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Versatile N-methylaminooxy functionalized polypeptides for preparation of neoglycoconjugates

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Abstract The preparation and characterization of a new set of well-defined polypeptides containing N-methylaminooxy side-chain functionality is described. These functional groups enabled the direct coupling of polypeptides with a variety of unmodified reducing saccharides in water to give neoglycopolypeptides in high yields. The use of different polypeptide scaffolds resulted in neoglycoconjugates with tunable chain conformations, hydrophobicity, and charge. These new neoglycopolypeptides were also found to be stable in aqueous media at pH 7.4 and 37 °C for 1 week. The combination of straightforward synthesis using unmodified saccharides, high yields of saccharide conjugation, and conjugate stability make these polypeptides attractive candidates for development of degradable glycoprotein mimics.

Introduction

Natural glycoproteins possess abundant biological functionality as well as structural and physical properties that enable their ability to provide cellular support in extracellular matrix.[1] Due to the large size and complexity of glycoproteins, it is desirable to obtain structurally defined synthetic glycoprotein mimics for study of cell-substrate interactions and for regenerative medicine biomaterial applications.[2,3] While many polymeric neoglycoconjugates have been reported, many rely on non-peptidic backbones,[4-6]

require tedious multistep syntheses,[7-12] or utilize chemically modified saccharide building blocks,[13-16] where many are limited to conjugation of simple monosaccharides. In order to better mimic glycoproteins in biomaterial applications, use of degradable peptidic polymer backbones and incorporation of complex oligosaccharides from natural glycoprotein components are preferred.[2,3] Further, to allow facile preparation of a variety of glycoprotein mimics for analysis of structure-property relationships, straightforward and robust synthetic methods are needed. Here, we describe the preparation of a new set of well-defined polypeptides containing N-methylaminooxy side-chain functionality, which enables direct coupling with unmodified reducing saccharides in water to give neoglycopolypeptides in high yields. The ability to react different saccharides to these polypeptides to produce a variety of stable conjugates provides a useful means for preparation of well-defined, highly functional neoglycoconjugates.

There has been much recent activity toward preparation of neoglycopolypeptides. These methods are based on two strategies: (i) ring-opening polymerization of glycosylated amino acid N-carboxyanhydride (NCA) monomers,[7-12] or (ii) post-polymerization conjugation of saccharides to reactive precursor polypeptides.[13-16] Both of these methods are able to provide polypeptides with high degrees of glycosylation, and many examples have been reviewed. The glycosylated NCA route provides advantages of 100% saccharide functionalization, and the ability to utilize natural glycoside linkages (e.g. to serine residues). The main disadvantage of this method is the need for multistep monomer synthesis, use of protecting groups, and tedious monomer purification. Consequently, use of this methodology for incorporation of complex oligosaccharides containing additional functionality, e.g. sulfonate and carboxylate groups often present in glycoproteins, is challenging. Post-polymerization neoglycoconjugate formation offers the advantage of simple and often more readily prepared polypeptide chains, but conjugations can suffer from incomplete saccharide functionalization.[13-16] Most of the reported methods also require use of saccharides that have been modified to contain reactive functionality, such as azide,

alkyne, thiol, epoxide, alkyl halide, or isothiocyanate groups.[13-16] These modifications require additional synthetic steps, and also result in incorporation of unnatural linkages such as triazole groups into the neoglycoconjugates that may affect biological properties.

Based on these studies, we sought to develop a facile method for neoglycopolypeptide synthesis utilizing a post-polymerization conjugation strategy, but wanted to improve conjugation to allow incorporation of more complex saccharides, and remove the need for saccharide chemical modification. Our strategy was focused on use of the oxime ligation,[17,18] a chemoselective and efficient method that has been used in many different polymer systems for post-polymerization functionalization, including neoglycoconjugate formation.[4-6,19-21] While oxime ligation has not been used to prepare neoglycopolypeptides,[22] it has been utilized to prepare neoglycoconjugates in short peptide and peptoid sequences.[23-26] A significant advantage in the use of aminooxy functionalized peptides and polymers is their ability to react directly with unmodified reducing saccharides. Another important feature is that while saccharide conjugation to aminooxy groups typically results in ring-opened saccharides, the use of N-alkylaminooxy or N-alkoxyamino groups results in ring closed saccharide conjugates that better mimic natural glycan presentation.[27] Using this knowledge, Godula's lab recently showed that N-methylaminooxy functionalized polyacrylamides can be used to prepare neoglycoconjugates with a variety of unmodified complex oligosaccharides derived from glycoproteins.[4-6] Here, we build upon this work by development of new water soluble polypeptides containing side-chain N-methylaminooxy functionality. The conjugation of saccharides to these polypeptides was evaluated, and physical properties of neoglycoconjugates with different side-chain structures were compared. These new polypeptides were found to enable straightforward preparation of neoglycopolypeptides with high degrees of saccharide conjugation in aqueous media.

Experimental Section

Materials and Instrumentation THF, hexanes, and DCM were dried by purging with nitrogen and passage through activated alumina columns prior to use. TEA and TMSCl were purified by distillation and stored over 3 Å molecular sieves. $Co(PMe_3)_4$, L-methionine NCA, and N_ε-Z-L-lysine NCA monomers were prepared according to literature procedures.[28-30] Poly(L-methionine sulfoxide)$_{70}$ (M^O_{70}), poly(S-methyl-L-methionine sulfonium)$_{70}$ (M^M_{70}), and poly(L-lysine·HCl)$_{70}$ (K_{70}) were prepared according to literature procedures.[31,32] All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Selecto silica gel 60 (particle size 0.032–0.063 μm) was used for flash column chromatography. FTIR measurements were taken using a Perkin Elmer RX1 FTIR calibrated using polystyrene film. ^{1}H NMR spectra were acquired on a Bruker ARX 400 spectrometer. GPC/MALS was performed at 25 °C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 μm columns at 30 °C with 0.5% (w/w) KTFA in HFIP as eluent and sample concentrations of 10 mg/mL. Zeta potential data were collected using a Zetasizer NanoZS. Pyrogen free DI water was obtained from a Millipore Milli-Q Biocel A10 purification unit. Dialysis was conducted using regenerated cellulose dialysis tubing (Spectrum Labs, MWCO 2000 Da). All CD spectra were collected using an OLIS RSM CD spectrophotometer (OLIS, USA) using conventional scanning mode. Samples were characterized by recording spectra (185-260 nm) within a quartz cuvette of 0.1 cm path length. Mass spectroscopy data was obtained by dissolving the sample in acetonitrile (50 ng/mL) and analysis in negative mode using a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with Dionex Ultimate 3000 RSLCnano System. Resolving power was 70,000 at m/z 200. Multiple spectra (50) were averaged to improve signal to noise.

2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine Synthesis of this functionalized amino acid was accomplished by adapting a procedure from literature.[33] L-methionine (0.94 g, 6 mmol, 1.0 eq) was dissolved in liquid ammonia (30 mL) followed by addition of sodium metal (0.57 g, 24 mmol, 4.0 eq) in

small pieces until the solution became heterogeneous and a blue color persisted for 15 minutes. The blue color was then quenched by adding ammonium chloride (1.0 g, 42 mmol, 7 eq) until the reaction mixture was colorless and homogenous. At this point, 2-(N-Boc-N-methylaminooxy)ethyl bromide (see supporting information (SI), 1.6 g, 6 mmol, 1.0 eq) was slowly added and the reaction was allowed to stir for 1 hour. The ammonia was then removed under reduced pressure to yield a crude white solid. The white solid was dissolved in a minimal amount of DI water (15 mL), and 3 M HCl (5 mL) was added until the pH was between 6 and 7. The water was then removed under reduced pressure to yield a crude white solid which contained salts (*ca*. 60% salt wt/wt) and used without further purification (5.8 g (*ca*. 2.3 g amino acid product), 100% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.15 (t, J = 6.2 Hz, 2H), 3.93 (dd, J = 7.0, 5.5 Hz, 1H), 3.23 (s, 3H), 2.92 (t, J = 6.2 Hz, 2H), 2.80 (t, J = 7.6 Hz, 2H), 2.32-2.14 (m, 2H), 1.56, (s, 9H). ^{13}C NMR (100 MHz, D_2O, 25 °C): 174, 157, 83.8, 72.7, 53.9, 35.9, 30.4, 29.1, 27.6, 27.2.

2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine N-carboxyanhydride, Boc-mao-Ehc NCA

To a solution of crude 2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine (3.0 g (*ca*. 1.2 g amino acid), 3.3 mmol, 1.0 eq) in dry THF (30 mL) in a Schlenk flask was added TEA (0.92 mL, 6.6 mmol, 2.0 eq) and TMSCl (0.84 mL, 6.6 mmol, 2.0 eq) via syringe. The reaction was stirred under N_2 at room temperature for 1 hour. Upon addition of TEA and TMSCl, precipitation of TEA·HCl was observed. A solution of 15% (w/v) phosgene in toluene (4.7 mL, 6.6 mmol, 2.0 eq) was then added via syringe and the reaction was stirred under N_2 at 50 °C for 2 hours. *Caution!* Phosgene is extremely hazardous and all manipulations must be performed in a well-ventilated chemical fume hood with proper personal protection and necessary precautions taken to avoid exposure. After 2 hours, the reaction was cooled and evaporated to dryness then transferred into a N_2 filled glovebox. In the fume hood, the condensate in the Schlenk line vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene. In the glove box, THF was added to the crude product and the insoluble salts were removed by vacuum filtration and were washed with THF. The filtrate was evaporated under reduced pressure to yield an

orange/red oil that was purified by passing it through vacuum dried silica using 10% THF/hexanes to 30% THF/hexanes.[29] Fractions containing the NCA were combined and concentrated under reduced pressure to yield a pale yellow oil (0.73 g, 67% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.58-7.46 (bs, 1H), 4.57 (ddd, J = 9.2, 4.1, 0.8 Hz, 1H), 4.10 (m, 1H), 4.01 (m, 1H), 3.12 (s, 3H), 2.92 (m, 2H), 2.74 (t, J = 5.6 Hz, 2H), 2.22 (m, 1H), 2.06 (m, 1H), 1.50 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 170, 157, 152, 82.6, 74.1, 56.3, 36.9, 31.3, 29.9, 28.4, 28.1. FTIR: NCA bands at 1857, 1787 and Boc bands at 1730 and 1705 cm^{-1}. MS *m/z* = 333.1128 [M-H]$^-$ (calcd 333.1120 for $C_{13}H_{21}O_6N_2S$).

poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine)$_{70}$, Boc-mao-Ehc$_{70}$ All polymerization reactions were performed in a N_2 filled glove box using anhydrous THF. To prepare Boc-mao-Ehc$_{70}$ at *ca*. 100 mg scale, a solution of $Co(PMe_3)_4$ (3.6 mg, 0.010 mmol, 1.0 eq) in THF (20 mg/mL) was quickly added to a solution of Boc-mao-Ehc NCA (100 mg, 0.30 mmol, 30 eq) in THF (50 mg/mL) at 20 °C. After *ca*. 60 minutes, complete consumption of NCA was confirmed by FTIR spectroscopy. In order to determine the length of Boc-mao-Ehc$_{70}$, a small aliquot (200 μL) of the polymerization mixture was removed for end-group analysis (see SI). The reaction mixture was then removed from the glove box and the polypeptide was precipitated into DI water (100 mL), collected using centrifugation, and dried under reduced pressure to yield an off white solid with yields ranging from 85 to 90%. ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.87-4.73 (br m, 1H), 4.42-4.29 (br m, 2H), 3.20 (s, 3H), 2.92-2.81 (br m, 2H), 2.75-2.61 (br m, 2H), 2.20-2.01 (br m, 2H), 1.57 (s, 8H). FTIR: polypeptide bands at 3288, 1652, and 1548 cm^{-1}.

poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, Boc-mao-Ehc$^{O}_{70}$ The oxidation of thioether groups was performed before removal of the N-Boc groups. To convert thioether groups to sulfoxides, a volume of 70 wt. % TBHP (32 eq per residue) was added to a solution of Boc-mao-Ehc$_{70}$ in HFIP (20 mg/mL). The reaction was stirred at room temperature for 48 hours and then it was transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free deionized milli-Q

water (3.5L) containing sodium thiosulfate (1.2 g, 2.16 mM) for 2 days to neutralize residual peroxide, (ii) pyrogen free milli-Q water (3.5L) for 2 days to remove residual sodium thiosulfate. For each step above, dialysate was changed every 12 hours. Within the first couple hours of dialysis, the polypeptide precipitated within the dialysis bag. The contents of the dialysis bag were removed and freeze dried to yield dense white solids with average yields of 90 to 95%, and complete conversion of thioether groups to sulfoxide groups. ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.97-4.59 (br m, 3H), 3.53-3.28 (br m, 4H), 3.22 (s, 3H), 2.69-2.47 (br m, 1H), 2.47-2.26 (br m, 1H), 1.56 (s, 8H).

poly(2-(N-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, mao-Ehc$^{O}_{70}$ The removal of N-Boc groups was performed after oxidation of the thioether groups. To remove the N-Boc groups, Boc-mao-Ehc$^{O}_{70}$ was dissolved in TFA (20 mg/mL) and allowed to stir at room temperature for 2 hours. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free milli-Q water (3.5L) acidified to pH 2 with HCl for 2 days to aid cobalt ion removal/counter ion exchange, and (ii) pyrogen free milli-Q water (3.5L) for 2 days to remove residual HCl. For each step above, dialysate was changed every 12 hours. The polypeptide solution remained clear throughout dialysis. The contents of the dialysis bag were removed and then freeze dried to yield the polypeptide as a white fluffy solid (95% yield) with > 99% removal of Boc groups. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.58-4.37 (br m, 1H), 4.26-4.06 (br m, 2H), 3.28-2.95 (br m, 4H), 2.69 (s, 3H), 2.44-2.17 (br m, 2H).

poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, Boc-mao-Ehc$^{M}_{70}$ The alkylation of thioether groups was performed before removal of the N-Boc groups. To convert thioether groups in Boc-mao-Ehc$_{70}$ to S-methyl sulfonium groups, iodomethane (5 eq per thioether group) was added to a 20 mg/mL suspension of Boc-mao-Ehc$_{70}$ in 0.1 M phosphate buffer (pH 7). The reaction was covered with aluminum foil to protect iodomethane from light and the suspension was vigorously stirred for 48 hours at room temperature. Afterwards, the reaction mixture was transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free deionized milli-Q water (3.5 L) containing

NaCl (7 g, 35 mM) for 2 days to facilitate counterion exchange, (ii) pyrogen free milli-Q water (3.5 L) for 2 days to remove residual NaCl. For each step above, dialysate was changed every 12 hours. Within the first couple hours of dialysis, the polypeptide became soluble. The contents of the dialysis bag were removed and freeze dried to yield a dense white solid (93% yield), and complete conversion of thioether groups to S-methyl sulfonium groups. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.66-4.55 (br m, 1H), 4.49-4.30 (br m, 1H), 3.83-3.37 (br m, 3H), 3.25-3.15 (br m, 3H), 3.14-3.01 (br m, 3H), 2.46-2.19 (br m, 2H), 1.61-1.38 (br m, 8H).

poly(2-(N-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, mao-Ehc$^M{}_{70}$ The removal of Boc-mao-Ehc$^M{}_{70}$ was performed after alkylation of the thioether groups. To remove the N-Boc groups, Boc-mao-Ehc$^M{}_{70}$ was dissolved in TFA (20 mg/mL) and allowed to stir at room temperature for 2 hours. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free deionized milli-Q water (3.5 L) containing NaCl (7 g, 35 mM) for 2 days to facilitate counterion exchange, (ii) pyrogen free milli-Q water (3.5L) for 2 days to remove residual NaCl. For each step above, dialysate was changed every 12 hours. The polypeptide solution remained clear throughout dialysis. The contents of the dialysis bag were removed and then freeze dried to yield the polypeptide as a dense white solid (96% yield) with > 99% removal of Boc groups. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.68-4.52 (br m, 1H), 4.28-4.14 (br m, 2H), 3.83-3.37 (br m, 4H), 3.12-2.95 (br m, 3H), 2.73-2.61 (br m, 3H), 2.50-2.23 (br m, 2H).

poly(6-(2-N-Boc-N-methylaminooxy)ethylthio)-L-norleucine)$_{70}$, Boc-mao-Etn$_{70}$ poly(L-homoallylglycine)$_{70}$, Hag$_{70}$, (11 mg), and DMPA (5 mg, 0.02 mmol, 0.2 eq per residue) were placed in a 1 dram screw top vial. THF was then added to give a 4 mg/mL resulting polymer concentration. 2-(N-Boc-N-methylaminooxy)ethyl mercaptan (see SI, 82 mg, 0.39 mmol, 4 eq per residue) was then added via micropipette and the solution was degassed via sparging with N_2 for 10 minutes. The vial was then sealed with parafilm and the solution was irradiated with UV light for 2.5 hours (Exo Terra Reptile Lamp) and

let stir overnight. The solution was then transferred to a 2000 MWCO dialysis bag and dialyzed against methanol for 24 hours with one change of dialysate, followed by dialysis in water for 24 hours with one water change. The solution was lyophilized to dryness to give the product as a white solid (31 mg, 95% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.66 (br s, 1H), 4.44 (d, J = 5.4, 2H), 3.28 (s, 3H), 2.94 (t, J = 5.1, 2H), 2.78-2.58 (br m, 2H), 2.24-1.27 (br m, 15H).

poly(6-(2-N-Boc-N-methylaminooxy)ethylthio)-L-norleucine sulfoxide)$_{70}$, Boc-mao-Etn$^{O}_{70}$ The oxidation of thioether groups was performed before removal of N-Boc groups. Boc-mao-Etn$^{O}_{70}$ (81 mg) was dissolved in HFIP (5.0 mL). 70% TBHP in water was then added (1.14 mL, 8 mmol, 32 eq per residue). The solution was let stir overnight and then dialyzed against water for 48 h with two water changes daily. The resulting solution was lyophilized to dryness to give the product as a sticky, white solid (84 mg, 99% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.95-4.76 (br m, 2H), 4.67 (br s, 1H), 3.56-3.39 (br m, 2H), 3.35-3.16 (br m, 4H), 3.09 (br s, 1H), 2.30-1.52 (br m, 15H).

poly(6-(2-N-methylaminooxy)ethylthio)-L-norleucine sulfoxide)$_{70}$, mao-Etn$^{O}_{70}$ The removal of N-Boc groups was performed after oxidation of thioether groups. Boc-mao-Etn$^{O}_{70}$ (31 mg) was dissolved in TFA (2.0 mL). The reaction was let stir for 3 hours at room temperature and then dialyzed against a pH 2 HCl solution (24 hr, 2 water changes), then against deionized water (24 hr, 2 water changes). The resulting solution was lyophilized to dryness to give the product as a white solid (13 mg, 62% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.40-3.97 (br m, 3H), 3.30-2.84 (br m, 4H), 2.68 (s, 3H), 2.18-1.45 (br m, 6H).

Saccharide conjugation to mao-Ehc$^{O}_{70}$, mao-Ehc$^{M}_{70}$, and mao-Etn$^{O}_{70}$

Procedure A (saccharide-mao-Ehc$^{O}_{70}$) A sample of mao-Ehc$^{O}_{70}$ was dissolved at 5 mg/mL in a 0.1 M buffer (acetate pH 4-6, phosphate pH 7) with or without 0.1 M aniline. Upon complete dissolution of mao-Ehc$^{O}_{70}$, the desired saccharide (5-500 eq) was added to the reaction mixture. The reaction was adjusted to the appropriate temperature (25-50 °C) and stirred for 4 days. The reaction

mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against pyrogen free milli-Q water (3.5L) for 1 day to remove ions and excess saccharide. Dialysate changes were performed every 12 hours. The resulting solution was removed and freeze dried to yield a white fluffy solid with isolated yields ranging from 79 to 100%, and saccharide conjugation yields ranging from 15 to 93%. The degree of saccharide conjugation was quantified by comparing ^{1}H NMR integrals of the N-methyl resonances of unconjugated methylaminooxy groups (2.69 ppm) with the saccharide conjugated methylaminooxy groups (2.72-2.80 ppm).[34]

Procedure B (Glc-mao-Ehc$^{M}_{70}$) A sample of mao-Ehc$^{M}_{70}$ was dissolved at 5 mg/mL in a 0.1 M buffer (acetate pH 5-6, phosphate pH 7) with or without 0.1 M aniline. Upon complete dissolution of mao-Ehc$^{M}_{70}$, D-glucose (100 eq) was added to the reaction mixture. The reaction was adjusted to 25 °C and stirred for 4 days. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against (i) pyrogen free milli-Q water (3.5L) containing NaCl (7.0 g, 35mM) for 1 day to facilitate counterion exchange (ii) pyrogen free milli-Q water (3.5L) for 1 day to remove excess NaCl. Dialysate changes were performed every 12 hours. The resulting solution was removed and freeze dried to yield a white fluffy solid with isolated yields ranging from 91 to 99%, and saccharide conjugation yields ranging from 37 to 93%. The degree of saccharide conjugation was quantified by comparing ^{1}H NMR integrals of the N-methyl resonances of unconjugated methylaminooxy groups (2.69 ppm) with the saccharide conjugated methylaminooxy groups (2.82 ppm).[34]

Procedure C (Glc-mao-Etn$^{O}_{70}$) A sample of mao-Etn$^{O}_{70}$ was dissolved at 1.5 mg/mL in a 0.1 M buffer (acetate pH 4-6, phosphate pH 7) with or without 0.1 M aniline to give a turbid solution. The desired saccharide (50-500 eq) was then added to the reaction mixture. The reaction was stirred at 50 °C for 4 days. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against pyrogen free milli-Q water (3.5L) for 1 day to remove ions and excess saccharide. Dialysate changes were performed every 12 hours. The resulting solution was removed and freeze dried to yield a white fluffy

solid with isolated yields ranging from 79 to 100%, and saccharide conjugation yields ranging from 60 to 96%. The degree of saccharide conjugation was quantified by comparing ^{1}H NMR integrals of the N-methyl resonances of unconjugated methylaminooxy groups (2.69 ppm) with the saccharide conjugated methylaminooxy groups (2.80 ppm).[34]

Stability of Glc-mao-Ehc$^{O}_{70}$, Glc-mao-Ehc$^{M}_{70}$ and Glc-mao-Etn$^{O}_{70}$ neoglycoconjugates Samples of Glc-mao-Ehc$^{O}_{70}$, Glc-mao-Ehc$^{M}_{70}$, and Glc-mao-Etn$^{O}_{70}$ (each having > 90% saccharide functionalization) were dissolved in a 0.2 M phosphate buffer (1.0 mg/mL) at either pH 5.5 or 7.4. These solutions were then transferred to 2000 MWCO dialysis bags and dialyzed at 37 °C against 200 mL of a 0.2 M phosphate buffer at either pH 5.5 or 7.4. Samples were dialyzed for 1 week with dialysate changes every day. After 1 week the contents of the dialysis bags were collected and lyophilized. ^{1}H NMR spectra of the resulting solids were obtained and the integrals of N-methyl group resonances at 2.80 and 2.69 ppm were compared to determine the relative amounts of glucose neoglycoconjugate residues and free methylaminooxy residues, respectively.[34] This data was used to calculate the percentage of glucose released from the polypeptide neoglycoconjugates after 1 week. Error bars are consistent with the standard deviation for n = 3.

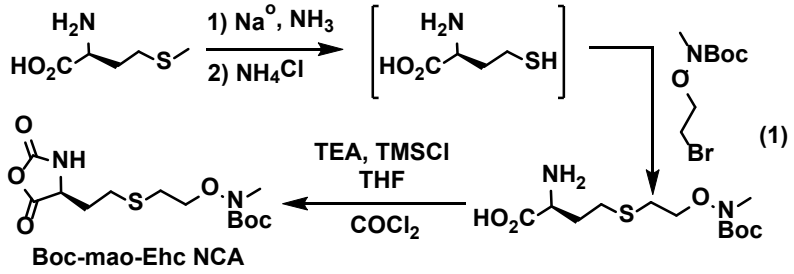
Circular dichroism Spectroscopy Samples of mao-Ehc$^{O}_{70}$, mao-Ehc$^{M}_{70}$, mao-Etn$^{O}_{70}$, Glc-mao-Ehc$^{O}_{70}$, Glc-mao-Ehc$^{M}_{70}$ and Glc-mao-Etn$^{O}_{70}$ were prepared at concentrations of 0.1 mg/mL in aqueous buffers containing 100 mM phosphate and 10 mM Tris. The buffered solutions were adjusted to pH 2.0, 3.0, 4.0, 5.0, and 9.0 using HCl (0.1 M) or NaOH (0.1 M). The spectra were reported in units of molar ellipticity [θ] (deg·cm^{2}·dmol^{-1}), which was calculated using $[\theta] = (\theta \times 100 \times M_W)/(c \times l)$ where θ is the measured ellipticity (millidegrees), M_W is the average residue molecular mass (g/mol), c is the polypeptide concentration (mg/mL), and l is the cuvette path length (cm).

In order to test the effect of methanol on polypeptide conformations, a stock solution of mao-Ehc$^{O}_{70}$ dissolved in 0.001 M NaOH (0.5 mg/mL) was prepared. This stock solution was diluted with the

appropriate volumes of DI water and methanol to give final mao-Ehc$^{O}_{70}$ concentrations of 0.1 mg/mL and overall methanol concentrations ranging from 0 to 80 volume %. Percent α-helical content was calculated using % helicity = ((-[θ_{222}] + 3000)/39000) x 100 where [θ_{222}] is the molar ellipticity at 222 nm.[35]

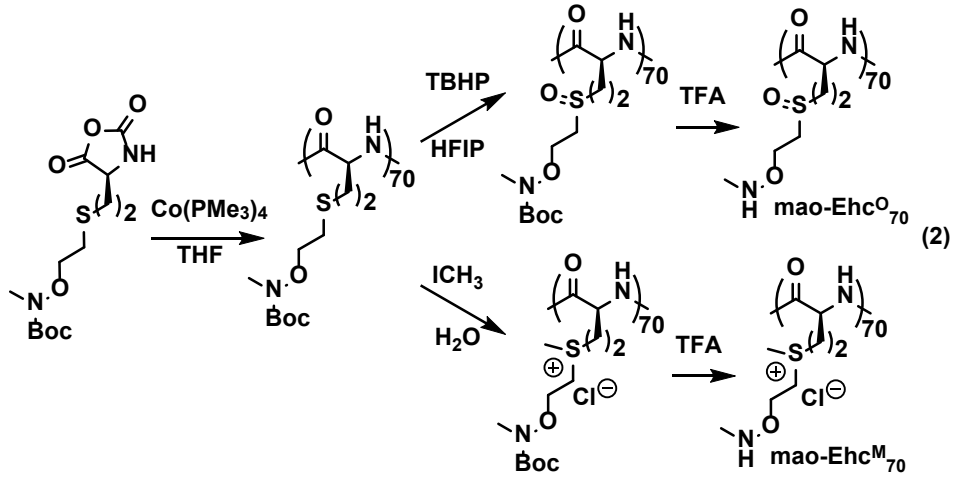
Zeta potential measurements Samples of mao-Ehc$^{O}_{70}$, M$^{O}_{70}$, M$^{M}_{70}$, and K$_{70}$ were dissolved at 5 to 15 mg/mL in filtered (0.45 μm) water containing 20 mM NaCl. Polypeptide solutions were adjusted to pH 2.0 using filtered (0.45 μm) aqueous 1.0 M HCl. The solutions were titrated to pH 12 with filtered (0.45 μm) aqueous 1.0 M NaOH. Aliquots (1 mL) were removed from the solutions at specific pH values, and zeta potentials of samples were measured using a Zetasizer NanoZS instrument (Malvern Instruments Ltd., United Kingdom). Due to the high conductivity of the samples, the electrophoretic mobility was calculated in monomodal mode where only the fast field reversal (FFR) technique was applied to avoid degradation of the cuvette electrodes. The software (Malvern Zetasizer Software) was used to calculate electrophoretic mobility using the Henry equation.

Results and Discussion



A N-methylaminooxy functionalized amino acid derived from L-homoserine has been previously reported for preparation of neoglycoconjugates in peptides.[24,25] While this residue may be amenable for polypeptide synthesis, we initially chose to develop a new N-methylaminooxy functionalized derivative of L-homocysteine since previous work suggests the resulting polypeptide would possess good solubility during polymerization, and the side-chain thioether groups would allow further modification by oxidation or alkylation to adjust polymer properties.[36] The target 2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine amino acid was prepared in two steps from readily available L-methionine in high yield

(Eq 1, see SI Eq S1). Subsequent phosgenation in the presence of acid scavengers gave the desired monomer, 2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine NCA, Boc-mao-Ehc NCA, as an oil that was purified by chromatography on dried silica (Eq 1, see SI Figure S1).[29]



(2)

Boc-mao-Ehc NCA was found to polymerize readily in THF to complete monomer conversion using $Co(PMe_3)_4$ initiator (Eq 2).[28] Homopolypeptide chain lengths were controlled by adjustment of monomer to initiator ratios enabling preparation of poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine)$_n$, Boc-mao-Ehc$_n$, with degrees of polymerization up to 145 with low dispersity (Figure 1, see SI Table S1 and Figure S2). Subsequent deprotection of a model sample, Boc-mao-Ehc$_{70}$, under a variety of acidic conditions was found to yield only intractable crosslinked solids. We suspect that protonation of free N-methylaminooxy groups formed during acidic deprotection results in sulfur mustard type chemistry where N-methylhydroxylamine can eliminate, yielding electrophilic cyclic sulfonium intermediates that can be attacked by N-methylaminooxy groups of other chains to give crosslinks (see SI Eq S2).[37] To circumvent this issue, Boc-mao-Ehc$_{70}$ was first oxidized or methylated before deprotection to give the more polar derivatives poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, Boc-mao-Ehc$^O_{70}$, or poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, Boc-mao-Ehc$^M_{70}$, respectively (Eq 2, see SI Figures S3 and S4). Both of these reactions gave essentially quantitative modifications of the parent thioether groups, similar to previously described

results on related polypeptides.[36] Subsequent deprotection of Boc-mao-Ehc$^{O}_{70}$ and Boc-mao-Ehc$^{M}_{70}$ using trifluoroacetic acid gave the desired water soluble, N-methylaminooxy functionalized poly(2-(N-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, mao-Ehc$^{O}_{70}$, and poly(2-(N-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, mao-Ehc$^{M}_{70}$, without crosslinking (Eq 2, see SI Figures S3 and S4).

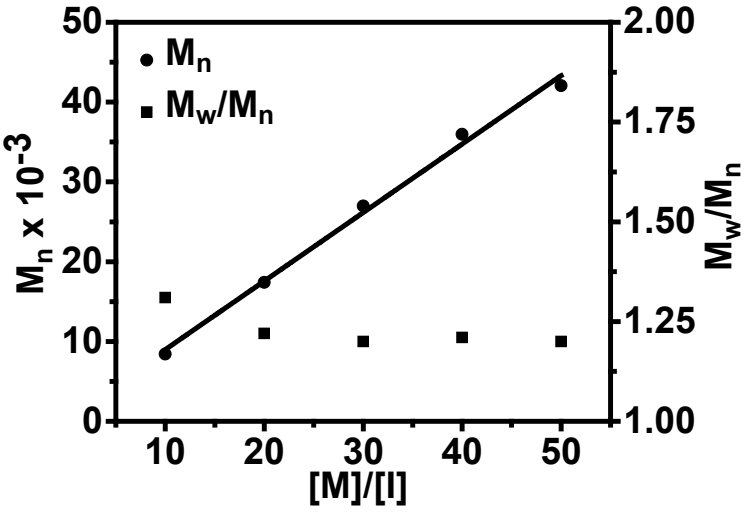
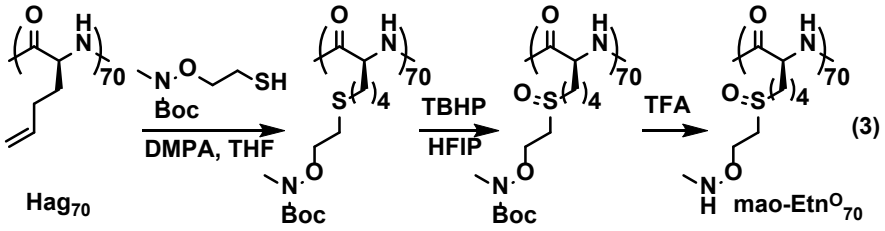


Figure 1. Molecular weight (M_n, circles) and dispersity (M_w/M_n, squares) of Boc-mao-Ehc$_n$ as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. M_n values were calculated via end group analysis using ^{1}H NMR integrations, and GPC/MALS was utilized to determine M_w/M_n.



With these N-methylaminooxy functionalized polypeptides in hand, one non-ionic and one cationic, we also prepared a homologous polypeptide using a different synthetic route. This target polypeptide, poly(6-(2-N-methylaminooxy)ethylthio)-L-norleucine sulfoxide)$_{70}$, mao-Etn$^{O}_{70}$, was prepared by coupling poly(L-homoallylglycine)$_{70}$, Hag$_{70}$, with a N-methylaminooxy containing thiol,

followed by oxidation and deprotection as described above (Eq 3, see SI Eq S3 and Figure S5).[38] In mao-Etn$^{O}_{70}$ the terminal N-methylaminooxy groups are two additional methylenes further from the polypeptide backbone compared to mao-Ehc$^{O}_{70}$. This difference was expected to lead to greater side-chain hydrophobicity and α-helical conformational stability in water for mao-Etn$^{O}_{70}$. To compare aqueous solution properties of these polypeptides, they were analyzed using circular dichroism (CD) spectroscopy.

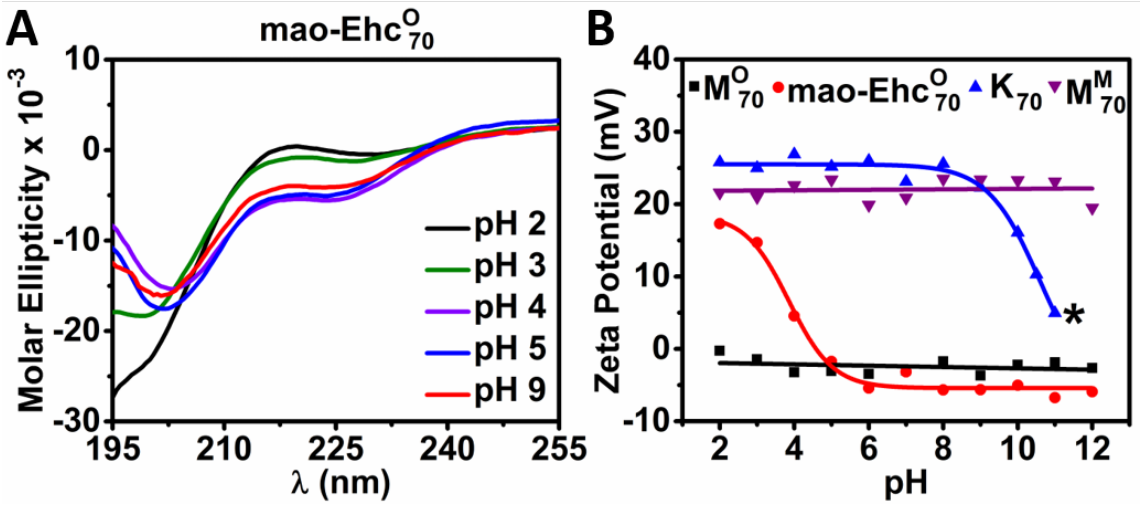


Figure 2. A) Circular dichroism spectra of mao-Ehc$^{O}_{70}$ at 20 °C and 0.1 mg/mL concentration in aqueous buffers containing 100 mM phosphate and 10 mM Tris adjusted to pH 2 (black), 3 (green), 4 (purple), 5 (blue), or 9 (red) using HCl (0.1 M) or NaOH (0.1 M). **B)** Zeta potentials as a function of pH for aqueous solutions of mao-Ehc$^{O}_{70}$ (red circles), M$^{O}_{70}$ (black squares), M$^{M}_{70}$ (purple down triangles), and K$_{70}$ (blue up triangles). * = K$_{70}$ was observed to precipitate above pH 11.

First, mao-Ehc$^{O}_{70}$ solutions were prepared in aqueous buffers ranging in pH from 2 to 9. Analysis of these solutions using CD spectroscopy showed that the chains primarily adopt disordered conformations across this pH range, yet a subtle change occurs between pH 3 and 4 where the chains appear to adopt a partial 3$_{10}$-helical conformation above pH 4 (Figure 2A).[39] Measurement of Zeta potential for solutions of mao-Ehc$^{O}_{70}$ as a function of pH revealed a transition from positive to slightly negative values between pH 3 and 5, indicative of a change from protonated to non-protonated N-methylaminooxy groups over this pH range (Figure 2B). This transition correlates well with the expected

pK_a range of N-methylaminooxy groups (*ca*. 3.65 to 4.75),[40] and was corroborated by analysis of other control samples that included non-ionic poly(L-methionine sulfoxide) (M^O_{70}), pH invariant cationic poly(S-methyl-L-methionine sulfonium) (M^M_{70}), and poly(L-lysine·HCl)$_{70}$ (K_{70}) that undergoes a cationic to uncharged transition at a much higher pH (pK_a *ca*. 9 to 10) (Figure 2B).[30,36] These results suggest that mao-Ehc$^O_{70}$ is uncharged above pH ~ 5 where it also retains good water solubility, which contrasts sharply with K_{70} that precipitates readily upon neutralization.

To further analyze the chain conformation of uncharged mao-Ehc$^O_{70}$, CD analysis was performed in water containing NaOH and increasing concentrations of methanol. As the methanol fraction increased, chains transitioned to α-helical conformations, up to a plateau of *ca*. 60% helicity (see SI Figure S6). Increased α-helical content in methanol likely reflects the weaker solvation of sulfoxide groups by this solvent compared to water, which is known to bind sulfoxides strongly and disrupt H-bonding as previously observed in M^O polypeptides.[36] To evaluate how side-chain length affects properties, a CD spectrum of mao-Etn$^O_{70}$ was collected at pH 2 for comparison to data obtained under identical conditions for mao-Ehc$^O_{70}$. At this pH, mao-Etn$^O_{70}$ displays higher α-helical content compared to mao-Ehc$^O_{70}$ likely due to its longer hydrophobic tether (see SI Figure S7). However, the increased hydrophobicity of mao-Etn$^O_{70}$ also impacts solubility such that it only possesses good water solubility in its protonated form below pH of *ca*. 4 to 5. CD spectroscopy was also used to study the conformation of cationic mao-Ehc$^M_{70}$. Since this polypeptide is charged independent of pH, it was found to always adopt a disordered conformation similar to most highly charged polypeptides in water (see SI Figure S8).[36]

The three N-methylaminooxy functionalized polypeptides were next evaluated for their ability to conjugate a model monosaccharide, D-glucose, under different conditions. First, non-ionic sulfoxide containing mao-Ehc$^O_{70}$ and mao-Etn$^O_{70}$ were reacted with D-glucose in the presence of aniline catalyst in water,[41,42] where pH, D-glucose concentration, and temperature were varied (Eq 4, Table 1). Entries 1-4 and 13-16 in Table 1 show that pH had little effect on saccharide conjugation yields after 4 days for mao-

Ehc$^{O}_{70}$, and a modest effect on yields with mao-Etn$^{O}_{70}$ possibly due to the diminished solubility of this polypeptide at higher pH. Although lower pH accelerates the coupling reaction, it also accelerates the hydrolysis of the product, leading to equilibrium levels of saccharide conjugation that are less than quantitative. As such, we found it useful to perform conjugations mainly at pH 7 in order to disfavor hydrolysis of the neoglycoconjugates and obtain high levels of saccharide functionalization (*ca*. 80 to 90%).[27,34] As a control experiment to highlight the significance of the N-methylaminooxy functionality for neoglycoconjugate formation, attempts were also made to conjugate D-glucose to poly(L-lysine·HCl)$_{70}$ under similar conditions. For all conditions attempted no glycoconjugate formation was observed for poly(L-lysine·HCl)$_{70}$, showing that glycoconjugation is selectively efficient for N-methylaminooxy functionality (see SI Figure S9).

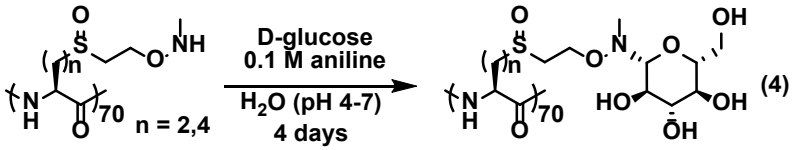
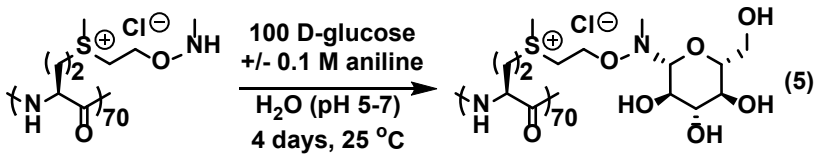


Table 1. Isolated and saccharide conjugation yields for reactions of mao-Ehc$^{O}_{70}$ (n = 2) or mao-Etn$^{O}_{70}$ (n = 4) with D-glucose. Experiments with mao-Etn$^{O}_{70}$ were performed at lower polypeptide concentrations due to low solubility. All reaction conditions as shown in Eq 4, except * = without aniline.

Entry	n	Polypeptide Concentration (mg/mL)	Glucose (eq)	Buffer (pH)	Temperature (°C)	Saccharide Conjugation (%)	Yield (%)
1	2	5	50	4	25	78	100
2	2	5	50	5	25	81	100
3	2	5	50	6	25	86	89
4	2	5	50	7	25	83	90
5	2	5	5	7	25	18	100
6	2	5	10	7	25	31	100
7	2	5	100	7	25	89	90
8	2	5	500	7	25	93	99
9	2	5	5	7	50	65	79
10	2	5	10	7	50	70	98
11	2	5	100	7	50	90	92
12	2	5	500	7	50	93	100
13	4	1.5	50	4	50	80	86*
14	4	1.5	50	5	50	83	97
15	4	1.5	50	6	50	79	86
16	4	1.5	50	7	50	60	79
17	4	1.5	500	5	50	93	100
18	4	1.5	500	7	50	96	99

At pH 7, saccharide conjugation is slow even in the presence of aniline, so the equivalents of D-glucose per N-methylaminooxy group were increased from 5 to 500 (Entries 5-8 and 17-18). A large excess of glucose was found to give high levels of saccharide conjugation for both mao-Ehc$^{O}_{70}$ and mao-Etn$^{O}_{70}$, although this strategy may not be practical for conjugation of expensive saccharides. Changing the temperature of mao-Ehc$^{O}_{70}$ conjugations from 25 to 50 °C was also found to be an effective way to increase saccharide conjugation yields when using fewer equivalents of D-glucose (Entries 9-12), and may be a better strategy for conjugation of expensive saccharides. For comparison to the non-ionic polypeptides discussed above, cationic mao-Ehc$^{M}_{70}$ was also reacted with D-glucose under a select set of conditions (Eq 5, Table 2). At pH 7, significantly lower saccharide conjugation to mao-Ehc$^{M}_{70}$ (37%) was observed under identical conditions used for mao-Ehc$^{O}_{70}$ (89%; see Table 1, Entry 7). However, at pH 5 with aniline catalyst, high levels of saccharide conjugation to mao-Ehc$^{M}_{70}$ (93%) were readily obtained.



(5)

Table 2. Isolated and saccharide conjugation yields for reactions of mao-Ehc$^{M}_{70}$ with D-glucose. Reaction conditions as shown in Eq 5.

Aniline	Buffer (pH)	Saccharide Conjugation (%)	Yield (%)
-	5	47	99
+	5	93	93
+	7	37	91

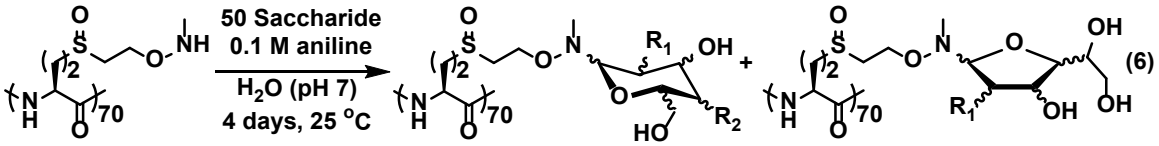


Table 3. Isolated and glycoconjugate yields, as well as number of glycoconjugate isomers formed after saccharide conjugation to mao-Ehc$^{O}_{70}$. Reaction conditions as shown in equation 6, except for * = mao-Ehc$^{O}_{70}$ and saccharide (50 eq) in water (pH 4) without aniline for 4 days at 25 °C.

Name	Saccharide	Saccharide Conjugation (%)	Yield (%)	# of Isomers
Glc-mao-Ehc$^{O}_{70}$	D-glucose	83	90	1
Gal-mao-Ehc$^{O}_{70}$	D-galactose	89	88	2
Man-mao-Ehc$^{O}_{70}$	D-mannose	81	84	4
GlcNAc-mao-Ehc$^{O}_{70}$	N-acetyl-D-glucosamine	15	96	1
GalNAc-mao-Ehc$^{O}_{70}$	N-acetyl-D-galactosamine	79	91	2
lac-mao-Ehc$^{O}_{70}$	β-lactose	38	98	1
mal-mao-Ehc$^{O}_{70}$	β-maltose	40	99	1
lac-mao-Ehc$^{O}_{70}$	β-lactose	74	97*	1
mal-mao-Ehc$^{O}_{70}$	β-maltose	79	99*	1

Based on its good water solubility and non-ionic character, mao-Ehc$^{O}_{70}$ was chosen for further studies on conjugation of a variety of mono- and disaccharides. Using optimized conditions from above, 50 equivalents of each saccharide were reacted with mao-Ehc$^{O}_{70}$ in the presence of aniline catalyst in

water at 25 °C (Eq 6, Table 3). The simple monosaccharides D-galactose and D-mannose gave high levels of neoglycoconjugate formation comparable to results obtained above using D-glucose. N-acetyl-D-galactosamine and N-acetyl-D-glucosamine saccharides, relevant to natural glycoproteins, were found to give significantly different levels of neoglycoconjugate formation, with high levels of saccharide conjugation in GalNAc-mao-Ehc$^{O}_{70}$ and low levels in GlcNAc-mao-Ehc$^{O}_{70}$. These results are consistent with trends observed for conjugation of these saccharides in related systems.[27,34] The disaccharides β-lactose and β-maltose were both found to give low levels of saccharide conjugation at pH 7, but efficient neoglycoconjugate formation was obtained at pH 4, suggesting that reaction of the disaccharides is slow at higher pH yet they can readily form stable conjugates under acidic conditions. Overall, in accord with results obtained in related N-methylaminooxy containing systems,[4,24,26,27] we observed that a variety of unmodified reducing saccharides can be conjugated in high yields to mao-Ehc$^{O}_{70}$ polypeptides.

As shown in Eq 6, the neoglycoconjugates formed from different saccharides can consist of isomers that differ in type of anomer and ring size. The number and types of isomers formed can be readily analyzed by examination of methyl group resonances from N-methylaminooxy groups using ^{1}H NMR as described in literature (see SI Figures S10, S11).[34] Based on assignments of related neoglycoconjugates, it was confirmed that D-glucose and disaccharides with D-glucose at the reducing end gave only β-pyranoside conjugates (Table 3, see SI Figure S10). Conjugation to the reducing ends of D-galactose or D-mannose based saccharides gave multiple isomers that varied in both ring size and anomer, with the β-pyranoside isomer typically being dominant, consistent with prior studies in related systems (Table 3, see SI Figure S11).[34]

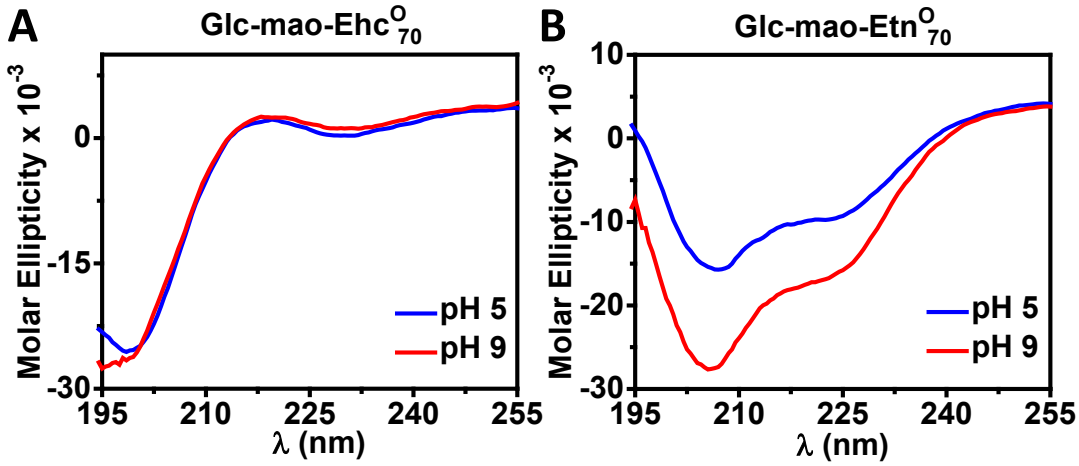


Figure 3. Circular dichroism spectra of **A)** Glc-mao-Ehc$^{O}_{70}$ and **B)** Glc-mao-Etn$^{O}_{70}$ at 20 °C. Samples were prepared at 0.1 mg/mL concentration in aqueous buffers containing 100 mM phosphate and 10 mM Tris adjusted to pH 2 (black), 5 (blue), or 9 (red) using HCl (0.1 M) or NaOH (0.1 M).

To evaluate physical properties of the neoglycopolypeptide conjugates, Glc-mao-Ehc$^{O}_{70}$ and Glc-mao-Etn$^{O}_{70}$ were chosen as model samples for CD spectroscopy analysis. CD spectra of Glc-mao-Ehc$^{O}_{70}$ collected at pH 5 and 9 were nearly identical (Figure 3A), and suggest this polypeptide adopts highly disordered conformations over this pH range. Increased solvation of this glucose functionalized polypeptide in water as compared to mao-Ehc$^{O}_{70}$ may explain the loss of partial 3_{10}-helical content observed in the parent sample. CD spectra of Glc-mao-Etn$^{O}_{70}$ collected at pH 5 and 9 showed this polypeptide also adopted partial 3_{10}--helical conformations with helical content increasing with pH (Figure 3B).[39] The longer hydrophobic tether in Glc-mao-Etn$^{O}_{70}$ likely helps stabilize the α-helical conformation in these polypeptides despite the addition of polar glucose groups. As expected for a polyelectrolyte, cationic Glc-mao-Ehc$^{M}_{70}$ was found to be disordered between pH 5 and 9, similar to parent mao-Ehc$^{M}_{70}$ (see SI Figure S8).[32]

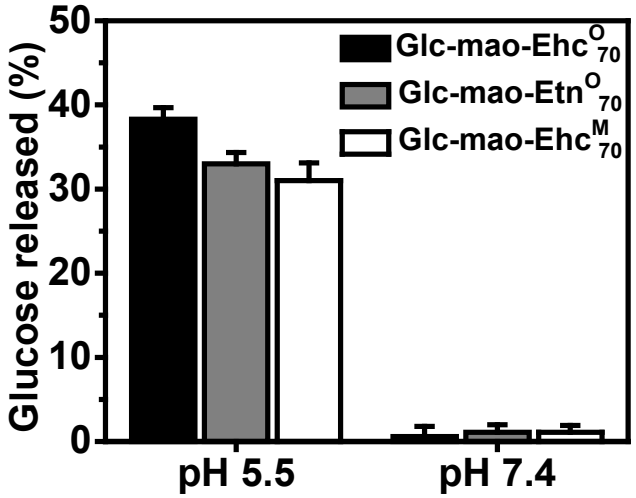


Figure 4. Release of free D-glucose from Glc-mao-Ehc$^{O}_{70}$ (black), Glc-mao-Etn$^{O}_{70}$ (grey) and Glc-mao-Ehc$^{M}_{70}$ (white) at pH 5.5 (200 mM phosphate) and 7.4 (200 mM phosphate). All samples were incubated at 37 °C for 1 week. Error bars are consistent with standard deviations of n = 3.

Stability of model neoglycoconjugates at different pH in water was also evaluated. Aqueous solutions of Glc-mao-Ehc$^{O}_{70}$, Glc-mao-Etn$^{O}_{70}$ and Glc-mao-Ehc$^{M}_{70}$ (all greater than 90% glycosylation) were individually dialyzed at 37 °C for 1 week at pH of either 5.5 or 7.4 (Figure 4). Subsequent analysis of the samples by ^{1}H NMR was used to quantify the loss of D-glucose from each sample. Since acid is known to catalyze both glycoconjugate formation and hydrolysis, and free D-glucose was removed during reactions, all samples were partially deglycosylated at pH 5.5.[43] Although the degree of deglycosylation (*ca*. 30-40%) was comparable for these samples, the more polar Glc-mao-Ehc$^{O}_{70}$ showed greater loss of D-glucose compare to less polar Glc-mao-Etn$^{O}_{70}$. The cationic neoglycoconjugate Glc-mao-Ehc$^{M}_{70}$ was also found to be more stable at pH 5.5 compared to Glc-mao-Ehc$^{O}_{70}$, where the nearby cationic sulfonium group in Glc-mao-Ehc$^{M}_{70}$ may be hindering N-glycoside protonation, which is the first step in hydrolysis. All three samples showed high neoglycoconjugate stability at pH 7 for 1 week at 37 °C, which makes them promising for use in downstream biological studies.

Conclusions

A set of N-methylaminooxy functionalized polypeptides have been prepared and characterized. These new functional polypeptides are water soluble and were found to react with unmodified reducing saccharides to form neoglycoconjugates in high yields under a variety of aqueous conditions. While N-methylaminooxy functionalized and related polymers have been reported, this approach has not been previously demonstrated in synthetic polypeptides. The variation of polypeptide scaffolds resulted in neoglycoconjugates with different chain conformations, hydrophobicity, and charge. The combination of straightforward synthesis, high yields of saccharide conjugation, and conjugate stability make these polypeptides attractive candidates for use as degradable glycoprotein mimics.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.#######.

Additional equations, figures and tables, synthesis procedures, and spectral data for all new molecules (PDF).

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Notes

The authors declare no competing financial interest.

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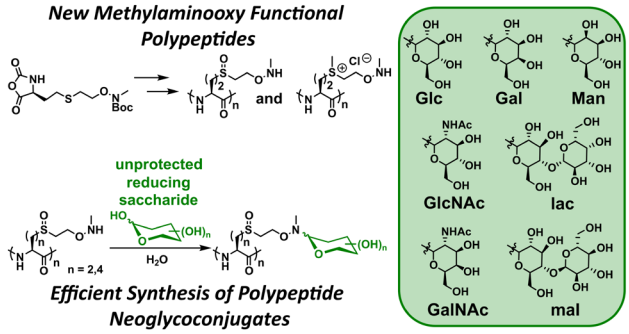
TOC Graphic:


New Methylaminooxy Functional Polypeptides
and
unprotected reducing saccharide
n = 2,4
H2O
Efficient Synthesis of Polypeptide Neoglycoconjugates
Glc
Gal
Man
GlcNAc
lac
GalNAc
mal