Oligosaccharide Analysis

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Solid-Phase Oligosaccharide Tagging (SPOT): Validation on Glycolipid-Derived Structures**

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Glycosylation is the most abundant form of post-translational modification of proteins. Over 50% of all protein sequences are glycosylated in eukaryotic systems.[1] Glycosylated lipids are also widespread in animal cells: they constitute up to 5–10% of the cell membrane content and are responsible for a wide array of pathological disorders.[2]

The structural analysis, or sequencing, of the oligosaccharide chains of glycoproteins and glycolipids is much more complex than that of DNA or proteins, as the molecules are generally branched and the linkage between sugar residues introduces a new α or β stereocenter. The major analytical approaches[3] rely on the detection of oligosaccharides either by mass spectrometry (MS) or pulsed amperometry. Oligosaccharides released from glycoproteins or glycolipids by chemical or enzymatic methods are also frequently labeled at the reducing end to afford increased sensitivity of detection.[4] The insertion of fluorescent tags followed by analysis by chromatography or electrophoresis is common practice. The above approaches, however, remain complex endeavors that require a combination of sophisticated equipment and a high degree of expertise in both sample handling and data analysis.

There would be clear advantages in any technique that could sequester a reducing oligosaccharide from solution and allow its further manipulation (including tagging) while immobilized on a solid phase, for example, on beads or glass surfaces such as slides. This is the rationale behind solid-phase oligosaccharide tagging (SPOT) that would involve only pipetting and washing. The advantages are:

- Once the oligosaccharide is "captured" it remains covalently bound to the beads, so there would be no sample loss during subsequent manipulations.
- Large excesses of tagging (or other) reagents could be used to drive reactions to completion, as they would be washed away without sample loss or dilution.
- If the immobilized oligosaccharide remained sterically accessible to enzymes and lectins (or antibodies), biological oligosaccharide recognition and enzyme-assisted

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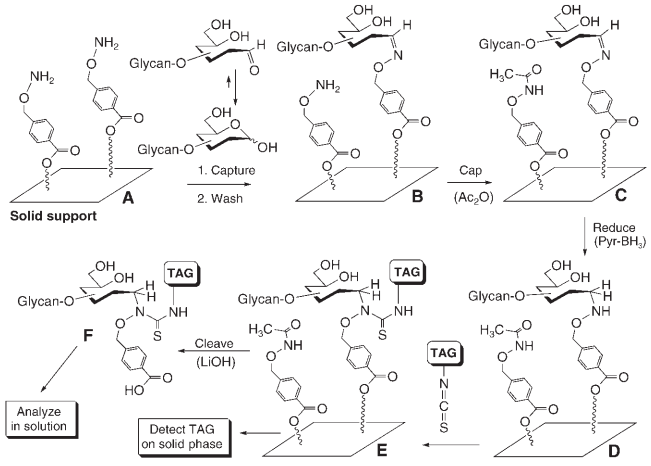
sequencing procedures already in use in glycomics could be applied.

- If a cleavable linker was present in the capture molecule, a "processed" (for example, fluorescently tagged) oligosaccharide could be released into solution in a small volume for further analysis.

Recently, Nishimura and co-workers[5] showed that polymeric beads functionalized with hydroxylamine groups could be used to capture reducing oligosaccharides from complex samples. After washing away the non-carbohydrate debris, the oligosaccharide could be released back into solution for further analysis. This convenient cleanup procedure, termed "glyco-blotting", yielded superior MS data for use in fragmentation sequencing. Shin and co-workers[6] later showed that oligosaccharides could be captured on glass array slides functionalized with hydrazide or hydroxylamine groups, and detected on the slides using fluorescently tagged lectins.

Herein, we show that a system can be devised where solid supports bearing hydroxylamine groups can capture oligosaccharides from solution. Furthermore, the immobilized product can be efficiently chemically manipulated while on the solid phase, and then released back into solution for analysis. We demonstrate the SPOT process on glycolipid-derived structures.

The chemistry described in Scheme 1 was developed for SPOT. Structure **A** shows benzyl hydroxylamine capture

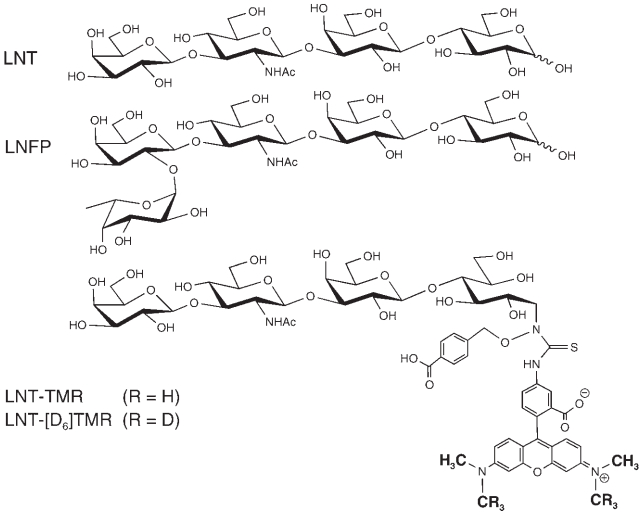


Scheme 1. The chemistry underlying SPOT.

groups attached through a cleavable ester linkage to a solid support, optionally through a spacer molecule. An appropriate spacer would be needed if access of a captured sugar molecule to a protein active site, such as that of a glycosidase or lectin, was required. Incubation of **A** with a solution containing a reducing sugar results in its capture to form an oxime (**B**), along with an excess of unreacted capture groups. The oxime is expected to be a mixture of tautomers including cyclic forms (not shown).[7] The excess unreacted hydroxylamines in **B** could now be capped (for example, by reaction with an amine-reactive compound such as acetic anhydride (Ac_2O)) to give **C**, under conditions in which neither the oxime nor the immobilized sugar OH groups react. Reduction of the oxime double bond in **C** would then give the *N*,*O*-alkylhydroxylamine **D**, now with a reactive sp^3 nitrogen atom. Tagging of the active nitrogen atom in **D** can be accomplished under conditions in which the sugar OH groups do not react (for example, by using aryl isothiocyanates) to give **E**. Base treatment of **E** then releases the tagged sugar **F** into solution for analysis. In the process, the immobilized oligosaccharide(s) in **B**–**E** should be detectable by lectins or antibodies, thus yielding potentially valuable information on their structure(s).

The process described has, as its key step, the selective inactivation of excess capture groups prior to reduction and insertion of a tag on the sugar. In this way, a single tag is added only to the sugar molecule, even though an excess of tagging agent is used. The excess tagging agent, and indeed all reagents used, are easily washed away from the immobilized sugar, which thereby remains in a small volume.

The feasibility of the SPOT process was evaluated by using two simple, model reducing oligosaccharides derived from glycolipids (Scheme 2): the linear tetrasaccharide lacto-

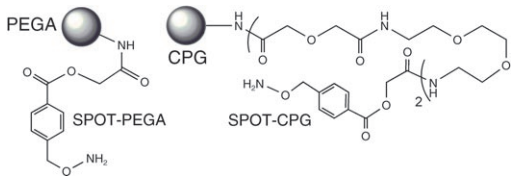


Scheme 2. Structures of LNT, LNFP, and fluorescently tagged products of LNT. TMR = tetramethylrhodamine.

N-tetraose (LNT) and its fucosylated product lacto-*N*-fucopentaose (LNFP). These specific structures were selected because they are commercially available in pure form, as are the glycosidases required to cleave their terminal sugar residues for eventual enzyme-assisted sequencing.

Two different solid supports were investigated in the evaluation of SPOT: PEGA-1900,[8] a polyethylene glycol–polyacrylamide resin that swells in both organic and aqueous solvents and has functionalizable amino groups incorporated in the range of 0.5 mmol g^{-1}; and controlled-pore glass (CPG), which comprises nonswellable, rigid particles having pores of size >1400 Å and near 30 μmol g^{-1} incorporated amino groups. The PEGA beads should be more useful when a high density of capture groups is required, whereas the CPG beads would be favored when an immobilized oligosaccharide should be accessible to a protein probe. For these reasons, the

amino groups on the PEGA beads were functionalized directly with the cleavable hydroxylamine capture groups (no spacer) to give SPOT-PEGA beads (Scheme 3), whereas the silylpropylaminated CPG was functionalized with a 40-



Scheme 3. Structures of solid supports, showing the capture group, cleavable linker, and optional spacer.

atom polyethylene glycol derived spacer prior to the incorporation of the capture groups to give SPOT-CPG beads (Scheme 3). Experimental details are provided in the Supporting Information.

Examination of the optimal conditions for the capture of oligosaccharides showed that the conditions for >95% capture differed between the two bead types. For SPOT-PEGA beads, dimethyl sulfoxide/acetic acid (DMSO/AcOH, 7:3), 70°C, and reaction for 3 h were found to be optimal. Capping was achieved with acetic anhydride/methanol (Ac_2O/MeOH; 1 h) and reduction was effected with pyridine/borane (Pyr-BH_3; 10 min). For SPOT-CPG beads, incubation overnight at 55°C in aqueous citrate/phosphate buffer (pH 5.0) proved ideal for the capture process. The capping and reduction steps were performed as for the SPOT-PEGA beads. Extensive washing of the beads was performed between steps, with the beads placed in syringes with frits on a vacuum manifold.

In a typical experiment, the capture of LNT (70 μg) on SPOT-PEGA beads (5 mg, ca. 30 μL) at 70°C for 3 h was followed by capping, reduction, fluorescence tagging with tetramethylrhodamine-5-isothiocyanate (TMR-NCS), and cleavage with an aqueous solution of LiOH. The brilliant red solution obtained was neutralized with aqueous AcOH and diluted to a final volume of 3 mL. An aliquot of this sample was further diluted 100 to 1000-fold with water, and a 22-nL portion was injected for micellar capillary electrophoresis (CE) with conventional (non-laser) fluorescence detection at 570 nm. A portion of about 10^{-8} of the sample was used for the analysis.

The electropherogram of the SPOT-labeled product is shown in Figure 1. A highly efficient chemical sequence is indicated, with the LNT-TMR (Scheme 2) eluting near 16 min (the structure was verified by MS and was independently confirmed by solution synthesis), and the only significant impurity (marked X, Figure 1) being a peak at 23 min. This latter impurity was isolated by HPLC, was found by MS to have a m/z of 624, and has the structure shown for LNT-TMR but with the sugar replaced by a methyl group. This compound arises from the scavenging of trace contaminating formaldehyde from the air and/or the solvents used. It does not interfere in the present analysis, but efforts are under way to minimize its formation.

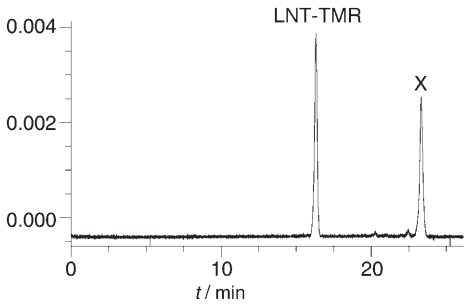


Figure 1. Capillary electropherogram of crude LNT-TMR. X denotes a non-carbohydrate side product.

We next examined the suitability of SPOT-PEGA beads for the insertion of a tag that would be of use in MS. We chose the *p*-bromophenyl (BrPh) group, as the unique isotope pattern (^{79}Br/^{81}Br 1:1) of the bromine atom can confirm that a product is indeed labeled. Incorporation of a bromine label also allows the very efficient "editing" of mass spectra as a result of the mass defect properties of this atom.[9] We therefore tagged the intermediate **D** (Scheme 1) with *p*-bromophenyl isothiocyanate, washed the beads, cleaved the linker with an aqueous base, and neutralized the sample as before. The removal of salts from the product LNT-BrPh (Figure 2) prior to MS analysis was effected by adsorption on C-18 resin, washing with water, and elution with an aqueous solution of MeOH. The mass spectrum of the crude product is shown in Figure 2 and confirms the quality of the tagged sample.

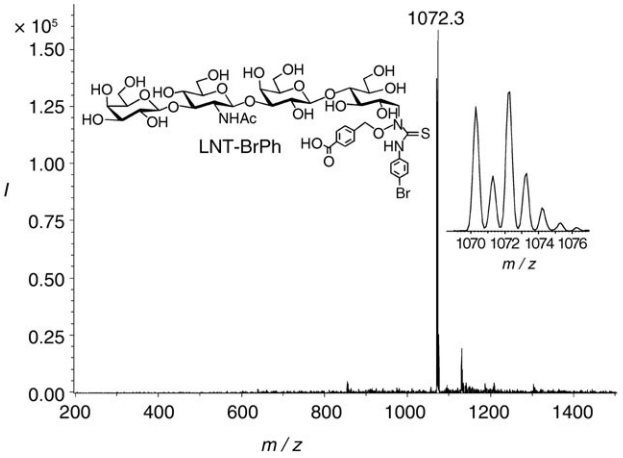


Figure 2. Structure and mass spectrum of LNT-BrPh, with the ^{79}Br and ^{81}Br isotopes highlighted in the inset.

We then set up a "dummy run" to evaluate a potential dual application of SPOT in both fluorescence tagging and CE analysis as just described, and in a differential glycomics[10] setting where the concentration of the same compound in two different samples would be estimated by MS using stable, isotope-encoded tagging species (Figure 3). For this experiment, we chemically synthesized TMR-NCS with two trideuteromethyl groups ([D_6]TMR-NCS). Separate samples containing LNT at concentrations of 1.0 and 0.5 μM

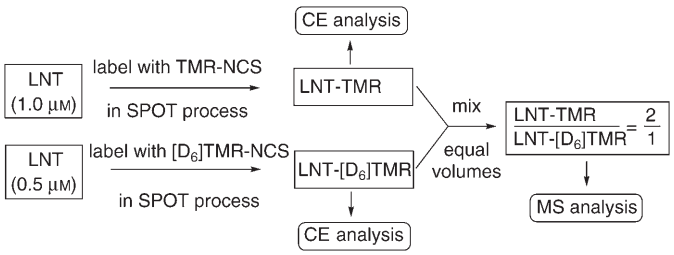


Figure 3. Flow chart for estimating the relative concentrations of LNT in two different samples, by both CE and MS analysis.

were run through the SPOT process (Scheme 1), each using 5 mg of SPOT-PEGA beads. The more concentrated sample was labeled with TMR-NCS and the other by using [D_6]TMR-NCS. CE analysis of the two cleaved and neutralized samples revealed identical retention times for LNT-TMR and LNT-[D_6]TMR (see Scheme 2 for structures). As expected, the integral of the peak for LNT-[D_6]TMR was half that of the unlabeled compound (Figure 4).

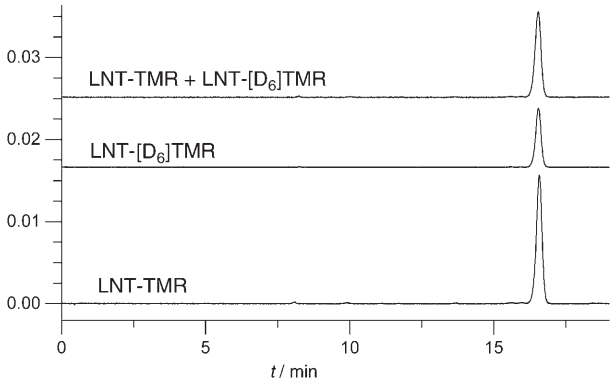


Figure 4. Capillary electropherograms of the LNT samples labeled as in Figure 3.

Identical volumes of each sample were then mixed together, following the flow diagram of Figure 3, and the resulting solution was desalted on C-18 resin prior to analysis by electrospray MS. CE analysis of the mixture (Figure 4, top trace) confirmed their expected co-elution. The mass spectrum obtained in the negative-ion mode is shown in Figure 5, and unambiguously reports the relative abundance of LNT in the two investigated samples.

To show that the composition of a mixture of oligosaccharides could be quantitated by the SPOT method, an equimolar mixture of LNT and LNFP was prepared by careful weighing of the commercial compounds. This time, SPOT-CPG beads were used. Surprisingly, the electropherogram of the product obtained after SPOT labeling with TMR-NCS showed that the compounds were present in the ratio LNT/LNFP 100:74 (Figure 6). We initially thought that either the labeling of the smaller oligosaccharide was more efficient, or that the excitation/emission properties of the labeled compounds differed. However, the fully relaxed 600-MHz

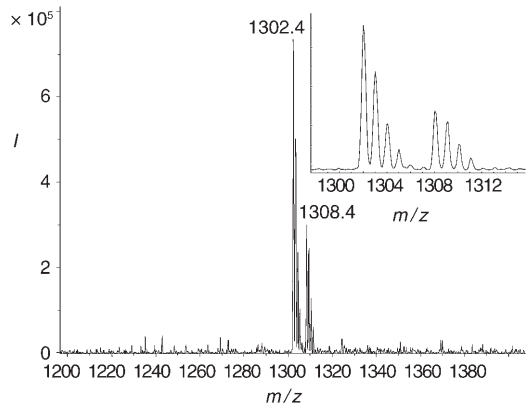


Figure 5. Electrospray mass spectrum of a mixture of equal volumes of the LNT solutions labeled as in Figure 3. The inset highlights the isotope patterns for nonlabeled and [D_6]-labeled LNT-TMR.

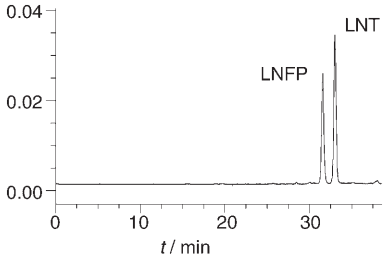
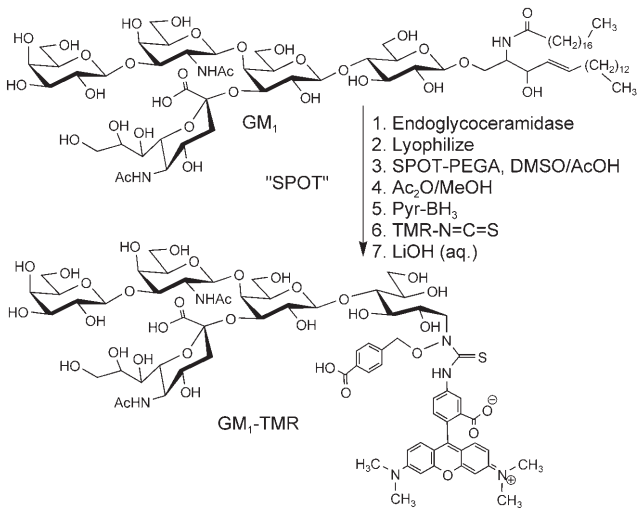


Figure 6. Capillary electropherogram of a mixture of LNT and LNFP SPOT-labeled with TMR.

^{1}H NMR spectrum of the mixture used for capture and tagging revealed that the compounds were in fact present in the ratio 100:79 (data not shown). The conclusion is therefore that the SPOT process does not significantly discriminate between the oligosaccharides of different size used here in the capture, subsequent chemistry, and analysis steps. The LNFP sample was most likely contaminated with salt and/or moisture, or an error in weighing was made.

Finally, we evaluated the entire SPOT process on a real-life glycolipid, the brain-derived ganglioside GM_1 (Scheme 4). The ganglio series of glycolipids are known to be good substrates for the commercial recombinant endoglycoceramidase II (Takara Bio), and the ceramide was indeed completely cleaved in an overnight incubation. The reaction mixture was lyophilized, the residue was redissolved in DMSO/AcOH, SPOT-PEGA beads were added, and the entire sequence of reactions developed on LNT was repeated. The CE and MS of the product GM_1-TMR are shown in Figure 7. The quality of the product validates the SPOT process.

We have demonstrated that reducing oligosaccharides can be captured on both swellable resins like PEGA, which are water and organic solvent compatible, and on rigid surfaces such as glass (CPG). Herein, immobilized hydroxylamine groups have been used as the capture reagents to produce immobilized oximes. Following a sequence of capping, reduction, and tagging with aryl isothiocyanates, a single tag is installed at the reducing end. Excess tagging (and other)



Scheme 4. Sequence of reactions for SPOT labeling of ganglioside GM_1.

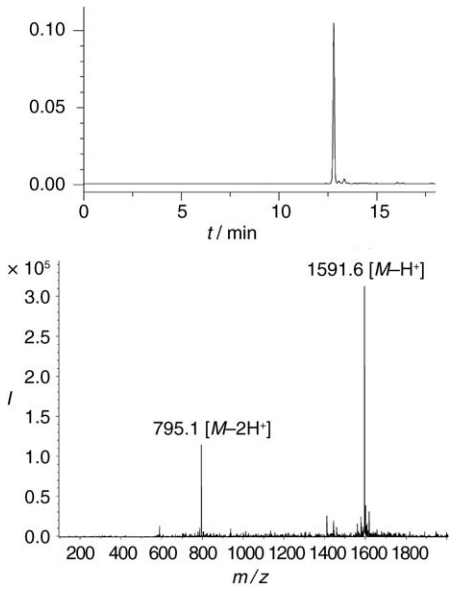


Figure 7. Characterization of the SPOT-labeled GM_1-TMR obtained as described in Scheme 4. Top: capillary electropherogram. Bottom: electrospray mass spectrum.

reagent is removed by simple washing. As a cleavable linker is installed between the capture group and the solid support, the tagged oligosaccharide can be released into solution for analysis by a variety of techniques. The types of tags used were selected to permit detection by fluorimetry and/or MS.

The two key advantages of SPOT are: 1) the simplicity of all the manipulations—which involve only pipetting and washing, and require no prior experience in the handling of oligosaccharides—means that valuable oligosaccharides cannot be lost once they are reduced after immobilization; and 2) a single protocol is used for labeling and release, irrespective of the tag used. The same captured oligosaccharide can therefore be rapidly derivatized with several different tags, and analyzed by a variety of different techniques.

The results presented are clearly those of a model study, designed only to test the feasibility of the chemistry used for capture, capping, reduction, tagging, and release. The central consideration for the chemistries developed is that, in real-life situations, very little oligosaccharide may be available for analysis. This means that the concentration of oligosaccharide in solution might be very low. Thus, it is essential that the concentration of immobilized capture groups is high for a bimolecular reaction to occur, and these capture groups must therefore be present in a large excess. In all of the experiments presented, the hydroxylamine capture groups were in fact present in at least a tenfold molar excess of the target oligosaccharide. As a result, it was necessary to be able to inactivate this excess prior to the tagging step, which would, for the same reasons, require a large excess of tagging agent. The sequence of chemical reactions developed proved to be highly efficient at achieving these objectives.

As presented herein, the capture and tagging of oligosaccharides using the SPOT process is highly efficient ($>90\,\%$) with SPOT-PEGA beads down to the range of 1 nmol oligosaccharide/mg beads. The lower level of capture group incorporation in SPOT-CPG requires a tenfold higher concentration of oligosaccharide to approach the same efficiency. The release of tagged oligosaccharide from the beads was judged to be complete under the saponification conditions used, as all of the visible red color was removed from CPG-SPOT beads.

There are certainly many challenges that remain before SPOT can be applied to real-life situations, especially to crude samples such as cell or tissue extracts. However, these are the same challenges that exist today for solution labeling techniques: complex mixtures of glycans will be present and their release from glycoproteins or glycolipids, by either enzymatic or chemical means, must be quantitative, as should their labeling. Furthermore, the labeling of N-linked oligosaccharides at the reducing end GlcNAc residue is more difficult than at the reducing Glc residue used herein. An additional challenge will be learning to eliminate trace compounds containing aldehydes and ketones, whether present in the biological samples or in the solvents and reagents used. These same compounds are present in the solution labeling techniques used today, but they are either removed by "sample processing" prior to labeling or react and become labeled. These undesired reaction products, which include excess labeling agent, are then removed in subsequent sample processing steps with attendant potential losses of oligosaccharide components. It is hoped that such sample processing steps, with their potential selective sample losses, can be avoided with the further development of the SPOT technique.

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