Michael Addition of Poly-L-lysine to *N*-Acryloylated Sialosides. Syntheses of Influenza A Virus Haemagglutinin Inhibitor and Group B Meningococcal Polysaccharide Vaccines†

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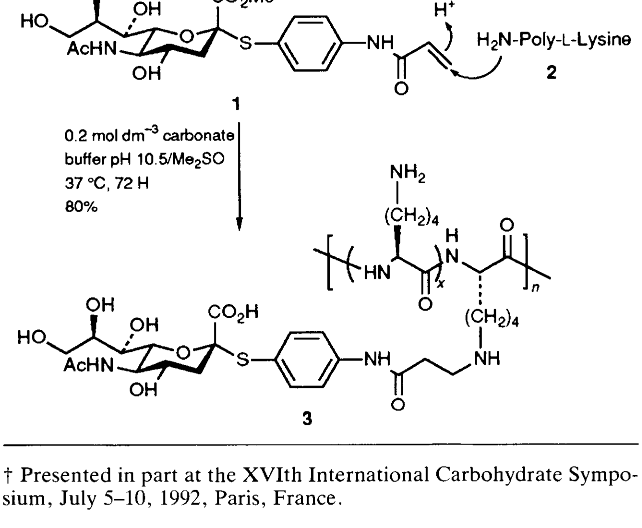
N-Acryloylated sialoside derivatives are directly conjugated to poly-L-lysine and protein carriers by the 1,4-conjugate additions of their N^{ε}-lysine residues to provide new glycoconjugates with potential therapeutic utilities.

Sialic acids constitute an important family of carbohydrate derivatives found on glycolipids, glycoproteins and capsular polysaccharides.[1] The intense research activities devoted to the sialic acids result from their implications in a wide range of cell adhesion and differentiation phenomena.[2,3] Moreover, inhibitions of influenza virus haemagglutinins have been demonstrated with entirely synthetic sialic acid glycoconjugates.[4–7] Also noteworthy are the efforts to produce immunogenic forms (vaccines) of poly-α-(2,8)-linked sialic acid (colominic acid) as this capsular polysaccharide is an integral cell-wall component of the pathogenic *Escherichia coli* K1 and group B *Neisseria meningitidis* bacteria.[8]

Previous reports from this group have successfully demonstrated the usefulness of *N*-acryloylated carbohydrate precursors for the syntheses of antigenic glycoconjugates.[4,5,9] The general strategy allowed direct access to both neoglycoproteins and glycopolymers from single precursors. The conjugated double bond of the *N*-acryloyl residues can serve as Michael acceptor for the nucleophilic additions of thiol and amine groups (Scheme 1) and concomitantly, can also be used in copolymerization processes.

Whereas our former glycoconjugate carriers were based on immunogenic globular proteins such as bovine serum albumin (BSA) and tetanus toxoid or non-immunogenic, non-biodegradable polyacrylamides, the present study describes the use of non-immunogenic but biodegradable poly-L-lysine of relatively low molecular mass carrier. In the actual investigation, *N*-acryloylated carbohydrate derivatives of mono- and poly-α-(2,8)-sialic acid antigens were used as Michael acceptors with poly-L-lysine and proteins.

The 4-*N*-acrylamidophenyl 2-α-thiosialoside ester **1**[4] was first treated with poly-L-lysine hydrobromide **2** (M_r 69 000, $DP_{(vis)}$ 333) (DP = degree of polymerisation) in 0.2 mol dm^{-3} sodium carbonate buffer pH 10.5 containing dimethyl sulfoxide (5 : 1) at 37 °C for 72 h (Scheme 1). The ratio of **1** to **2** in the reaction mixture was fixed at one for every 18–20 amine residues in the conjugate (4–5% molar basis). The reaction mixture was filtered, dialysed and freeze dried to provide the conjugate **3** in 80% yield. The homogeneity of **3** was demonstratred on an HPLC chromatogram using a TSK G4000 SW XL column (30 cm × 7.5 mm) using phosphate buffer saline (PBS) as the mobile phase and detection by the UV absorbance at both 210 and 260 nm (diode-array detector). The poly-L-lysine **2** had a retention time of 10.5 min as compared with **3** (t_R 11.3 min), which ruled out possible cross linking through the methyl ester. Under the above conditions, the sialic acid methyl ester residues of **1** were hydrolysed. The ^{1}H NMR spectra of the conjugate **3** showed the characteristic signals of both precursors in a molar ratio of one sialic acid residues to seventeen free lysine groups.‡



The conjugations of the poly-α-(2,8)-sialic acid **4** to poly-L-lysine **3** and to the protein carriers were accomplished through its reducing end after derivatization to an *N*-acrylamide functionality. The Michael acceptor residues were regioselectively introduced by reductive amination of the sole hemiketal function (reducing end terminus) with an excess of ammonium bicarbonate and reduction of the transient ketimine **5** with sodium cyanoborohydride (37 °C, 3 days) in 0.2 mol dm^{-3} sodium borate buffer pH 9.2. The intermediate α-amino acid derivative **6** was obtained in 95% yield after dialysis and lyophilization. High field ^{1}H NMR spectra of **6** showed it to be a 1 : 1 mixture of diastereoisomers at the α-amino acid methine position.§ *N*-Acryloylation of **6** was accomplished by the dropwise addition of a solution of acryloyl chloride in dioxane to an aqueous solution of **6** maintained at a constant pH of 9–10 with 0.5 mol dm^{-3} NaOH. The reaction was stopped when a ninhydrin test showed no amine function present. The *N*-acryloylated polysaccharide **7** was obtained pure in 74% yield after dialysis, lyophilization and purification on a BioGel A 0.5 column (2.5 × 100 cm) using PBS as eluent and dialysis of the pure fractions. A total of four fractions were pooled, which corresponded to polysaccharides of M_r 4000, 6000, 8000 and 10 000, respectively.

Conjugation of **7** (M_r 6000) to bovine serum albumin and to tetanus toxoid under the above conditions afforded the Michael adduct conjugates **8**, **9** and **10**, respectively. The polysaccharide contents of the protein conjugates **9** and **10** were estimated at 10–15 units per protein molecules as estimated by the resorcinol (sialic acid)[10] and by the bicinchoninic acid (protein)[11] colorimetric assays. For biological assay purposes, the poly-L-lysine conjugate **8** was further derivatized with *N*-acryloylated biotinamidocaproylhydrazide¶ by a

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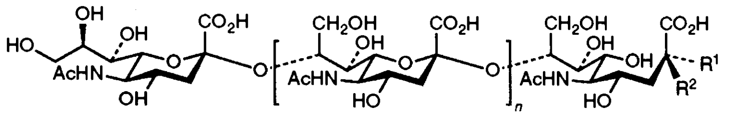
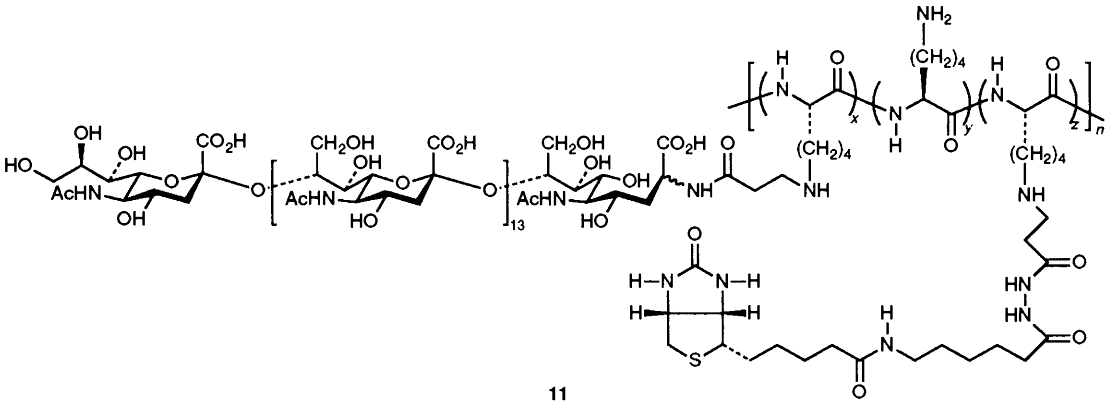
‡ *Selected data* for compound **3**: ^{1}H NMR (D_2O, 200 MHz), δ 4.20 (t, lysyl α-CH), 3.47–3.90 (m, Neu5Ac H_4-H_9), 2.91 (t, lysyl ε-CH_2), 2.03 (s, Neu5Ac N-Ac), 1.68 (m, lysyl β-δ CH_2, H_3 eq Neu5Ac), 1.36 (m, lysyl γ-CH_2, H_{3ax} Neu5Ac). The integrations were measured relative to the aromatic, Neu5Ac ring and *N*-Acetyl proton signals.

§ *Selected data* for compound **6**: ^{1}H NMR (D_2O, 500 MHz): δ 4.2–4.4 (m, H-2 reducing end), 2.07 (s, *N*-acetyl); ^{13}C NMR: δ 54.5 (C-2 reducing end), 97.7 (C-2, non reducing end). For more details see: R. Roy and R. A. Pon, *Glycoconjugate J.*, 1990, **7**, 3. For **7**: ^{1}H NMR ($COCH_c$=C-H_a *trans*, C-H_b *cis*): 6.37 (dd, H_c), 6.19 (dd, H_b), 5.77 (dd, H_a), 4.33 (dd, H-2′), 4.24 (dd, H-2), 2.07 (s, N-Ac); ^{13}C NMR δ 174.1 (C=O), 130.8 (CH=), 128.2 (=CH_2), 102.8 (C-2 non-reducing end), 5.47 (C-2 reducing end).

¶ Prepared by *N*-acryloylation of commercial biotinamidocaproylhydrazide (78% yield); m.p. (MeOH) 185.7–187.1 °C, $[\alpha]_D^{23}$ (Me_2SO) + 35.4°. All intermediates showed satisfactory spectral and/or analytical data.



4; R^1, R^2 = O, reducing sugar
5; R^1, R^2 = NH, ketimine
6; R^1, R^2 = H,NH, mixture of diastereoisomers
7; R^1,R^2 = H,NH–CO–CH=CH_2
8; R^1,R^2 = H,NH–CO–CH_2CH_2–poly-L-lysine
9; R^1,R^2 = H,NH–CO–CH_2CH_2–BSA
10; R^1,R^2 = H, NH–CO–CH_2CH_2–NH–tetanus toxoid



11

second Michael addition under the above conditions. The three-component conjugate **11** was obtained after dialysis and freeze drying of the reaction mixture. It was further purified on a DEAE ion-exchange column (0.75 cm × 10 cm, acetate form) and eluted with 5 mmol dm^{-3} sodium chloride. After dialysis and lyophilization, pure **11** was recovered in 65–70% yield. ^{1}H NMR spectra of **11** showed it to contain a polysaccharide : lysine : biotin ratio of 1.4 : 100 : 3.2.[‖]

The glycoconjugates thus obained by 1,4-Michael additions of preformed *N*-acryloylated carbohydrate derivatives have been evaluated in their respective biological assays. Thus, for the first time mouse (Balb/c) monoclonal antibodies (2 IgM, 6 IgG) were obtained against the colominic acid–tetanus toxoid conjugate **10**. These results together with those obtained with the influenza A virus haemagglutinin inhibitors will be presented in due course.

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[‖] Compound **11**: ^{1}H NMR (D_2O, 300 MHz): δ 2.10 (s, N-Ac), 3.00 (t, lysyl ε-CH_2).

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