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Oxidative Release of Natural Glycans for Functional Glycomics

Xuezheng Song, **Hong Ju**, **Yi Lasanajak**, **Matthew R. Kudelka**, **David F. Smith**, and **Richard D. Cummings**

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, USA

Abstract

Glycans have essential roles in biology and the etiology of many diseases. A major hurdle in studying glycans through functional glycomics is the lack of methods to release glycans from diverse types of biological samples. Here we describe an elegant yet simple oxidative strategy using household bleach to release all types of free reducing N-glycans and O-glycan-acids from glycoproteins, and glycan nitriles from glycosphingolipids. Released glycans are directly useful in glycomic analyses and can be derivatized fluorescently for functional glycomics. This chemical method overcomes the limitations in glycan generation and promotes archiving and characterization of human and animal glycomes and their functions.

Keywords

Functional glycomics; glycan release; sodium hypochlorite

Introduction

Genomics and proteomics have made tremendous advances in part due to facile technologies developed to promote high-throughput studies. In regard to complex carbohydrates, advances in technologies to aid in defining their structures and functions have been limited. Thus, glycomics, while it has received considerable interest in recent years [1, 2], severely lags behind genomics and proteomics because of analytical and preparative difficulties. Glycomics focuses on analyses of glycan structures [3], whereas functional glycomics requires studies on the recognition of glycans by glycan-binding proteins (lectins) either free or on cells, bacteria, and viruses [4, 5], as well as glycan binding by antibodies. Detailed glycomics analyses require sufficient amounts of glycans for studies by nuclear magnetic resonance

Correspondence to: Xuezheng Song, Ph.D., Department of Biochemistry, Emory University School of Medicine, O. Wayne Rollins Research Center, 1510 Clifton Road, Suite 4025, Atlanta, GA 30322, Tel: 404-727-3664, Fax: 404-727-2738, xsong2@emory.edu, Richard D. Cummings, Ph.D., Director, National Center for Functional, Glycomics, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, CLS 11087 - 3 Blackfan Circle, Boston, MA 02115, Tel: 1-617-735-4643, rcummin1@bidmc.harvard.edu.

Contributions

X.S. and R.D.C. conceived the method; X.S., H.J. and Y.L. performed experiments; X.S., D.F.S., and R.D.C. analysed the data and wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

(NMR), crystallography, and other methods including high performance liquid chromatography (HPLC) [6-8], MS [9-15], and glycan microarrays [16-21]. However, rapid advances in glycomics are frustrated by the lack of practical methods to generate large quantities of natural glycans from unprocessed biological samples to represent all glycan structures within human and animal glycomes for analysis and functional studies. Obviously, glycans cannot be 'amplified', since there is no template that encodes their sequences, as exists for nucleic acids and proteins. While recent developments in chemical/enzymatic syntheses of glycans are impressive [22-26], detailed structures of glycans within animal glycomes are not yet available, and it is not feasible to generate thousands of highly complex compounds identical to those found in nature, and chemical synthesis of milligram quantities of pure glycans is prohibitively costly.

While natural glycans can potentially be used to study protein-glycan interactions [17, 19, 21, 27-30], the current approaches to release glycans typically are only useful with small amounts of materials, require expensive enzymes, and/or corrosive or toxic chemicals [31-35]. N-glycanases including PNGase F and A remain the most widely used reagents for N-glycan analysis. However, they are too expensive for large scale preparation, which also necessitate enzymatic proteolysis [35-37]. Hydrazinolysis has been used for both N- and O-glycan analysis and preparation but it is limited due to the reagent toxicity and glycan structural integrity [31, 38]. Ammonium salt treatment was originally developed for O-glycans and found to also be effective for N-glycans [32, 39]. Although the reagents are inexpensive, long reaction times and large amounts of excess reagents make it difficult for large scale preparation, and the peeling reaction is also difficult to control [40]. Pronase digestion of glycoproteins can release glycoamino acids, which can be directly functionalized [30] or further trimmed with of N-bromosuccinimide (NBS) [41] for further functionalization. However, complete pronase digestion is difficult to achieve and may be impossible for mucins and mucin-like glycoproteins with multiple O-glycans. One-pot β-elimination and tagging with 1-phenyl-3-methyl-5-pyrazolone (PMP) have shown to be effective analytical methods for O-glycan release, but the products cannot be further functionalized [42, 43]. Endoceramidases are available to release the lipid moiety from glycosphingolipids (GSLs) for glycan analysis [33, 34]. However, large scale preparations are not feasible due to the high costs of reagents. Ozone initiated glycan release from glycosphingolipids under basic or neutral conditions can be potentially used for large scale preparation [44, 45], but ozone generators are not commonly available in biological laboratories.

Here we report our discovery of a simple approach to prepare N- and O-glycans and GSL-derived glycans from kilogram quantities of animal samples. Controlled treatment of biological samples with sodium hypochlorite (NaClO) in commercial bleach, a process termed "Oxidative Release of Natural Glycans" (ORNG) selectively releases intact N-, O- and GSL-glycans, which can be tagged specifically for chromatographic separation and structural elucidation (**Fig. 1**). This novel strategy, which is inexpensive, rapid, and scalable, overcomes the current technical and conceptual limitations in functional glycomics and opens the possibility to identify and archive all glycans in biological samples and accomplish the goals of sequencing human and animal glycomes.

Results

NaClO releases free reducing N-glycans from glycoproteins

We previously reported that N-bromosuccinimide (NBS), a mild oxidant, oxidatively decarboxylates small N-glycopeptides with short peptide chains without affecting glycan structures [46]. This method, however, requires prior proteolysis of intact glycoproteins for effectiveness. In exploring other oxidative release/degradation approaches, we discovered that sodium hypochlorite (NaClO), the active ingredient in household bleach, which is known to degrade proteins [47, 48], effectively and selectively degrades the aglycon portion of native glycoconjugates to release intact glycans. Brief treatment of glycoproteins with NaClO releases free N-glycans, which can be specifically derivatized through the reducing end. Thus, the released N-glycans by ORNG can be easily fluorescently tagged by reductive amination (**Fig. 2a**). NaClO degrades glycoproteins in minutes (Method 1) and released glycans are easily visualized by increased mobility compared to untreated glycoproteins, e.g. ovalbumin, on thin layer chromatography (TLC) (**Fig. 2b**). Analysis of treated glycoproteins by SDS-PAGE and Coomassie staining showed complete loss of intact glycoprotein material after NaClO treatment. Matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS analysis showed that N-glycans are produced as glycosylamines, which spontaneously convert to free reducing glycans in aqueous conditions at room temperature (**Supplementary Fig. 1**). We found that ORNG is applicable to all types of common glycoproteins (**Fig. 2c**), including ovalbumin, bovine IgG, and horseradish peroxidase (HRP), the latter of which is resistant to PNGase F digestion due to core α3-fucose modification of N-glycans [37]. The typically labile sialic acid residues are preserved during ORNG, as we observed that bovine fetuin-derived released glycans, which are highly sialylated, after permethylation, showed similar profiles to those released by PNGase F (**Fig. 2d**).

As noted, N-glycans derived by ORNG react with amine-based tags, such as the bifunctional fluorescent linker 2-amino-N-(2-aminoethyl)benzamide (AEAB) or other fluorescent tags to facilitate chromatographic separation and purification. AEAB conjugates of glycans released from bovine fetuin by ORNG showed the same glycans as those released by PNGase F (**Fig. 2e**). MALDI-TOF-MS profiles of permethylated glycans from human plasma, released either by ORNG or by PNGase F were very similar, and showed comparable contents of multi-sialylated glycans (**Fig. 2f**). Several fucosylated glycans, presumably from serum IgG, were more abundant in the ORNG-released glycans, probably because some N-glycans may be somewhat resistant to PNGase F digestion. Note that PNGase F typically is not highly reactive with intact glycoproteins, and in most cases it is desirable to first degrade glycoproteins by trypsin or other proteases to generate glycopeptides as substrates for PNGase F treatment. We also obtained an N-glycan profile of human saliva following a brief NaClO treatment (**Supplementary Fig. 2**). We observed that saliva-derived N-glycans had masses predicting they are highly fucosylated, which is consistent with previous reports [49]. These results demonstrate that ORNG has great potential in high-throughput analyses of N-glycosylation of large-scale biological samples with minimal sample preparation and no prior proteolysis.

To determine the large-scale utility of ORNG, we analyzed hen eggs as a readily available and inexpensive source of animal glycoproteins [50, 51]. We separated egg white and yolk and treated several hundred grams of these materials directly with NaClO (Method 3). MALDI-MS profiles (**Fig. 2g**) showed very different N-glycan profiles for egg white versus yolk. While egg white showed a profile including high-mannose and hybrid type N-glycans similar to that of ovalbumin as expected (ovalbumin constitutes ~80% of egg white), the egg yolk profile indicated an abundance of high-mannose and complex type N-glycans. Several hundred grams of animal tissues were also treated with NaClO to easily obtain gram scale crude N-glycans (**Supplementary Table 1**), which can be readily conjugated with AEAB. Thus, while it is not possible to directly determine the yield of material from such natural sources because it is not possible currently to measure the total glycan content, the amounts of recoverable N-glycans from wet animal tissue was 0.5-1%, which approximates the predicted carbohydrate content, which for egg yolk is 0.7-1% [52].

As to the potential mechanism of NaClO release of N-glycans, we propose that the glycan-peptide amide bond is likely chlorinated to form an N-chloroamide (**Supplementary Fig. 3)**. A pericyclic reaction involving an asparagine proton gives a glycan-isocyanate intermediate, which is hydrolyzed to a glycosylamine and then to a free reducing glycan. This mechanism is consistent with the occurrence of glycosylamines as the major products after reaction (**Supplementary Fig. 1**). Because of the presumed instability of the glycosylamine towards further oxidation, we also observed some N-glycans that have lost the reducing terminal GlcNAc residue (**Fig. 2f**, **Supplementary Fig. 3**). Such glycans resemble those that would be released by Endo F, which cleaves within the chitobiosyl core of N-glycans. Thus, while this partial degradation may increase the complexity of the glycan profiles and structural diversity of the glycan products, it can also provide valuable sequence information at the reducing end, such as core fucosylation. In addition, the Endo F-like released N-glycans, whose yield can be relatively controlled by time and temperature, could serve as novel substrates for various endoglycosidases, e.g. Endo M, which is useful in reverse synthesis of glycans by reattachment of glycans to GlcNAc-Asn residues in synthetic glycopeptides [53].

Because ORNG is simple and useful for generating large amounts of natural glycans from glycoproteins or biological samples, we further applied this method for preparative release of N-glycans from various other sources, including ovalbumin, bovine IgG, and fetal bovine serum (FBS). Released glycans were AEAB tagged and separated by multidimensional HPLC using a combination of preparative C18-reverse phase (RP), semi-preparative amino-normal phase (NP) and analytical porous graphitized carbon (PGC)-reverse phase columns. These separations yielded individual glycans of >95% purity (**Supplementary Fig. 4**), which are salt-free and useful for MS characterization and microarray printing. A library of 67 complex N-glycans with significant quantities (multi-micrograms to milligrams) was readily obtained from gram quantities of these glycoproteins (**Supplementary Table 2**). For example, from 2 g of ovalbumin we obtained ~6 mg of glycans (~3.5 μmol) comprised of 25 purified individual glycan-AEAB conjugates (Oval-01 to Oval-25, **Supplementary Table 1**). These glycans were analyzed by MS and MS/MS to obtain significant structural information (**Supplementary Fig. 5**). It is often not possible to characterize natural glycans by NMR, because only small amounts of samples are used that require enzymatic release

and the glycans are not typically purified. To confirm the robustness of ORNG, we selected several purified N-glycans, including high-mannose, hybrid and complex type, neutral and acidic glycans for characterization by ^{1}H NMR spectroscopy (**Supplementary Fig. 6a**). Clear spectra were obtained and anomeric region chemical shifts of six N-glycans isolated from egg yolk were tentatively assigned (**Supplementary Fig. 6b**) based on comparison with predicted value of methyl β-glycosides using CASPER (http://www.casper.organ.su.se/casper/) (**Supplementary Fig. 7**) [54].

To confirm that glycans can be recognized by glycan-binding proteins, as is used in functional glycomics strategies, we printed an N-glycan microarray from this library and analyzed binding of immobilized glycans to several plant lectins (**Fig. 3**, **Supplementary Table 3**). The interactions showed consistent and predictable results, demonstrating that these nonenzymatically and chemically-prepared glycans retain their conformation and ability to be recognized. The binding profiles of lectins toward glycans, which provides information about specific glycan determinants [55], aids in the structural characterization (**Supplementary Fig. 5)**. Thus, ORNG permits the exploitation of 'shotgun glycomics' [56, 57], which we have shown is an effective method to both identify potential glycan ligands for glycan binding proteins and anti-glycan antibodies, and sequence the glycans within the relevant metaglycomes under study [46, 58].

Although NaClO was previously considered to be a non-selective oxidant of organic molecules, our discovery indicates that glycans are degraded much more slowly than peptide backbones. To test this directly, we treated a free reducing glycan lacto-N-neotetraose with 1% NaClO. We only observed slight degradation after a long treatment of 15 minutes (**Supplementary Fig. 8**). The only observed degradation product, through permethylation and MS analysis, was a sugar lactone/acid derivative with the reducing end oxidized. Coupled with the evidence on similarity of N-glycans produced by PNGase F or ORNG above, these results indicate that under controlled conditions, glycans are stable to NaClO treatment.

NaClO treatment of glycoproteins releases O-glycan acids

Unexpectedly, we also found that ORNG is useful for the preparation of O-glycan derivatives (**Fig. 4a**). As might be predicted given the above data, O-glycosidic linkages are more stable than N-glycoside upon NaClO treatment (Method 4), while the protein is quickly degraded (**Fig. 4b**), as shown by TLC analysis. MALDI-MS analysis of the NaClO treatment product from porcine stomach mucin, a glycoprotein rich in O-glycans linked to Ser or Thr residues, showed the recovered glycans were linked at the reducing end to glycolic acid/lactic acid (O-glycan-acids) as the major products (**Fig. 4c**). The acidic aglycons, glycolic acid and lactic acid, presumably arise from Ser and Thr residues, respectively, to which the glycans are attached. Significantly larger amounts of NaClO are needed to degrade peptide chains more completely to release O-glycans compared to that for N-glycans. MALDI-TOF-MS analysis of O-glycan-acids released from porcine stomach mucin and fetuin after permethylation showed two sets of signals clearly matching these glycan-glycolic acid/lactic acid products, along with some permethylated free reducing glycans (**Fig. 4d**). The availability of O-glycans that retain their linkage to the aglycon

peptide products is an unanticipated advantage, and facilitates studies on glycan recognition, especially where O-glycan linkage and anomericity is necessary. For glycomic analyses of O-glycan-acids, permethylation under typical highly basic conditions, is accompanied by release of O-glycans from incompletely degraded O-glycopeptides through β-elimination, as reported previously for Pronase digested glycoproteins [59]. To test whether modified sialic acids in O-glycans were stable to ORNG, we treated bovine submaxillary mucin (BSM) and derived primarily the O-glycan-acids GalNAcα-R and Neu5Acα2-6GalNAcα-R (**Supplementary Fig. 9**), in which it is known that some sialic acid residues carry a 9-O-acetyl group. Importantly, the 9-OAc moiety of the sialic acid, which is labile under prevailing β-elimination methods, is retained in ORNG. The 9-OAc does not survive the usual permethylation conditions, but was stable to a partial methylation procedure used only to methylate carboxylic acids. To facilitate the separation and purification of the products, the glycolic acid/lactic acid aglycon can be easily derivatized using common EDC/NHS activation and a fluorescent linker with an amino group, such as monoFmoc ethylenediamine, which can be analyzed by MALDIMS and separated by HPLC (**Fig. 4e,f**) to prepare O-glycan libraries for microarray preparation. This derivatization does not significantly affect sialic acids as reported previously [60].

Using these approaches, we used ORNG to prepare an O-glycan library from porcine stomach mucin (Method 4). Such a library is impractical with common methods of O-glycan release, which typically require $NaOH/NaBH_4$ treatment and results in reduction of O-glycans. We treated 10 g of porcine stomach mucin with NaClO in consecutive treatments to give 4.3 g O-glycan-acids, which were easily tagged with monoFmoc ethylenediamine and separated by multidimensional chromatography, including size exclusion chromatography (SEC), C18 RPHPLC, and amino NP-HPLC. A library of 65 major O-glycan-acids were obtained (**Supplementary Table 4**) and analyzed by MS and MS/MS (**Supplementary Fig. 10**). We noted that many sulfated glycans were identified in the library, confirming the compatibility of ORNG with O-sulfation of glycans. These glycans were deprotected by piperidine to expose the amino group, printed onto microarray slides, and analyzed by plant lectins. Distinct and specific lectin binding (**Supplementary Fig. 11**) was observed, validating the ORNG approach with O-glycans.

Mouse gastrointestinal tract glycan analysis—To demonstrate the utility of ORNG in comparative tissue Glycomics analysis, we prepared O-glycans from the gastrointestinal tract tissues of a C57BL/6 wild type female mouse (6 months old), including stomach, small intestine, and colorectum (Method 2). Glycans were released by NaClO, purified by C18 and carbon SPE cartridges, and permethylated for MS analysis (**Supplementary Fig. 12 and Supplementary Table 5**). Contamination of hexose oligomers (m/z 681, 885 etc.) presumably from food digest, was found, as expected, in all tissues in the elution from hypercarb cartridge. Only low abundance N-glycans were observed as high-mannose structures for all three tissues. Interestingly, the three tissues showed quite different O-glycan profiles in both C18 and hypercarb elutions. Under the NaClO treatment condition used for these tissues (Method 2), the mucin glycoproteins were not fully degraded to give O-glycan acids due to the limited amount of NaClO used. Instead, partially degraded glycopeptides were obtained and retained on both C18 and hypercarb SPE cartridges. These

O-glycopeptides, upon treatment with DMSO/NaOH/iodomethane, release and permethylate O-glycans efficiently [59]. Mouse stomach showed abundant fucosylated O-glycans, similar to that reported for porcine stomach mucin [42] with nearly no sialylated O-glycans. Small intestine showed much less complex profiles with more monosialylated glycans. Colorectum showed even more sialylation, including many disialylated glycans that were not observed in stomach and small intestine. These results demonstrate that mucin O-glycan expression and sialylation is highly regulated in different regions of the murine gastrointestinal tract.

NaClO treatment of GSLs releases glycan nitriles

We also made the unexpected discovery that ORNG can be used to degrade GSLs, a challenging class of glycoconjugates (**Fig. 5a**). When unmodified porcine brain gangliosides containing the common ceramide lipid moiety were treated with NaClO in aqueous conditions (Method 5) and products were analyzed by MS, we observed loss of the lipid moiety and the major products have a 39Da molecular mass increase over the corresponding free reducing glycans (**Fig. 5b**). Based on structures of GSLs and the oxidative nature of NaClO, we deduced that the products are cyanomethyl glycosides (**Fig. 5a**), consistent with the 39Da mass increase. Importantly, this reaction can be used to directly treat porcine brain tissue in aqueous conditions, avoiding the tedious and noxious organic solvent extraction [61]. Consistently, the permethylated product gave a MS profile matching cyanomethyl glycosides (**Fig. 5c**). Similarly, we also discovered that NBS treatment of gangliosides at 65°C also yielded glycan nitriles (**Supplementary Fig. 13**). However, NBS cannot release glycan nitriles directly from brain tissue. To further confirm the glycan nitrile structure of ORNG products, a nickel chloride/$NaBH_4$ reduction was carried out. The resulting product showed a 4Da mass shift, matching exactly the expected aminoethyl glycosides (**Fig. 5d**, top). When this product was permethylated, an expected profile was observed to match the expected tertiary ammonium cations (**Fig. 5d**, bottom), confirming that NaClO treatment of GSLs in aqueous buffer oxidatively delipidated the glycan moieties, converting them to cyanomethyl glycosides.

This reaction of NaClO and GSLs is unprecedented and we propose a mechanism (**Supplementary Fig. 14)** as a potential route. Two consecutive oxidative elimination steps through pericyclic intermediates are proposed, which convert the amide of the lipid moiety to a nitrile, which is stable towards further oxidation. Nitriles are reasonably stable and can survive many normal reactions. Nevertheless, a nitrile can be specifically activated under certain reductive conditions, which makes it a useful functional group for further modification and conjugation. We applied the mild, specific nickel chloride/sodium borohydride reduction to the glycan-nitriles. The resulting alkylamine was easily protected with an Fmoc group as a reversible fluorescent tag, which greatly facilitated detection during chromatographic separation. This strategy permits an easy functional labeling of gangliosides, which was validated by MALDI-TOF-MS (**Fig. 5e**) and a clean HPLC profile (**Fig. 5f**). The reaction is essentially quantitative, as no nitriles were observed in the products. A C18 SPE column is useful to purify the glycan-Fmoc products based on Fmoc hydrophobicity. Fmoc protecting groups can be easily removed by simple piperidine treatment. The resulting glycans with a primary amino group can be used in further

modifications including microarray immobilization or linkage to amine-reactive aglycon moieties.

While Fmoc serves as a reversible fluorescent tag for amino groups, we also developed a method to directly utilize the nitrile functionality for fluorescent tagging. Nitriles have been used in palladium/carbon (Pd/C) catalyzed N-alkylation of amines, although often nitriles are used in large excess [62-65]. We demonstrated that glycan-nitriles generated from N-glycans by pronase/NBS can be efficiently tagged with 2-aminobenzamide (2-AB) [46]. When nitriles were directly treated with 2-AB along with Pd/C catalyst and ammonium formate, we observed efficient tagging of the glycan-nitriles, as demonstrated by MALDI-TOF-MS and HPLC profiles (**Supplementary Fig. 15**). No nitriles were found after the conjugation and only trace amounts of glycan-amines (from direct reduction of nitriles) were observed on mass spectra.

Discussion

Our results demonstrate a novel approach to liberate glycans from glycoproteins and glycolipids from large amounts of biological samples, and provide a robust and original solution to the obstacle of identifying human and animal glycomes. While it is predicted that vertebrate animal glycomes are highly complex [3], complete structural elucidation of glycans in human and animal glycomes, as well as explorations of their functional interactions (*functional glycomics*) have been exceedingly difficult due to lack of methods to obtain sufficient quantities of natural material for study and the lack of high-throughput sequencing. This surprisingly easy method to release glycans with cheap and safe reagents significantly improves the competitive advantages of studying glycoconjugates relative to other types of biological molecules.

A key development in our search for a simple, aqueous-based reagent that directly releases glycans from mg-g scales of biological materials was the finding that NaClO (bleach) differentially degrades proteins versus carbohydrates, as well as specifically degrading ceramide. In **Supplementary Table 6**, we compared the ORNG method with several traditional glycomics approaches. Bleach is commonly used for sterilization through its strong oxidative power toward proteins in microorganisms. However, there has been no prior systematic study on NaClO in the preparation and release of glycans from glycoproteins and GSLs. NaClO can be used to solubilize yeast cell walls to facilitate extraction of glucan polysaccharides [66], and it was found that NaClO can degrade some glycosaminoglycans [67-69]. Although an oxidative reagent, we have found that NaClO under controlled conditions degrades glycans very slowly due to the already high oxidation state of carbohydrates, including commonly occurring modifications of glycans such as O-acetylation, O-sulfation, etc. By its chemical nature of attacking peptide bonds, ORNG should be applicable to plant and other non-mammalian glycans that might not be cleavable using traditional methods. O-glycans released by ORNG retain their α-linkage to Ser or Thr derivatives and the glycolic acid/lactic acid (O-glycan-acids) are directly useful for derivatization and characterization in which the glycan retains its anomeric configuration. ORNG releases GSL-glycans without the limitation of organic solvents extraction, endoglycoceramidases with unwanted specificities, or ozone [44, 70, 71]. Furthermore, ORNG is directly applicable to biological

samples such as brain in aqueous solution, which is easily automated and scalable. The three major classes of glycans released by ORNG can be tagged easily and specifically using fluorescent tags that are either commercially available or can be easily prepared. Therefore, we succeeded in developing ORNG in a controlled oxidative degradation for rapid and large-scale production of glycans from animal glycoconjugates in forms that can be directly purified, derivatized, and functionally explored, as in glycan microarrays. Due to the chemical nature of NaClO, glycans with certain functional groups such as primary amines, e.g. as heparin/heparan sulfate, and sulfhydryl or C=C bonds might be partly degraded by this approach.

The sensitivity of glycoconjugates to oxidative degradation may have biological implications beyond the technical discoveries described here. Since hypohalous acids are known to be important natural oxidants with bioactivities [72, 73], the generation of N-, O-, and GSL-glycans from natural glycoconjugates raises the possibility of glycans as secondary metabolites, a process that might occur naturally. Studies have shown that free glycans occur in body fluids, such as N-glycans with a cleaved chitobiosyl core [74], as we observed upon prolonged NaClO treatment. Although our studies here deal with *in vitro* analysis and preparation of these glycans, our findings warrant further investigation of potential *in vivo* glycans that may be generated through a similar oxidative degradation pathway.

Methods

Materials

All chemicals and HPLC solvents were purchased from Sigma-Aldrich, Acros, Oakwood chemicals, and Fisher Scientific. Potentially, any commercial source of NaClO, including local markets, may be used, but should be compared to NaClO from a chemical supplier for validation. Milli-Q water was used to prepare all aqueous solutions. Sodium hypochlorite solutions are from Clorox (6.15% NaClO), Pure Bright (6% NaClO), Up & up (8.25% NaClO) or Sigma-Aldrich (5% chlorine) and prepared freshly by addition of water. Bleach stored for more than 6 months under room temperature as 6% NaClO has been used successfully. Pd/C: 10%Palladium on C (Sigma-Aldrich). C18 Sep-pak (Waters); Hypercarb cartridge (Thermo Scientific).

Mass spectrometry (MS)

A Bruker Daltonics Ultraflex-II MALDI-TOF/TOF system and an anchorchip target plate were used for MS analysis. Reflective positive mode was used for glycans before and after permethylation. 2,5-dihydroxybenzoic acid (DHB) (5 mg/mL in 50% acetonitrile with 0.1% trifluoroacetic acid) was used as matrix.

High performance liquid chromatography (HPLC) analyses

A Shimadzu HPLC CBM-20A system with UV detector SPD-20A and fluorescence detector RF-10Axl was used for HPLC analysis. UV absorption at 330 nm or fluorescence at 330 nm excitation (Ex) and 420 nm emission (Em) was used for detection of anthranilic acid (AA), 2-aminobenzamide (AB), and 2-amino-N-(2-aminoethyl)benzamide (AEAB) tags. For Fmoc-protected glycans, UV absorption at 330 nm or fluorescence at 330 nm excitation (Ex)

and 420 nm emission (Em) was also used. Agilent amino columns were used for normal phase HPLC. Both C18 and PGC columns were used for reverse phase HPLC separation. Detailed methods are described in individual experiments. The mobile phases were acetonitrile, water, and aqueous ammonium acetate buffer at pH 4.5. A linear gradient from 20 mM ammonium acetate in 80% acetonitrile to 200 mM ammonium acetate in 10% acetonitrile in either 25 or 50 minutes was used.

Thin layer chromatography (TLC) analyses

Glycoproteins or glycans in aqueous solutions (2 μL) were spotted on silica-gel TLC plates, dried, and developed using two different solvent systems as described in figure legends. After air drying, the plates were sprayed with 0.1% orcinol in 5% sulfuric acid. The plates were heated on a hot plate until a clear pattern appeared.

Permethylation and MS analysis

Permethylation of glycan samples was carried out according to reported procedures [75] to increase the sensitivity of MS analysis. Briefly, a lyophilized sample was treated with DMSO/NaOH slurry (100-200 μL) and methyl iodide (25-50 μL) for 10-30 minutes. The supernatant was then partitioned between water (500 μL) and chloroform (500 μL). The organic layer was washed with 500 μL water, dried, and redissolved in 50% methanol for MS analysis.

Sodium hypochlorite release of N-glycans for analysis

Method 1—Glycoprotein (50 μL, 10 mg/mL) was mixed with 50 μL saturated borax solution. 100 μL 1% NaClO was added and the mixture was shaken for 1 minute. Formic acid (10 μL) was added to quench the reaction. After briefly cooling on ice (2 minutes), the mixture was centrifuged at 10,000g for 2 minutes and the supernatant was transferred into a suspension of 5 mg 10% Palladium on C (Pd/C) in 200 μL water in a centrifuge filter with 0.2 μm Nylon membrane. For unknown reasons, Pd/C stabilized multisialylated glycans compared to normal activated carbon for absorption of glycans. After shaking for 5 minutes at room temperature, the mixture was filtered by centrifugation and the filtrate was discarded. The Pd/C powder was washed with 3 × 250 μL 1% formic acid. To the Pd/C powder, 100 μL 0.1% formic acid was added and the mixture shaken at 37°C for 1 hour and centrifuged to remove the filtrate. The Pd/C powder was washed with 250 μL 0.1% trifluoroacetic acid. Glycans were eluted with 50 μL acetonitrile/0.1% trifluoroacetic acid and analyzed by MALDI directly. The eluate was dried and permethylated for MALDI analysis. For human plasma samples, a 50 mg/mL protein concentration was assumed. Human plasma (10 μL) was mixed with 40 μL water and processed as described above.

Method 2—Animal tissue was treated with pestle and mortar. For x mg of tissue, $4x$ μL water and $5x$ μL saturated borax solution and $10x$ μL 1% NaClO were grinded together for 2 minutes under room temperature and quenched with $0.2x$ μL formic acid. The mixture was centrifuged to remove insoluble material and the supernatant was passed through a Sep-pak C18 cartridge (500 mg) and washed with 5 × 3 mL. The C18 cartridge was eluted with 3 mL 50% acetonitrile/0.1% trifluoroacetic acid. The elution was dried by SpeedVac, permethylated and analyzed by MALDI. The flow-through fraction and first 3 mL wash

fraction were collected and passed through a Hypercarb carbon SPE cartridge (50 mg). The carbon cartridge was further washed with 5 × 1 mL water. The C18 cartridge is eluted with 1 mL 50% acetonitrile/0.1% trifluoroacetic acid. The elution was dried by SpeedVac, permethylated and analyzed by MALDI.

Preparative Sodium hypochlorite treatment for the production of N-glycans

Method 3—Glycoproteins (1 – 10 g) were dissolved in water to 20 mg/mL. To this solution, 0.2 volume of 6% NaClO was added under stirring. After 15 minutes at room temperature, 0.01 volume of formic acid was added to the reaction mixture slowly and stirred for another 5 minutes, and centrifuged to remove insoluble material. The supernatant was dried on a rotary evaporator and the residue was suspended in water and centrifuged to remove insoluble material. The supernatant was desalted over a Sephadex G-25 column (1.6 × 60 cm), and the desalted solution was passed through a C18 Sep-Pak column (2-10 g resin). The flow through solution was dried and ready for AEAB conjugation as described previously [17].

For egg yolk, egg white, and other animal tissues, tissues were homogenized with ice cold water using a Waring blender so that the final protein concentration was ~20 mg/mL based on average protein content estimation. For example, 18 egg yolks (345 g) were mixed with 2,400 mL water in a mechanical stirrer. 6% NaClO (550 mL) was added and the mixture was stirred. NaClO was quickly consumed along with a quick drop of pH from 12 to 9 within 5 minutes. The mixture was stirred for 15 minutes under room temperature. Octanol (3 mL) and formic acid (30 mL) were added slowly and the mixture was stirred for 5 minutes. The mixture was centrifuged at 9,500g for 30 minutes. The supernatant was collected and dried on rotary evaporator. The residue was resuspended in 200 mL water, filtered and dialyzed in MWCO 1K tubes for 4 hours against running water. The dialysate was made to 1,100 mL by addition of water and pH was adjusted to 9 by addition of 50% sodium hydroxide solution. To this mixture, 46 mL 6% NaClO was added slowly over 10 minutes and the solution stirred for another 2 minutes. Formic acid (10 mL) was added and the mixture was again dried on rotary evaporator. The residue was dissolved in 100 mL water and filtered through 0.45 μm membrane. The filtrate was desalted with a Sephadex G25 column (5 × 100 cm). Fractions positive with phenol-sulfuric acid assay were collected and lyophilized to give 4.7 g crude glycans. For solid animal tissue/organ, a 20% protein concentration was used for calculation.

Sodium hypochlorite treatment for the production of O-glycans

Method 4—As an example, porcine stomach mucin (10 g dry weight) was dissolved/ suspended in 500 mL water. To this, 250 mL of 6% NaClO was added under stirring. After 30 minutes at room temperature, formic acid (7.5 mL) was added to the reaction mixture slowly. The mixture was stirred for another 5 minutes, and centrifuged to remove insoluble material. The supernatant was dried on a rotary evaporator and the residue was suspended in water and filtered through 0.45 μm membrane. The filtrate was made to 500 mL by addition of water and adjusted to pH 7.6 by addition of NaOH. To this mixture, 16.6 mL 6% NaClO was added and the mixture was stirred for 24 hours at room temperature. Formic acid (2mL) was added and the mixture was dried on rotary evaporator. The residue was dissolved in 100

mL water and desalted with a Sephadex G25 column (5 × 100cm). Fractions positive with phenol-sulfuric acid assay were collected and lyophilized to give 4.3 g crude glycans.

Sodium hypochlorite treatment for the production of GSL-glycans from porcine brain

Method 5—Porcine brain (220 g wet weight), which was obtained from a local farmer's market as frozen blocks, was diced into small cubes blended with 440 mL cold water to a homogeneous mixture. To this suspension, 1,320 mL of 6% NaClO was added under vigorous stirring. After 30 minutes, octanol (10 mL) and formic acid (30 mL) was added. The mixture was stirred briefly and stored at 4°C overnight. The mixture was centrifuged to remove the upper, fatty layer. The residual aqueous material was dried in a rotary evaporator. The residue was dissolved in 100 mL water and desalted on a Sephadex G25 column (5 × 100cm). Fractions positive with phenol-sulfuric acid assay were collected and lyophilized to give 2.5 g crude GSL-derived glycans.

Fluorescent tagging of O-glycans using MonoFmoc-ethylenediamine

O-glycan-glycolic/lactic acids were dissolved in 0.5 M MES buffer (pH 5.5) to 25 mg/mL. An equal volume of freshly prepared N-hydroxysuccinimide (NHS) (100 mg/mL in DMSO) and an equal volume of EDC (100 mg/mL in DMSO) solutions were added. The mixtures were stirred at room temperature for 15 minutes. An equal volume of MonoFmoc-ethylenediamine (50 mg/mL in DMSO) was added followed by sodium bicarbonate (100 mg/mL of total volume). The mixture was stirred for 1 hour and centrifuged. The supernatant was precipitated into 10 volumes of acetonitrile at −20°C for one hour. After centrifugation, the pellet was collected and redissolved in water for HPLC purification.

Fluorescent tagging of glycan-nitriles with Fmoc by Pd/C catalyzed reduction and Fmoc protection

Crude porcine brain ganglioside nitriles (1.6 g) prepared by ORNG were mixed with 10 g of ammonium formate, 100 mL water and 100 mL methanol. To this solution, 500 mg Pd/C was added and the mixture was stirred at room temperature for 48 hours. The mixture was filtered and the filtrate was dried on rotary evaporator. The residue was desalted on Sephadex G25 column and lyophilized to give 1.3 g crude gangliosides-amines.

The ganglioside-amines were dissolved in 4 mL saturated sodium bicarbonate and 16 mL DMSO. Then 2.6 g Fmoc-OSu was added and the mixture was mixed at 37°C. After 30 minutes, 400 mg sodium bicarbonate and 1.3 g Fmoc-OSu were added and the mixture was mixed for another 30 minutes at 37°C. The mixture was centrifuged and the supernatant was precipitated into 200 mL acetonitrile at 4°C overnight. The pellet was dried and redissolved in water for HPLC separation.

Glycan release from GSLs by N-bromosuccinimide (NBS)

In a typical procedure for underivatized GSLs, GSL was dissolved in 1 M phosphate buffer pH 7.2 to ~5 mg/mL, then NBS was added to a final concentration of 20 mg/mL(saturated). The mixture was briefly mixed and heated at 65°C for 4 hours. Sodium sulfite (equal weight to NBS) solution was added and the mixture was centrifuged. The supernatant was passed

through C18 Sep-Pak. The flow through fraction was desalted using Sephadex G-25 or Carbograph column.

Nickel chloride/sodium borohydride reduction of nitrile and 9-Fluorenylmethoxycarbonyl (Fmoc) protection

The Nickel chloride/sodium borohydride reduction was carried out on dialyzed glycan-nitriles according to reported procedure [76]. After reaction, sodium bicarbonate was added followed by Fmoc chloride dissolved in acetonitrile. The mixture was stirred at room temperature for 1 hour and filtered. The filtrate was evaporated to remove organic solvents and filtered again. The final filtrate was loaded onto a C18 Sep-Pak, washed with a large amount of water to remove salts, and eluted with 50% methanol.

Fluorescent tagging of glycan-nitriles with 2-aminobenzamide (2-AB) by Pd/C mediated N-alkylation

In a typical procedure, desalted glycan-nitriles released from GSLs were mixed with 2-AB (25 mM) and ammonium formate (0.5-1 M) in 9:1 (water: methanol). Then Palladium (10% on Carbon (Pd/C)) was added (1-2 mg/mL). The mixture was mixed by rotation at 50 rpm at room temperature for 4 hours and more decolorizing carbon was added to absorb the glycans. The mixture was filtered, washed, and glycans were eluted from carbon by 50% acetonitrile with 0.1% TFA.

Microarray printing, binding assay and scanning

Non-contact printing on NHS-activated slides was used. N-glycans were printed at 100 μM in 100 mM sodium phosphate (pH 8.5) in replicates of 4. Biotinylated lectins (Vector Labs) were assayed at 10 μg/mL except Con A, which was assayed at 1 μg/mL. Alexa 488-streptavidin (Invitrogen) 5 μg/mL was used for the detection of binding using a fluorescent scanner (Molecular Diagnostics). For O-glycans, before printing, the Fmoc-tagged O-glycans were incubated with a mixture of water/DMF/piperidine (40/40/20 v/v/v) for 10 minutes. The mixture was then dried in speed-vac and reconstituted in 100 mM sodium phosphate (pH 8.5) to 50 μM for microarray printing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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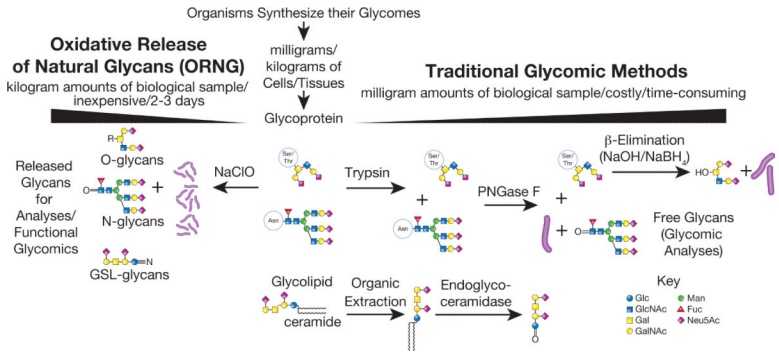

Figure 1.

The comparison of oxidative release of natural glycans by NaClO (ORNG) and the traditional glycomics methods. The traditional methods typically use milligram amounts or less of samples and can take days to weeks to complete, and require enzymes and organic solvents. The ORNG approach takes 1-3 days to complete and is useful with kilogram amounts of samples.

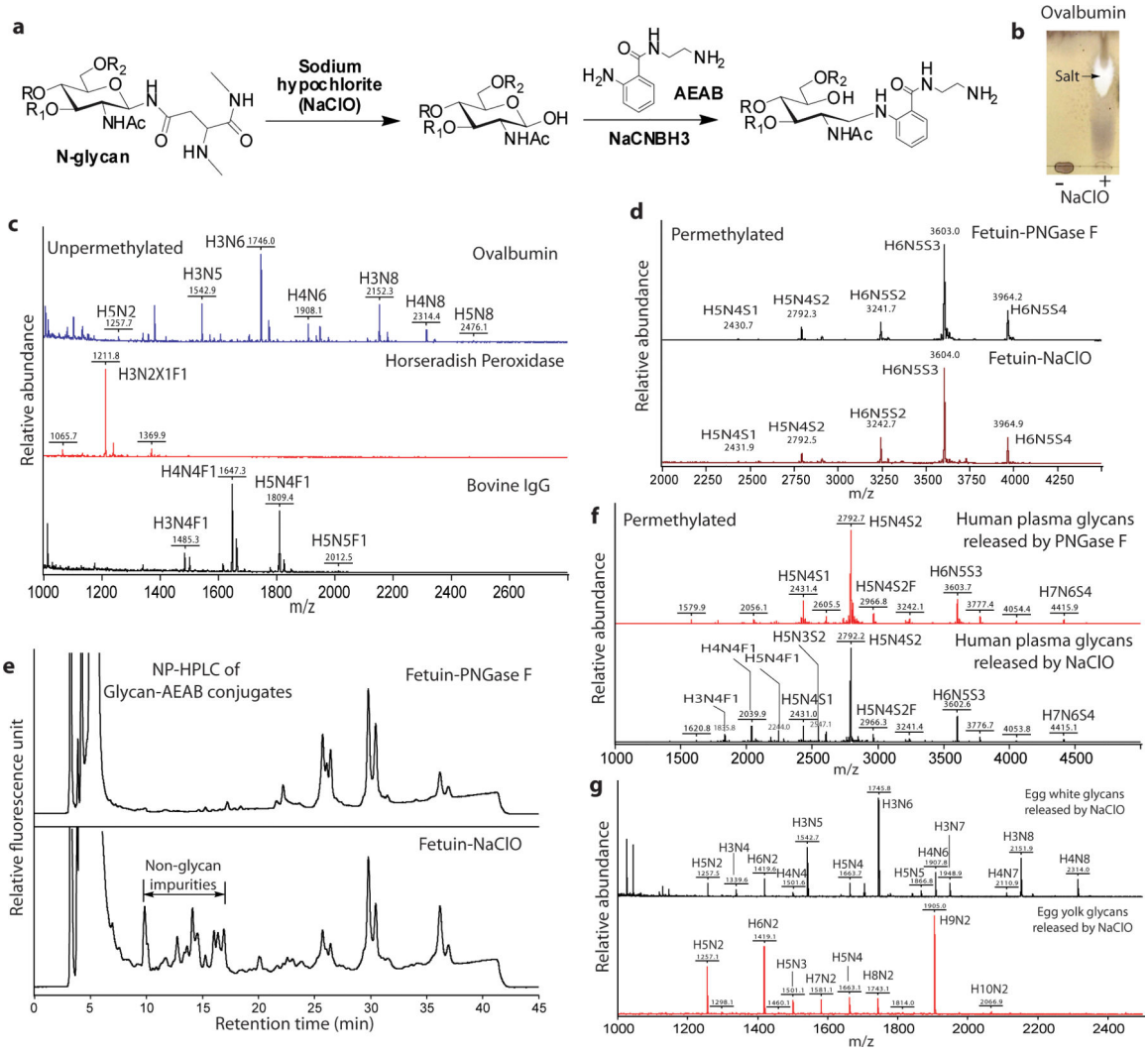

Figure 2.

Sodium hypochlorite treatment of glycoproteins to release N-glycans. a) Chemical scheme. b) TLC analysis of ovalbumin before and after bleach treatment. c) MALDI-MS profiles of N-glycans released from several glycoproteins by bleach treatment. d) MALDI-MS profiles of permethylated glycans released from fetuin by PNGase F digestion (top) or bleach treatment (bottom). e) Normal phase (NP) HPLC profiles of AEAB conjugated N-glycans released from fetuin by PNGase F digestion (top) or bleach treatment (bottom). Peaks at 10-17 min were nonglycan peaks presumably generated from oxidized peptide fragments. f) MALDI-MS profiles of human plasma permethylated N-glycans released by PNGase F digestion (top) and bleach (bottom). g) MALDI-MS profiles of N-glycans released by NaClO from egg white (top) and egg yolk (bottom). H: Hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid. The Y-axis scales are not calibrated to reflect quantitative comparison.

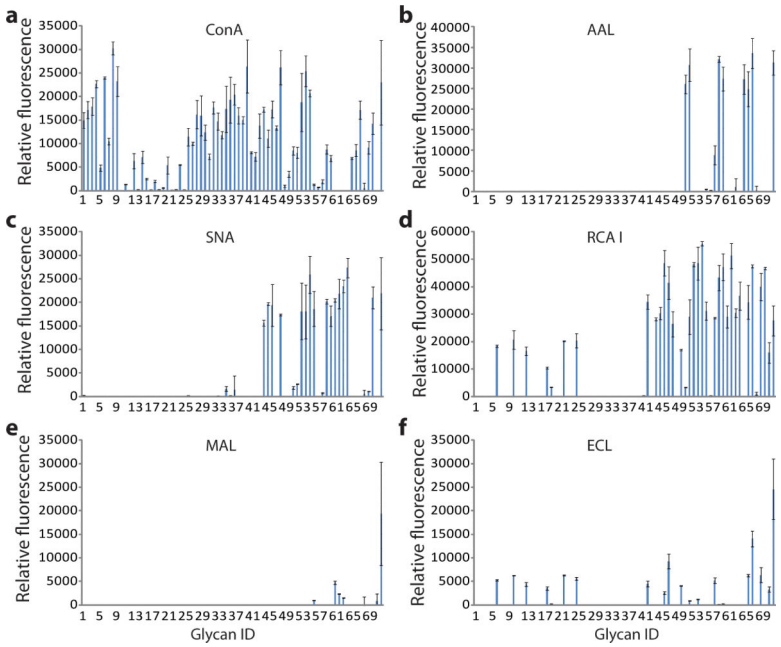

Figure 3.

Lectin binding on the microarray of N-glycan AEAB conjugates prepared from ovalbumin (1-25), egg yolk (26-60), fetal bovine serum (61-64) and bovine IgG (65-67). a) ConA b) AAL c) SNA d) RCA I e) MAL f) ECL.

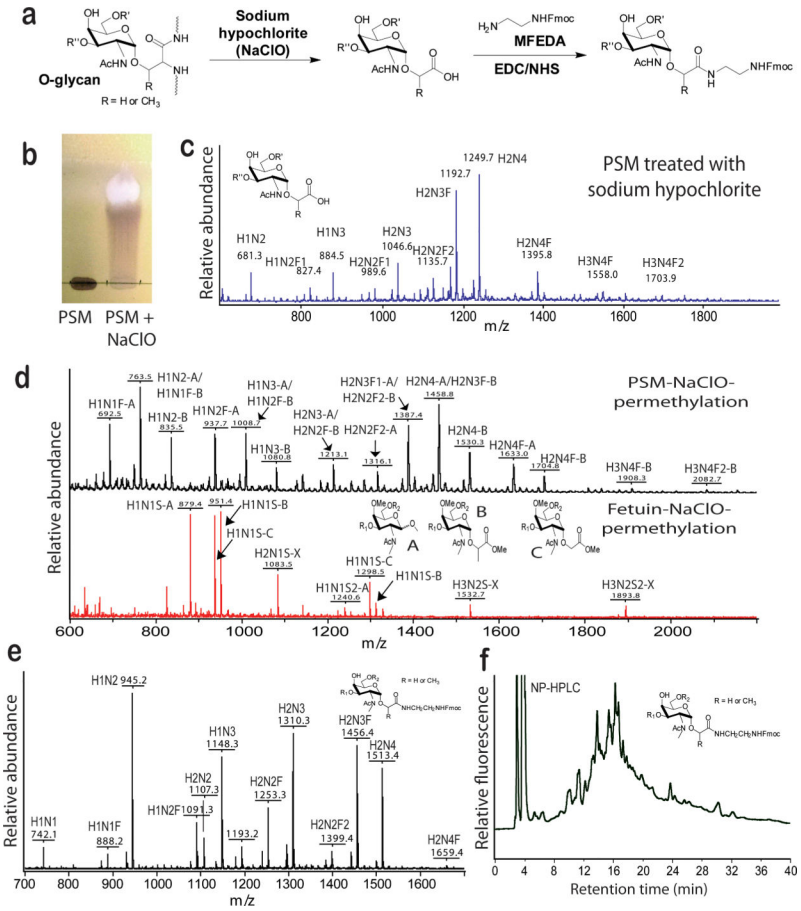

Figure 4.

Sodium hypochlorite treatment of glycoproteins to release O-glycans. a) Chemical scheme of O-glycan release and labeling. b) TLC analysis of porcine stomach mucin before and after NaClO treatment. c) MALDI-MS profile of O-glycans released from porcine stomach mucin. d) MALDI-MS profile of glycans from porcine stomach mucin and fetuin after NaClO treatment and permethylation. e) MALDI-MS profile and f) HPLC profile of O-glycans released from porcine stomach mucin by NaClO and labeled with monoFmoc-ethylenediamine. Fluorescence: Ex 254 xnm/Em 340 nm.

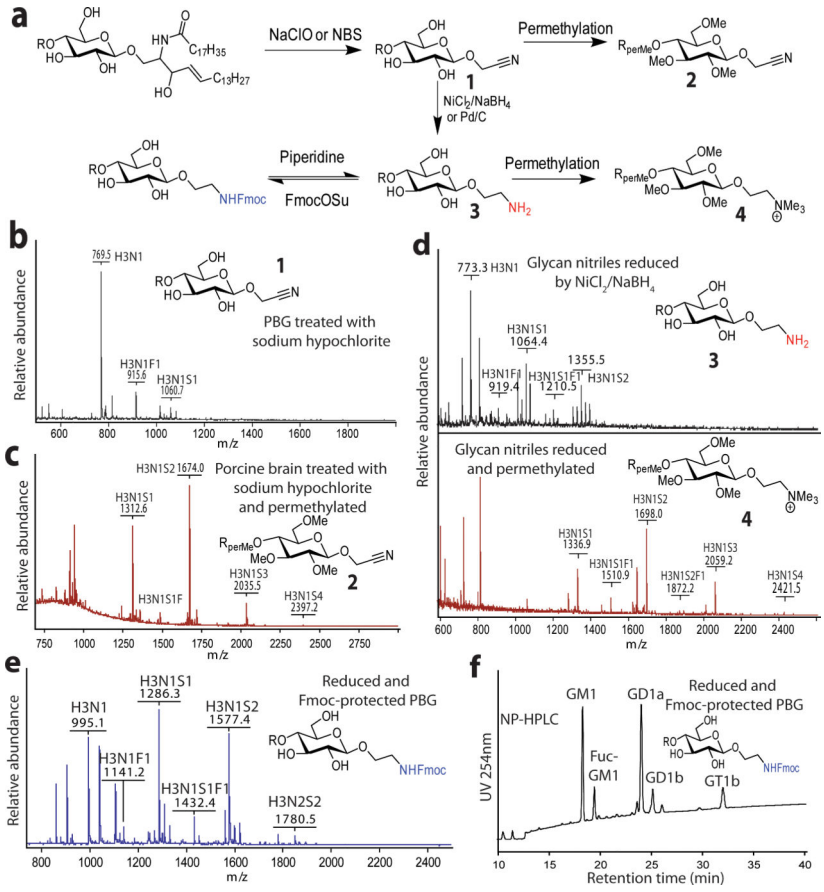

Figure 5.

Release and tagging of glycans from glycosphingolipids (GSL) by NaClO. a) Chemical scheme of GSL release and labeling. b) MALDI-TOF profile of porcine brain gangliosides after NaClO treatment. c) MALDI-TOF profile of porcine brain tissue after NaClO treatment and permethylation. d) MALDI-TOF analysis of reduction products of gangliosides -nitriles before permethylation (top panel) and after permethylation (bottom panel). e) MALDI-TOF profile of the Fmoc protected glycan amines from gangliosides. f) HPLC profile of the Fmoc protected glycan amines from porcine brain gangliosides.