

Resin- and magnetic nanoparticle-based free radical probes for glycan capture, isolation, and structural characterization

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ABSTRACT: By combining the merits of solid supports and free radical activated glycan sequencing (FRAGS) reagents, we develop a multi-functional solid-supported free radical probe (SS-FRAGS), which enables glycan enrichment and characterization. SS-FRAGS comprises a solid support, free radical precursor, disulfide bond, pyridyl, and hydrazine moieties. Thio-activated resin and magnetic nanoparticles (MNPs) are chosen as the solid support to selectively capture free glycans via the hydrazine moiety, allowing for their enrichment and isolation. The disulfide bond acts as a temporary covalent linkage between the solid support and the captured glycan, allowing the release of glycans via the cleavage of the disulfide bond by dithiothreitol. The basic pyridyl functional group provides a site for the formation of a fixed charge, enabling detection by mass spectrometry and avoiding glycan rearrangement during collisional activation. The free radical precursor generates a nascent free radical upon collisional activation and thus simultaneously induces systematic and predictable fragmentation for glycan structure elucidation. A radical-driven glycan deconstruction diagram (R-DECON) is developed to visually summarize the MS² results and thus allow for the assembly of the glycan skeleton, making the differentiation of isobaric glycan isomers unambiguous. For application to a real-world sample, we demonstrate the efficacy of the SS-FRAGS by analyzing glycan structures enzymatically cleaved from RNase-B.

INTRODUCTION

Glycosylation is one of the most important forms of protein post-translational modification, and it plays a vital role in biology. The analysis of glycans by mass spectrometry has been hampered by the trace amounts of glycan sample available from low-abundant glycoconjugates in biological sources. Alterations of glycan structures have been found in various types of diseases, such as cancers, diabetes, and immune disorders.¹⁻² Therefore, profiling disease-associated glycans is essential for the understanding of their functions at a molecular level, while also facilitating the identification of diagnostic glycan biomarkers and the better design of therapeutic drugs. Mass spectrometry (MS) has proven to be the most powerful and informative tool to elucidate glycan structures and inform their roles in biological processes. Prior to mass spectrometric analysis, glycan samples are prepared mainly in three steps: 1) glycoprotein isolation and/or digestion; 2) glycan release from glycoproteins; 3) glycan enrichment from the mixture, which consists of proteins, peptides, enzymes, and other chemicals involved in sample preparation. It is challenging to directly analyze glycans with even traces of these other components by MS since some species, such as proteins and peptides, are often much more readily ionized, and thus greatly suppress glycan ionization and detection. As a result, efficient glycan enrichment and separation from complex biological mixtures is crucial for mass spectrometric glycan characterization. Physical interaction based approaches, including lectin affinity chromatography,³⁻⁶ hydrophilic interaction liquid chromatography (HILIC),⁷⁻¹⁴ and graphite affinity chromatography^{7, 10, 15-16} have been widely utilized to enrich glycans from complex biological samples. Unfortunately, nonspecific binding and low enrichment efficiency often mitigate their effective application. Solid-phase chemical immobilization methods have emerged as promising alternatives for glycan enrichment.¹⁷⁻²⁵ After either enzymatic release or chemical release by β -elimination from glycoproteins, glycans have a single reducing terminus, which has both cyclic and open forms. The reducing terminus can covalently conjugate with hydrazide, amine, and oxyamine functionalities through its unique aldehyde functional group in its open form.^{17, 19, 26}

Mass spectrometric glycan structure elucidation is extremely challenging. Glycans can exhibit incredibly complicated branched structures with a large number of residues having both structural and stereochemical diversity. It has been stated that “small changes in environmental cues can cause dramatic changes in glycans produced by a given cell”.²⁷ Although significant advances have been made, glycomics remains much less developed than its siblings, genomics and

proteomics. Noted for its capability to perform multi-stage tandem mass spectrometry, minimal sample consumption, and high sensitivity, mass spectrometry has been broadly used for the elucidation of glycan structures. Many dissociation techniques, such as collision-induced dissociation (CID),²⁸⁻³⁰ infrared multiphoton dissociation (IRMPD),^{29, 31} higher-energy collisional dissociation (HCD),^{23, 32} ultraviolet multiphoton dissociation,³³⁻³⁵ electron capture dissociation (ECD),^{29, 36-38} electron transfer dissociation (ETD),³⁹⁻⁴⁰ electron detachment dissociation (EDD),^{29, 39, 41-43} and electron excitation dissociation (EED),⁴³⁻⁴⁵ have been demonstrated to provide complementary and extensive information for glycan structural analysis. Among these dissociation techniques, ECD, ETD, EDD, and EED are generally referred to as electron activated dissociation (ExD) since they usually involve fragmentation via low energy free radical dissociation pathways. ExD provides enhanced yields of cross ring fragmentations. However, such approaches require special instrumentation to allow for interaction of an electron source with targeted ions. Recently, free radical chemistry⁴⁶ has regained great attention in the field of biomolecular characterization.⁴⁷⁻

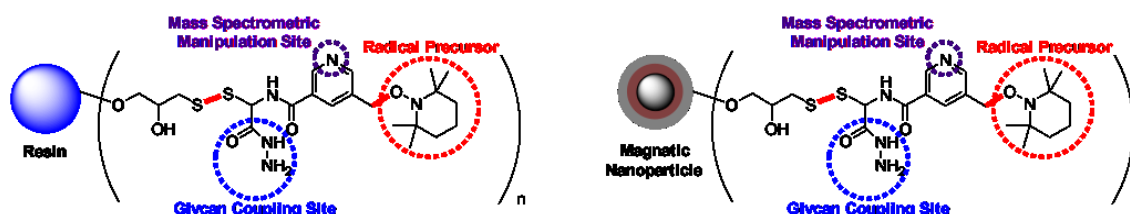
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Inspired by the achievement of electron activated dissociation (ExD) for glycan structure analysis, which usually involve fragmentation via low energy free radical dissociation pathways, we recently developed a novel free radical activation glycan sequencing reagent (FRAGS), along with a methylated free radical activated glycan sequencing reagent (Me-FRAGS) for glycan structural characterization.^{26, 52} Free radical-directed systematic glycan dissociation with high fragmentation efficiency has been obtained using these two free radical reagents. Moreover, the use of these free radical glycan sequencing reagents does not require multiply charged precursor ions. Furthermore, our reagents can be employed with a wide variety of instrumentation, with the main requirement being the availability of collisional activation of mass selected ions to achieve fragmentation. However, use of these two reagents is hampered by the low sensitivity for glycan detection when applied to glycans released enzymatically from glycoproteins, subsequently coupled with the FRAGS or Me-FRAGS reagent, and then directly analyzed by mass spectrometry without any enrichment or purification. Proteins and peptides, which are the major matrix, significantly suppress the ionization of glycans.

For solid supports, we employ thio-activated resin beads and magnetic nanoparticles (MNPs). Resin beads and magnetic nanoparticles (MNPs) have been broadly used as solid-supports and

receive significant attention for their potential biomedical applications, such as sample purification and enrichment.^{25, 53-57} Thiol-activated resin (Thiopropyl SepharoseTM 6B) is a medium for reversible immobilization of molecules containing thiol groups under mild conditions via the formation of a disulfide bond. For instance, it has been commonly used to enrich thiol-containing compounds including thiolated proteins, thiolated peptides, thiolated RNA, thiophosphates, and aliphatic thiols. More recently, attempts have been made to exploit its reactivity with thiols to generate resin-supported probes for the analysis of biomolecules.²⁵ However, the relatively low surface-to-volume ratio and large particle size (100 μm) of resin hinders its further application in the biomedical field, especially for in vivo diagnosis and therapy. Magnetic nanoparticles (MNPs) have attracted much attention due to their high magnetic susceptibility, biocompatibility, and stability, along with other important relevant characteristics. Their unique physical properties make MNPs the ideal medium for in vivo biomedical applications, such as drug delivery, hyperthermia treatment, and magnetic resonance imaging (MRI) of cancer cells.⁵⁸ Considering the potential biomedical application of SS-FRAGS, we designed and prepared both resin- and MNPs-supported free radical probes.

Overall, glycan structural intricacy, low abundance, and masked detection are the main barriers to direct application of our sequencing reagents to real world analyses. To address these issues, we have developed a multi-functional solid-supported free radical probe (SS-FRAGS, Scheme 1). The probe comprises of a solid support, disulfide bond, free radical precursor, pyridyl, and hydrazine moieties. SS-FRAGS selectively captures free glycans, allowing for their enrichment and purification. The disulfide bond acts as a temporary covalent linkage between the solid support and the free radical reagent, allowing the release of glycans via the cleavage of this bond after enrichment and purification. The free radical precursor generates a nascent free radical upon collisional activation, which subsequently initiates low energy free radical dissociation pathways, fragmenting the glycan. The pyridyl functional group provides a fixed charge, allowing glycan structure determination from analysis of the systematic fragmentation of the glycan back towards the reducing terminus. The hydrazine functional group selectively targets bioconjugation with the reducing terminus of the glycan, serving as a coupling site between the reagent and the glycan.



Scheme 1. Structures of solid-supported free radical probes.

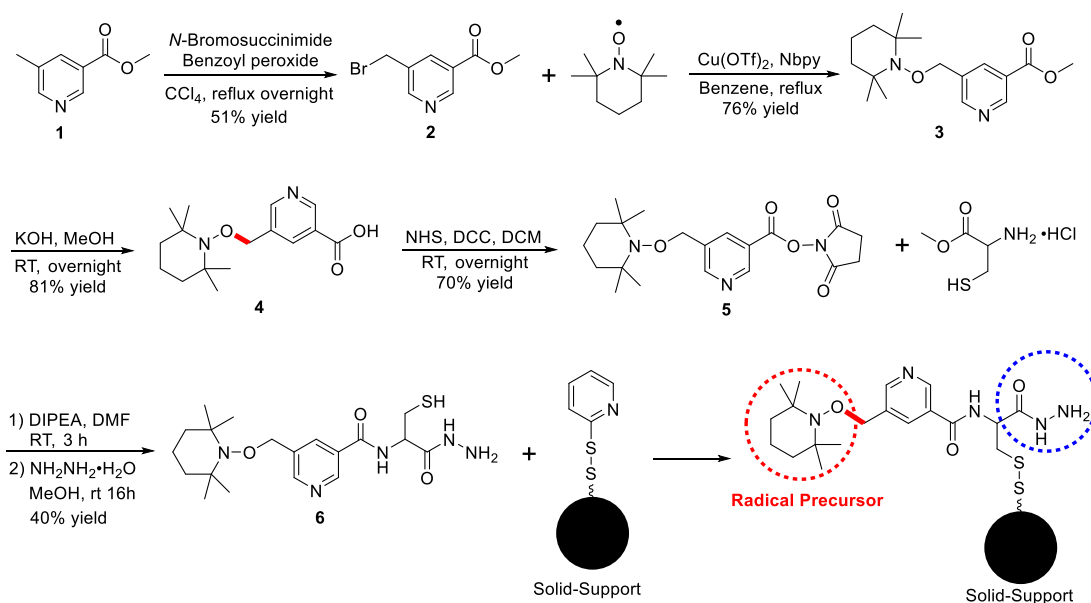
EXPERIMENTAL SECTION

Glycans

Lacto-*N*-difucohexaose I (LNDFH I), lacto-*N*-difucohexaose II (LNDFH II), and Ribonuclease B (RNase B) from bovine pancreas were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron(III) chloride hexahydrate and iron(II) chloride tetrahydrate were purchased from Alfa Aesar (Tewksbury, MA, USA). All solvents are HPLC grade and were purchased from EMD Merck (Gibbstown, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Solid-Supported Free Radical Probes

The preparation of the solid-supported free radical probe (SS-FRAGS) is described with details in the supporting information (Scheme 2). Briefly, the synthesis of SS-FRAGS is accomplished by benzylic bromination with NBS, coupling with NBS, hydrolysis of the ester group, condensation between the carboxylic acid and cysteine, hydrazinolysis of the imide group, and finally disulfide bond formation between the resin coupling reagent and thiol activated resin.^{26, 52} The preparation of magnetic nanoparticle (Scheme S1) was achieved by following previously reported procedures.⁵⁹ Electron microscopy reveals the average size of the magnetic nanoparticles as 10 nm (Figure S1).



Scheme 2. Preparation of the solid-supported free radical probe (SS-FRAGS).

Mass Spectrometry

A Thermo-Fisher Scientific linear quadrupole ion trap (LTQ-XL) mass spectrometer (Thermo, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source was employed. Derivatized glycan sample solutions were directly infused into the ESI source of the mass spectrometer via a syringe pump at a flow rate of 5-10 $\mu\text{L}/\text{min}$. Critical parameters of the mass spectrometer include spray voltage of 5~6 kV, capillary voltage of 30~40 V, capillary temperature of 275 $^{\circ}\text{C}$, sheath gas (N_2) flow rate of 10 (arbitrary unit), and tube lens voltage of 80~200 V. Other ion optic parameters were optimized by the auto-tune function in the LTQ-XL tune program for maximizing the signal intensity. Collision-induced dissociation (CID) was performed by resonance excitation of the selected ions for 30 milliseconds. The normalized CID energy was 10~45 (arbitrary unit).

RESULTS AND DISCUSSION

All product ions are classified according to the Domon and Costello nomenclature.⁶⁰ Greek letters α and β are employed to differentiate a branched glycan wherein α indicates the heavier branch while β indicates the lighter branch.

Glycan Characterization

To test the capability of both resin- and MNPs-supported free radical probes for glycan structure elucidation, maltopentaose and one pair of isobaric glycan isomers, lacto-*N*-difucohexaose I (LNDFH I) and lacto-*N*-difucohexaose II (LNDFH II), were selected. Maltopentaose presents a

linear chain of five identical glucose residues. LNDFH I and II are branched difucosylated hexasaccharides differing only in the location of the terminal fucose residue.

Maltopentaose: The general procedure for glycan purification and enrichment is illustrated in Figure 1. Briefly, glycans are selectively captured through covalent conjugation to the SS-FRAGS via the reduction reaction between the unique glycan reducing terminus and the probe hydrazide moiety (glycan coupling site of the probe, Figure 1). To achieve glycan purification and enrichment, the impurities and/or excess reactants are thoroughly washed away by water and acetonitrile. After enrichment, the conjugated glycans are released by the selective cleavage of the disulfide bond using the chemical scissors, dithiothreitol (DTT). Then, the conjugated glycans are methylated by reacting with iodomethane at the pyridine nitrogen site to avoid glycan rearrangement during collisional activation, ionized by electrospray ionization (ESI), and subjected to collision-induced dissociation (CID) in the ion trap.

As expected, collisional activation generates systematic and predictable glycan dissociation (Z, Y, and ^{1,5}X) with the charge retained on the reducing terminus, which significantly decreases the complexity of the CID spectra (Figure 2). The nascent free radical is generated by the loss of TEMPO at the radical precursor site. Without the need for subsequent collisional activation, all ions resulting from glycan fragmentation are generated via a free radical initiated mechanism. The formation of these three types of ions are proposed to result from hydrogen abstraction, initiated by the nascent free radical, followed by a series of β -eliminations.^{26, 52} The free radical activated glycan dissociation is systematic and predictable due to the narrow range of the C–H bond dissociation enthalpies (BDEs) of the glycan.^{26, 52}

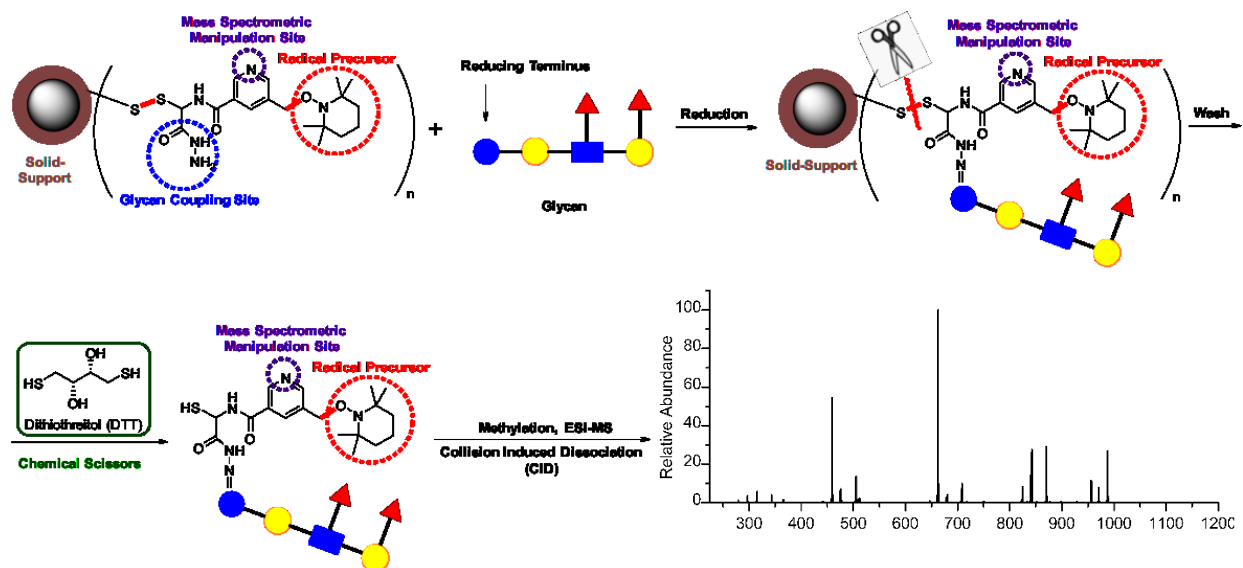


Figure 1. Schematic diagram of glycan capture, release, and MS analysis.

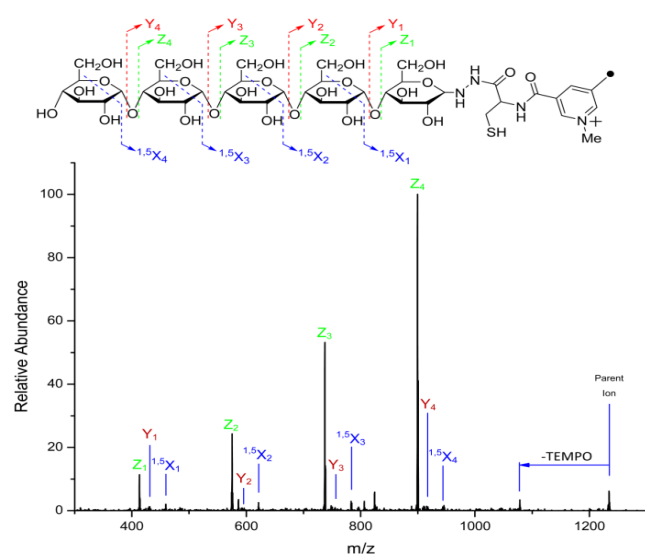
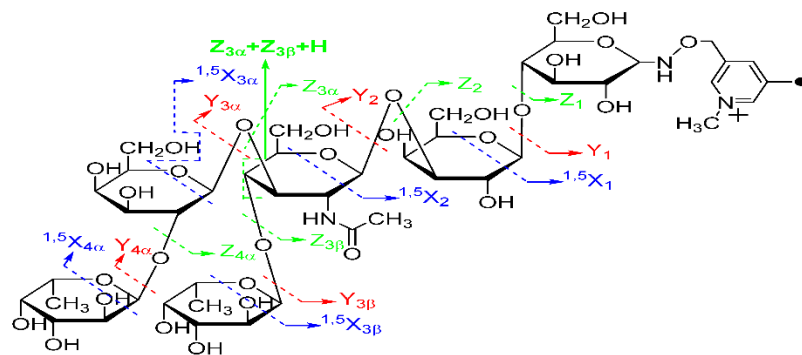


Figure 2. The fragmentation patterns and MS² CID spectrum of SS-FRAGS-derivatized maltopentaose.

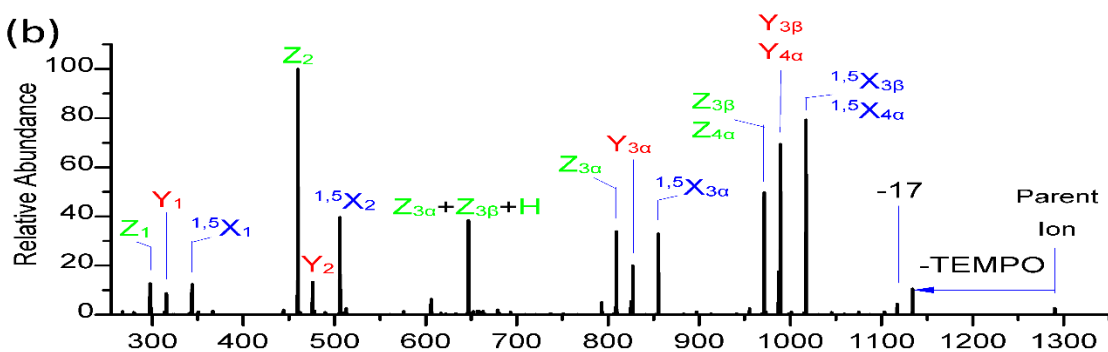
LNDFH I and II: LNDFH I and II were employed as highly branched isobaric glycans to assess the capability of SS-FRAGS to analyze more complicated glycan structures and differentiate glycan isomers. Similarly, systematic and predictable radical-directed glycan fragment ions are generated upon collisional activation, including Z, Y, ^{1,5}X, and Z_α+Z_β ions retaining the charge on the reducing terminus. More importantly, the unique fragmentation pattern Z_α+Z_β (Z_{3α}+Z_{3β} for LNDFH I and Z_{1α}+Z_{1β} and Z_{3αα}+Z_{3αβ} for LNDFH II, Figure 3) is observed only at the branch site, providing the information to confirm the presence and location of the branch structure. It is

essential to note that the two glycosidic linkages need to be adjacent to each other to observe the unique $Z_{\alpha}+Z_{\beta}$ ion. The glycan LNDFH I has β 1-3 and α 1-4 linkages on the branch site while LNDFH II has α 1-3 and β 1-4 linkages on the first branch site and β 1-3 and α 1-4 linkages on the second branch site. The determination of branch sites with two glycosidic linkages which are not adjacent to each other will be discussed in the case of glycans released from RNase B (*vide infra*). The mechanism for the formation of this unique ion has been proposed to be hydrogen abstraction followed by β -elimination.²⁶ Meanwhile, the radical-driven glycan deconstruction diagram (R-DECON diagram, Figure 4) visually summarizes the MS² results and thus allows for the assembly of the glycan skeleton, making the differentiation of these two isobaric glycan isomers unambiguous.

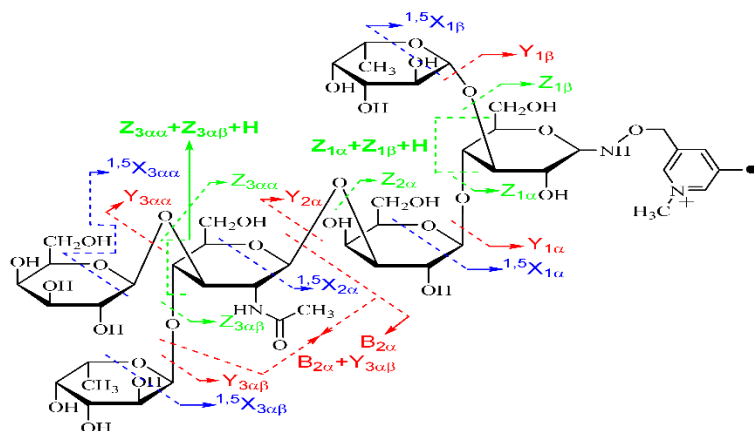
(a)



(b)



(c)



(d)

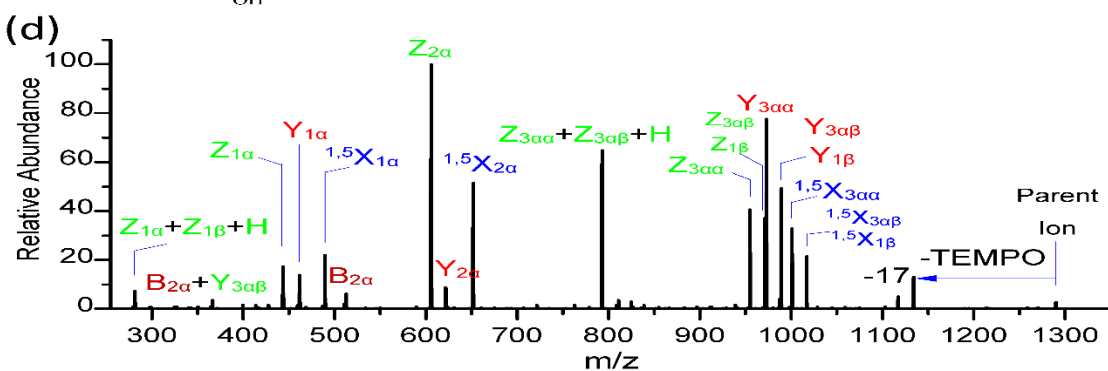


Figure 3. The fragmentation patterns observed following MS² CID of MNPs-FRAGS-derivatized LNDFH I (a), LNDFH II (c), and the CID spectra of MNPs-FRP-derivatized LNDFH I (b), LNDFH II (d).

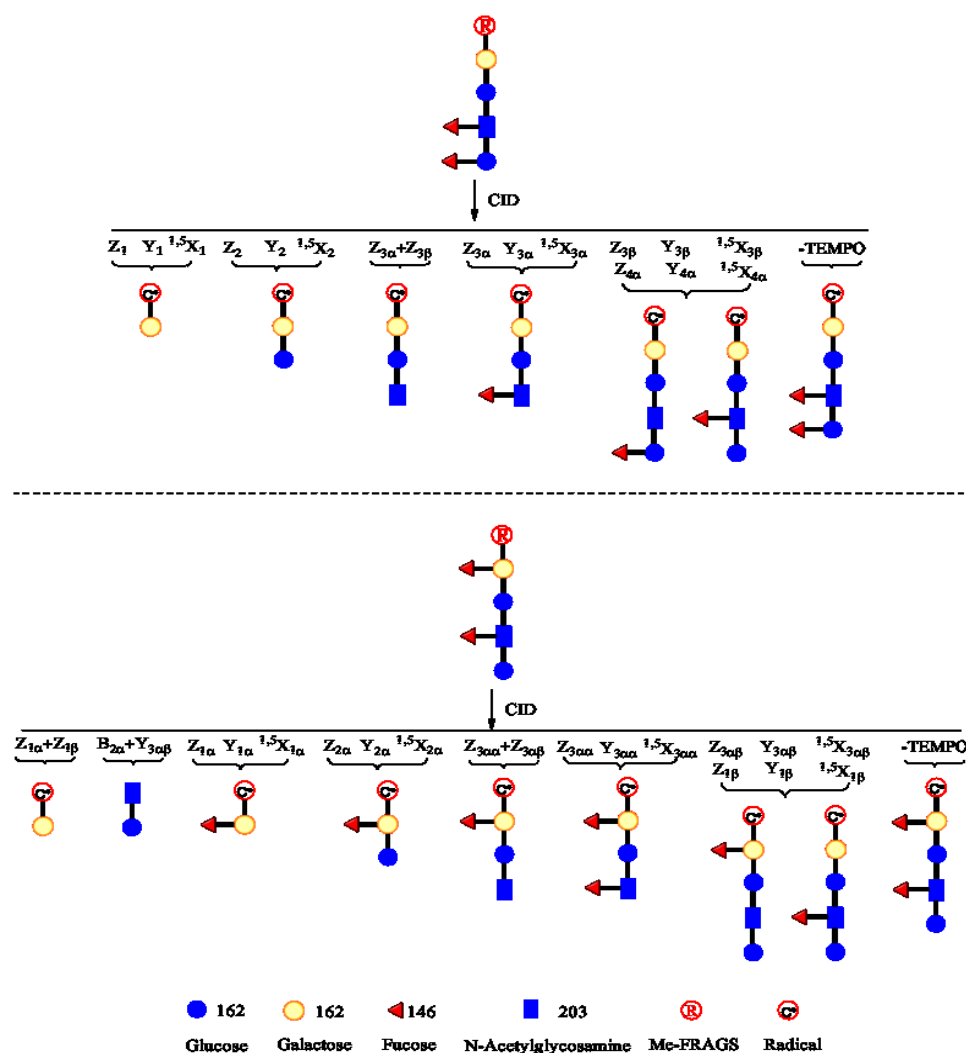


Figure 4. Radical-driven glycan deconstruction (R-DECON) diagrams for LNDFH I and II. In each case the precursor ion is subjected to MS² to generate a series of product ions, allowing the assembly of the glycan skeleton and differentiation of these two isobaric glycan isomers.

Glycan Enrichment

To test the capability of SS-FRAGS for the enrichment of glycans from biological samples, analysis of glycans released from Ribonuclease B (RNase B) from bovine pancreas was performed. Bovine pancreatic RNase B is a glycoprotein that contains a single glycosylation site at Asn³⁴. Due to the heterogeneity in the glycosylation at Asn³⁴, RNase B has five glycosylated variants, with an average molecular weight of approximately 15 kDa. The general procedure for glycan purification and enrichment is illustrated in Figure S2. The RNase B (1 mg) was denatured at 90 °C for one hour. Then the glycans were enzymatically released from RNase B by PNGase F followed by the enrichment protocol described in Figure 1. Briefly, without any pre-purification, the SS-FRAGS

reagent selectively captures the glycans to the probe via the reductive coupling reaction. Glycan enrichment is achieved by simply washing all the impurities away with water and acetonitrile. The pyridyl functional group of the probe is then reacted with iodomethane to form a fixed positive charge. Finally, the disulfide bond is cleaved by dithiothreitol (DTT) to release the derivatized glycans for mass spectrometric analysis.

By utilizing SS-FRAGS and following the procedure described in the protocol above, all the impurities including proteins, peptides, salt, and detergent were easily washed away by water and acetonitrile, allowing the purification and enrichment of glycans. As shown in Figure 5, abundant ions were detected, enabling subsequent collision induced dissociation for further structure characterization of glycans released from RNase B, which is further discussed below. FRAGS (b, Figure 5), which were proved to provide systematic and predictable cleavages for glycan structure elucidation, were used as a point of comparison to prove the capability of solid-supported free radical probe to enrich glycans. No signal was observed for the parallel control test (b, Figure 5). To further compare SS-FRAGS with SPE, we run the SPE purification after derivatization of FRAGS. It is clear that SS-FRAGS obtains better glycan purification than SPE.

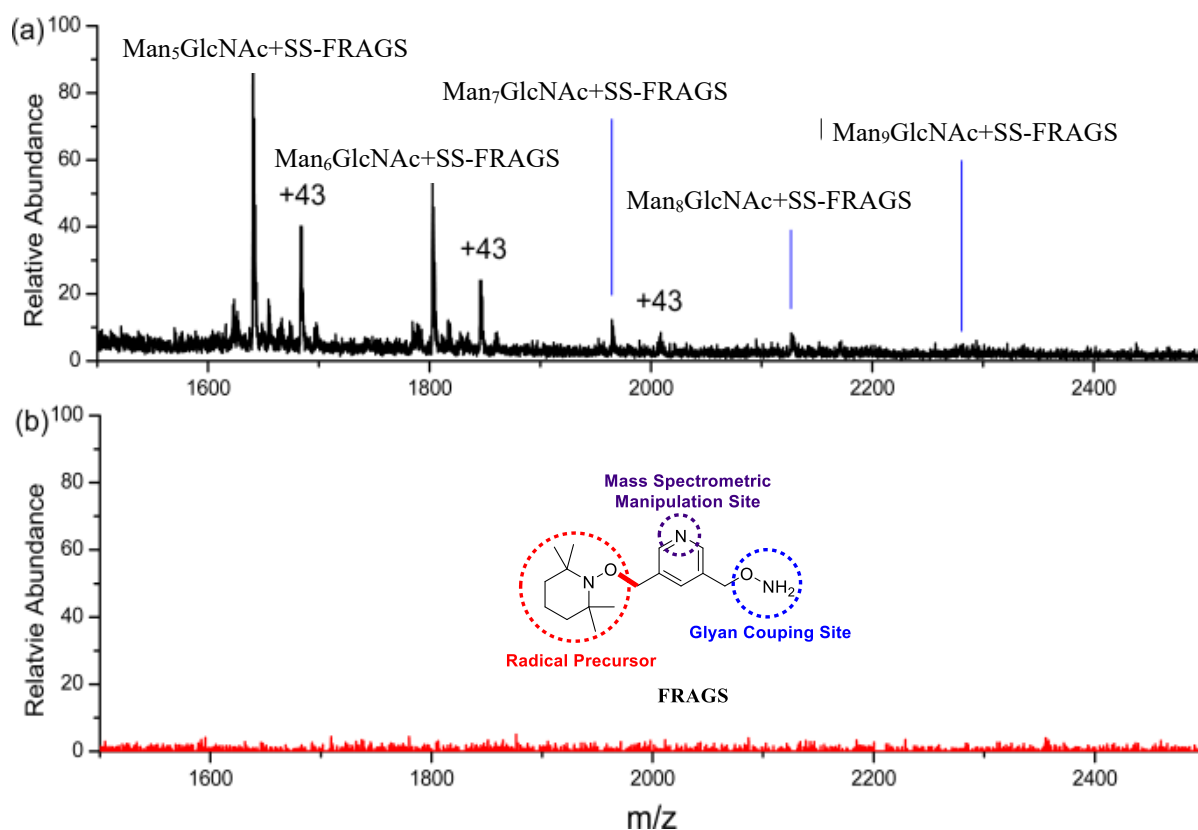


Figure 5. Enrichment of glycans released from RNase B by SS-FRAGS (a), and control test by using FRAGS (b).

Abundant mass spectrometric signals were observed for target glycans which were released from RNase B. It has been reported that the high-mannose structures released from RNase B exist as a mixture of isomers.^{61, 62} For instance, three permethylated Man₇GlcNAc₂ isomers have been reported by using porous graphite LC/MS (quadrupole orthogonal time-of-flight mass spectrometer).⁶¹ Moreover, ion trap mass spectrometry has been reported to define RNase B glycan topology and isomers by coupling precursor isolation with sequential MSⁿ disassembly.⁶² Generally, prior separation of the glycans before structural analysis is certainly desirable, since isobaric isomers can often be resolved and characterized individually. We tried to use HPLC to separate derivatized glycans and found that the two isomers from the coupling of the reagent to the reducing end of the glycan, adding complexity to the use of HPLC by increasing the number of peaks. This can be rationalized by considering the α and β forms of the reducing terminus. Reduction after amination was also tried to avoid the α and β forms but cannot be achieved. Without the prior HPLC separation, further collisional activation was still conducted to induce fragmentation for structural elucidation of the most abundant isomers of glycans released from

RNase B, as illustrated below. The free radical probes developed here provide more structural information to further elucidate the structures of the most abundant isomer of glycans released from RNase B. Figure 6 shows the CID mass spectra of free radical probe derivatized glycans released from RNase B. As expected, only systematic Z, Y, and 1,5 X cleavages were generated due to the presence of the well-defined site of radical generation. The R-DECON diagram makes possible the straightforward visualization of the assembly of the glycan skeleton (Figure 7). For instance, the proposed structure of the most abundant isomer of $\text{Man}_5\text{GlcNAc}_2$ can be confirmed by the loss of one mannose, three mannose, and five mannose residues, as shown in Figure 6. We have reported the unique ion, $Z_\alpha + Z_\beta$, for the adjacent branch site of glycans, such as LNDFH I and II.^{26, 52} The disappearance of the $Z_\alpha + Z_\beta$ ions indicates the two glycosidic linkages in the branch site are distal to each other. However, the branch sites can still be determined by analyzing the fragmentation patterns since each fragment ion is a product of single cleavages (Z, Y, and 1,5 X) induced by the nascent free radical. The loss of five mannose residues indicates the cleavage of the glycosidic bond between the mannose and *N*-acetylglucosamine (GlcNAc). The losses of one and three mannose subunits denote that one mannose residue is on one side and three mannose residues are on the other side of the first branch site. Moreover, the first branch site can also be confirmed by the absence of the loss of four mannose residues. The loss of four mannose residues would indicate the presence of four connected mannose residues which can be released by a single glycosidic bond cleavage, such as the four mannose residues enclosed in the red box in the proposed structure of $\text{Man}_5\text{GlcNAc}_2$ (**a** and **b** in Figure 8). Meanwhile, the second branch site is determined by the absence of the loss of two mannose residues. The loss of two mannose residues would indicate the presence of two mannose residues which can be released by a single glycosidic bond cleavage, such as the two mannose residues enclosed in the blue box in the proposed structure of $\text{Man}_5\text{GlcNAc}_2$ (**a**, **b**, and **c** in Figure 8). As expected, MS² CID generates one mannose, two mannose, three mannose, and six mannose losses for the most abundant isomers of $\text{Man}_6\text{GlcNAc}_2$. The presence of two and three mannose residue losses provides information to decipher the composition of the first branch site: two mannose residues on one side and three on the other side. Again, the first branch site configuration can be confirmed by the absence of the loss of four or five mannose residues. Two structures have been proposed for $\text{Man}_6\text{GlcNAc}_2$. Unfortunately, the existence of the second branch site cannot be confirmed due to the fact that these two proposed glycans can have the same radical directed fragmentation patterns. Similarly, two structures have

been proposed for the most abundant isomers of $\text{Man}_7\text{GlcNAc}_2$. Three $\text{Man}_7\text{GlcNAc}_2$ isomers has been reported by Costello et. al. by coupling HPLC separation with Q-TOF mass spectrometry.⁶¹ Therefore, the proposed structure on the left in Figure 6 is the structure of the most abundant isomer of $\text{Man}_7\text{GlcNAc}_2$ while the right structure is not the correct. The masses of the radical probe derivatized glycans $\text{Man}_8\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$ exceeded the nominal mass range of the instrument. They were detected but low signal intensity did not provide useful MS^2 results for these products.

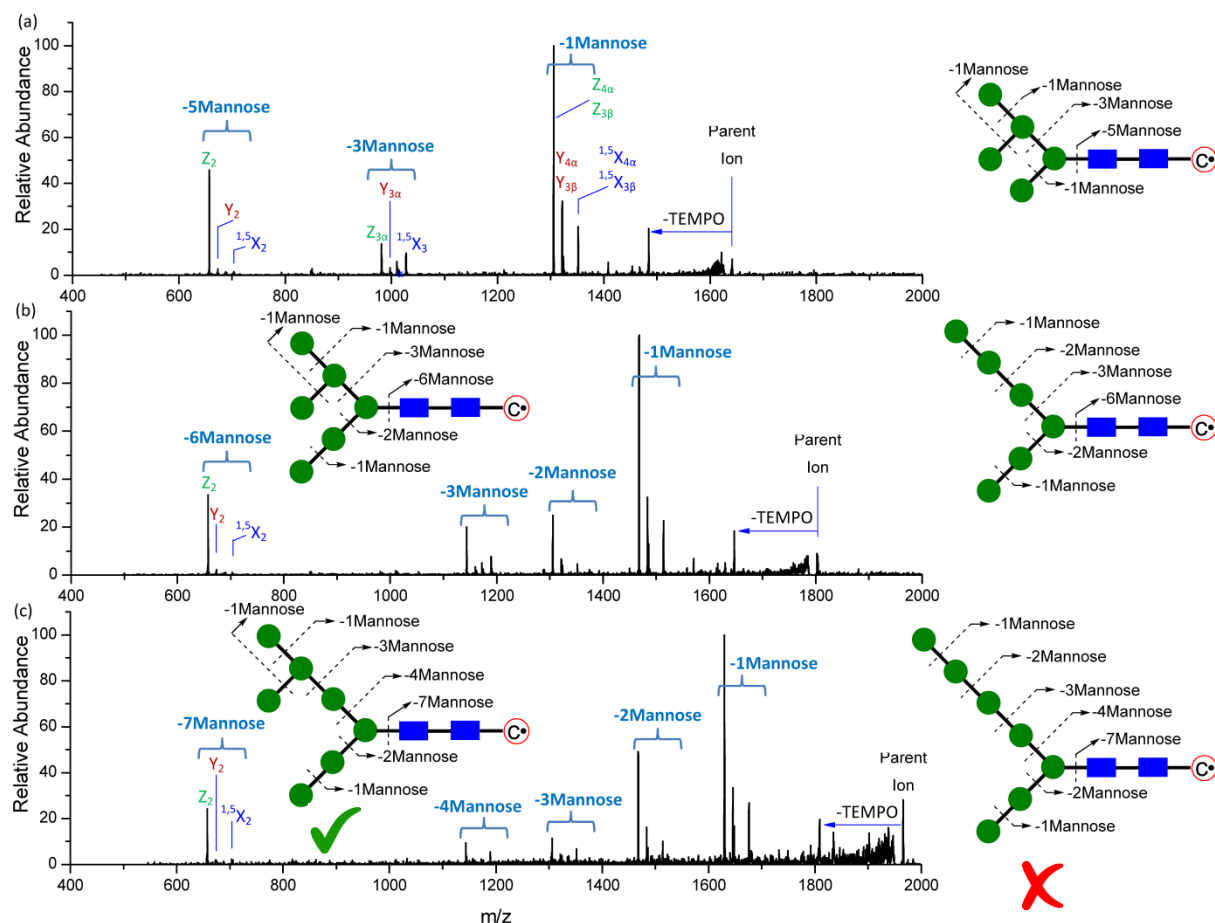


Figure 6. The fragmentation patterns observed following CID of MNPs-FRP-derivatized $\text{Man}_5\text{GlcNAc}_2$ (a), $\text{Man}_6\text{GlcNAc}_2$ (b), and $\text{Man}_7\text{GlcNAc}_2$ (c). Parent ion refers to the methylated molecular ion.

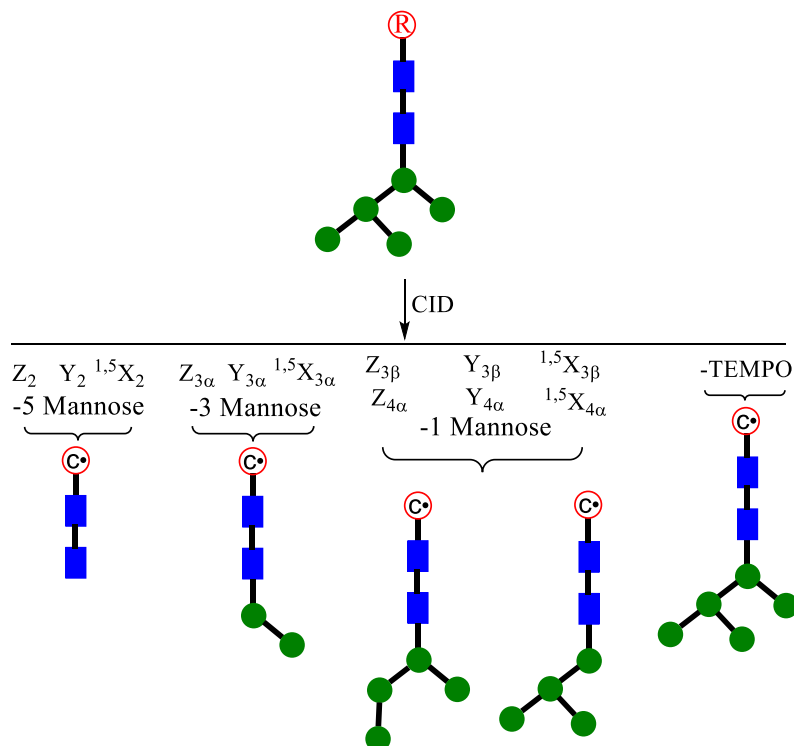


Figure 7. Radical-driven glycan deconstruction (R-DECON) diagram for $\text{Man}_5\text{GlcNAc}_2$.

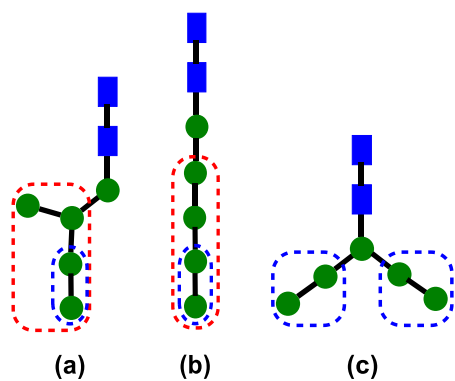


Figure 8. Proposed structures of $\text{Man}_5\text{GlcNAc}_2$ that would be able to lose two (enclosed in blue box) or four (enclosed in red box) mannose residues. Structures (a), containing one branch with one and three mannose residues on two sides, can have loss of two and four mannose residues. Structure (b) with no branches can have loss of two and four mannose residues. Structure (c), containing one branch with two mannose residues on each side, can have loss of two mannose residues.

CONCLUSION

In this study, we describe and demonstrate the effective application of multi-functional solid-supported free radical probes for glycan enrichment and characterization. Glycans which are enzymatically cleaved from proteins can be easily enriched by temporary and selective immobilization on resin beads or magnetic nanoparticles via the reduction reaction between a hydrazide moiety of the probe and the reducing terminus of the target glycans. Glycan characterization is achieved by the systematic and predictable fragmentation induced by a well-defined nascent free radical which is generated following cleavage of the captured glycan from the solid support. Major fragmentation processes include the formation of $^{1,5}X$, Y, and Z ions for both model glycans and glycans released from RNase B. In addition, the unique $Z_{\alpha}+Z_{\beta}$ ions ($Z_{3\alpha}+Z_{3\beta}$ for LNDFH I and $Z_{1\alpha}+Z_{1\beta}$ and $Z_{3\alpha\alpha}+Z_{3\alpha\beta}$ for LNDFH II) can be used for the identification of the branch sites with adjacent linkages, such as the 1-3 and 1-4 linkages on the branch site for LNDFH I. Glycan structures can be visually assembled by the systematic development of radical directed DECON diagrams using MS^n data. The systematic radical-directed fragmentation aids in the determination of glycan structures and in particular facilitates discrimination of isomeric glycans that differ only in the connectivity of their component sugars. The determination of branch sites with two glycosidic linkages which are distal to each other can also be confirmed. For the glycans released from RNase B, the structure of the most abundant isomer of $Man_5GlcNAc_2$ is proposed and validated to have two branch sites with distal glycosidic linkages. The configuration of these two branch sites is confirmed by the corresponding loss of mannose subunits on each side of the branch site. For the most abundant isomers of $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ the configuration of the first branch can be determined, although the existence and/or configuration of the second branch site cannot be ascertained. The development of new free radical reagent, which allows the HPLC separation without increasing the peak numbers, is underway.

The enhancement of glycan enrichment, high fragmentation efficiency, and systematic radical-directed dissociation facilitates the application of SS-FRAGS in addressing problems in structural glycobiology.

Supporting Information Available: [Details about the preparation of the solid-supported free radical probe (SS-FRAGS), schematic illustration of glycan enrichment and MS analysis using solid-supported free radical probes, and NMR spectroscopy of synthesized compounds]

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