A PEGA Resin for use in the Solid-phase Chemical–Enzymatic Synthesis of Glycopeptides

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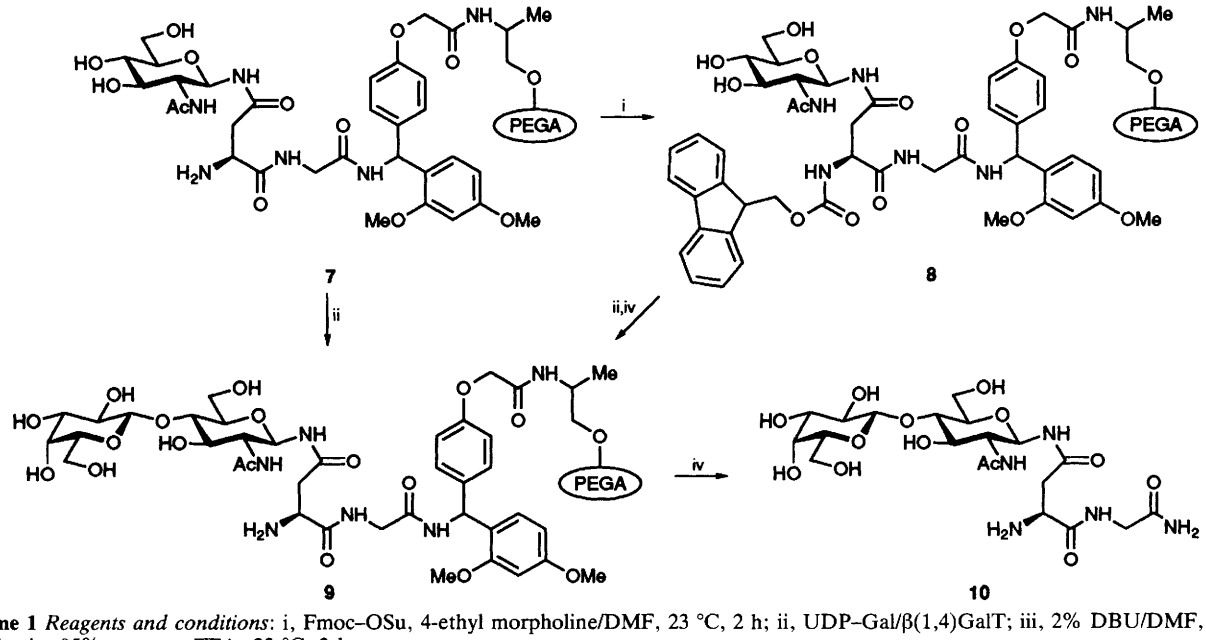
The successful application of a new resin consisting of beaded polyethylene glycol polyacrylamide copolymer **5** ($PEGA_{1900}$) as a solid support for the chemical–enzymatic synthesis of glycopeptides is reported; the resin is mechanically stable, yet highly swelling in both organic solvents and aqueous buffers.

The solid-phase synthesis of peptides is commonly used in organic chemistry. By comparison, the solid-phase synthesis of oligosaccharides remains highly exploratory and, by its very nature, requires the protection of hydroxyl groups and the development of stereospecific glycosylation methodology.[1] When the objective is the solid-phase synthesis of glycopeptides, the two chemistries must be combined and remain compatible, which is a daunting challenge.

One alternative, now available, for the rapid synthesis of glycopeptides is to combine well developed peptide chemistry with enzymatic glycosylation which, by its very nature, is regio- and stereo-specific. The compatibility of biomolecules towards solid supports used in chemical synthesis are, however, generally quite poor, in particular for polystyrene-based resins, which have been shown to exclude enzymes completely.[2] Enzymatic solid-phase syntheses with glycosyltransferases have previously been explored[3,4] but the yields and quantities of oligosaccharides produced were too low to be acceptable. Soluble polyacrylamide polymers have also been employed.[5] Recently, this situation was improved by Schuster *et al.*[6] who reported the successful use of a non-swelling silica-based resin where peptide synthesis and subsequent enzymatic glycosylation takes place at the surface of the silica matrix. We report here that copolymerization of a mixture of mono- and di-acryloylated polyethylene glycol **1** and **2** (diamino-PEG, 0.77 equiv. acryloyl chloride) with acrylamide **3** or *N,N*-dimethylacrylamide **4** produce resins **5** and **6** which are compatible with both chemical peptide synthesis [using *e.g.* fluorenylmethoxycarbonyl (Fmoc) protocols in dichloromethane or *N,N*-dimethylformamide (DMF)] and swell 10–20 fold in aqueous buffers to allow full access of internal immobilized glycopeptides to glycosyltransferases with a molecular weight on the order of 50 000. The open interior of this flexible PEG polymer[7,8] enables full access for enzymatic glycosylation.

The first amino acid was anchored to 500 mg of resin using the acid-labile 4-(α-amino-2′,4′-dimethoxybenzyl)phenoxyacetic acid linker,[7] which had been added by the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) procedure.[10] In principle, a nucleophile-labile[11] or enzyme-labile[6] linker could also have been employed when more acid-sensitive residues such as fucose or sialic acid would have been present. The linker was derivatized with Fmoc–Gly-*O*-pentafluorophenyl (Pfp) ester (3 equiv.) and after removal of the Fmoc group, Fmoc–Asn-(*O*-Ac_3-β-D-GlcNAc)–OPfp (1.6 equiv.)[12] was added. The couplings were performed with standard conditions and were quantitative as monitored visually by addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dbht–OH).[13,14] The Fmoc group was cleaved off with 2% 1,8-diazabicyclo[5.4.0]-undec-1-ene (DBU) in DMF and *O*-acetyl groups were removed with hydrazine hydrate in methanol. The resulting resin **7a** was washed with methanol, DMF and CH_2Cl_2 and dried *in vacuo*. A small amount (10 mg) of the resin-bound glycopeptide was cleaved off with 95% aqueous trifluoroacetic acid (TFA) (2 h, 23 °C) and the crude material (1.2 mg) shown to be pure Asn(β-D-GlcNAc)-Gly–NH_2 by ^1H NMR spectroscopy. It has previously been demonstrated that with hexopyranoses both the interglycosidic bonds and the *N*-linkage to the peptide are stable to 95% aqueous TFA at 20 °C for 24 h.[15] Quantitative amino acid analysis of the resin revealed an incorporation of 0.16 mmol g^{-1}. Cleavage and NMR spectroscopy showed that the product was pure.

Similarly a second batch of **7b** was prepared on a more



Scheme 1 *Reagents and conditions*: i, Fmoc–OSu, 4-ethyl morpholine/DMF, 23 °C, 2 h; ii, UDP–Gal/β(1,4)GalT; iii, 2% DBU/DMF, 23 °C, 20 min; iv, 95% aqueous TFA, 23 °C, 2 h

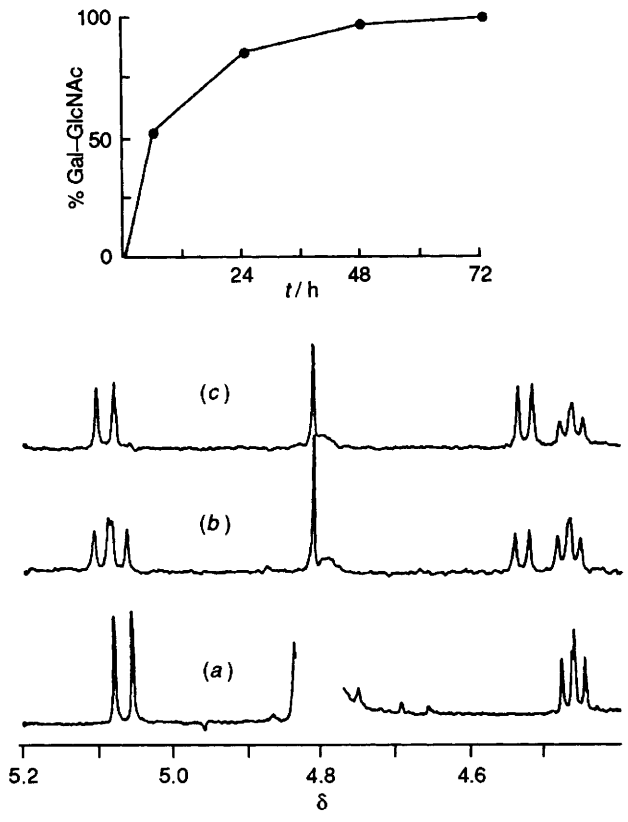


Fig. 1 Time course of the galactosylation followed by NMR spectroscopy of the product after cleavage off the resin with TFA

crosslinked $PEGA_{1900/300}$ **6**[16] and an aliquot was derivatized at the *N*-terminal amino group with Fmoc–OSu (Su = succinimide) to give **8**.

The resin **7a** (4 aliquots, 20 mg each) was swollen at 23 °C in enzyme and substrate containing buffer (150 mm^3 of 100 mmol dm^{-3} sodium cacodylate buffer, pH 7.4, 5 mmol dm^{-3} $MnCl_2$, 2 mg UDP–Gal, 1.1 units bovine β-(1 → 4)-galactosyltransferase) causing a ≈20 fold swelling (m/v) of the resin (Scheme 1). Additional 0.5 mg portions of UDP–Gal were added after 5, 10, 18, 24 and 40 h. The time course of the reaction at 23 °C was monitored by taking aliquots of the resin **9**, cleaving the immobilized glycopeptide for 2 h with 95% aqueous TFA and directly recording the NMR spectra of the cleaved product **10** without purification. According to the spectra the glycopeptides were completely stable under the acidic conditions of the cleavage. Within 48 h, the reaction forming Asn(β-*N*-acetyllactosamine)-Gly–Resin **9** was essentially complete (>95%). During this period the transferase was remarkably stable and the activity did not decrease significantly. Thus an aliquot was removed from the incubation mixture and assayed radiochemically at the start of the reaction and again after 48 h. After 48 h at least 80% of the activity remained. The extended reaction time is probably required for the PEG-polymer to refold around the protein allowing it to move around inside the gel-like resin in its folded form and access all reaction sites, and indeed a single experiment performed with preincubation of resin with enzyme and buffer for 3 d at 4 °C before addition of UDP–Gal afforded >90% conversion in 6 h.

The same conditions as above were used for glycosylation reactions carried out on the more crosslinked resin **7b** and its Fmoc-derivative **8** as shown in Scheme 1. The reactions were in these cases only 50% complete after 72 h and would be difficult to drive to completion indicating the importance of the right choice of resin components. The degree of conversion was not affected by the presence of the Fmoc-group in resin **8**.

Immobilization of an enzyme substrate on a solid support offers a number of advantages in addition to the obvious simplified removal of reagents and by-products. This is particularly true when quantitative and specific reactions can be performed leading to virtually uniform molecules linked to the solid support for use in bioassays. Reactions may be performed on very small amounts of resin in the case of rare enzymes of limited availability. Ideally steps of chemical synthesis carried out in organic media can be combined with the enzymatic synthesis. The advantage of a swelling resin over a surface is clearly the homogeneous character of the resin interior allowing equal access to all substrate molecules. Furthermore, the transparent character of the beads, their uniform spherical shape and mechanical stability make them ideal for synthesis of portion-mixing libraries and subsequent screening for molecular interaction with biomolecules. As demonstrated in this work the possible variations of the PEGA resins with chain-length of the PEG used as a crosslinker and with the resin polarity depending on the type of the acrylic diluent will allow the preparation of resins specially designed for particular biochemical purposes.

In summary, we have demonstrated that synthetic glycopeptides immobilized in PEGA resins are accessible to both chemical reagents and to large proteins or glycoproteins, in this instance galactosyltransferase. Conversions were essentially quantitative in contrast to previous attempts to glycosylate substrates on polystyrene based resins. When the preparation of a series of homologous glycopeptides is required, the decision can be made at any stage of the synthesis as whether to change the amino-acid or the sugar sequence, or both, opening up many possibilities of structural variations. The suitability of this resin in the preparation and screening of glycopeptide libraries is in progress.

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