of oxygen (p_{O_2}) at which half the HBOC is saturated with oxygen. The cooperativity coefficient (n) of Hb is associated with the allosteric transition from the tense (T) to the relaxed (R) quaternary state upon oxygenation.[45] For native bHb, P_{50} was determined to be 27.61 mmHg, while the glycosylated Hb's had values ranging from 12 to 16 mmHg (Table 1). It is apparent from the oxygen equilibrium curves (Figure 5A), decreased cooperativity coefficients, and decreased P_{50} values (Table 1) that the glycosylated Hb's possess a higher affinity for oxygen compared to native Hb. The observed reduction in cooperativity upon glycosylation of Cys-β93 indicates a reduction in the available motion of the $\alpha_1\beta_2$ interface in which the Cys-β93 is located. Similar modifications of Cys-β93 by other chemical agents have been shown to have an analogous effect by influencing the heme pocket of the β chain.[46−48]

Interestingly, the low P_{50} of glycosylated Hb's may be beneficial in any future development of glycosylated Hb's as potential HBOCs. This lies in the fact that one of the few HBOCs to reach phase III clinical trials, Hemospan (PEGylated human Hb), has a low P_{50} value of 5–6 mmHg, whereas other HBOCs that have failed in phase II clinical trials typically lie in the range of 26–38 mmHg.[49,50] It has been postulated that lower P_{50} values may be beneficial by limiting arteriole oxygen transport, thus targeting oxygen transport to tissues and organs with low partial pressure and avoiding induced high blood

Table 1. Oxygen Affinity (P_{50}) and Cooperativity Coeffient (n) of Native bHb and Glycosylated bHb's

	bHb	maltotriose–bHb	maltopentaose–bHb	maltohaptaose–bHb	dextran–bHb
P_{50} (mmHg)	27.61 ± 0.02	15.75 ± 0.01	12.57 ± 0.01	14.09 ± 0.01	14.91 ± 0.01
n	2.862 ± 0.010	2.106 ± 0.004	1.835 ± 0.004	2.002 ± 0.004	2.065 ± 0.004

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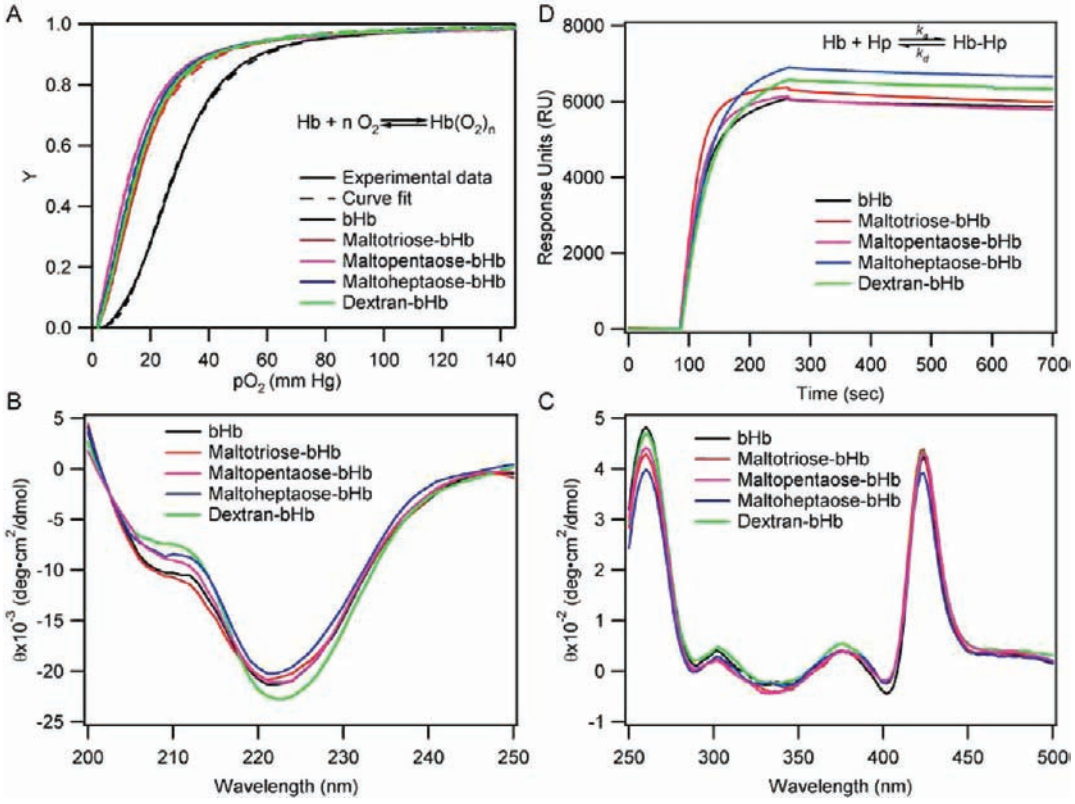

Figure 5. (A) Oxygen equilibrium curves of bHb, maltotriose−bHb, maltopentaose−bHb, maltoheptaose−bHb, and dextran−bHb. CD spectra of native and glycosylated Hb's in the (B) far-ultraviolet region and (C) near-ultraviolet region. (D) SPR analysis of native and glycosylated bHb's.

Table 2. Association/Dissociation Rate Constants and Equilibrium Dissociation Constant of Native and Glycosylated bHb's

	bHb	maltotriose−bHb	maltopentaose−bHb	maltohaptaose−bHb	dextran−bHb
k_a ($M^{-1}\cdot s^{-1}$)	$(1.30 \pm 0.01) \times 10^5$	$(1.83 \pm 0.13) \times 10^5$	$(1.49 \pm 0.07) \times 10^5$	$(1.12 \pm 0.05) \times 10^5$	$(1.00 \pm 0.04) \times 10^5$
k_d (s^{-1})	$(5.23 \pm 0.04) \times 10^{-5}$	$(10.00 \pm 0.05) \times 10^{-5}$	$(8.85 \pm 0.06) \times 10^{-5}$	$(6.45 \pm 0.04) \times 10^{-5}$	$(8.23 \pm 0.07) \times 10^{-5}$
K_D (M)	$(4.02 \pm 0.03) \times 10^{-10}$	$(5.46 \pm 0.10) \times 10^{-10}$	$(5.94 \pm 0.07) \times 10^{-10}$	$(5.76 \pm 0.05) \times 10^{-10}$	$(8.23 \pm 0.06) \times 10^{-10}$

pressure caused by an autoregulatory response to high arteriole O_2 levels, resulting in vasoconstriction.[51]

Circular Dichroism (CD) Analysis. To investigate the effects of glycosylation on the structural characteristics of Hb and its glycosylated variants, the CD spectra were obtained, in the far-ultraviolet (200−250 nm) region (Figure 5B) and near-ultraviolet (250−500 nm) region (Figure 5C). An initial concern in performing CD spectroscopy was the potential of the conjugated carbohydrates themselves to alter the obtained spectra of the modified Hb; however, it is known that the absorbance of unsubstituted polysaccharides does not lie above wavelengths greater than 200 nm, and thus, the spectra obtained were expected to yield accurate information about the secondary structure and heme environment of glycosylated Hb's.[52] Comparison of the glycosylated and native Hb spectra in the far-ultraviolet region reveals that glycosylation does not adversely affect the structure of bHb as indicated by the analogous spectra. In the near-ultraviolet spectra, the L band (in the vicinity of 260 nm) provides information indicative of the heme ligand, and examination of this region shows no significant change in intensity upon glycosylation. Likewise, investigation of the Soret region (around 420 nm), which provides information regarding the interactions of the heme prosthetic group with surrounding aromatic residues, shows little deviation for the glycosylated Hb compared to native Hb. Overall, glycosylation does not appear to alter the secondary structure and heme environment of bHb, as indicated by examination of the CD spectra.

Surface Plasmon Resonance (SPR) Analysis. Haptoglobin−hemoglobin (Hp−Hb) complexation is an important biological process in which Hp binds to free Hb in the blood to effectively remove it from systemic circulation.[53] Thus, SPR analysis was used to investigate the effect of glycosylation at Cys-β93 on the formation of Hp−Hb complexes. The binding−dissociation curves (Figure 5D) indicate that glycosylation at Cys-β93 has only a slight influence on the interaction between glycosylated bHb and Hp. Compared to bHb with an association rate constant (k_a) of 1.30×10^5 $M^{-1}\cdot s^{-1}$, maltotriose and maltopentaose modification resulted in a slightly higher association rate constant (1.83×10^5 and 1.49×10^5 $M^{-1}\cdot s^{-1}$, respectively), while maltoheptaose and dextran modification resulted in a lower association rate constant, 1.12×10^5 and 1.30×10^5 $M^{-1}\cdot s^{-1}$, respectively (Table 2). Furthermore, glycosylation increased the dissociation rate constant (k_d) from 5.23×10^{-5} s^{-1} for native bHb to a value greater than 6.45×10^{-5} s^{-1} for all four forms of glycosylated bHb (Table 2). Collectively, glycosylation increased the equilibrium dissociation constant (K_D) from 4.02×10^{-10} M to a value above 5.46×10^{-10} M for the lower MW glycosylated forms of bHb, with the dextran−bHb reaching as high as 8.23 ×

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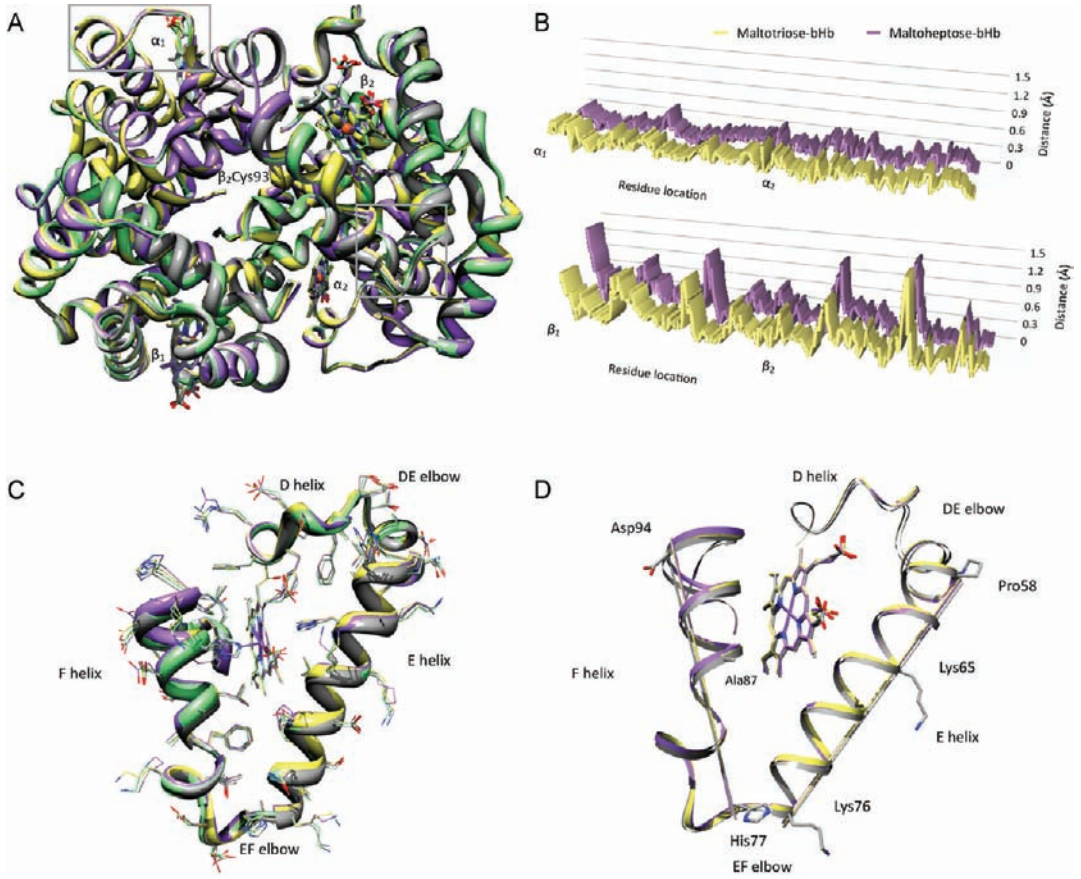

Figure 6. (A) Superposition of bHb (gray), maltotriose−bHb (yellow), maltopentaose−bHb (green), and maltoheptaose−bHb (purple). The overall tertiary and quaternary structures of all three glycosylated bHb's are quite similar to those of native bHb. Gray rectangles enclose two regions exhibiting conformational change: the CE elbow on the α subunit (top left rectangle) and the EF elbow on the β subunit (bottom right rectangle). (B) Variable deviation from native bHb in the α and β subunits of maltotriose−bHb (yellow) and maltoheptaose−bHb (purple). Five residues at the N- and C-termini of each of the four subunits were deleted, and the C_α atoms of all of the remaining residues were used to generate an optimal superposition. The peaks represent the separation of a C_α atom of each of the glycosylated Hb's from the corresponding C_α atom of native bHb. (C) Overall preservation of the side chain conformations in the "EF" region of the β_1 subunit. The side chains of bHb (gray), maltotriose−bHb (yellow), maltopentaose−bHb (green), and maltoheptaose−bHb (purple) are depicted as thin wires. (D) Axial displacement of E and F helices of the β_1 subunit upon glycosylation. Axes were computed through the geometric centroid for C_α atoms of three residues on the E helix and C_α atoms of residues selected within the EF elbow, middle of the F helix, and the carboxy end of the F helix. The axes passing through the maltotriose−bHb (yellow) and maltoheptaose−bHb (purple) are in a very similar position, especially on the F helix. This position is distinct from the axes passing through the corresponding position of native bHb (gray).

10^{-10} M (Table 2). Consequently, it can be inferred that glycosylation appears to slightly interfere with the formation of Hp−Hb complexes, which may be expected considering the glycans rest on the surface of Hb and likely interfere with Hp's recognition of Hb.

X-ray Crystallography. Hb is a $\alpha_2\beta_2$ tetramer having one true 2-fold rotational symmetry axis and two 2-fold pseudosymmetry axes. Functional properties of Hb, such as its equilibrium oxygen binding characteristics, are regulated at the structural level by the relative orientation and interaction of its constituent subunits.[54,55] For example, interactions at the $\alpha_1\beta_1$ (and the corresponding $\alpha_2\beta_2$) intersubunit interface as well as the interactions at the $\alpha_2\beta_1$ (and the corresponding $\alpha_1\beta_2$) interface launch Hb into quaternary conformations that have distinct oxygen affinities.[56−59] Furthermore, in the absence of certain salt bridges Hb's "central cavity" assumes a more relaxed, high oxygen affinity state referred to as the R state.[55,60] Consequently, the quaternary structure of Hb directly reflects its functional behavior, making it paramount to investigate any significant changes upon glycosylation. Accordingly, the crystal structures of maltotriose−bHb, maltopentose−bHb, and maltoheptose−bHb were obtained and reveal that the tetrameric state was preserved upon glycosylation. More importantly, a superposition of only the $\alpha_1\beta_1$ subunits of tetrameric bHb and glycosylated bHb's shows that the quaternary state of the glycosylated bHb's corresponds exactly to the quaternary state of native bHb (data not shown). A complete structural alignment of the 572 C_α atoms of the $\alpha_2\beta_2$ subunits of bHb and the glycosylated bHb's shows that their tertiary and quaternary features correspond well (Figure 6A). The average root-mean-square deviations from bHb are 0.337 Å for maltotriose−bHb and 0.371 Å for maltoheptose−bHb. These analyses strongly suggest that the overall tertiary and quaternary features of bHb are preserved upon glycosylation.

The comparison between the structures of glycosylated bHb's and the structure of native bHb reveals that the deviations from the structure of native bHb are not uniformly distributed throughout the bHb molecule, but instead localized (Figure 6B). These deviations were found to be present mostly in the β_1 and β_2 subunits. However, this was expected to a

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Table 3. Dimensions of the Heme Pocket for Selected Hb's

	bHb				maltotriose−bHb				maltoheptaose−bHb			
	α_1	α_2	β_1	β_2	α_1	α_2	β_1	β_2	α_1	α_2	β_1	β_2
heme pocket surface area (Å^2)	800	680	600	600	730	640	580	530	730	820	530	520
heme pocket volume (Å^3)	960	950	760	740	920	900	790	740	880	1010	700	710

certain degree, since it is the β Cys-93 residue located near the carboxy edge of the F helix which is glycosylated. Although the electron density for the glycan linker is not defined, the position of structural deviations suggests that the glycan linker interacts closely with the β subunit. The $\alpha_2\beta_1$ interface is defined by a close interaction between the C helix and the CE elbow on the α_1 subunit and the carboxy edge of the F helix and the FG elbow on the β_2 subunit (Figure 6A). Given the minor structural deviations observed in C-helices on α subunits, it is possible that the interaction influences the conformation of the $\alpha_2\beta_1$ interface. Inspection of the β subunit region of glycosylated bHb's for structural deviation from the corresponding region in native bHb reveals the region is comprised mainly of the E helix, the EF elbow, the F helix, and the FG elbow. Interestingly, the side chain conformations in this region closely resemble each other (Figure 6C). This raises the intriguing possibility of a global movement of the helices in the region that accommodates glycosylation, which likely serves as a buffer to restrict transmission of these changes to other regions of the quaternary structure of the glycosylated bHb's.

The C_α atoms of three residues contained in the E helix of the β_1 subunit, namely, Pro-57, Lys-66, and Lys-76, were selected for calculating an axis passing through the geometric centroid of the atoms. This procedure was repeated for bHb, maltotriose−bHb, and maltoheptaose−bHb. In a similar fashion, axes were computed through the geometric centroid of C_α atoms of three residues on the β_1 subunit, namely, His-77 that is located at the EF elbow, Ala-87 that is located in the middle of the F helix, and Asp-94 that is located at the carboxy end of the F helix. These two sets of axes revealed some interesting features (Figure 6D) that may have profound implications. First, the axes drawn through the β_1 subunits of maltotriose−bHb and maltoheptaose−bHb are in almost identical positions. Second, the axes computed for the β_1 subunit of native bHb are in a position that is close to, yet distinctly apart from, the corresponding axes in maltotriose−bHb and maltoheptaose−bHb. Third, these analyses reveal the structure contained within the EF helices undergoes a "global" movement farther away from the $\alpha_2\beta_1$ interface. Finally, the β_1 superposition in the region of the FG elbow and amino edge of the G helix as well as the β_1 superposition in the region of the D helix and the DE elbow indicates that these conformational changes are not transmitted further. Conformational changes in the F helix of β subunits, especially near its carboxy edge, are deemed important in determining the oxygen affinity of Hb.[61] Thus, upon oxygen binding, this region is displaced farther from the $\alpha_2\beta_1$ interface, which in turn "relaxes" Hb and increases its oxygen affinity. The fact that glycosylation at Cys-β93 also results in a small displacement farther from the $\alpha_2\beta_1$ interface rationalizes the observation that glycosylated Hb's have a higher oxygen affinity than native Hb. A measurement of the surface area and volume of the heme pocket of bHb, maltotriose−bHb, and maltopentose−bHb further reveals these values are quite similar across all three structures (Table 3), suggesting the conformational changes upon glycosylation are accommodated and compensated for in such a manner that allows the quaternary structure to be preserved.

CONCLUSIONS

In this work, we have presented a novel method allowing for the facile site-selective glycosylation of proteins with carbohydrates of variable MWs. To the best of our knowledge, this is the first reported methodology for the site-selective glycosylation of proteins with oligosaccharides of such a high MW. To demonstrate the feasibility of the reported methodology, Hb was site-selectively glycosylated with four glycans of increasing MW, up to 10 000. Although the present study was limited to oligosaccharides with a maximum MW of 10 000, it is envisioned—due to the aqueous nature of the reactions employed in both the synthesis of the carbohydrate linkers and the bioconjugation reaction—that larger saccharides could be utilized. Furthermore, upon examination of the biophysical properties of the glycosylated Hb, it was discovered that glycosylation of Hb lowered P_{50} to levels similar to that of the commercially developed oxygen therapeutic Hemospan, now in phase III clinical trials. It has been postulated that a low P_{50} value may be critical to the development of HBOCs because it facilitates oxygen transport to tissues with low levels of oxygen and decreases arteriole oxygen transport, potentially eliminating undesired cardiovascular effects such as induced high blood pressure.[51] Consequently, the presented methodology may be of significant importance for the future development of glycosylated Hb's as potential HBOCs. Specifically, the dextran-conjugated bHb has a significant potential to serve as a "red blood cell substitute", for through site-specific glycosylation it combines Hb, an oxygen carrier, and dextran, a plasma expander.

Moreover, the method developed has the ability to be applied toward the site-specific glycosylation of other therapeutic proteins with high MW oligosaccharides/polysaccharides, thus serving as a viable alternative to site-specific PEGylation. Glycans carry with them a vast array of unique biological properties spread out over a wide range of MWs; consequently, the method reported herein, which easily allows for high MW glycan conjugation to a desired therapeutic protein, could be of significant usefulness. Furthermore, glycans are quite biodegradable in comparison to PEG, making their use advantageous when administration must occur over long periods of time or in large doses, thus preventing long-term accumulation. Future potential applications range from the conjugation of high MW polysacchardies to carrier proteins used in vaccine conjugation—a task that has been daunting in the past—to the conjugation of large oligosaccharides/polysaccharides to therapeutic proteins as a means to extend the circulation half-life and thus their pharmacological effectiveness.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, compound characterization, biophysical characterization methods, and data and crystallographic

information files. This material is available free of charge via the Internet at http://pubs.acs.org

AUTHOR INFORMATION

Corresponding Author

palmer.351@osu.edu; pwang11@gsu.edu

Notes

The authors declare no competing financial interest.

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