Glycoconjugate Journal (1996) **13:** 315–319

Use of diethyl squarate for the coupling of oligosaccharide amines to carrier proteins and characterization of the resulting neoglycoproteins by MALDI-TOF mass spectrometry*

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Received 2 August 1995; revised 27 September 1995

The 8-methoxycarbonyloctyl glycosides of GlcNAcβ, Galβ1-4Glcβ, Fucα1-2Fucα1-3GalNAcβ and Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ were converted to primary amines by reaction with neat ethylenediamine and then coupled to bovine serum albumin (BSA) using diethyl squarate as the connector. The average degree of incorporation of the sugar onto the protein, as well as the molecular weight distribution, could be conveniently determined using matrix assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry thus avoiding cumbersome structure-dependent colour-tests or analysis of cleaved ligand. The present coupling method has the advantages of proceeding under very mild conditions, yielding controlled incorporation values and can reliably be used for the coupling of very small amounts (mg) of oligosaccharide.

Keywords: neoglycoprotein, glycoconjugate, diethyl squarate, BSA-conjugates, matrix assisted laser desorption ionization/time of flight (MALDI-TOF).

Introduction

The primary interest in neoglycoproteins [1] bearing oligosaccharides of well defined structure is in their use as polyvalent ligands for the study of biologically important carbohydrate-protein recognition [2] or carbohydrate-carbohydrate interactions [3]. Simple methods for the controlled preparation of neoglycoconjugates and the analysis of their structures are therefore highly desirable.

Many coupling methods have been employed for the preparation of neoglycoconjugates using both isolated and synthetic oligosaccharides attached to various spacer arms (for reviews, see [1, 4, 5]). Many of these coupling methods employ reaction conditions that can be harsh, most often either oxidative or reductive, and other methods require precise control of reagent stoichiometry. The net result is that such methods can be incompatible with many proteins and difficult to implement when only small quantities of oligosaccharide are available. For many of these methods, the chemical manipulations are difficult to reproduce and it can therefore be difficult to predict the degree of incorporation of the sugar onto the carrier protein, typically bovine serum albumin (BSA). Determination of the average incorporation value usually requires either performing a colour-test specific for the attached carbohydrate structure directly on the dialysed neoglycoprotein or cleavage of the carbohydrate followed by sugar analysis.

We report here a simple, efficient and reproducible method for the preparation of neoglycoproteins which makes use of diethyl squarate, a reagent introduced by Tietze *et al.* [6], to couple amino-derivatized sugars to amino groups on proteins. The method is efficient even on very small scales (~1 mg). The degree of incorporation of oligosaccharide onto the protein can be determined very simply using matrix assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry (MS), thereby avoiding the need for sugar and protein assays [1, 7–9]. In addition to providing the

*This paper is dedicated to Sen-itiroh Hakomori on the occasion of his 65th birthday

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average incorporation value, MALDI-TOF MS also provides information on the range of distributions making up this value. The method is particularly useful in our own research where we have built up a large inventory of 8-methoxycarbonylactyl glycosides for use in the preparation of glycoconjugates.

Materials and methods

Synthetic glycosides

GlcNAcβ-O$(CH_2)_8$COOMe, Galβ1-4Glcβ-O$(CH_2)_8$COO Me, Fucα1-2Fucα1-3GalNAcβ-O$(CH_2)_8$COOMe and Fucα1-2 Galβ1 - 3[Fucα1 - 4]GlcNAcβ - O$(CH_2)_8$COOMe were available from previous work [10–13]. Analytical TLC was performed on Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by charring with 5% sulfuric acid. Millex-GV filter units were from Millipore (Missisuaga, ON), C-18 Sep-Pak cartridges were from Waters Associates (Missisuaga, ON). Diethyl Squarate was from Aldrich. ^{1}H NMR spectra were recorded at ambient temperature at 300 MHz (Bruker AM-300) with DOH (δ4.80, D_2O). Crystalline bovine serum albumin (BSA) was obtained from Miles Laboratories, Inc., dialysed against Milli-Q water and lyophilized before use. Anhydrous ethanol and diethyl squarate was obtained from Aldrich. Ethylenediamine was dried first over CaO/KOH, then distilled over 4 Å molecular sieves and redistilled from sodium metal. MALDI-TOF mass spectra was recorded on an HP G2020A (LD-TOF) instrument using gentisic acid (2,5 dihydroxy benzoic acid) as the matrix. Fast atom bombardment (FAB) mass spectra were recorded on a Kratos AEIMS9 instrument with Xe as the bombarding gas and glycerol as the matrix.

General procedure for the preparation of oligosaccharide amines

The oligosaccharide (1–10 mg) was dissolved in neat anhydrous ethylenediamine (2 ml) and heated at 70 °C for 2 days in a screw capped tube with a Teflon liner. The solution was cooled, diluted with deionized water (6 ml) and placed in an ice-bath. The cooled solution was then passed through a C-18 Sep-Pak cartridge which was washed with water (30 ml). The aqueous flow-through was loaded again onto a fresh C-18 Sep-Pak cartridge which was also washed with deionized water (20 ml). The product was eluted from each Sep-Pak with methanol (15 ml), the eluates were combined and the methanol evaporated. The residue was dissolved in deionized water (8 ml) and the solution passed through a Millipore filter (0.22 μm) and the filtrate was lyophilized to provide white powders. ^{1}H NMR and FAB mass-spectral data for the oligosaccharide amines thus obtained are presented in Table 1.

Table 1. ^{1}H-NMR (chemical shifts and coupling constants) and mass data for oligosaccharide amines.

SM[a]	*Oligosaccharide-amine*	*^{1}H-NMR data*	*Mass data*
1	GlcNAcβ1-O$(CH_2)_8$CO-NH$(CH_2)_2NH_2$	4.53 (GlcNAc, J = 8.5 Hz, 1H), 3.29 (CONHCH_2, J = 6.5 Hz, 2H), 2.80 (CH_2NH_2, J = 6.5, 2H), 2.25 (CH_2CO, J = 7.5 Hz, 2H), 1.93 (NAc, 3H).	*m/z* 420.0 $[M+H]^+$ 442.0 $[M+Na]^+$
2	Galβ1-4GLcβ1-O$(CH_2)_8$CONH$(CH_2)_2NH_2$	4.40 (Gal, J = 8.5 Hz, 1H), 4.39 (Glc, J = 8.5 Hz, 1H), 3.25 (CONHCH_2, J = 6.5 Hz, 2H), 2.78 (CH_2NH_2, J = 6.5 Hz, 2H), 2.23 (CH_2CONH$_2$, J = 6.5 Hz, 2H).	*m/z* 541.1 $[M+H]^+$ 563.1 $[M+Na]^+$
3	Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-O$(CH_2)_8$-CONH$(CH_2)_2NH_2$	5.10 (Fuc, J = 4.0 Hz, 1H), 5.00 (Fuc, J = 4.0 Hz, 1H), 4.60 (Gal, J = 8.0 Hz, 1H), 4.32 (GlcNAc, J = 8.1 Hz, 1H), 3.29 (CONHCH_2, J = 6.5 Hz, 1H), 2.80 (CH_2NH_2, J = 6.5 Hz, 1H), 2.22 (CH_2CONH$_2$, J = 7.5 Hz, 1H), 2.00 (N*A*c, 3H).	*m/z* 874.5 $[M+H]^+$ 896.4 $[M+Na]^+$
4	Fucα1-2Fucα1-3GalNAcβ1-O$(CH_2)_8$CONH-$(CH_2)_2NH_2$	5.29 (Fuc, J = 3.5 Hz, 1H), 4.96 (Fuc, J = 4.0 Hz, 1H), 4.50 (GalNAc, J = 8.5 Hz, 1H), 3.29 (CONHCH_2, J = 6.5 Hz, 2H), 2.80 (CH_2NH_2, J = 6.5 Hz, 2H), 2.26 (CH_2CONH$_2$, J = 7.5 Hz, 2H).	*m/z* 712.3 $[M+H]^+$ 734.3 $[M+Na]^+$

[a]Starting Material

General procedure for diethyl squarate coupling

3,4-Diethoxy-3-cyclobutene-1,2-dione (diethyl squarate, 0.95 equivalents) was added to a stirred solution of the oligosaccharide amine in anhydrous ethanol (1 ml). After 4 h, silica gel TLC ($CHCl_3$:MeOH:H_2O 23:9:1) showed complete conversion to a faster moving spot. The reaction mixture was concentrated and the crude residue was dissolved in deionized water (5 ml). The solution was passed through a C-18 Sep-Pak cartridge and washed with water (20 ml) and the product was eluted with methanol (10 ml) which was evaporated. The residue was dissolved in water (4 ml), filtered through a 0.22 μm filter and lyophilized to yield the oligosaccharide-squarate adduct as a white powder.

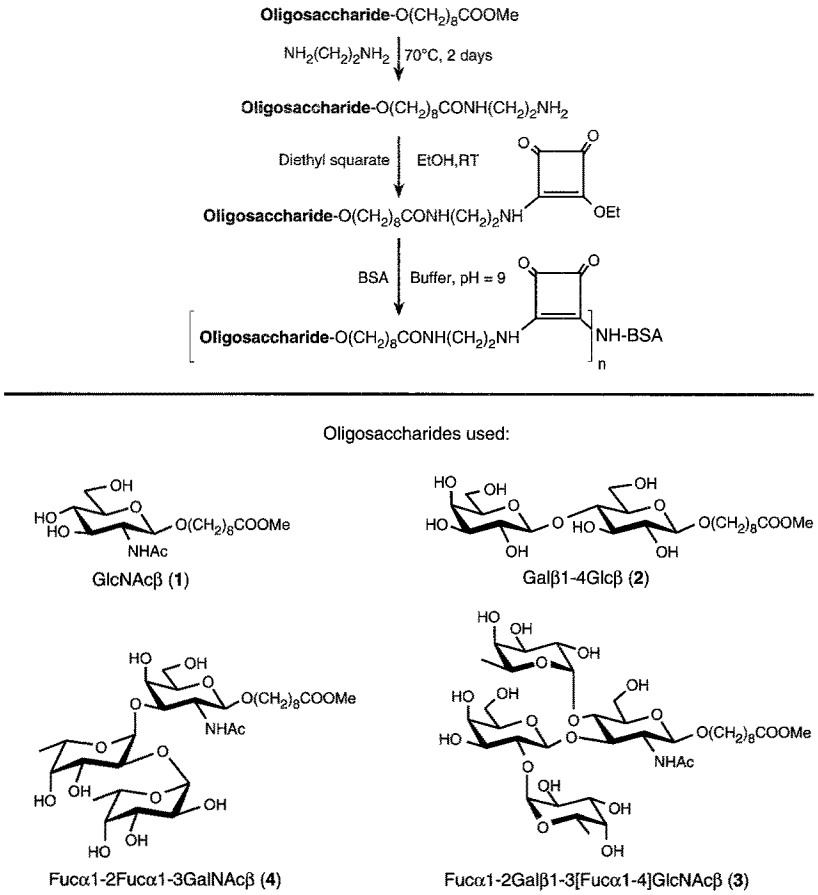
General procedure for coupling to BSA (Scheme 1)

The chosen amount of bovine serum albumin was dissolved in buffer (0.35 M $KHCO_3$ in 0.07 M $Na_2B_4O_7$, pH = 9.0, 2.5 ml), the oligosaccharide-squarate adduct was added and the reaction mixture was stirred at RT for 16 h. The reaction mixture was dialysed against five changes of deionized water in an Amicon ultrafiltration cell (50 ml) equipped with a PM-10 membrane, and then lyophilized to provide the neoglycoprotein.

For MALDI-TOF MS, the neoglycoproteins were dissolved in deionized water to yield a concentration of 0.14 mM, which resulted in a delivered quantity of 70 pmol being delivered onto the probe in 0.5 μl. Gentisic acid (2,5 dihydroxy benzoic acid, 0.5 μl of a 10 mg ml^{-1} in water) was added to the sample probe and dried under vacuum. Then, 0.5 μl of the neoglycoprotein solution was placed on the probe followed by an additional 0.5 μl of matrix solution and the probe was again dried under vacuum before the spectra were recorded. The average degree of incorporation was estimated by MALDI-TOF MS (Table 2 and Fig. 1) using the centre of the distribution of the singly-charged molecular ion.

Results and discussion

The synthetic mono- or oligosaccharides used in this study contained the 8-methoxycarbonyloctyl aglycone introduced by Lemieux as a linking arm for the attachment to proteins and solid supports [7]. Previously, such compounds have been coupled to proteins by a sequence of reactions involving the reaction with hydrazine to form acyl hydrazides which are then oxidized by nitrous acid formed *in situ* [7] or, more conveniently, N_2O_4 [8] to give



Scheme 1. General reaction scheme for the coupling of oligosaccharides to BSA.

Table 2. Degree of incorporation of BSA-conjugates.

Oligosaccharide used (mg)	*Targeted incorporation (n value)*	*Structure of BSA-conjugate formed*	*Incorporation achieved (n value)*	*Incorporation efficency (%)*
1 (3.7 mg)	10	[GlcNAcβ-O(CH$_2$)$_8$CONH(CH$_2$)$_2$NH- squarate -NH-BSA]$_{8.4}$	8.4	84
2 (2.1 mg)	5	[Galβ1-4Glcβ-O(CH$_2$)$_8$CONH(CH$_2$)$_2$NH- squarate -NH-BSA]$_{3.5}$	3.5	70
2 (1.0 mg)	20	[Galβ1-4Glcβ-O(CH$_2$)$_8$CONH(CH$_2$)$_2$NH- squarate -NH-BSA]$_{8.2}$	8.2	50
3 (4.6 mg)	15	[Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ-O(CH$_2$)$_8$CONH(CH$_2$)$_2$NH- squarate -NH-BSA]$_{11}$	11	73
4 (5.8 mg)	15	[Fucα1-2Fucα1-3GalNAcβ-O(CH$_2$)$_8$CONH(CH$_2$)$_2$NH- squarate -NH-BSA]$_{13}$	13	86

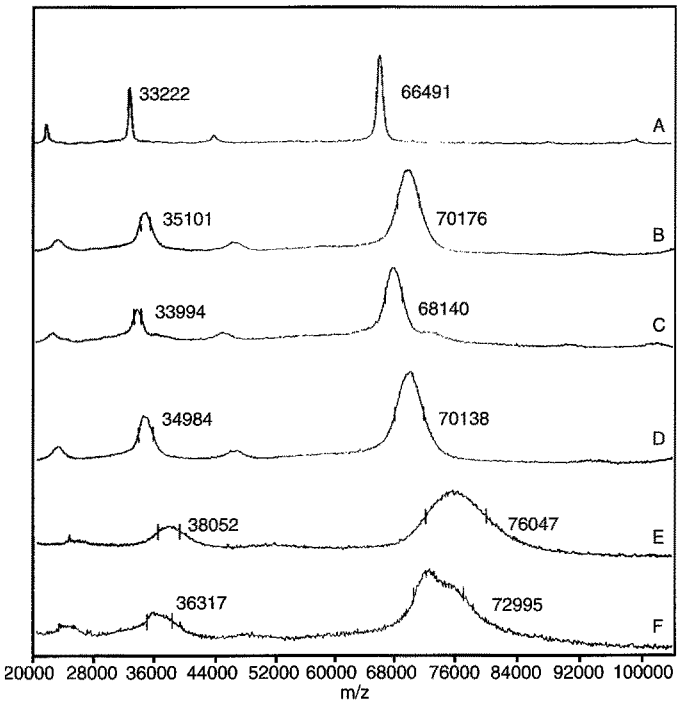


Figure 1. MALDI-TOF spectra of: (A) BSA calibration standard; (B) GlcNAc-BSA (n = 8.4); (C) lactose-BSA (n = 3.5); (D) lactose-BSA (n = 8.2); (E) Fucα1-2Galβ1-3[Fucα1-4]GlcNAc-BSA (n = 11); (F) Fucα1-2Fucα1-3GalNAc-BSA (n = 13).

the labile acyl azides. The acyl azides couple to proteins by acylation of amino groups, typically lysine residues. These procedures are effective for large scales, but on smaller scales traces of residual hydrazine can drastically lower yields, an excess of nitrous acid must be used and the labile acyl azide undergoes a competing reaction, the Curtius rearrangement, leading to difficulties in obtaining reproducible coupling yields. In the most commonly used procedure of Pinto and Bundle [8] the percentage of the oligosaccharide attached to BSA varied in the range 15–58% with an average incorporation of 39%.

In the present method, the esters were converted to primary amines by reaction with neat anhydrous ethylenediamine at 70 °C for 2 days [14]. The use of hydrazines was also considered, and would most likely work, but since hydrazine reacts with double bonds the generality of the method for analogue coupling would be restricted to saturated compounds. Upon completion of the reaction, as monitored by TLC, the excess ethylenediamine was quantitatively removed by simple passage through a C-18 Sep-Pak cartridge onto which the hydrophobic glycosides are adsorbed. Elution with methanol then yielded the oligosaccharide amines as white powders with recoveries in the range of 85–95%. ^{1}H-NMR data of these amines showed two characteristic triplets at δ2.80 and 3.29 (J = 6.5 Hz) ppm for the CH_2 groups of the ethylenediamine unit. The capacity of Sep-Pak cartridges varies somewhat with the oligosaccharide structures, but is normally near 10 mg for over 90% retention. We therefore routinely use one Sep-Pak cartridge for up to 5 mg of oligosaccharide to err on the safe side.

The oligosaccharide amines were then treated with 0.95 equivalents of diethyl squarate in anhydrous ethanol at room temperature [6, 15]. The reaction is readily monitored by TLC, or by UV spectroscopy [6], and the oligosaccharide-squarate adduct is stable to chromatography. The reaction was complete in approximately 4 h. The compounds used here were all soluble in ethanol, which is not the case for some sialylated derivatives. In those cases, water can be added to the mixture until a clear solution is obtained without interference to the reaction. The product can be isolated on a C-18 Sep-Pak, and further characterized if desired. The yields of this reaction were in the range of 80–85%. The oligosaccharide-squarate adduct was then coupled to BSA by incubation in buffer (pH 9.0) at ambient temperature for 16 h. The BSA-conjugates were obtained as white powders after ultrafiltration and lyophilization.

The degree of incorporation of the oligosaccharides

onto the neoglycoconjugates was established by MALDI-TOF MS using gentisic acid as the matrix. The mass spectra (positive ion mode) obtained using ca. 70 pmol of sample are shown in Fig. 1. The spectrum obtained for BSA itself (A) shows a sharp peak for the singly-charged protein (*m/z* 66 491). The peak at *m/z* 33 222 arises from the doubly charged protein and the very minor peaks at ca. *m/z* 22 000 and *m/z* 44 000 are assigned to the triply charged protein and the triply-charged protein dimer, respectively. The corresponding peaks are also seen in the mass spectra of the neoglycoproteins.

Panels B-F (Fig. 1) show the corresponding MALDI-TOF mass spectra obtained from the diethyl squarate-coupled neoglycoproteins. The peaks are much broader than that for BSA since each neoglycoprotein sample is made up of a collection of oligosaccharide-protein conjugates with a range of different incorporation values centred around the average. The average molecular weight, and this distribution, are obtained from the spectra in Fig. 1. This average molecular weight allows the calculation of the average number of oligosaccharide residues added to the BSA.

Table 2 summarizes the results obtained using the diethyl squarate coupling procedure for the preparation of neoglycoproteins. For scales using 2.1–5.8 mg of the 8-methoxycarbolyoctyl glycosides, efficiencies of 70–86% were obtained, with the incorporation values determined by MALDI-TOF MS. In the case of the trisaccharide BSA conjugate prepared from **4**, the incorporation value of 13 obtained from MALDI-TOF MS was verified using the phenol/sulfuric acid method of Dubois [9] with soluble **4** as the reference. This colorimetric method yielded an incorporation value of 14.

Both low incorporation ($n = 3.5$) or more normal values ($n = 13$) were obtainable by this method; higher incorporations were not attempted. The method is also shown to work with as little as 1 mg of oligosaccharide, though only 50% of the oligosaccharide was coupled on this small scale. The coupling yields reported are for all three of the chemical steps combined, i.e. from the 8-methoxycarbonyloctyl glycosides. It is likely that most of the observed losses in coupling arise from the manipulations involved in adsorption/desorption of the samples from the Sep-Pak cartridges. Coupling to give higher incorporation was not examined because our need was for a method that would reliably work when only very small quantities of valuable oligosaccharide, such as 1 mg produced in a glycosyltransferase reaction, are available.

It is interesting to note that the peak shapes in the mass spectra shown in Fig. 1 (C and F) appear to be bimodal suggesting the possible presence of two populations of neoglycoconjugates with different incorporations. We do not know the cause of this phenomenon, which is not seen in the remaining spectra, but such information is not available from other sugar-quantitating techniques which give simply the ratio of carbohydrate to protein with no distribution information.

In summary, a simple method has been developed for coupling 8-methoxycarbonyloctyl glycosides to BSA, with high efficiency and on a small scale, and the incorporation values of the neoglycoconjugates thus obtained can be deduced by MALDI-TOF MS.

Acknowledgements

We wish to thank Professor Monica M. Palcic and her group for the use of their facilities and for all their help in the isolation of the BSA-conjugates. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

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