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Synthesis of homogeneous glycopeptides and their utility as DNA condensing agents

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Abstract

Two glycopeptides were synthesized by attaching purified glycosylamines (*N*-glycans) to a 20 amino acid peptide. Triantennary and Man9 Boc-tyrosinamide *N*-glycans were treated with trifluoroacetic acid to remove the Boc group and expose a tyrosinamide amine. The amine group was coupled with iodoacetic acid to produce *N*-iodoacetyl-oligosaccharides. These were reacted with the sulfhydryl group of a cysteine-containing peptide (CWK_{18}), resulting in the formation of glycopeptides in good yield that were characterized by ^1H NMR and ESIMS. Both glycopeptides were able to bind to plasmid DNA and form DNA condensates of approximately 110 nm mean diameter with zeta potential of +31 mV. The resulting homogeneous glycopeptide DNA condensates will be valuable as receptor-mediated gene-delivery agents.

Keywords: Glycopeptide; *N*-Glycan; Gene delivery; Glycoconjugate

1. Introduction

Nonviral gene delivery utilizes carrier molecules designed to bind ionically to plasmid DNA, resulting in the formation of DNA colloids of approximately 100 nm diameter. Targeted nonviral gene delivery relies on the incorporation of ligands into the DNA carrier, which are presented on the surface of the DNA colloid and function to mediate receptor recognition and cellular uptake of the DNA–carrier complexes [1]. One of the earliest ligands used for DNA delivery was the glycoprotein asialoorosomucoid (ASOR) possessing Gal-terminated *N*-glycans that bind to the asialoglycoprotein receptor (ASGP-R) on hepatocytes with high affinity [2]. ASOR was conjugated to high-molecular-weight (HMW) polylysine (DP 100–250) to prepare a DNA carrier capable of ionically binding to and condensing plasmid DNA [3]. Although this carrier protected DNA from metabolism and targeted DNA to hepatocytes via the ASGP-R [4], it was difficult to purify from free polylysine [5], and it proved to be antigenic [6].

To circumvent problems associated with HMW carriers, lower-molecular-weight neoglycopeptides have been prepared by coupling multiple lactose (40–50%), Gal (1%), or Man (1%) residues to HMW polylysine (DP 146–190) [7–11]. Although chemically simpler to prepare, these carriers are still heterogeneous, making further modification with polyethylene glycol [12] or subcellular targeting peptides difficult to control [13].

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Further refinements in carrier design were reported by Wagner and co-workers, who utilized a synthetic tetraantennary neoglycopeptide ligand covalently linked to polylysine (DP 190 or 290) to achieve receptor-mediated gene expression in hepatocytes [14]. Although the ligand portion was chemically defined and possessed high affinity for the ASGP-R, the resulting carriers were also HMW and heterogeneous due to random coupling of the neoglycopeptide to polydisperse polylysine.

A carrier composed of a high-affinity neoglycopeptide prepared by attaching three terminal GalNAc residues to a tripeptide backbone (YEE) was also reported as a ligand for ASGP-R-mediated gene delivery [15]. Although the ligand was well defined, its attachment to low-molecular-weight (LMW) polylysine (DP 10–30) using human serum albumin as an intermediate spacer produced a heterogeneous carrier of approximately 70 kDa.

To date, the most chemically defined carrier developed for ASGP-R-mediated gene delivery is a 4–6 kDa triantennary glycopeptide [16]. The glycopeptide is composed of a single natural triantennary N-linked oligosaccharide attached to a short polylysine (DP 10–30) chain. Even though this LMW glycopeptide mediated specific gene transfer to HepG2 cells relative to LMW polylysine, the heterogeneity within polylysine [17] makes it less desirable for further development into a carrier for in vivo applications.

To develop structurally defined LMW glycopeptides useful for targeted gene delivery, we first synthesized a panel of peptides and determined that a minimal polylysine chain of Cys-Trp-Lys_{18} (CWK_{18}) was sufficient to condense DNA into small condensates and protect DNA from metabolism [18,19]. In the present study, we demonstrate the derivatization of CWK_{18} with purified *N*-glycans to form homogeneous glycopeptide carriers that bind and condense DNA into small colloids. The ability to substitute different oligosaccharides into these glycopeptide DNA condensates should provide a means to design a variety of chemically well-defined gene targeting agents for in vivo use.

2. Experimental

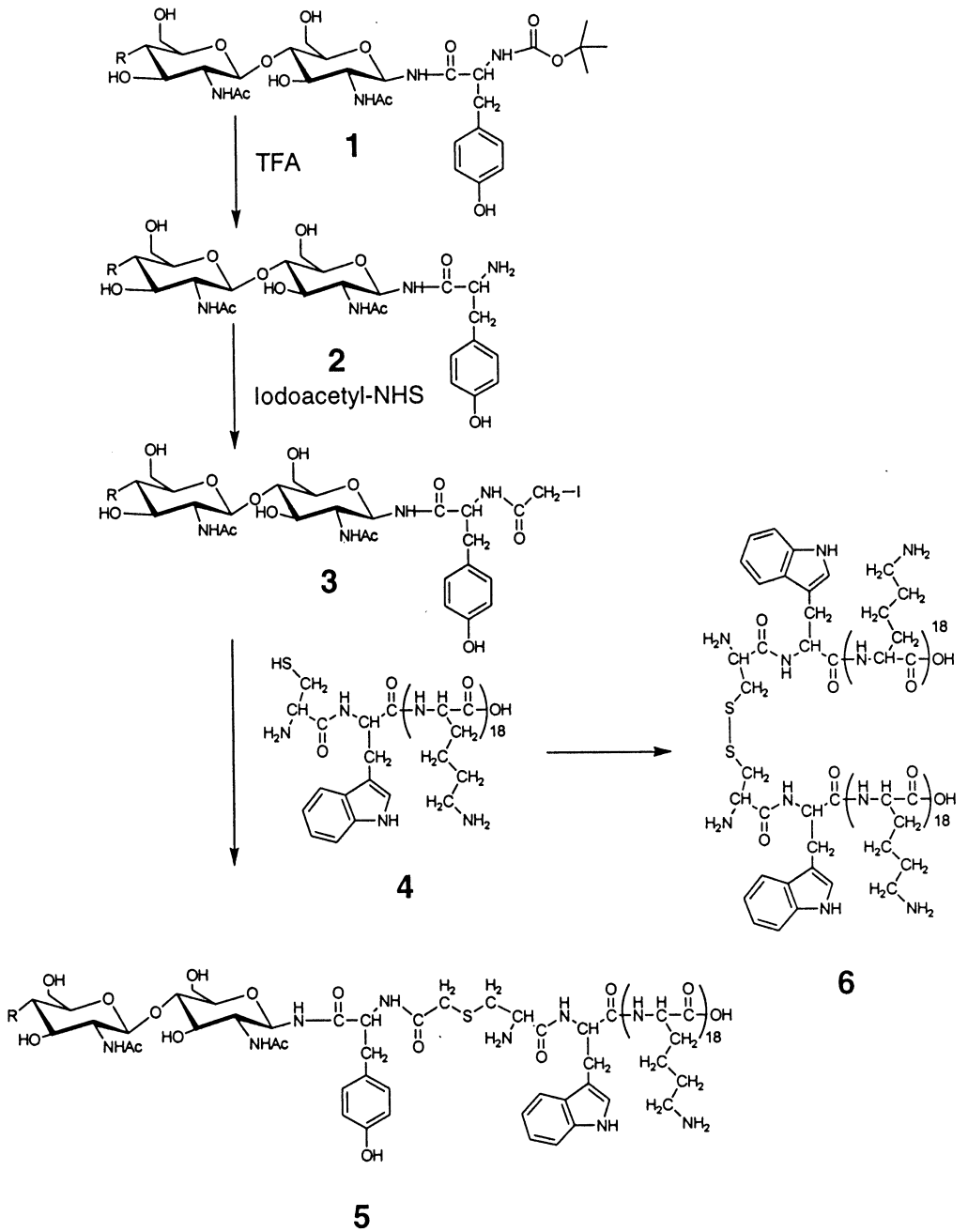
Materials.—tris(2-Carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Iodoacetic acid *N*-hydroxysuccinimide ester was purchased from Sigma (St. Louis, MO). The 5.6 kb plasmid (pCMVL) encoding the luciferase gene under the control of the cytomegalovirus promoter was produced in *E. coli* and purified using a Qiagen Ultrapure-100 kit (Santa Clarita, CA). Preparative and analytical C_{18} reversed-phase HPLC columns were purchased from Vydac (Hesperia, CA). High-performance liquid chromatography (HPLC) was performed using a computer-interfaced HPLC and fraction collector from ISCO (Lincoln, NE).

Synthesis and characterization of glycopeptides.—CWK_{18} was synthesized on solid phase and purified as previously reported [18]. The terminal cysteine was either alkylated with iodoacetic acid to prepare $AlkCWK_{18}$ [18] or used as an attachment site for oligosaccharides. A triantennary and a Man9 *N*-glycan were purified as Boc tyrosinamide derivatives from bovine fetuin and soy bean agglutinin, respectively [20,21]. The Boc group was removed by treating the dry tyrosinamide oligosaccharide (0.5 μmol) with 100 μL of neat trifluoroacetyl (TFA) for 10 min at room temperature (rt), followed by freeze-drying. The resulting amine terminus was N-iodoacetylated by dissolving 0.5 μmol of the tyrosinamide oligosaccharide in 0.5 mL of 100 mM sodium bicarbonate pH 8, followed by reaction with 20 μmol of iodoacetic acid *N*-hydroxysuccinimide ester in 50 μL of DMF. After 3 h at rt, an additional 20 μmol of iodoacetic acid *N*-hydroxysuccinimide ester was added and reacted for an additional 12 h. The reaction was acidified with 50 μL of 10% (v/v) AcOH and purified on a Sephadex G-25 column (2.5×45 cm) eluted with 0.1% (v/v) AcOH while detecting absorbance at 280 nm. The peak eluting at 75–125 mL was pooled and freeze-dried, resulting in 80–90% yield of *N*-iodoacetyl-oligosaccharide that eluted as a single peak on RP-HPLC as described below.

CWK_{18} (1 μmol) was reduced by reaction with 25 μmol of TCEP in 0.5 mL of 0.1 M

sodium phosphate pH 7 for 4 h at rt. Reduced CWK_{18} was purified by injecting 0.5 μmol onto a semipreparative C_{18} RP-HPLC column (2 × 25 cm) eluted at 10 mL min^{-1} with 0.1% TFA and a gradient of 5–25% MeCN over 25 min, while detecting by Abs_{280nm}. The peak eluting at 14 min was collected, freeze-dried, and stored frozen in 0.1% TFA.

N-iodoacetyl-oligosaccharide (200 nmol) was conjugated to CWK_{18} (250 nmol) by reaction for 12 h at rt in 200 μL of 0.2 M Tris pH 8.0. The resulting glycopeptide was purified by injecting up to 200 nmol onto a semipreparative RP-HPLC (2 × 25 cm) column eluted at 10 mL min^{-1} with 0.1% TFA and a gradient of 5–25% MeCN over 25 min. The glycopeptide peak eluting at 23 min was pooled, freeze-dried, reconstituted in water and quantified by absorbance ($\varepsilon_{280nm} = 6930\ M^{-1}\ cm^{-1}$), resulting in an isolated yield of 40%. Purified gly-



Scheme 1. Glycopeptide synthetic scheme. R represents the remainder of the triantennary or Man9 *N*-glycan illustrated in Fig. 2.

copeptides (triantennary-CWK_{18} and Man9-CWK_{18}) rechromatographed as single peaks and were characterized as described below.

Glycopeptides (500 nmol) were prepared for 500-MHz ^1H NMR spectroscopy by repeated freeze-drying in D_2O. Samples were prepared in 0.5 mL of 99.98% D_2O containing 0.01% acetone as an internal standard and analyzed on a Bruker 500 MHz NMR spectrometer operating at 23 °C. Glycopeptides were characterized by ESIMS by injecting 2 nmol onto a RP-HPLC column eluted at 1 mL min^{-1} with 0.1 v/v% AcOH and 0.02% TFA and a gradient of 5–25% MeCN over 30 min. The eluting glycopeptide was directly infused into the electrospray source of a Finnigan LCQ mass spectrometer and ions were collected in the positive mode.

Preparation and characterization of glycopeptide DNA condensates.—Glycopeptide DNA condensates were formed at a plasmid DNA concentration of 50 μg mL^{-1} in HBM (5 mM Hepes 0.27 M mannitol pH 7.4). DNA condensates were formed by combining 750 μL of plasmid DNA (100 μg mL^{-1}) with 750 μL of triantennary-CWK_{18} (50 nmol mL^{-1}), Man9-CWK_{18}, or AlkCWK_{18} while vortexing. The particle size of glycopeptide and peptide DNA condensates were analyzed at a DNA concentration of 50 μg mL^{-1} in HBM by quasielastic light scattering (QELS) [22]. The particle surface charge was determined at 50 μg mL^{-1} in HBM by zeta potential analysis using a Brookhaven ZetaPlus (Brookhaven Instruments).

3. Results

The synthetic scheme used to prepare glycopeptides is illustrated in Scheme 1. Boc-tyrosinamide oligosaccharide **1** was deprotected with TFA to produce tyrosinamide oligosaccharide **2** with an exposed primary amine. The amine was selectively derivatized with iodoacetic acid, resulting in the formation of *N*-iodoacetyl-oligosaccharide **3**. The iodo group was readily displaced by the sulfhydryl on CWK_{18} **4**, resulting in the formation of either triantennary-CWK_{18} or Man9-CWK_{18} glycopeptide **5**.

Each reaction was monitored by RP-HPLC (Fig. 1). Removal of Boc from **1** under acidic conditions (TFA) produced an earlier-eluting product **2** (Fig. 1(B)). Attachment of iodoacetic acid to form **3** resulted in a shift back to a longer retention time (Fig. 1(C)). Reaction of **3** with **4** (Fig. 1(D)) resulted in the formation of **5** and **6** (Fig. 1(E)) with the complete disappearance of **3**. Product **5**, eluting at 23 min, was identified as the desired glycopeptide, even though it eluted coincident with starting material **4**. Byproduct **6**, eluting at 28 min, resulted from the oxidation of residual **4** at pH 8. When studying the reaction of **3** with **4**, a reduction in pH to 7.5 decreased the yield of **5** due to the increased formation of byproduct **6**. Likewise, at a stoichiometry of 1:1, the reaction of **3** and **4** was judged incomplete due to residual **3**. Under optimal conditions, the reaction was complete in 2 h at pH 8 at a stoichiometry 1:1.25 of **3**:**4**. Glycopeptides were purified by RP-HPLC, resulting in a 40% yield that was $>95\%$ pure on re-chromatography by analytical RP-HPLC (Fig. 1(F)).

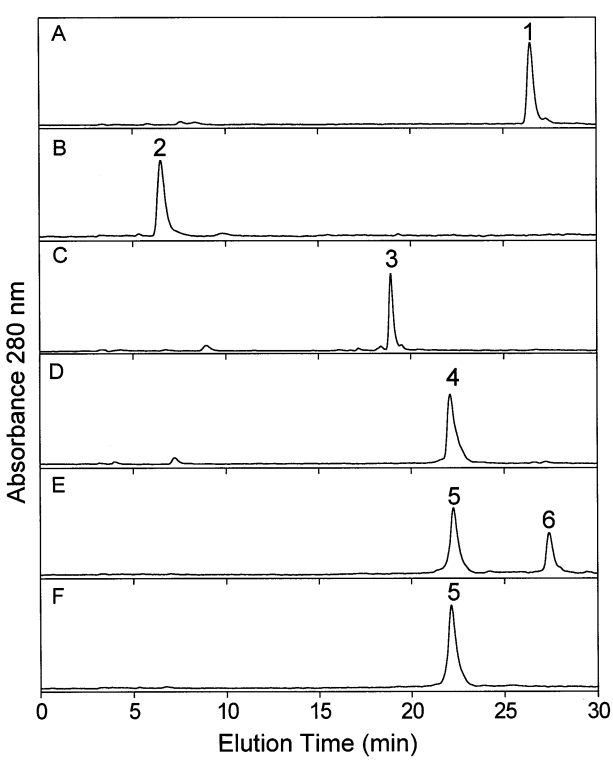


Fig. 1. RP-HPLC analysis of Man9-CWK_{18} synthesis. An example of RP-HPLC monitoring of glycopeptide synthesis is illustrated. Peaks are labeled according to numbering in Scheme 1.

^{1}H NMR spectroscopy was used to verify the presence of signals arising from both the peptide and the oligosaccharide of triantennary-CWK$_{18}$ and Man9-CWK$_{18}$ (Fig. 2). Resonances arising from the 18 Lys side chains [CH_2 1.48 (γ), CH_2 1.69 (δ), CH_2 1.77 (β), and CH_2 2.99 ppm (ε)], and CH α protons (4.29 ppm) were present in the spectra of both glycopeptides (Fig. 3(A) and (B)). Likewise, the tryptophan and tyrosine resonances at 6.8–7.7 ppm were indistinguishable in both glycopeptides. In contrast, the anomeric protons of each glycopeptide were characteristic of triantennary or Man9 oligosaccharide. Triantennary-CWK$_{18}$ possessed Gal (6, 6′ and 8), GlcNAc (5, 5′ and 7) and Man (4 and 4′) anomeric resonances that closely matched the chemical shifts identified for Boc tyrosinamide triantennary oligosaccharide (Table 1), with only subtle shifts in the anomeric resonance for GlcNAc 1 and 2 [19] (Fig. 3(A)). In addition, the chemical shifts of all five *N*-acetyl groups were readily assigned by comparison with the same resonance in a Boc tyrosi-

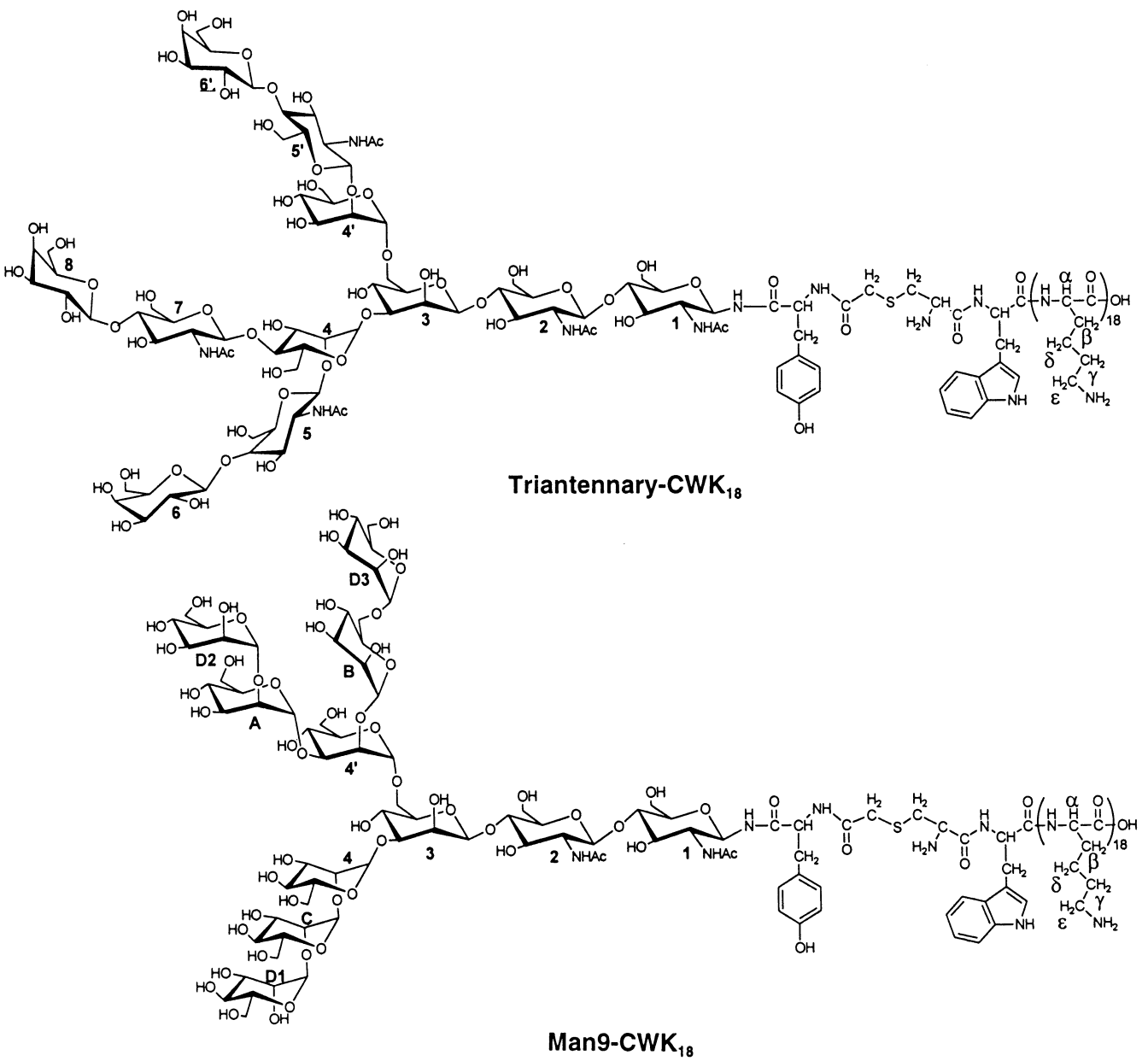


Fig. 2. Structures of synthetic glycopeptides. The structures of triantennary-CWK$_{18}$ and Man9-CWK$_{18}$ are illustrated along with nomenclature used in assigning NMR spectra.

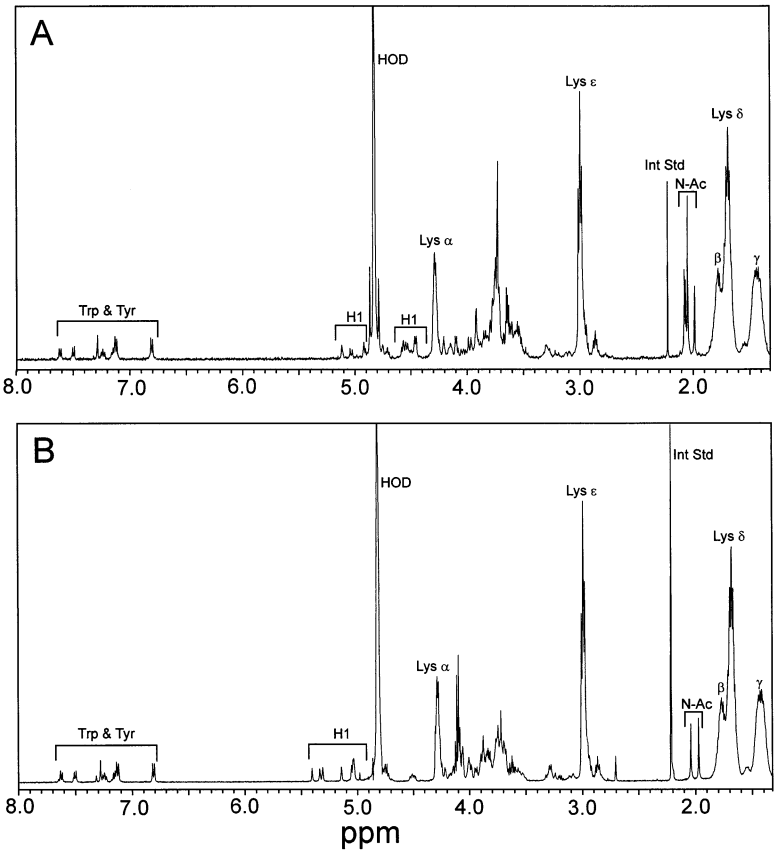


Fig. 3. ^{1}H NMR analysis of triantennary-CWK$_{18}$ and Man9-CWK$_{18}$. The 500-MHz ^{1}H NMR spectra of triantennary-CWK$_{18}$ (panel A) and Man9-CWK$_{18}$ (panel B) are illustrated. The α, β, γ, δ and ε lysine resonances are assigned along with the anomeric and *N*-acetyl resonances of the oligosaccharide using the nomenclature presented in Fig. 2.

namide triantennary. The NMR spectrum of Man9-CWK$_{18}$ also possessed similar chemical shifts for the anomeric protons of Man D1-3, A-C, and 4, 4′ relative to that reported for Boc tyrosinamide Man9 oligosaccharide (Table 1), with only minor perturbation of the anomeric resonances of GlcNAc 1 and 2 [20] (Fig. 3(B)). Likewise, the two *N*-acetyl groups were well resolved from the lysine signals in the glycopeptide and possessed nearly identical chemical shifts as the Boc tyrosinamide oligosaccharide.

LC–MS analysis of triantennary-CWK$_{18}$ produced ions at 1608.7, 1206.7 and 965.5, which identified a molecule of 4822.8 amu (Fig. 4(A)). This correlated well with the calculated average mass of 4823.2 for triantennary-CWK$_{18}$. Man9-CWK$_{18}$ produced ions of 940.9, 1175.9 and 1567.4 (Fig. 4(B)) corresponding to a molecule of 4699.2 amu, which agrees with the calculated average mass of 4700.3.

To establish that each glycopeptide could bind to plasmid DNA and form small DNA condensates, the particle size and zeta potential of glycopeptide DNA condensates were compared with those of alkylated-CWK$_{18}$ (AlkCWK$_{18}$) DNA condensates using QELS. Previous titration studies involving displacement of an intercalated dye from DNA established that AlkCWK$_{18}$ binding to plasmid DNA was complete at a stoichiometry of 0.3 nmol per μg or greater [18]. We performed the same titration with each glycopeptide and found these to have identical binding affinity to DNA as AlkCWK$_{18}$. The mean particle size of DNA condensates prepared at an identical stoichiometry (0.5 nmol of peptide or glycopeptide per μg of DNA) was slightly greater when using triantennary-CWK$_{18}$ (107 nm) and Man9-CWK$_{18}$ (109 nm) as condensing agents when compared with AlkCWK$_{18}$ (81 nm). Each glycopeptide and peptide condensed DNA to form two populations of particles.

Table 1
^{1}H NMR chemical shifts [a] for glycopeptides

Proton [b]		Triantennary-CWK$_{18}$	Boc-Tyr-triantennary [c]	Man9-CWK$_{18}$	Boc-Tyr-Man9 [d]
H-1 of GlcNAc	1	5.036	5.017	5.039	5.012
	2	4.724	4.616	4.560	4.602
	5	4.563	4.568		
	5′	4.582	4.583		
	7	4.536	4.547		
Gal	6	4.467	4.463		
	6′	4.471	4.475		
	8	4.464	4.469		
Man	4	5.121	5.119	5.341	5.336
	4′	4.925	4.923	4.866	4.869
	A			5.410	5.409
	B			5.148	5.147
	C			5.316	5.312
	D1			5.039	5.040
	D2			5.059	5.056
	D3			5.039	5.040
NAc	1	1.987	1.979	1.989	1.971
	2	2.077	2.082	2.059	2.070
	5	2.048	2.049		
	5′	2.048	2.046		
	7	2.067	2.076		
Lys	α	4.292		4.295	
	β	1.778		1.775	
	γ	1.438		1.426	
	δ	1.690		1.693	
	ε	2.998		2.995	
Trp	b	7.312		7.296	
	d	7.504		7.507	
	e	7.251		7.255	
	f	7.157		7.163	
	g	7.614		7.624	
Tyr	a	7.124	7.138	7.131	7.111
	b	6.805	6.850	6.815	6.813

[a] Reported as the ppm relative to an internal standard of acetone (2.225 ppm).
[b] See Fig. 2 for residue nomenclature.
[c] Data reproduced from Ref. [20].
[d] Data reproduced from Ref. [21].

The major population (75–85%) possessed a mean diameter less than 100 nm (Fig. 5), whereas a minor population (15–25%) having a slightly larger size (150–200 nm diameter) was present for AlkCWK$_{18}$ DNA condensates and both glycopeptide DNA condensates. In addition, the surface charge of glycopeptide DNA condensates ($+31 \pm 5$ mV) was indistinguishable from that of AlkCWK$_{18}$ DNA condensates.

4. Discussion

The design of glycoconjugate-targeted drug-delivery systems for in vivo use requires an understanding of the specificity of mammalian lectins to properly cluster and orient nonreducing residues for optimal multivalent recognition by the target lectin [23]. Although many Gal- and Man-terminated glycoconjugates have been prepared for targeting DNA to liver

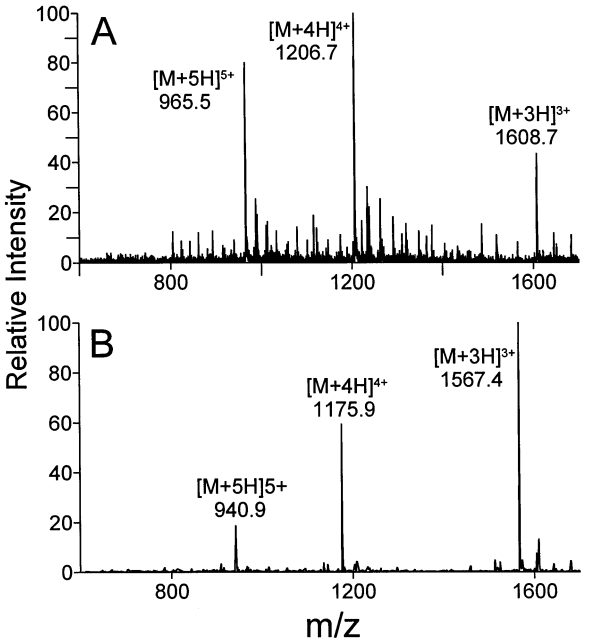


Fig. 4. Positive-ion ESIMS analysis of (A) triantennary-CWK_{18} and (B) Man9-CWK_{18}.

hepatocytes [3,4,6–10,15,16] and macrophages [11], few studies have attempted to design LMW carriers that bind and condense DNA while possessing high-affinity ligands for their target receptor.

Our strategy differs significantly from others in that natural *N*-glycans are used as ligands that are conjugated site specifically to a LMW DNA condensing peptide. The triantennary oligosaccharide used has been studied extensively for its ability to bind to the ASGP-R both in vitro and in vivo [23,24]. Likewise, CWK_{18} was selected as the minimal polylysine peptide that could bind and condense DNA into small condensates that mediate nonspecific in vitro gene delivery [18]. The present study demonstrates the synthesis of homogeneous glycopeptides by forming a conjugate between well-characterized *N*-glycans and CWK_{18} to create carriers of less than 5000 Da.

N-glycans were prepared as *N*-iodoacetyl-tyrosinamide oligosaccharides to allow conjugation to a cysteine-containing peptide. This required the removal of Boc with acid, which may be accomplished without hydrolysis of glycosidic linkages (even NeuAc and Fuc), provided neat TFA is used. Once exposed, the single amine group acts as a regioselective conjugation site to attach iodoacetic acid. The *N*-iodoacetyl-tyrosinamide oligosaccharides were found to be stable products that reacted at near 1:1 stoichiometry with CWK_{18}. A slight excess of peptide was found to be optimal to consume the oligosaccharide completely. Surprisingly, the attachment of an *N*-glycan to CWK_{18} did not influence its retention time on RP-HPLC. This was true of both triantennary-CWK_{18} and Man9-CWK_{18}, which were chromatographically equivalent to CWK_{18} despite numerous attempts to resolve the glycopeptides and peptide. However, their coelution did not preclude their purification from excess CWK_{18}, which readily formed dimeric-CWK_{18} at pH 8.0, resulting in its complete resolution on RP-HPLC.

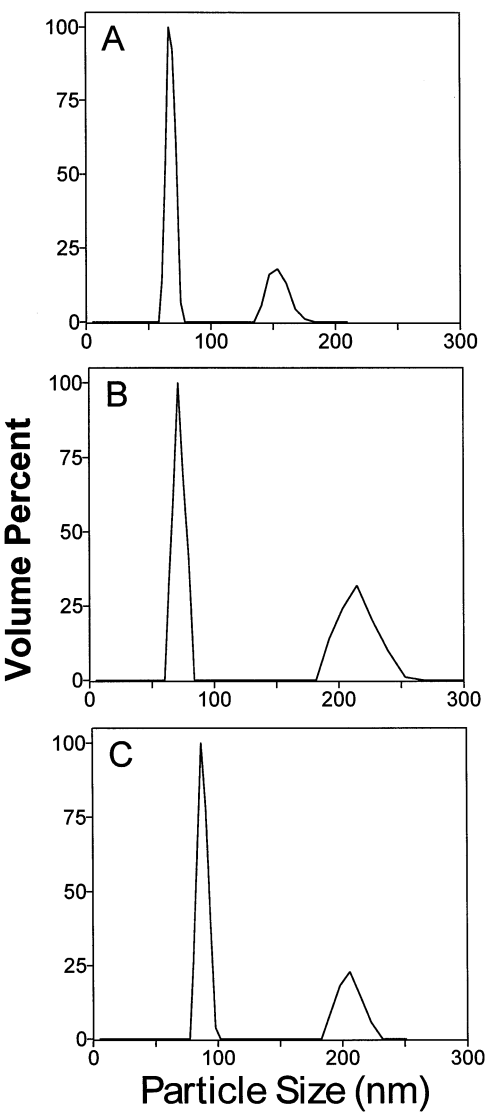


Fig. 5. Particle size analysis of glycopeptide DNA condensates. The particle size of AlkCWK_{18} (A), triantennary-CWK_{18} (B) and Man9-CWK_{18} DNA condensates (C) are compared by QELS analysis.

Purified glycopeptides were determined to be $>95\%$ pure by HPLC analysis and produced ^{1}H NMR spectra in which the integration of carbohydrate anomeric signals to the lysine side chain resonances provided evidence of a 1:1 conjugate free of unmodified CWK_{18}. Likewise, each glycopeptide produced multiply charged ions on ESIMS that closely corresponded to the predicted mass for each glycopeptide.

The ability of glycopeptides to ionically bind and condense DNA is a critical parameter for their use in gene delivery. We have previously demonstrated that polylysine peptides shorter than $AlkCWK_{18}$ have low DNA binding affinity and produce large (>500 nm) DNA condensates [18]. Likewise, derivatization of CWK_{18} with polyethylene glycol (5000 Da) failed to alter the binding affinity of CWK_{18} for DNA but did slightly increase the particles size and significantly decreased the zeta potential determined for PEG-peptide DNA condensates [25].

The present study compared the particle size and zeta potential when using $AlkCWK_{18}$, triantennary-CWK_{18} or Man9-CWK_{18} as the DNA condensing agent. QELS analysis revealed two populations of particles for glycopeptides and peptides, also resulting in slightly larger mean diameters such as determined for PEG-peptide DNA condensates [25]. However, unlike PEG-peptides, the zeta potential was found to be equivalent for both glycopeptide and peptide DNA condensates, a fact that demonstrates that incorporation of an oligosaccharide into the carrier did not alter DNA condensate surface charge. These results suggest that a variety of neutral *N*-glycans may be substituted onto CWK_{18} without interfering with DNA condensate formation.

The present study established an efficient route to the synthesis of glycopeptides, resulting in glycoconjugates that form small plasmid DNA condensates, which may find utility as targeted gene-delivery systems. Given the diversity of carbohydrate lectin interactions in nature [26], it should be possible to alter the oligosaccharide structure within these glycopeptides to select unique target sites in vivo.

Acknowledgements

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