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## Novel cleavage of reductively aminated glycan-tags by N-bromosuccinimide to regenerate free, reducing glycans

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### Abstract

Glycans that are fluorescently tagged by reductive amination have been useful for functional glycomics studies. However, the existing tags can introduce unwanted properties to the glycans and complicate structural and functional studies. Here we describe a facile method using N-bromosuccinimide (NBS) to remove the tags and efficiently regenerate free reducing glycans. The regenerated free reducing glycans can be easily analyzed by routine mass spectrometry or re-tagged with different tags for further studies. This new method can be used to efficiently remove a variety of fluorescent tags installed by reductive amination, including 2-aminobenzoic acid and 2-aminopyridine. NBS treatment essentially transforms the commonly used 2-aminobenzoic linkage to a cleavable linkage. It can be used to cleave printed glycans from microarrays and cleave neoglycopeptides containing a 2-aminobenzoic linker.

### Keywords

Glycomics; free reducing glycan; fluorescent tag; reversible cleavage

### Introduction

Functional glycomics is the systematic study of glycan structures and functions, and is attracting increased attention from the larger scientific community. A wide variety of evidence shows that glycoproteins, proteoglycans, and glycolipids within the glycocalyx at the animal cell membrane are involved in a variety of molecular interactions and that the glycan moieties are structurally and functionally important(1–9). Functional studies of glycans have been greatly impeded, however, by glycan structural complexity, arising from their non-template-driven biosynthesis. Glycans are often branched making them difficult to fully sequence as well as difficult to chemically and/or enzymatically synthesize. In addition, the structural and functional analysis of glycans requires special analytical and chemical modification techniques, which lag far behind those developed for sequencing and synthesis of proteins and nucleic acids. The techniques for exploring glycan structures and

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functions have advanced greatly in recent years due to the analytical power of high resolution and specialized mass spectrometry (MS), high performance liquid chromatography (HPLC), genetic and cell biological approaches, and the newly developed glycan microarrays(3, 10).

Due to the lack of an intrinsic chromophore and strong hydrophilicity, underivatized glycans are usually very insensitive towards commonly used analytic tools such as MS and HPLC. A variety of fluorescent tags have been widely used to increase the sensitivity for HPLC detection and ionization in MS. The most widely used tags, such as 2-aminopyridine(11–13), 2-aminobenzoic acid(14), aminoacridone(15), PMP (16, 17) and others(18–20), are installed at the reducing end of the free glycan by reductive amination. These fluorescent tags are essential for HPLC analysis and depending on the tag can increase MS sensitivity compared with unmodified glycans. However, under certain situations when the tags have fulfilled their usage, it would be ideal to remove the tags for further structural or functional studies. Although a fluorescent tag can increase MS sensitivity compared with unmodified glycans, it often complicates permethylation of glycans and limits more detailed structural analysis. A fluorescent tag with an aromatic ring installed at the reducing end might interfere with some functional studies such as binding with glycan binding proteins (GBPs) or cellular uptake experiments. Furthermore, reports using different tags can be difficult to compare and transformation of one tag to another is not currently practical. There have been very limited studies on tag cleavage and only inefficient and non-general approaches have been reported(21, 22). In a broader viewpoint, cleavable linkages have been a very useful tool to bring versatility to bioconjugates. There are few general cleavable linkages developed for glycoconjugates, but they often require sophisticated chemistry(23). Here, we report not only the development of a new method to easily remove the fluorescent tags to greatly increase the versatility of fluorescent glycoconjugates, but also the discovery of a cleavable linkage of glycoconjugates.

## Results and Discussion

### Free glycans can be generated by N-bromosuccinimide treatment of glycan-2-amino-N-(2-aminoethyl)benzamide (AEAB) conjugates

The C-N bond generated by reductive amination of glycans with aromatic amines are generally considered to be stable. We envisioned, however, that the aromatic amine might still be prone to oxidation followed by degradation. N-bromosuccinimide (NBS) is a widely used oxidative reagent for protecting groups and anomeric leaving group manipulation in carbohydrate chemistry(24–27). It is known not to affect modifications such as O-acetyl group. Treatment of GlcNAc-6-sulfate with NBS does not alter its structure, suggesting the stability of O-sulfate. As a mild oxidant, it generally does not cause degradation or have other adverse effects on glycan structures. We therefore explored the treatment of glycan-AEAB (GAEAB) conjugates with NBS using LNnT-AEAB as a model compound for our study.

Figure 1a shows the chemical scheme of treatment of LNnT-AEAB with NBS. When LNnT-AEAB was treated with NBS in water, the starting material disappeared nearly instantly (Fig. 1b and c) as monitored by HPLC and mass spectrometry. MALDI-TOF

analysis of the product mixture showed two products; the higher mass matched exactly with the mass of free reducing LNnT. Two products were seen; a major product at  $700.1[M+Na]^+$  and a minor product at  $730.0[M+Na]^+$ . The product with 30Da mass decrease matched a new derivative from LNnT with the reducing end trimmed to a pentose (Fig. 1b). To confirm this, the NBS treated product mixture was lyophilized and re-conjugated with AEAB and compared with the starting material by MALDI (Fig. 1b) and HPLC (Fig. 1c). While the minor compound was confirmed to be LNnT, the new product from NBS treatment of LNnT-AEAB was fully conjugated with AEAB generating a new derivative with expected mass and HPLC (Fig. 1b and c). To further confirm the nature of this new compound, we used glucose-AEAB as a model compound. Based on the proposed reaction scheme, treatment of glucose-AEAB with NBS should trim the reducing end of glucose to an arabinose structure (Fig. 1d). High pH anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) analysis (Fig. 1e) confirmed the results by showing the production of arabinose and glucose by treatment of glucose-AEAB with NBS.

To explore the general applicability of this approach, NA2-AEAB, prepared from a complex-type asialo-biantennary N-glycan, was treated with NBS and analyzed by MALDI (Fig. 2a and b). NBS treatment efficiently removed the AEAB tag and generated free reducing NA2 along with the corresponding glycan having a reducing end pentose. In this case, the original free reducing sugar NA2 is the major product, likely due to protection afforded by the 2-acetoamido group. Re-conjugation with AEAB regenerated NA2-AEAB as expected, confirming the reaction (Fig. 2c). As expected, the other product with a reducing end pentose, protected by N-acetyl at the anomeric position, could not be re-conjugated with AEAB. Thus, with glycans containing reducing GlcNAc, the reversible cleavage and re-conjugation with AEAB selects for those glycans with a regenerated, intact reducing GlcNAc, which is the major product of the regenerated glycans. We expect O-glycans with an O-GalNAc reducing end would behave in a similar way to N-glycans due to the HexNAc reducing end. Therefore this reaction would be useful to regenerate both N-glycans and mucin-type O-glycans from their AEAB conjugates.

We found that the NBS reaction is not dependent on the pH of the aqueous solution and is efficient in a wide variety of buffering conditions, which is of great advantage when certain labile sugars such as sialic acids were involved. NBS treatment of disialylated NA2-AEAB and re-conjugation with AEAB demonstrated that the NBS treatment does not affect the integrity of the sialic acid in buffered conditions and there is no obvious loss or modification of sialic acid, which would generate NA2-AEAB after re-conjugation with AEAB. Therefore, the simple NBS treatment of GAEAB can quickly and efficiently remove the fluorescent tag and leave fully reducing glycans that are more amenable to structural analysis.

### The mechanism of tag removal of GAEAB conjugates by NBS

This new and unexpectedly efficient reaction is likely to go through a mechanism of oxidation followed by elimination (Fig. 3). After the initial N-bromination, through either radical or cationic mechanism, the elimination process actually diverts to two routes. In **Route a**, a pericyclic reaction occurs through a 6-membered ring intermediate to break the

C1-C2 carbon-carbon bond to generate a glycan with a pentose reducing end. It is also possible that this process is facilitated by another molecule of NBS. In **Route b**, a simple  $\beta$ -elimination generates a Schiff's base, which after hydrolysis, regenerates the original free reducing glycan. It is clear for LNnT-AEAB and glucose-AEAB that **Route a** is the dominant process. It is also not surprising that the 2-acetoamido group in N-glycans interferes with **Route a** and changes the preference towards **Route b**. To our knowledge, the cleavage of C-C bond of a  $\beta$ -aminoalcohol through NBS oxidation (**Route a**) have not been reported. Although not in the scope of this study, it could be very useful in synthetic chemistry. Based on the mechanism, we expect that this method is likely incompatible with glycans with free amino groups.

### Free glycans can be regenerated from various fluorescent glycoconjugates prepared by reductive amination

Since the proposed mechanism is not directly related to the structures of the tags, we reasoned that this method should be a universal method for all fluorescent glycoconjugates prepared by reductive amination. A panel of tags was tested as LNnT conjugates (Fig. 4a and b and Table 1), including 2-aminobenzoic acid tags to other aromatic rings, such as aniline and 2,6-diaminopyridine (DAP). Simple alkylamine ethylenediamine was also tested. Results show that the reaction was general to all tested tags, although in certain cases elevated temperature was needed (Table 1). Interestingly, the ratio between the two products **2** and **3** (Fig. 4b) is highly dependent on the nature of the tag. Nevertheless, this does not undermine the practicability of this reaction, since both products are fully compatible towards further analysis or derivatizations such as permethylation or fluorescent conjugation. The results also suggest that previously prepared glycan conjugates through 2-AA, 2-AB and DAP, which are not very useful for microarray analysis due to less their reactive aryl or secondary amine used for less efficient printing, can now be readily transformed to GAEAB conjugates for more efficient solid phase immobilization of other bioconjugation. It is also now possible to compare and confirm glycan structures prepared using different tags.

### Development of a new cleavable linkage for glycoconjugates

While being able to remove fluorescent tags from glycoconjugates is clearly advantageous, the implication of using a simple chemical treatment to break an easily installed and commonly used linkage is of greater importance. We reasoned that glycoconjugates that are immobilized to solid surfaces or attached to other biomolecules through the 2-aminobenzoic linkage should be easily cleaved to remove the tag and recover the key moieties for further analysis. LNnT-AEAB was printed on NHS-activated microarray glass slides for protein binding analysis (Fig. 5a). As expected, we found that the plant lectin RCA-I, which recognizes terminal non-reducing  $\beta$ -linked galactose, bound to LNnT in a concentration-dependent pattern. If the printed slide is treated with NBS before the binding assay, the RCA-I binding is significantly reduced. This result demonstrates both that the linkage of tag to the glycan can be easily cleaved on the microarray slides, and that the method can be practically used to validate the glycan printing. We also explored the utility of this method to examine reversible conjugation of glycans to solid microspheres. To this end, LNnT-AEAB was conjugated to Ultralink beads, comprised of azlactone-activated, beaded-

polyacrylamide resin (Fig. 5b). When the glycan-coated beads were treated with NBS, glycan was released and analyzed by MS after permethylation. This provides an approach to analyze glycan structures that are immobilized onto solid surfaces. We also generated a glycan-peptide neoconjugate by reaction of a peptide with the LNnT-PNPA conjugate (Fig. 5c)(28). This neoglycoconjugate was then treated with NBS to cleave the two molecules and release the free peptide and glycan, which were analyzed by MS before and after permethylation, respectively (Figure 5d). The results show the recovery of the intact starting peptide and the glycan LNnT.

In conclusion, we have developed a new method to efficiently remove fluorescent tags from glycoconjugates prepared from glycans by reductive amination. By a simple treatment with NBS, free reducing glycans can be released from glycoconjugates tagged through reductive amination. This method is general to all the tags tested, including all 2-aminobenzoic tags, 2,6-diaminopyridine tag, aniline tag, and simple ethylenediamine tag. The regenerated free reducing glycans can be analyzed by permethylation/MS and/or re-functionalized at the reducing end for functional study, bring versatility to fluorescently tagged glycans. Furthermore, this new method is applied to convert known glycan/glycopeptide tags into a “new” cleavable linkage of such derivatives. Solid phase immobilized glycans or glycan-peptide conjugates with 2-aminobenzoic linkages can be efficiently cleaved off for structural analysis and further derivatizations. Although only exemplified here for glycoconjugates, the 2-aminobenzoic linkages can now be essentially considered a cleavable linker for other bioconjugates. In a previous study, Suzuki et. al. explored the usage of hydrogen peroxide to remove fluorescent tags from glycoconjugates(21). While such treatment was effective, the reaction was slow with greatly varying efficiency, and some tags, such as 2-aminopyridine, were not removable. The NBS reaction proposed here is much faster, more efficient, and more universal. In addition, the ability to add and remove tags in a facile manner will promote both glycomics studies and functional glycan recognition, and could lead to new tools aimed at identifying novel glycan-binding proteins.

## Materials and Methods

### Materials

HPLC solvents were purchased from Fisher Scientific, Pittsburgh, PA. A Bruker Daltonics Ultraflex-II TOF/TOF system was used for mass spectrometry analysis. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO and Fisher Scientific, Pittsburgh, PA. The model peptide was kindly provided by David Live (Complex Carbohydrate Research Center, University of Georgia, Athens, GA).

### HPLC and HPAEC analyses

A Shimadzu HPLC CBM-20A system was used for porous graphitized carbon (PGC)-HPLC analysis and separation of fluorescently tagged glycans, which was coupled with a UV detector SPD-20A and a fluorescence detector RF-10Ax1. UV absorption at 330 nm or fluorescence at 330 nm excitation (Ex) and 420 nm emission (Em) was used for detection. A Hypercarb HPLC column (150mm × 4.6mm) was used. The mobile phase was acetonitrile and water with 0.1% TFA. The concentration of acetonitrile increased from 15% to 40% in

25 minutes. HPAEC-PAD analysis was carried out with a Dionex ICS-3000 system on a Carbowac PA-10 column. An isocratic program with 20 mM sodium hydroxide was used for monosaccharide analysis.

### Free glycan release from GAEAB conjugates

In a typical procedure for the release of free glycans from GAEAB conjugates, NBS solution (2mg/mL in 0.1% TFA in water) was added to give a final GAEAB concentration of 0.1–1mg/mL. The solution was incubated at room temperature for 30 minutes and directly analyzed by MALDI or HPLC, or was lyophilized and directly conjugated with AEAB or other fluorescent tags.

### Permethylation analysis and fluorescent labeling

After treatment to release free glycan, the lyophilized glycan mixture was directly permethylated as described(29). Fluorescent tagging of lyophilized glycan mixtures was carried out as described previously(19).

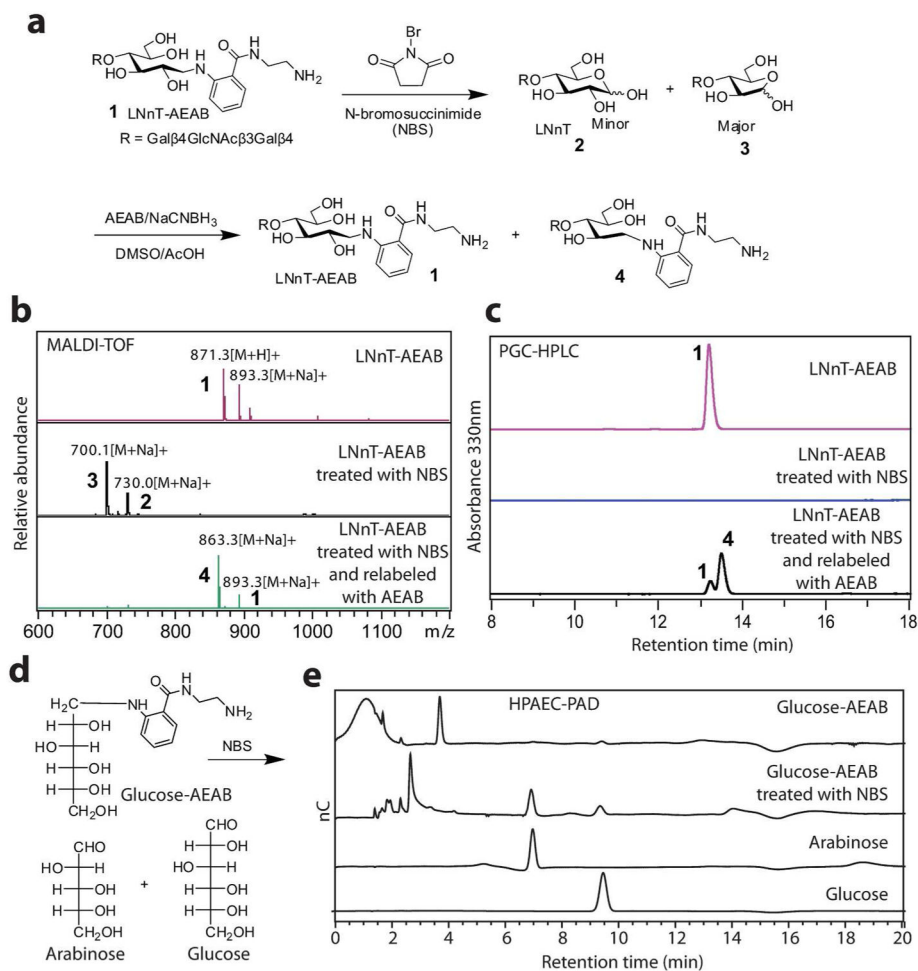
### Acknowledgments

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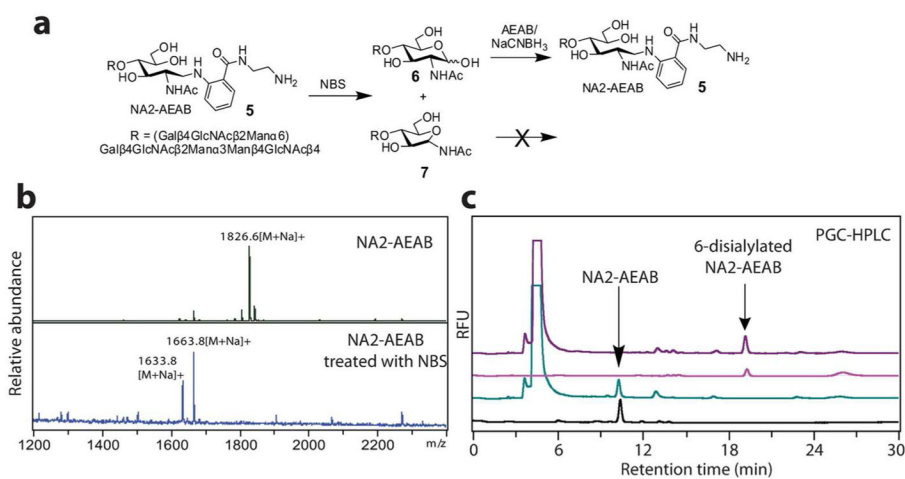
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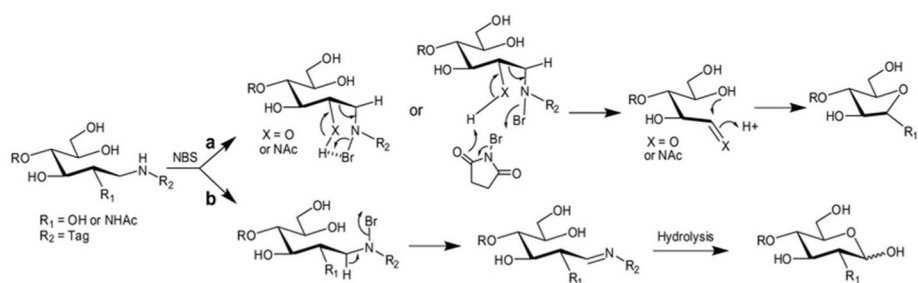


**Figure 1.** The regeneration of free glycans from GAEAB by NBS treatment. a) The treatment of LNNt-AEAB with NBS generates two products (**2** and **3**), both of which can be re-labeled with AEAB. b) MALDI-TOF spectra of LNNt-AEAB (top), LNNt-AEAB treated with NBS (middle) and the AEAB-reconjugation of the NBS treatment products (bottom). c) HPLC profiles of LNNt-AEAB (top), LNNt-AEAB treated with NBS (middle) and the AEAB-reconjugation of the NBS treatment products (bottom). d) The schematic of NBS treatment of glucose-AEAB to generate arabinose and glucose. e) The HPAEC-PAD profiles of glucose-AEAB, glucose-AEAB treated with NBS, arabinose, and glucose (from top to bottom).

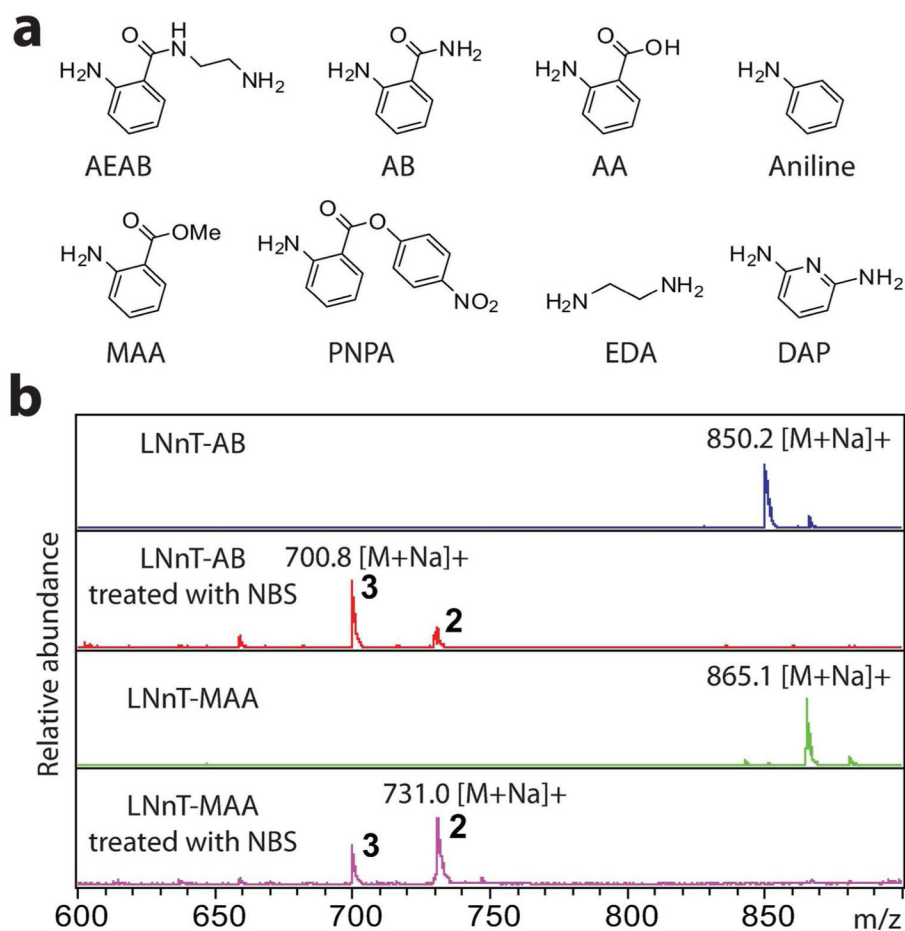


**Figure 2.**

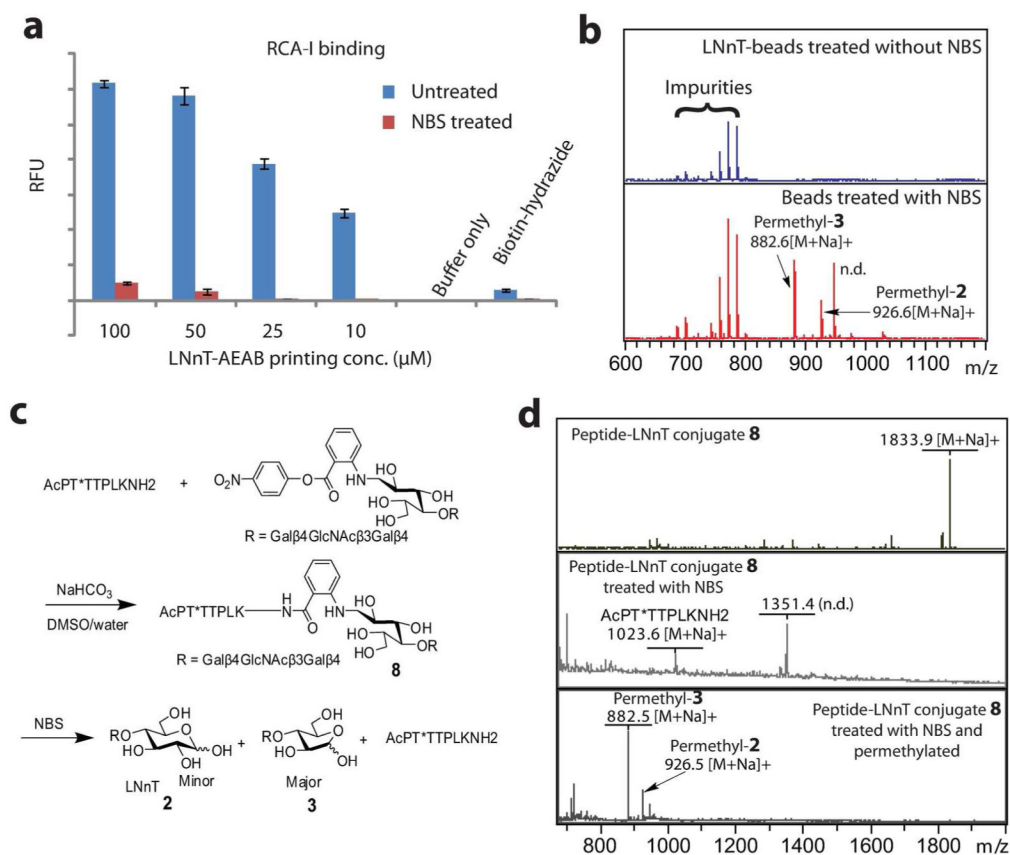
The NBS treatment of AEAB conjugates of N-glycans. a) The NBS treatment of NA2-AEAB generates two compounds, including free reducing NA2 which can be labeled with AEAB. b) MALDI-TOF spectra of NA2-AEAB (top) and the NBS treatment products of NA2-AEAB (bottom). c) PGC-HPLC profiles (from bottom to top) of NA2-AEAB, NA2-AEAB treated with NBS and labeled with AEAB, 6-disialylated NA2-AEAB, and 6-disialylated NA2-AEAB treated with NBS and labeled with AEAB.



**Figure 3.** The proposed mechanisms of the NBS treatment of glycan-AEAB conjugates and the release of untagged glycans. **Routes a** and **b** are shown above and below, respectively.



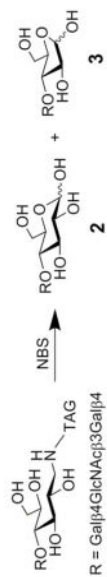
**Figure 4.** NBS treatment is a general method to removed different tags from tagged glycans. a) Various tags tested in this study. b) MALDI-TOF spectra of two typical experiments of NBS treatment of tagged glycans as examples. From top to bottom: LNnT-AB, NBS treatment of LNnT-AB, LNnT-MAA, and NBS treatment of LNnT-MAA.

**Figure 5.**

2-Aminobenzoic acid based linkers used as a cleavable linker upon NBS treatment. a) LNnT-AEAB was printed on a microarray at different concentrations. NBS pre-treatment of the slides before binding assay significantly reduced RCA-I binding. RFU = relative fluorescence units, Error bars =  $\pm$  1 SD from the average of 4 printed replicates. b) LNnT-AEAB conjugated Ultralink beads were treated without (top) or with (bottom) NBS 1mg/mL in 0.1% TFA. The filtrates were lyophilized and permethylated for MALDI analysis. c) The generation of a peptide-glycan conjugate through PNPA linker, which can be cleaved by NBS treatment to regenerate glycan and peptide. d) MALDI-TOF profiles of the peptide-LNnT conjugate before (top) and after (middle) NBS treatment, and after permethylation (bottom).

Table 1

The NBS treatment of conjugates of LNnT with various tags.



Tag	AEAB	AA	AB	Aniline	MAA	PNPA	EDA	DAP
Temp (°C)	22	22	22	22	22	22	65	65
Time (h)	1	1	1	1	1	1	1	1
Yield (%)	100	100	100	100	75	100	50	80
Ratio (3 : 2)	3:1	8:1	5:1	4:1	1:2	1:4	1:4	1:1