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A solid-phase glycosyltransferase assay for high-throughput screening in drug discovery research

Rob S. Donovan[1], Alessandro Datti[1*], Myung-Gi Baek[2], Qingquan Wu[2], Ian J. Sas[1], Bozena Korczak[1], Eric G. Berger[3], René Roy[2] and James W. Dennis[4*]

[1]*GlycoDesign Inc., 480 University Ave., Suite 900, Toronto, ON, Canada M5G 1V2*
[2]*Department of Chemistry, University of Ottawa, Ottawa, ON, Canada K1N 6N5*
[3]*Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland*
[4]*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave. M5G 1X5, and Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON*

Glycosyltransferases mediate changes in glycosylation patterns which, in turn, may affect the function of glycoproteins and/or glycolipids and, further downstream, processes of development, differentiation, transformation and cell-cell recognition. Such enzymes, therefore, represent valid targets for drug discovery.

We have developed a solid-phase glycosyltransferase assay for use in a robotic high-throughput format. Carbohydrate acceptors coupled covalently to polyacrylamide are coated onto 96-well plastic plates. The glycosyltransferase reaction is performed with recombinant enzymes and radiolabeled sugar-nucleotide donor at 37°C, followed by washing, addition of scintillation counting fluid, and measurement of radioactivity using a 96-well β-counter. Glycopolymer construction and coating of the plastic plates, enzyme and substrate concentrations, and linearity with time were optimized using recombinant Core 2 β1-6-N-acetylglucosaminyltransferase (Core 2 GlcNAc-T). This enzyme catalyzes a rate-limiting reaction for expression of polylactosamine and the selectin ligand sialyl-Lewis^x in O-glycans. A glycopolymer acceptor for β1-6-N-acetylglucosaminyltransferase V was also designed and shown to be effective in the solid-phase assay.

In a high-throughput screen of a microbial extract library, the coefficient of variance for positive controls was 9.4%, and high concordance for hit validation was observed between the Core 2 GlcNAc-T solid-phase assay and a standard solution-phase assay. The solid-phase assay format, which can be adapted for a variety of glycosyltransferase enzymes, allowed a 5–6 fold increase in throughput compared to the corresponding solution-phase assay.

Keywords: **glycosyltransferase, assay, high-throughput screening, drug discovery**

Abbreviations: **HTS, high-throughput screening; UDP, uridine 5′-diphosphate; GlcNAc, D-N-acetylglucosamine; Gal, D-galactose; GalNAc, D-N-acetylgalactosamine; Man, D-mannose; Glc, D-glucose; T, transferase; PSGL-1, P-selectin glycoprotein ligand-1; ESL-1, E-selectin ligand-1; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PCR, polymerase chain reaction; MES, 2-(N-morpholino) ethanesulfonic acid; DMSO, dimethyl sulfoxide; pNp, *para*-nitrophenyl**

Introduction

Glycoproteins bearing N- and/or O-glycan chains are generally found on the cell surface or are secreted; many are molecules that mediate cell-cell communication such as growth factors and their receptors, cell adhesion molecules and extracellular matrix proteins [1]. The structural diversity of N- and O-linked glycans is due to variations in linkages, anomeric configuration, and monosaccharide composition, dictated in large part by glycosyltransferase expression and enzyme specificities [2,3]. Variations in glycan structure can affect glycoprotein distributions and function. For example, terminal sialylation and SO_4-GalNAc on peptide hormones [4,5] and the degree of sialylation and GlcNAc-branching of N-linked oligosaccharides on erythropoietin have been shown to regulate their half-lives and activity in vivo [6]. GlcNAc-branching of N-glycans due to increased GlcNAc-T V (i.e.

*Corresponding authors: A Datti, E-mail: adatti@glycodesign.com
JW Dennis, E-mail: Dennis@MSHRI.on.ca

UDP-GlcNAc: α-mannoside β1-6-N-acetylglucosaminyltransferase V, EC 2.4.1.155) activity in many rodent and human cancers is closely associated with invasion and metastasis [7].

The fucosylated O-glycans of PSGL-1, ESL-1, GlyCAM-1, CD43 and MAdCAM-1 serve as ligands for the selectins, a family of carbohydrate-binding receptors [8]. Sialyl-Lewis^x tetrasaccharide on O-linked glycans of PSGL-1 glycoprotein in activated leukocytes is required for selectin-mediated adhesion of leukocytes to the vascular endothelium of inflamed tissues. Mice lacking α1,3 fucosyltransferase VII (α1,3 Fuc-T VII) are deficient in leukocyte extravasation into areas of inflammation, and show elevated peripheral leukocyte counts, similar in phenotype to mice deficient in both P- and E-selectins [9].

Core 2 GlcNAc-T (i.e. UDP-GlcNAc:Galβ1-3GalNAc-R β1-6-N-acetylglucosaminyltransferase [GlcNAc to GalNAc], EC 2.4.1.102) also appears to be a key enzyme for selectin-mediated adhesion, as ligand expression on Chinese hamster ovary cells requires co-transfection and expression of PSGL-1, α1,3 Fuc-T VII and Core 2 GlcNAc-T genes [10,11]. The product of Core 2 GlcNAc-T provides the preferred acceptor intermediate for substitution with polylactosamine and sialyl-Lewis^x sequences on O-linked glycans [12].

Although sialyl-Lewis^x tetrasaccharide and mimics have been shown to block neutrophil invasion and acute inflammation in animal models of injury [13], the short serum half-life and low affinity of the monomeric sugar ligand in solution limits this approach to treatment. Alternatively, inhibitors of Core 2 GlcNAc-T, α1,3 Fuc-T VII or other glycosyltransferases that are required for selectin ligand expression on leukocytes might prove to be useful anti-inflammatory agents.

With similar rationale, inhibitors of GlcNAc-T V may be useful in the treatment of cancer. To test these hypotheses, candidate glycosyltransferase inhibitors must be identified, and this requires efficient high-throughput assay methods. Current solution-based glycosyltransferase assays are sensitive, but limited by the requirement for the separation of product from unreacted substrates by means of solid-phase extraction or chromatographic or electrophoretic approaches [14–18].

We have developed a rapid solid-phase glycosyltransferase assay for high-throughput screening and drug discovery research. The assay utilizes multivalent oligosaccharide acceptors linked to poly[N-(acryloyloxy)-succinimide] polymers coated onto 96-well plastic plates, thereby eliminating the need for chromatographic separation of product. Although developed for Core 2 GlcNAc-T and GlcNAc-T V, the assay method can readily be adapted for use with other glycosyltransferases.

Materials and methods

Chemicals

Poly[N-(acryloyloxy)succinimide] (pNAS) (Fig. 1, structure **1**) with a viscosity-average molecular weight M_v of 42.1 kDa (degree of polymerization ~250) was prepared according to Mammen et al. [19]. Disaccharide Galβ1-3GalNAcα-$O(CH_2)_3S(CH_2)_2NH_2$ (i.e. Core 2 GlcNAc-T acceptor) and trisaccharide GlcNAc(β1-2)Man(α1-6) Glc(β-$O(CH_2)_3S(CH_2)_2NH_2$) (i.e. GlcNAc-T V acceptor) were prepared from the corresponding allyl glycosides (i.e. Galβ1-3GalNAcα-pNp [Toronto Research Chemicals] and GlcNAc(β1-2) Man (α1–6)Glc(β-O-$(CH_2)_7CH_3$) [Rose Scientific] following a procedure described by Roy and Tropper [20].

Glycopolymer syntheses

As shown in Figure 1, poly [N-(acryloyloxy) succinimide] (**1**) was first treated at room temperature with the amine-terminated T-antigen disaccharide **2** in DMSO (16 h) to provide a core copolymer containing one sugar residue for every ten N-substituted acrylamide residues. The active ester-containing polymer was then treated at room temperature for three hours with excess primary amines (ammonia, methylamine, ethylamine, or propylamine) to give four different copolymers **3**–**6** having the same comonomer ratios but differing by the lipophilicity of the copolymer backbones. The glycopolymers were then purified by size exclusion chromatography over BioGel P-10 using water as eluent. Alternatively, disaccharide **2** was treated with methacryloyl chloride and the resulting monomer was copolymerized with methacrylamide to provide copolymer **8**, while direct copolymerization of allyl glycoside precursor of **2** with acrylamide gave copolymer **10**. Using the same strategy, copolymer acceptors **11**–**14** for GlcNAc-T V were prepared using the same core pNAS (**1**) and molar ratios of acrylamide : sugar of 10 : 1.

Recombinant Core 2 GlcNAc-T

A truncated form of Core 2 GlcNAc-T cDNA [21], lacking 37 amino acids from the N-terminus was prepared by PCR. The truncated cDNA was cloned in-frame into pPROTA vector [22] for expression as a secreted protein A (ProtA) chimeric protein. The expression vector was co-transfected into Chinese hamster ovary cells, along with pSV2neo, in a 10 : 1 molar ratio, using the calcium phosphate method. Cells were cultured in the presence of 800 μg/ml of geneticin (antibiotic G418), and resistant cell clones were selected, and tested for Core 2 GlcNAc-T activity in culture medium. The representative clone 614 C2 showed stable expression of Core 2 GlcNAc-T activity, and was selected for enzyme production. The cells were routinely propagated in minimum essential medium (α-MEM) containing 5% fetal bovine serum and G418 (0.2 mg/ml). To partially purify the enzyme, IgG-Sepharose Fast Flow^{TM} beads (Pharmacia Biotech) were added in a ratio of 5 μl of a 50% bead slurry, 2.5 μl of 2 M Tris·HCl, pH 8.0, and 5 μl of 10% Tween-20 per ml of culture medium. Following incubation on a rocking platform at 4°C for 20 h, the beads were collected by centrifugation, washed with 10 volumes of TST buffer (50 mM Tris·HCl, pH 8.0, 150 mM

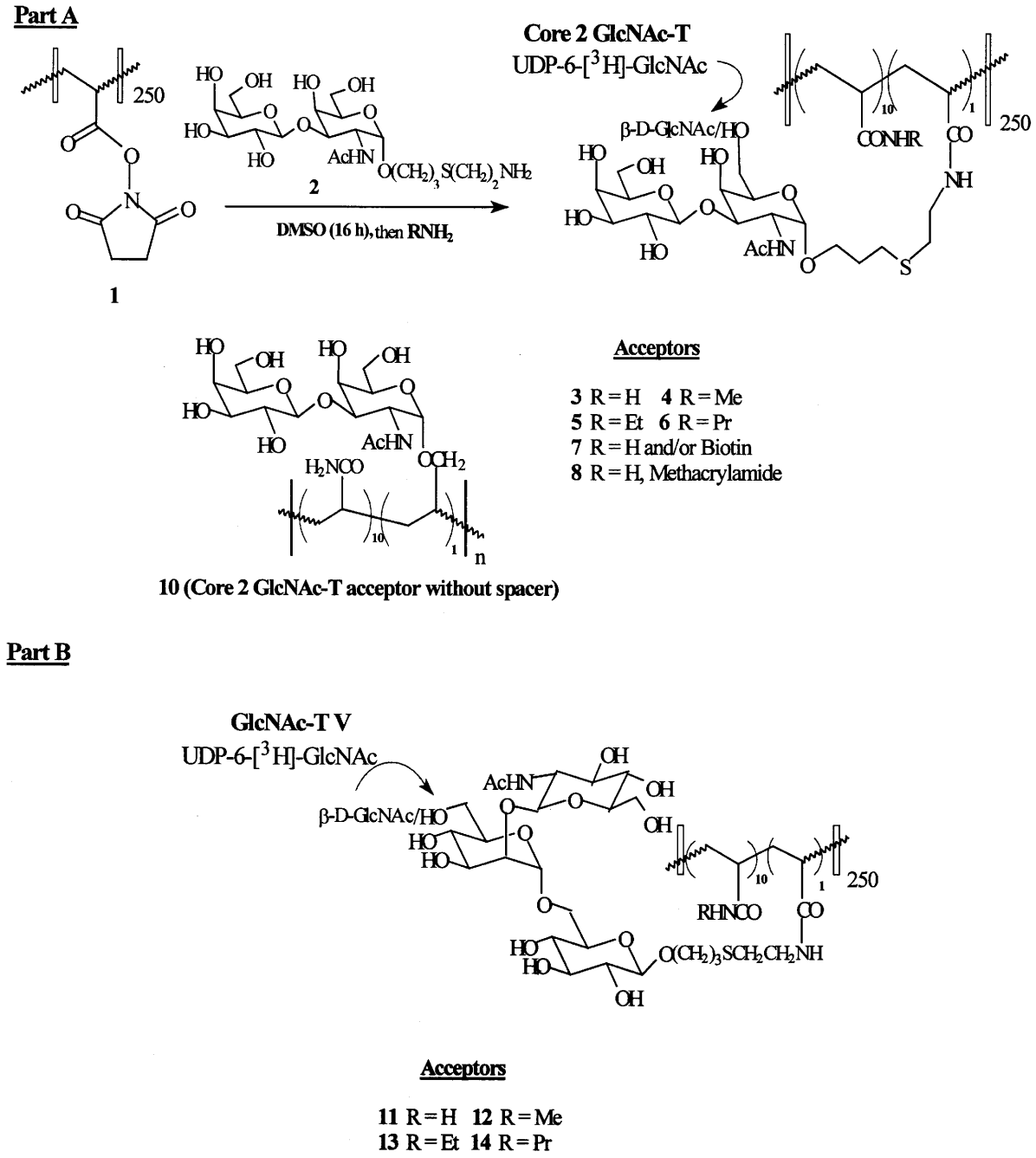

Figure 1. Copolymer syntheses and their use as acceptors in solid-phase Core 2 GlcNAc-T (Part A) and GlcNAc-T V (Part B) assays. The chemical synthesis of the glycopolymer acceptor, and the solid-phase glycosyltransferase assays are described in the Materials and methods section. Et, ethyl; Me, methyl; Pr, propyl.

NaCl, 0.05% Tween-20) and 2 volumes of 5 mM NH_4Ac, pH 5.0. The recombinant ProtA-Core 2 GlcNAc-T enzyme was then eluted with 1 volume 0.5 M acetic acid, pH 3.4, and resuspended in 3 volumes of 0.5 M MES, pH 7.5 (Calbiochem). One μU of enzyme activity is defined as the amount of protein forming 1 pmol/min of product at 37°C under optimal reaction conditions.

Solid-phase Core 2 GlcNAc-T assay and HTS of a natural product library

Glycopolymer **3** was used as the acceptor substrate; a stock solution was prepared by resuspending the compound in water to a concentration of 1.25 mg/ml and then incubating the solution at 60°C for 1 h. The solution was gently mixed at

15 min intervals during this time to allow the polymer to unwind and become fully dissolved. The glycopolymer solution was not vortexed since strong agitation may cause shearing of the polymer backbone. The preparation, containing sodium azide (0.05%), was then stored at room temperature.

Wallac 96-well Printed Rigid Sample Plates (#1450-511; Wallac, FI) were used in all cases for the solid-phase assay. To prepare the plates for coating with acceptor, the wells were washed twice with 100 µl of methanol and then rinsed 3 times with 200 µl water. After allowing the plates to dry at room temperature, the wells were coated by adding 60 µl of a 33.3 µg/ml glycopolymer solution and incubated overnight at room temperature. Following the incubation, unbound glycopolymer was removed by washing 3 times with 200 µl water and the remaining liquid in wells was allowed to evaporate by incubating the plates at 37°C (or room temperature) for approximately 1 h. Dried coated plates could be used immediately or sealed and stored for use at a later date.

The HTS Core 2 GlcNAc-T assay, carried out in a 96-well plate format, consisted of 20 µl of test compound, 20 µl of 3X assay buffer made of 90 mM MES, pH 6.7, 10 mM EDTA (Sigma), 0.0075 mM UDP-GlcNAc (Sigma) and 0.1 µCi of UDP-6-[^{3}H]GlcNAc (16 Ci/mmol; Toronto Research Chemicals), and 20 µl of recombinant Core 2 GlcNAc-T (160–200 µU).

To minimize pipetting, the enzyme and the 3X buffer were routinely combined and 40 µl of the enzyme-buffer mixture was added to the wells following the addition of the test compounds. After incubating the plates at 25°C for 60 min, the reactions were stopped by adding 175 µl of water to each well, aspirating the contents and washing 4 times with 190 µl water. The radioactive signal was measured using a MicroBeta plate counter (Wallac, FI) after adding 100 µl of OptiPhase Supermix scintillation cocktail (Wallac) to each well and incubating for >2 h, to allow for mixing. Each plate in HTS had 4 controls with vehicle added rather than test extracts, and background was determined with the omission of enzyme, also 4 wells per plate. Background was subtracted for each plate, and results were expressed as a percentage of control reactions on the plate. The HTS assays were run on a Beckman integrated robotic platform using a Biomek 2000 pipetting station and Zymark rotating robotic arm.

Panlabs (now Thetagen, Bothell, WA) supplied a collection of 30,000 bacterial and fungal extracts in 96-well plates. The dried extracts were resuspended in 100% DMSO, and diluted into water at 0.15% DMSO for the Core 2 GlcNAc-T HTS.

Solution-phase Core 2 GlcNAc-T assay

The Core 2 GlcNAc-T solution-phase assay mixture was similar to that used in earlier studies [12,14,23] but was adapted for automation on the Beckman robotic platform. Each reaction, carried out in a 96-well plate format, contained 10 µl of test extract, 10 µl of 3X assay mixture made of 90 mM MES, pH 6.7, 10 mM EDTA, 3 mM Galβ1-3GalNAcα-pNp as acceptor, 3 mM UDP-GlcNAc (Sigma) and 0.1 µCi of UDP-[^{3}H]GlcNAc (16 Ci/mmol; Toronto Research Chemicals), and 10 µl of recombinant Core 2 GlcNAc-T enzyme (4–5 µU). Reactions were incubated for 1–2 h at 37°C and stopped by adding 200 µl cold water. Plates were processed immediately or stored at −20°C. To recover the product, the assay mixture was aspirated through C_{18}-packed pipette tips (BGBS96C_{18} Biotips^{TM}, National Scientific) and the packing was then washed 3 times with 200 µl of H_2O. Bound product was eluted into a β-scintillation counting plate (Wallac 96-well Printed Rigid Sample Plate, #1450-511) by washing the C_{18} packing 3 times with 100 µl of 100% ethanol. The eluates were then dried overnight at room temperature to remove ethanol and radioactive signal was counted in a β-scintillation counter after the addition of counting fluid. Reaction products were found to accumulate in a linear manner for up to 2 hours of incubation. The C_{18}-packed tips were washed with 200 µl ethanol and re-equilibrated three times with 100 µl of H_2O.

Results

Glycopolymers for solid-phase glycosyltransferase assays

The disaccharide acceptor Galβl-3GalNAcα-R where R is either octylmethyl or *para*-nitrophenyl has been used routinely in solution Core 2 GlcNAc-T assays where UDP-[^{3}H]GlcNAc is the sugar-nucleotide donor. The product, Galβ1-3([^{3}H]GlcNAcβ1-6)GalNAcα-R is captured on C_{18} solid support, eluted with ethanol, and measured in a β-counter [12,14,23]. This procedure has been miniaturized and automated, but remains relatively slow compared to high-throughput ELISA-style assays. The glycopolymers with Galβ1-3GalNAcα- groups were prepared by chemical synthesis and reacted with recombinant ProtA-Core 2 GlcNAc-T to establish the condition for the solid-phase glycosyltransferase assays.

The water-soluble glycopolymer acceptors (**3**–**8**, **10**, and **11**–**14**) (Fig. 1) used in the solid-phase glycosyltransferase assay are polyvalent substrates composed of N-substituted polyacrylamide backbones containing one disaccharide Galβ1–3GalNAcα-O$(CH_2)_3$S$(CH_2)_2NH_2$ (**2**) or trisaccharide GlcNAc(β1–2)Man(α1–6)Glc(β-O$(CH_2)_3$S$(CH_2)_2NH_2$) residue for every ten acrylamide backbone monomers. The viscosity-average molecular weight M_v of the core polymer was determined to be 42.1 kDa based on polyacrylamide derived from **1** by treatment with aqueous ammonium alone. The ratio of sugar to acrylamide (one to ten) was determined using high field ^{1}H-NMR spectroscopy and was based on previous optimization experiments using analogous glycopolymers in enzyme-linked lectin assays (ELLA) [24]. The copolymer backbones were modified with various alkylamines to enhance their lipophilicity and thus, increase their adsorption behaviors to the surface of the polystyrene microtiter plates.

Based on the signal obtained in the corresponding enzyme assays, copolymers **6** and **14**, which have the most lipophilic N-propylacrylamide backbones, were found to be more sensitive acceptors than copolymers with either N-ethyl (**5**, **13**), acrylamide (**3**, **11**) or N-methyl substituents (**4**, **12**) (Table 1), a fact most likely due to differential coating efficiencies. Copolymer **10**, containing a shorter allyl spacer was also found unsuitable for the enzymatic glycosylation, presumably because of the inaccessibility of the GalNAc residues in the enzyme's active site. Similarly, copolymers **7** (co-biotin) or **8** (co-methacrylamide) provided either poor coating or poor enzymatic glycosylation. Biotin-containing copolymer **7** was initially designed to serve as coating substrate after capture by streptavidin/avidin pre-coating (manuscript in preparation).

Coating plastic wells with glycopolymer

Early in the development of the solid-phase assay, we observed variable results with different batches of plastic plates. A number of pre-wash solutions were tested for their ability to improve the consistency of the Core 2 GlcNAc-T reaction. As shown in Figure 2, prewashing the plastic with organic solvents improved the signal by 2–5 fold and eliminated variability between different lots of plates. Washing with non-ionic detergent reduced reaction efficiency. Based on the results shown in Figure 2, the 96-well plastic plates were routinely prewashed twice with methanol, then twice with water and stored dry prior to coating with the glycopolymer **3**. Washing of plastic plates was performed on the Beckman 2000 workstation with robotic arm. The time and temperature dependency for coating the wells with glycopolymer were determined as shown in Figure 3. Overnight coating with 2 μg/ml at 25°C was determined to be optimal. Glycopolymer **3** was determined to be in excess, as the recovered solution of

Table 1. Relative activity of glycopolymers in Core2 GlcNAc-T and GlcNAc-TV assays

Core 2 GlcNAc-T Glycopolymer	*Relative Activity (%)*
3	100
4	150
5	170
6	210
7	61.3
8	7.5
10	1.7
GlcNAc-TV Glycopolymer	*Relative Activity (%)*
11	100
12	50
13	100
14	830

Activity normalized to that measured with glycopolymer **3** for Core 2 GlcNAc-T and to glycopolymer **11** in the GlcNAc-TV assay.

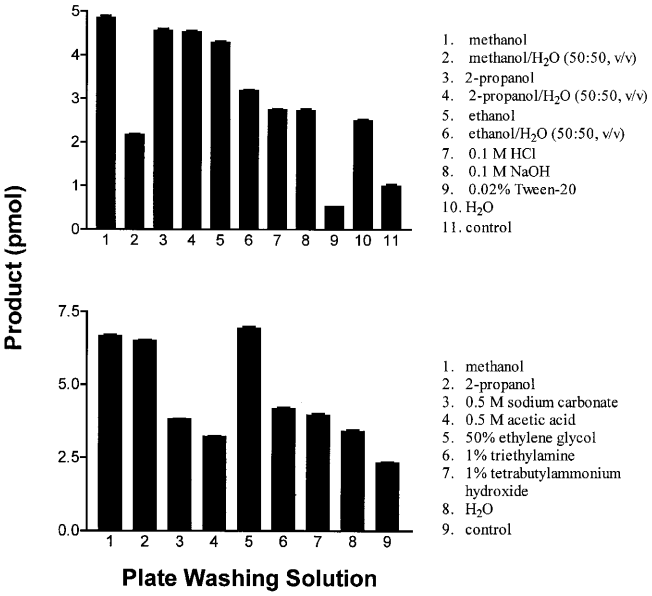

Figure 2. Preparation of 96-well plastic plates for coating with glycopolymer. Wells of Wallac 96-well Printed Rigid Sample Plates were washed twice with 200 μl of solvent as indicated in the legend for 2 min and then rinsed twice with 200 μl H_2O. The plates were coated with glycopolymer **3** at 2 μg/well in 60 μl water as described in the Materials and methods section. The assay was performed using 2.5 μM UDP-GlcNAc, and 200 μU/well of recombinant Core 2 GlcNAc-T for 50 min. The data in each bar represent the mean±SD of 4 replicate determinations. The graphs represent data which were obtained in two separate experiments.

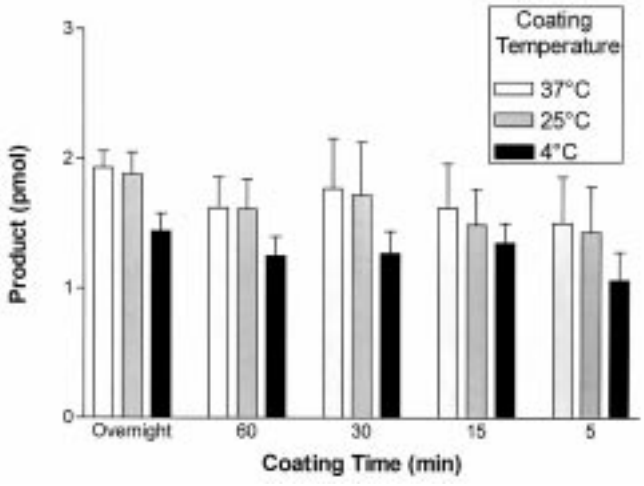

Figure 3. Coating of 96-well plastic plates with glycopolymer. Plates were washed with methanol and water, and wells coated with glycopolymer **3** at 2 μg/well in 60 μl water. The coating was performed at the temperature and time indicated in the legend and X-axis. The UDP-GlcNAc concentration was 2.5 μM, and recombinant Core 2 GlcNAc-T was added at 200 μU/well. The reactions were incubated for 30 min at 20°C. The data in each bar represent the mean±SD of 9 replicate determinations.

polymer was used to coat wells subsequently, and produce 80–90% of the reaction product realized with the first coating (data not shown). However, glycopolymer solutions were routinely used only once.

Characterization of the Core 2 GlcNAc-T solid-phase assay

In the initial experiments, 96-well plates were coated with a solution of 2 μg/well of glycopolymer acceptor **3**, and the reaction products were observed to be proportional up to 120 μU of added enzyme over 60 minutes (Fig. 4). However, the K_m for UDP-GlcNAc, and for acceptor using ProtA-Core 2 GlcNAc-T in the solution assay was determined to be 1.75 mM and 146 μM respectively (data not shown). In contrast, the solid-phase glycosyltransferase reaction conditions employ substrates well below K_m concentrations, as the amount of glycopolymer **3** bound to the plastic is limiting. Therefore, the sugar-nucleotide concentration is adjusted to optimize the detection of radioactive product and is also below K_m concentrations. To establish that the substrates are not exhausted during the 60 minute reaction, a time course and titration of UDP-GlcNAc were performed at 37°C (Fig. 5). As expected, at higher concentrations of UDP-GlcNAc, the reaction went to completion in less than 5 minutes. However, with UDP-GlcNAc at 2.5 μM, and 200 μU of enzyme activity, the Core 2 GlcNAc-T reaction product accumulated in a time-dependent manner for 30–60 minutes. The maximal product formed was 6–10 pmoles per well, and when 2.5 μM UDP-GlcNAc was used in the reaction, this represented approximately 4% utilization of the sugar-nucleotide donor. To simplify the HTS protocol, these conditions (2.5 μM UDP-GlcNAc, 200 μU enzyme) were then further tested at room temperature (approximately 20°C). Under these conditions, product accumulation was found to be linear with time for approximately 60 min (Fig. 6), and thus, the HTS assay was routinely performed at room temperature.

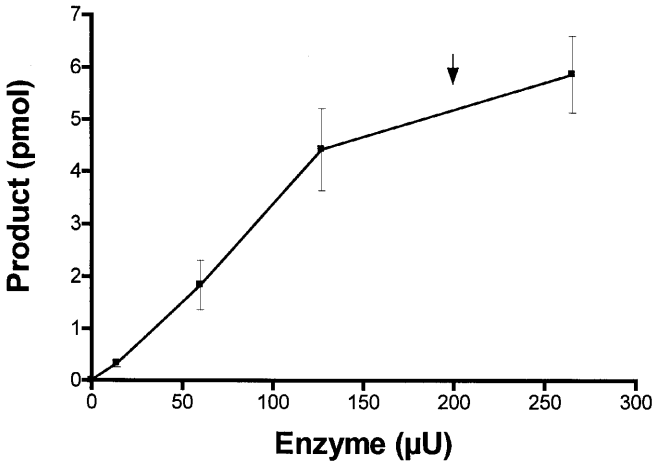

Figure 4. Core 2 GlcNAc-T reaction product dependence on enzyme concentration. Wells were coated overnight with glycopolymer **3** at 2 μg/well in 60 μl water. Recombinant Core 2 GlcNAc-T enzyme was added to each well, and the reaction, in a total volume of 60 μl, was incubated for 60 min at 37°C. The assay was performed using 2.5 μM UDP-GlcNAc, and the arrow indicates the enzyme concentration used in subsequent experiments.

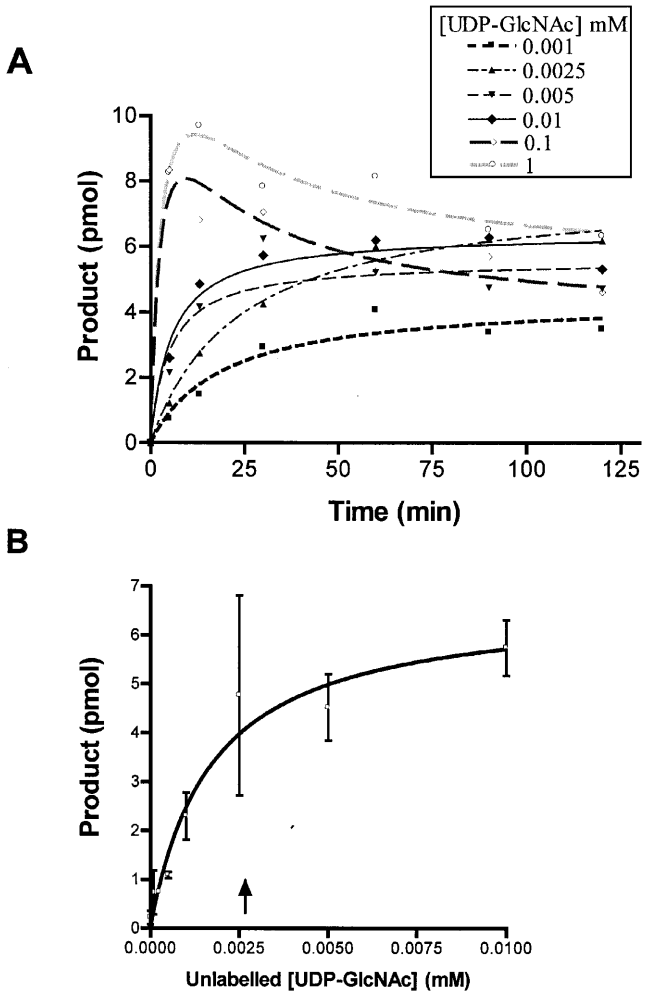

Figure 5. Core 2 GlcNAc-T reaction dependence on UDP-GlcNAc donor concentration. (A) The solid-phase Core 2 GlcNAc-T assay was performed as described in the Materials and methods section. Recombinant Core 2 GlcNAc-T enzyme was added at 200 μU/well. (B) Core 2 GlcNAc-T reaction product after 30 minutes as a function of UDP-GlcNAc concentration. The arrow indicates the concentration of sugar-nucleotide donor adopted for routine assays.

Core 2 GlcNAc-T high-throughput screen (HTS) of microbial extracts

A Panlabs microbial library of 30,000 extracts was subjected to HTS using the Core 2 GlcNAc-T solid-phase assay for the primary screen; to this end, glycopolymer **3** was employed as the acceptor substrate. The primary screen yielded hits (i.e. extracts showing >50% inhibition) at a frequency of 1.5%; normalized results from a typical run of 1,600 assays are shown in Figure 7. The signal-to-background ratio was 20-fold

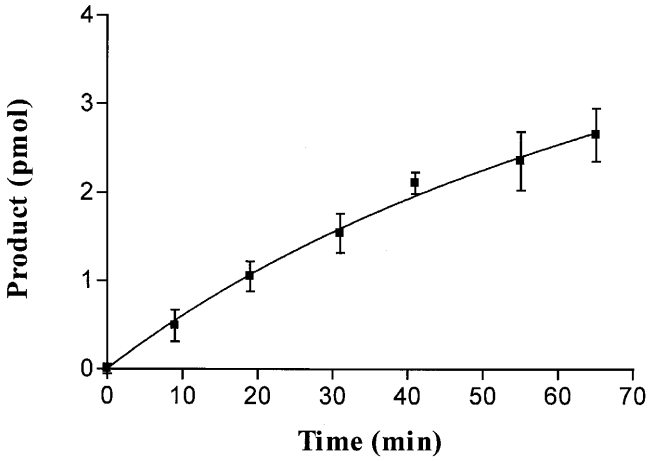

Figure 6. Time-dependent accumulation of Core 2 GlcNAc-T reaction product at 20°C. The reactions contained 2.5 μM UDP-GlcNAc and 200 μU/well of Core 2 GlcNAc-T enzyme. The data in each bar represent the mean±SD of 8 replicate determinations.

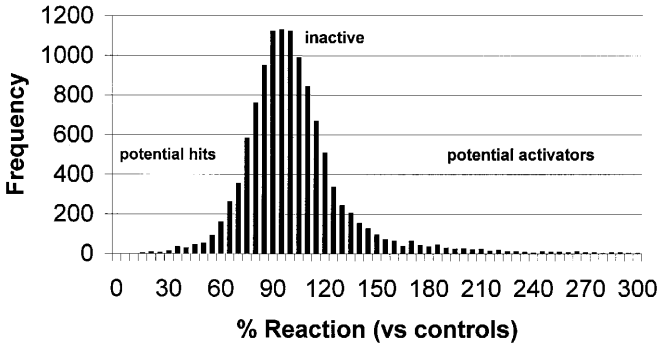

Figure 7. HTS for Core 2 GlcNAc-T inhibitors in a microbial extract library. Distribution of normalized assay results for 1,600 assays expressed as % of control. Assays were performed as outlined in the Materials and methods section. Extracts showing >50% inhibitory activity of the Core 2 GlcNAc-T reaction in the solid-phase glycopolymer assay were selected and re-tested using both the solid-phase assay and a standard solution assay.

and the coefficient of variance of the positive controls was 9.4%.

A series of 48 hits (approximately 10% of the total), chosen from the primary screen data, were placed on plates with 88 inactive extracts, and re-tested in a secondary screen using both the solid-phase and solution Core 2 GlcNAc-T assays. As summarized in Table 2, 17 of the 21 confirmed hits identified in the solid-phase assay matched 17 of the 18 hits detected with the solution assay. Nine other hits initially identified in the secondary screen using the solid-phase assay were rejected as false positives, based on titration analyses performed using a 5 point dilution series (Table 2).

Hit extracts identified in the Core 2 GlcNAc-T HTS are currently being fractionated to identify active molecules for further testing in cell culture and animal models of disease.

Use of glycopolymer for the GlcNAc-T V solid-phase assay

Glycopolymer acceptors for GlcNAc-T V were also made and tested using recombinant enzyme (as with Core 2 GlcNAc-T, a truncated form of GlcNAc-T V cDNA [25] was cloned in-frame into pPROTA vector, expressed in Chinese hamster ovary cells and purified using IgG Sepharose Fast Flow Beads, manuscript in preparation).

Similar to what was observed for Core 2 GlcNAc-T, glycopolymer **14**, with the most lipophilic N-propylacrylamide backbone was more effective than either N-ethyl (**13**) or acrylamide (**11**) glycopolymers (Table 1). Glycopolymer **12**, an N-methyl substituent linker was the least effective coating acceptor. The GlcNAc-T V reaction product using glycopolymer **14** accumulated in a time and enzyme dependent manner (Fig. 8).

Table 2. Concordance of hits in solid-phase and solution-phase Core 2 GlcNAc-T assays

Primary Screen	*Solution Assay*	*Solid Phase Assay*
Hit = ⩾50% inhibition at 0.05% stock extract concentration (single determination)	ND	48
Secondary Screen (hit confirmation)		
Hit = ⩾40% inhibition at 0.05% stock extract concentration (duplicate determination)	19	30
• "Confirmed hits"	18	21
• "Hits with partial titration"	1	8
• "Hits rejected due to poor titration"	0	1
Validated Hits	18	21
Concordance of assays "Confirmed Hits" in both solution phase and solid phase assays	17/18	17/21

The most potent 48 hits, detected with the solid-phase assay, were selected from the primary screen. Eighty-eight non-active extracts were also chosen and tested in parallel. The Secondary Screen consisted of duplicate determinations, using either assay method. Hits were then confirmed through titration analyses whereby single point tests were performed using a 5 point dilution series, with extract concentrations ranging between 0.0125 and 0.2%.

"Confirmed Hits" in either assay format inhibited at least 40% of enzyme activity at the 0.05% dilution and showed consistent titration of signal throughout the dilution range tested. "Hits with partial titration" inhibited ⩾40% of enzyme activity at the 0.05% dilution but did not demonstrate linearly decreasing signal throughout the entire dilution series. "Rejected hits" demonstrated no change in signal at any dilution. ND, not done.

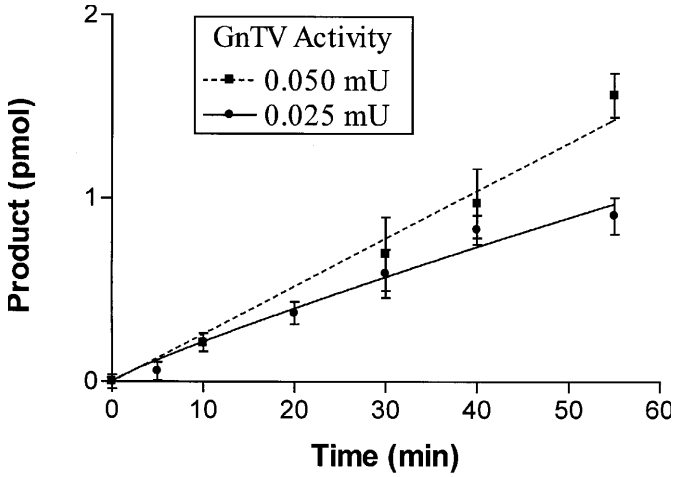

Figure 8. Time course reaction for GlcNAc-T V glycopolymer assay at 20°C. The reaction was performed using 2.5 μM UDP-GlcNAc and the level of enzyme indicated in the legend. The data in each bar represent the mean±SD of at least 3 replicate determinations. Recombinant GlcNAc-T V (a 2.5 μU/μL stock preparation) and glycopolymer **14** were used for this experiment.

Discussion

As the future of drug discovery will present challenges at all stages, the design of either new or improved assay technologies in HTSs has already been a major focus of attention [26].

In this report, we describe a solid-phase glycosyltransferase assay, and conditions for its use with recombinant Core 2 GlcNAc-T. The assay was also tested with a glycopolymer acceptor for GlcNAc-T V and shown to be adaptable for other glycosyltransferase enzymes. Of the glycopolymers tested in the Core 2 GlcNAc-T assay, the copolymer having the most lipophilic N-propylacrylamide backbone was more effective than that with the N-methyl substituent. In fact, efficient coating of the plastic and availability of the sugar moeity to the enzyme are critical factors, which may vary depending upon the sugar structure and enzyme of interest.

The solid-phase Core 2 GlcNAc-T assay was employed in a HTS of microbial extracts, and hit extracts were confirmed with a high degree of concordance to a conventional solution assay. The solid-phase assay format allowed a 5–6 fold increase in throughput compared to a solution phase assay, for a rate of 7,500 per day on our Beckman robotic system.

Natural product extracts might be expected to contain ionic or organic molecules that could remove the polymer from the plastic, or degrade the donor substrate UDP-GlcNAc present at low concentration in the reaction, thereby yielding more false positives. Indeed, in a controlled, small-scale comparison of the solid-phase and solution assays (Table 2), 11 more hits (30 vs 19) were observed with the former method. Of the 30 hits identified with the solid-phase assay, 9 were rejected as false positives, whereas only 1 of 19 was rejected in the solution assay. The higher frequency of false positives in the solid-phase assay is, however, balanced by the detection of additional hits. In fact, use of a concentration of UDP-GlcNAc well below the K_m (i.e. 700-fold) would be expected to facilitate the detection of hits representing competitive inhibitors of the enzyme.

This study shows that the frequency of false positives in the solid-phase assay is manageable, as 30,000 extracts yielded only 1.5% hits in total. Furthermore, the time required to eliminate false positives in secondary tests is negligible when compared to the time saved during the primary screen by using the solid-phase assay rather than the solution assay protocol, which includes a time-consuming, chromatographic step.

The solid-phase glycosyltransferase method described herein, as well as most previously reported solution assays, employs radiolabeled sugar-nucleotide donors. A likely improvement would, therefore, be the use of chemical detection tags linked to the sugar nucleotide donor, thereby eliminating the need for radioisotopes and converting a radiometric signal into a fluorescence readout. Dansyl chloride, for example, has already been shown to be a suitable tag for *para*-aminophenylsugars employed as acceptor substrates of glycosyltransferases. In fact, use of Galβ1-3GalNAcα-pNHDansylphenyl in Core 2 GlcNAc-T reactions [17,27] resulted in unaltered kinetic characteristics of the enzyme and a stable, highly fluorescent signal.

Gosselin et al. [28] have described a non-radioactive glycosyltransferase assay involving detection of nucleotide diphosphate reaction byproducts, in a coupled assay using pyruvate kinase and lactate dehydrogenase. This approach allows continuous measurement of reaction products without using radioisotopes, however, in our hands, the method gave rise to inconsistent results when adapted to HTS (Donovan RS and Datti A, unpublished observation).

In recent years, several other assay methods for glycosyltransferases, most of them based upon the immobilization of an enzyme substrate in microtiter plates, were reported as potential tools for HTS efforts [29–34]. However, to our knowledge, there is no information available regarding the practical application of such assays to the screening of libraries of compounds. Thus, given the unpredictability of adaptation to HTSs, it remains to be seen how the utility of these assays compares to the approach reported here.

In summary, we have developed a solid-phase glycosyltransferase assay method, which is sufficiently robust and sensitive to permit HTS of a natural product library, and capable of identifying hit extracts with inhibitory activity against recombinant Core 2 GlcNAc-T.

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