Notes & Tips

A new synthetic route for the preparation of glycoprobes

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Glycoprobes are a novel tool that we have recently developed to simplify analysis of lectin activity in complex biological samples [1]. Although proven effective, their shortcoming was the relatively complex procedure for the incorporation of ligands and the very low water solubility of preglycoprobe that did not allow incorporation of charged ligands such as sialylated carbohydrates. Here we describe a new approach for the synthesis of glycoprobes that enables simpler synthesis of functional glycoprobes and at the same time enables incorporation of water-soluble ligands.

Materials and methods

Digoxin and CNBr were purchased from Aldrich Chemical Co. (Milwaukee, WI), *N*-Hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA)[1] was purchased from Pierce (Rockford, IL), *N*-hydroxysuccinimide ester (NHS) and antibodies against digoxin labeled with alkaline phosphatase were purchased from Sigma (St. Louis, MO); α-9-fluorenylmethyloxycarbonyl–lysine (Fmoc–Lys) and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were purchased from Advanced ChemTech (Louisville, KY), PVDF membranes were purchased from Millipore (Bedford, MA). Man$_9$–oligosaccharide was obtained from soybean agglutinin as described [2]. Dimethylformamide (DMF) and triethylamine (TEA) were distilled under vacuum before use.

Thin-layer chromatography. Thin-layer chromatography (TLC) was performed on 0.2-mm Silica Gel 60 F254 precoated on aluminum sheets (Merck, Darmstadt, Germany) using chloroform:methanol:water (v/v/v) 80:20:1 as a developing solvent. Digoxin was identified by "charring" after spraying with 15% H$_2$SO$_4$ in 80% ethanol, and amino groups were identified by spraying with 2% ninhydrin in 95% ethanol.

Mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis was performed using a Voyager DE-STR Time-Of-Flight Mass Spectrometer (Applied Biosystems, Foster City, CA).

Dry samples of glycoprobe–NHS (100 nmol) and Man$_9$–glycoprobe (20 nmol) were resuspended in 30 μl of 50% acetonitrile. Glycoprobe–NHS solution was stored in the dark overnight to allow complete hydrolysis of *N*-hydroxysuccinimide ester. A 0.3-μl portion was deposited on the target, supplemented with 0.3 μl of saturated solution of 6-aza-2-thiothymine (matrix) and air-dried in the dark. Nitrogen laser pulses (337 nm) were used for desorption and ionization, while the extraction voltage was set to 20 kV.

Electrophoresis and blotting. Proteins were separated electrophoretically in SDS–polyacrylamide slab gels as described by Laemmli [3] and transferred onto Immobilon PVDF membrane using a semidry blotting system according to Towbin et al. [4]. After blotting, membranes were blocked overnight with 3% BSA, and digoxigenin-conjugated protein bands were visualized by incubation with antidigoxin antibodies conjugated with alkaline phosphatase. 5-Bromo-4-chloro-3-indolyl phosphate (0.02 mg/ml) in 50 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$ was used as a substrate, and the color was intensified with addition of 0.04 mg/ml nitroblue tetrazolium.

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[1] *Abbreviations used:* NHS-ASA, *N*-hydroxysuccinimidyl–4-azidosalicylic acid; NHS, *N*-hydroxysuccinimide ester; Fmoc–Lys, α-9-fluorenylmethyloxycarbonyl–lysine; EDAC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; PVDF, polyvinylidene difluoride; DMF, dimethylformamide; TEA, triethylamine BSA, bovine serum albumin.

Results

Synthesis of glycoprobe–NHS. α-Fmoc-ε-digoxin–Lys was prepared as described in the original synthesis procedure [1] (see Fig. 1). After removing the Fmoc group by incubating for 30 min in 20% piperidine in tetrahydrofuran, 10 volumes of the mixture of chloroform and hexanes (1:1, v/v) were added, vortexed for 5 min, and centrifuged at 10,000g for 10 min. A pellet was formed that consisted mostly of ε-digoxin–Lys. The pellet was further purified by dissolving in a small (ca. 100 µl) volume of methanol–tetrahydrofuran (1:1, v/v) and precipitating the product with 10 volumes of the mixture of chloroform and hexanes (1:1, v/v). The pellet was dried under reduced pressure and weighed to determine the yield of ε-digoxin–Lys.

ε-Digoxin–Lys (20 µmol) and ASA–NHS (30 µmol) were dissolved in 200 µl of DMF–TEA (95:5, v/v) and reacted at room temperature. This and all subsequent reactions were performed in the dark to prevent activation of the photoreactive cross-linker (ASA). The reaction progress was followed by TLC. After 4 h nearly all ε-digoxin–Lys ($R_f = 0.05$) was converted to α-ASA–ε-digoxin–Lys ($R_f = 0.2$). DMF–TEA was evaporated under reduced pressure and the reaction mixture was dissolved in 50 µl of tetrahydrofuran. Toluene (1.2 ml) was added and vortexed for 5 min. Gelatinous precipitate that contained mostly α-ASA–ε-digoxin–Lys was formed, while unreacted ASA–NHS was dissolved in toluene and nearly completely removed by two additional washes with 2 ml toluene.

The free COOH group in α-ASA–ε-digoxin–Lys was activated and made reactive with amino groups by esterification with *N*-hydroxysuccinimide. Approximately 20 µmol of α-ASA–ε-digoxin–Lys (final concentration 130 mM) was incubated with *N*-hydroxysuccinimide (600 mM) and EDAC (250 mM) in DMF. Small amount of anhydrous NaH_2PO_4 were added to the reaction vial to provide sufficient buffering capacity to prevent hydrolysis of digoxin. After 3–4 h, virtually all α-ASA–ε-digoxin–Lys ($R_f = 0.2$) was converted to a $R_f = 0.8$ material (NHS ester) and the reaction mixture was evaporated under reduced pressure.

After two extractions with chloroform:water (1:1) to remove free *N*-hydroxysuccinimide, EDAC, and phosphates, the combined chloroform phase was divided into small portions, freeze-dried, and stored at −20 °C in the dark. The structure of glycoprobe–NHS was checked by mass spectrometry and was found to correspond exactly to the expected mass (Fig. 2).

Synthesis of Man9–glycoprobe. Man9–oligosaccharide (10 nmol) was dissolved in 20 µl 0.1 M TEA–bicarbonate buffer, pH 9.5, and mixed with 100 nmol of glycoprobe–NHS dissolved in 20 µl of tetrahydrofuran in the dark. The reaction was followed by TLC, and the progress of reaction was determined by the disappearance of the ninhydrin reactivity at the baseline. Virtually all amino groups were consumed after 12 h of incubation at 25 °C. The reaction mixture was supplemented with 30 µl water and extracted three times with 300 µl chloroform. The Man9–probe that was extracted into the aqueous layer was divided into small portions, freeze-dried, and stored
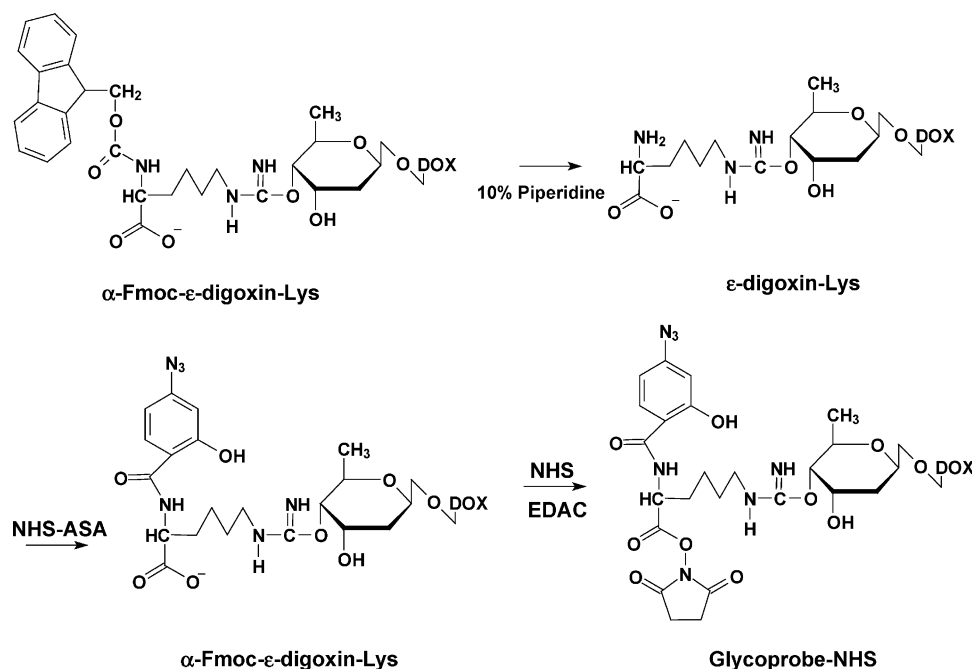

Fig. 1. Synthetic scheme of glycoprobe–NHS. The synthesis begins from α-Fmoc–ε-digoxin–lysine that was prepared as reported previously [1]. For the reaction details see text. The actual structure is a mixture of the structure shown above and the alternative structure where digoxin is attached through 3′-OH group of the last digitoxose.
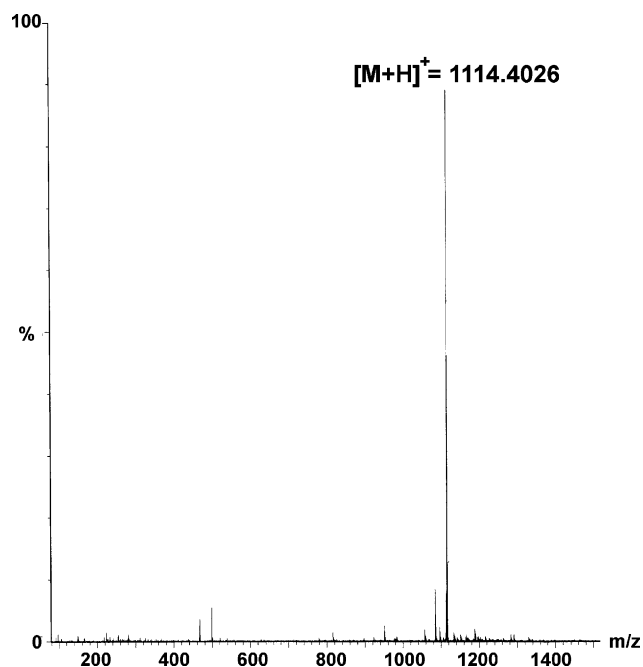
Fig. 2. Matrix-assisted laser desorption/ionization time-of flight mass spectrometry spectrum of glycoprobe–NHS.

Fig. 3. Application of Man$_9$–glycoprobe. Man$_9$-glycoprobe was prepared and used to detect concanavaline A (ConA) as described in the text. Concanavaline A (5 μg) and BSA (50 μg) were incubated for 120 min in the absence (lane A) or in the presence (lane B) of 0.5 nmol Man$_9$–glycoprobe, cross-linked by UV illumination, separated by SDS–PAGE, blotted onto PVDF membrane, and detected with anti-digoxin antibodies labeled with alkaline phosphatase. Even in the presence of 10-fold surplus of BSA, Man$_9$–glycoprobe exclusively labeled concanavaline A.

at −20 °C in the dark. The structure of Man$_9$-glycoprobe was checked by mass spectrometry and the measured mass ($M + H^+ = 2954.67$) was found to correspond exactly to the expected mass (data not shown).

Application of Man$_9$–glycoprobe. Concanavalin A (5 μg) and BSA (50 μg) in 5 μl phosphate-buffered saline, supplemented with 1 mM Ca^{2+} and Mg^{2+} were added to 0.5 nmol of the Man$_9$–glycoprobe in 10 μl of water and incubated at 37 °C in the dark for 120 min. Next, the reaction mixture was irradiated with an UV lamp (long wavelength) for 15 min at 4 °C to activate the cross-linker. After cross-linking, the mixture was separated by SDS–PAGE electrophoresis and Western blotted as described under Materials and methods. As is clearly visible in Fig. 3, Man$_9$–glycoprobe specifically labeled concanavalin A even in the presence of 10-fold surplus of BSA.

Discussion

Glycoprobes are a novel molecular tool that can identify active lectins in complex biological samples. In the original synthetic procedure, they are prepared by reacting glycans or glycopeptides containing a free amino group with a preprobe, followed by a removal of the Fmoc group and the incorporation of a photoreactive cross-linker [1]. In addition to being quite complicated, this process is additionally hampered with very poor water solubility of the preprobe. We have addressed this problem by developing a novel 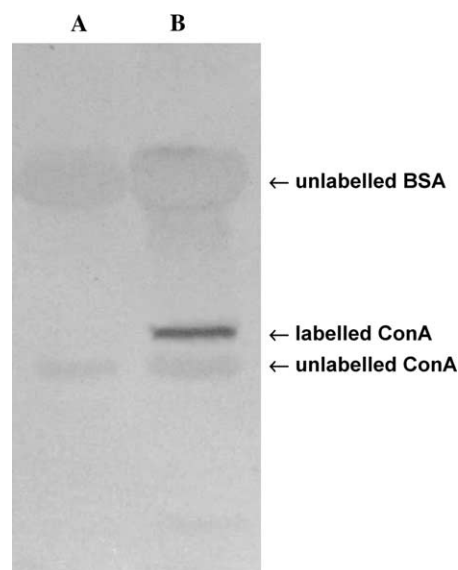synthesis procedure that enabled incorporation of the photo-reactive cross-linker in the structure before the addition of the ligand. The new synthetic scheme, summarized in Fig. 1, yielded glycoprobe–NHS, thus successfully resolving both of the above-mentioned problems. By eliminating the need of the subsequent removal of the protecting Fmoc group and the incorporation of the photoreactive crosslinker we have significantly simplified preparation of functional glycoprobes. At the same time, since the highly hydrophobic Fmoc group was replaced with a less hydrophobic azidosalicilic acid, glycoprobe–NHS was now soluble in mixtures of aqueous and organic solvents which enabled incorporation of glycans that are not soluble in organic solvents such as DMF or dimethylsulfoxide.

To confirm the expected structure of the synthesized glycoprobe–NHS and to test its functional properties, we prepared a functional Man$_9$–glycoprobe starting with only 20 nmol of Man$_9$–glycopeptide. The glycoprobe produced by the new method was able to specifically label concanavaline A (which recognizes Man$_9$ structure) in the presence of 10-fold surplus of BSA that was not labeled at all (Fig. 3).

Conclusion

In this paper we have described a new synthetic route for the synthesis of glycoprobes, that resolves the solu-

bility problem present in the original synthetic route. In addition, this approach provides a structure (glyco-probe–NHS) that can be converted to functional glycoprobe by simple one-step reaction with ligands, which significantly simplifies the preparation of functional glycoprobes.

Acknowledgments

References

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