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A Carbon Nanoparticles-based Solid-phase Purification Method Facilitating Sensitive MALDI-MS Analysis of Permethylated N-Glycans

Jieqiang Zhong, Alireza Banazadeh, Wenjing Peng, and Yehia Mechref*

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409, United States.

Abstract

In recent decades, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been extensively used for the analysis of glycans. However, native glycans usually have low ionization efficiency in MS, which hinders the direct analysis. Permethylation of glycans is a solution for this issue, but a significant amount of salt is introduced during this process, which can further suppress the MS signals. Thus, it is necessary to purify the glycans prior to MALDI-MS analysis. In this study, we developed a carbon nanoparticles-based solid-phase purification method to enable direct MALDI-MS analysis of permethylated glycans. Two carbon nanomaterials, carbon nanoparticles (CNPs) and graphene nanosheets (GNs), and two conventional carbon materials, activated charcoal and porous graphitic carbon (PGC), were investigated as sorbents to purify permethylated N-glycans derived from ribonuclease B and fetuin. The results confirmed the superior performance of CNPs over the other carbon materials. Additionally, our method was also employed to purify glycans released from human sera in different esophageal disease stages. The obtained data confirmed 16 and 18 structures in adenocarcinoma and Barret's sera with significantly different relative intensities *versus* disease-free sera. Comparing the performance of CNPs based solid-phase purification method employed in this study to on-line purification suggested more than 97% recovery rate. The results of this study demonstrate that CNPs have the potential to be a better alternative to existing solid-phase purification sorbents.

Keywords

Carbon nanomaterials; esophageal disease; glycomics; MALDI-MS; solid-phase purifications

1 Introduction

The glycosylation of proteins and lipids in many biological processes, such as protein folding and localization, cell signaling and adhesion, immune response, and pathogen

*Corresponding Author Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061, yehia.mechref@ttu.edu, Tel: +1-806-742-3059, Fax: +1-806-742-1289.

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interaction, is vitally important [1–7]. The glycosylation of proteins is also the most common posttranslational modifications (PTM) of proteins, and more than 50% of proteins are glycosylated [8]. Moreover, aberrant glycosylation has been defined as biomarkers for various diseases, such as hereditary disorders, cardiovascular disease, and cancers [9–14]. Therefore, reliable and sensitive methods for glycomics are essential to better understand the biological roles of glycans in disease development and progression.

Among various analytical methods, mass spectrometry (MS) has been proven to be a powerful tool routinely employed in glycomics analysis due to its sensitivity [15–18]. Specifically, MALDI has several advantages over other ionization techniques, including ease of operation, rapid data acquisition, reduced sample size requirements, and relative simplicity of spectral interpretation [19–21]. However, the use of MALDI-MS for direct glycan analysis is still hampered by glycan complexity, low abundance, and relatively low ionization efficiency compared to peptides and proteins. Additionally, it remains a challenging task to analyze sialylated glycans due to their possible sialic acid loss during the ionization. Derivatization of sialic acids using amidation and ethyl esterification reactions does not only stabilize sialic acid moiety but also improves the glycan signal and distinguishes linkages of sialic acids [22–24].

Permethylation of glycans developed in 1984 [25], is commonly employed to substantially enhance the ionization efficiencies of glycans. More recently, a simplified method with high-throughput sample preparation labeled solid-phase permethylation was developed [26], and it has been adapted to liquid chromatography-mass spectrometry (LC-MS) glycomic profiling methods [27–29]. However, during the permethylation process, plenty of salt is introduced to samples, and it is well known that high salt concentration generally suppresses the signals in MALDI-MS analysis, which significantly affects the sensitivity and ionization efficiency [30]. Thus, further purification for permethylated glycans prior to MALDI-MS analysis is necessary. There are several purification methods for glycans, including liquid-liquid extraction [31, 32] and dialysis [33]. However, liquid-liquid extraction suffers a considerable sample loss while dialysis is not suitable for permethylated glycan due to their poor solubility in water. Thus, there is a need for developing post-permethylation purification methods

Porous graphitic carbon (PGC) has been extensively used in the separation of N-glycans, due to its ruggedness and its ability to efficiently separate both neutral and acidic permethylated glycans [34–36], which implies its potential to purify glycans. Moreover, solid-phase extraction cartridge, including C18 cartridge, and activated charcoal cartridge, has been commercially available to purify glycans. In comparison to PGC, C18 can only be employed to separate glycans after derivatization with reagents that render, otherwise hydrophilic structures, hydrophobic enough to be separated on C18 columns. On the other hand, since PGC utilizes both polar and hydrophobic interactions, it has an ability to separate both native and permethylated glycans. When analyzing native glycan, polar interaction is responsible for the separation. However, the retention of permethylated glycans on PGC is based on hydrophobic interaction. Thus, PGC is capable to efficiently and effectively analyze both native and permethylated glycans. In the recent decades, carbon-based nanomaterials, such as carbon nanotubes (CNT), graphene nanosheets (GNs), and

carbon nanoparticles (CNPs), have attracted a vast amount of attention because of their superior mechanical, optical, electrical, and thermal properties [37]. However, the applications of carbon-based nanomaterials in glycomics are still limited and relatively unexplored.

In this study, we developed a robust, fast, solid-phase purification method to purify permethylated N-glycans using different carbon nanomaterials, including CNT, GN, and CNP. The potential of CNPs in purifying permethylated N-glycans was investigated and compared to other carbon materials of different size and morphologies, such as GNs, activated charcoal, and PGC. Both activated charcoal and PGC are routinely used for purification of glycans. All studied carbon materials were effectively employed to purify permethylated N-glycans derived from human sera representing diseased esophageal patients at different stages.

2 Materials and method

2.1 Chemicals and reagents

CNPs, GNs, ribonuclease B (RNase B) from bovine pancreas, fetuin from fetal bovine serum, 2,5-dihydroxybenzoic acid (DHB), iodomethane, and borane-ammonia complex were purchased from Sigma-Aldrich (St. Louis, MO). Human blood serum from disease-free subjects and patients with Barrett's esophagus disease and esophageal adenocarcinoma were obtained from Dr. Zane Hammoud (Henry Ford Hospital, Detroit, MI) with all the needed institutional review board (IRB) approvals for sample collection. Microspin columns were purchased from Harvard Apparatus (Holliston, MA) and PNGase F with 10×G7 reaction buffer (0.5 M sodium phosphate) was obtained from New England Biolabs (Ipswich, MA). PGC and charcoal powders were HyperSep PGC cartridge (Thermo Scientific, Waltham, MA) and charcoal Macro SpinColumn (Harvard Apparatus, Holliston, MA), respectively. HPLC-grade water, ethanol, and acetonitrile (ACN) were used for the preparation of samples.

2.2 Preparation of model glycoproteins

PNGase F was used to cleave the N-glycans from RNase B, and fetuin as previously reported [38–40]. Briefly, a 10 μ L aliquot of RNase B or fetuin stock solution (5 μ g/ μ L) was mixed with a 10 μ L aliquot of diluted G7 buffer (50mM sodium phosphate buffer, pH 7.5, diluted 10 times with HPLC-grade water). The resulting solution was then incubated in a water bath at 90 °C for 20 min to denature the proteins. After cooling down, 1.2 μ L PNGase F (500 units/ μ L) stock solution was added, and the samples were further incubated in 37 °C water bath for 18 h. A 180 μ L aliquot of ethanol was added to the mixtures of the released N-glycans and proteins, followed by incubation at –20 °C for 30 min to precipitate the proteins. Subsequently, the mixtures were centrifuged at 14,800 rpm for 10 min to separate glycans and proteins, and the supernatants which contained the glycans were collected and dried in a SpeedVac concentrator (Kansas City, MO).

2.3 Preparation of pooled human sera

The sera samples representing different esophageal disease cohorts were subjected to the following sample preparation method. A 10 μL aliquot of each sera samples representing different esophageal disease cohorts was mixed with a 10 μL aliquot of diluted G7 buffer (50mM sodium phosphate buffer, pH 7.5, diluted 10 times with HPLC-grade water) and 30 μL water before the incubation in 90 °C water bath for 20 min. The following enzymatic digestion procedure and protein precipitation procedure were the same as those of model glycoproteins. Finally, the resulting supernatants containing the released N-glycans were collected and dried in SpeedVac concentrator. The dried samples were then treated to eliminate N-glycan reducing ends as described next.

2.4 Reduction of released N-glycans

The reduction was performed as previously reported [27–29]. Briefly, a 10 μL aliquot of freshly prepared ammonia borane solution (10 $\mu\text{g}/\mu\text{L}$) was added to the dry released N-glycans samples, followed by incubation in 60 °C water bath for 1h. The reduced samples were then treated with 500 μL methanol, which produces the volatile methyl borate salt. The mixtures were then dried in SpeedVac concentrator. This step was repeated several times until no visible white precipitate was observed in the samples after evaporation. The repetitive addition and evaporation of methanol removed the excess ammonia borane completely.

The reduced N-glycans released from human sera were desalted by using dialysis membrane with a molecular cut of 500–1000 Da before permethylation, to remove the small molecules and salts in the sera matrix which might interfere with the following permethylation.

2.5 Solid-phase permethylation of N-glycans

Next, the reduced glycans were permethylated using solid-phase permethylation method as previously reported [27–29]. Briefly, spin columns packed with 2-cm depth sodium hydroxide beads were prepared and washed with 50 μL DMSO twice. The reduced glycans were resuspended in 1.2 μL water, 30 μL DMSO, and 20 μL methyl iodide was then added to the mixtures, followed by the transfer of the samples to the sodium hydroxide beads packed spin column. The spin columns were allowed to sit for 30 min before the addition of another 20 μL methyl iodide, allowing the spin columns to sit for another 20 min. Subsequently, the sample mixtures were centrifuged at 1,800 rpm, and 30 μL ACN was used to elute all the permethylated glycans remaining in the sodium hydroxide beads before the second centrifugation at 1,800 rpm. The permethylated glycans were dried under vacuum and stored at -20°C until use.

2.6 Offline solid-phase purification of N-glycans

The CNPs, GNs, charcoal, and PGC slurry were prepared at a concentration of 2 $\mu\text{g}/\mu\text{L}$ in 10% (v/v) ACN aqueous solution, respectively. All the slurries were sonicated with Fisherbrand™ sonicator (Waltham, MA) vigorously prior to use. The dry permethylated glycans were resuspended in 100 μL 10% (v/v) ACN. A 10 μL aliquot of each of the permethylated glycan samples was then mixed with 10 μL carbon slurry and 80 μL water, followed by sonication for 10 min to adsorb glycans on carbon. Then, the mixtures were centrifuged at 14,800 rpm for 10 min before the removal of supernatant. With this step, most

of the glycans can be adsorbed on the carbons, and the salt is removed along with the supernatant removal. After that, a 100 μ L aliquot of 80% (v/v) ACN was added, and the mixtures were re-dispersed with sonication for another 10min for elution. Centrifugation at 14,800 rpm for 10 min was applied again to separate carbon and solution. The resulting supernatant was collected and dried under vacuum.

2.7 MALDI-MS instrumentation and acquisition parameters

MALDI-TOF-MS analyses were performed on a 4800 MALDI TOF/TOF analyzer (AB SCIEX) equipped with a pulsed Nd:YAG laser at an excitation wavelength of 355 nm. 50 shots of 50 sub-spectra were acquired on the same spot with a total of 2500 shots for each sample. The mass spectra were analyzed using Data Explorer 4.9 software (AB SCIEX). The glycoworkbench software was applied for MS data interpretation and glycoform analysis. For MALDI analysis, dried permethylated glycan samples were dissolved in a 10 μ L aliquot of 50% ACN. Then 0.5 μ L of samples were added to the MALDI plate, followed by the addition of 0.5 μ L of 2,5-dihydroxybenzoic acid (DHB, 20 μ g/ μ L in 50% ACN), as a MALDI matrix.

3 Results and discussion

3.1 The extraction efficiency of permethylated N-glycans derived from model glycoproteins using different carbon nanomaterials

Permethylated glycans are high hydrophobic, thus prompting strong interaction with carbon materials. Initially, permethylated N-glycans derived from RNase B and fetuin were purified with CNPs, GNs, charcoal, and PGC to assess the efficiency of each material. Figure 1a illustrates the relative intensities of N-glycans derived from RNase B acquired by MALDI-MS. All the carbon materials show a similar distribution of glycans (Man5: Man6: Man7: Man8: Man9= 59: 23.5: 7: 8.5: 2), which is consistent with the known endogenous distribution of the glycans derived from RNase B [41]. Thus, we believe that these carbon materials have no preferences for any glycan structures. Comparing the different carbon purifications, the total signal intensity of permethylated N-glycans purified with CNPs is 1.6-, 2.1, and 2.2-fold higher than those purified with GNs, charcoal, and PGC, respectively. Additionally, the average relative standard deviation values of all glycan structures derived from RNase B purified with CNPs, GNs, charcoal, and PGC are 5.5%, 8.0%, 5.6% and 9.4%, respectively (Figure 1a). The purification method using CNPs exhibited the highest reproducibility. The relative intensities of sialylated N-glycans derived from fetuin samples are shown in Figure 1b. Similarly, the distribution of glycans derived from fetuin was not affected by the purification using different carbon nanomaterials and agrees with the previously reported distribution [42]. Again, the total intensity of permethylated N-glycans purified with CNPs is 1.7-, 2-, and 2.8-fold higher than those purified with GNs, charcoal, and PGC, respectively. Also, the CNPs based purification method displayed the highest reproducibility with a 6.1% relative standard deviation value (Figure 1b).

To evaluate the adsorption efficiency of different carbon materials, Man5 was chosen as a model structure, since it is the most abundant structure in RNase B and the other glycans in RNase B have similar structures as Man5, which we assumed they share similar adsorption

efficiency. To avoid the influence of salt on MALDI-MS, LC-MS was used. First, the unpurified samples were analyzed. Subsequently, the same aliquots of the supernatants after the adsorption with different carbon materials were analyzed. The results acquired by LC-MS are shown in Figure S1. The data shown in Figure S1 indicates the percentages of glycans which did not bind to the carbon sorbents. For all the four carbon materials we tested in this study, there were more than 97% (for detailed calculation, see Supporting Information) of glycans retained on different carbon materials, which has more than 2-time higher purification efficiency in comparison to the conventional liquid-liquid extraction [31]. Comparing the different carbon materials, CNPs showed the highest adsorption efficiency which was higher than 99.9%, and this result was consistent with that acquired by MALDI-MS, indicating the better performance of CNPs than the other three. This might be due to the better dispersibility of CNPs over the other carbon materials that we used, which can be resulted by their smaller particle size. GNs, charcoal, and PGC did not exhibit good dispersibility. Their slurries were stable only for a few minutes after ultrasonication.

3.2 MALDI-MS profiles of permethylated glycans derived from human sera in different esophageal diseases cohorts, purified with different carbon materials

The performance of CNPs and other carbon materials was further investigated with human sera samples in different esophageal diseases periods. MALDI-MS glycans profiles of sera samples derived from disease-free subjects, Barrett's esophagus patients, and esophageal adenocarcinoma patients, purified with different carbon materials, are shown in Figure 2a, b, and c, respectively. Similarly, intensities of the results obtained with CNPs were the most intensive, comparing to the other three carbon materials, and the distributions of glycans in different sera samples were slightly different (see below). Plus, to demonstrate the necessity of purifying samples before MALDI-MS analysis, the unpurified sera samples were tested. Also, as shown in Figure 2, no structures could be detected in all the traces of unpurified control samples.

When looking into the details of individual glycan quantitation, more information can be drawn from side-by-side comparisons among the different diseases using different carbon materials. Figure 3 depicts the butterfly charts for the results obtained for permethylated glycans derived from disease-free subjects, Barrett's esophagus, and esophageal adenocarcinoma patient's sera samples. In each case, the left-hand side shows the ratios of the relative intensities of permethylated glycans derived from Barrett's relative to the disease-free sera, while in the right-hand side, the ratios of the relative intensities of permethylated glycans derived from adenocarcinoma relative to that of the disease-free sera, are shown. Generally, more glycan structures were detected by using CNPs as the sorbent. For instance, 55, 50, 49 and 50 structures were identified in disease-free serum samples by using CNPs, GNs, charcoal, and PGC, respectively. Also, the number of detected glycans in cancer sera was higher than the disease-free serum samples. 60, 57, 54 and 58 structures were identified by using CNPs, GNs, charcoal, and PGC, respectively.

As shown in Figure 3, in comparison with Barrett's sera's glycan profiles, the profiles which were obtained from cancer sera show more differences relative to the disease-free samples. By looking at the results acquired using CNPs, as they show better performance for the

enrichment of permethylated glycans, it can be found that sialylated glycans, especially double, tri- and tetrasialylated glycans derived from sera of adenocarcinoma patients show higher intensities than those derived from disease-free sera (Figure 3 and Table S2). For example, the ratios of the relative intensities of HexNAc₅Hex₆NeuAc₃ and HexNAc₅Hex₆DeoxyHex₁NeuAc₃ in adenocarcinoma sera to the disease-free sera, are 1.68 (p -value = 0.004) and 2.44 (p -value = 0.008), which are consistent with our previously reported results [43]. Additionally, our results also confirmed under-expression of high mannose glycans derived from two diseased samples compared to the disease-free samples as reported previously [43]. Table S2 shows 18 and 16 structures in adenocarcinoma and Barrett's sera with significantly different relative intensities (p -value < 0.05) *versus* disease-free sera. These results demonstrate the benefits of utilizing CNPs for reliable purification of glycome samples derived from biological samples. The same trends were also observed by using GNs, charcoal, and PGC (Figure 3). However, the ratios of relative intensities which were obtained by using CNPs, are more consistent with the previously reported data [43, 44].

Principal component analysis (PCA) is a widely used chemometric technique that allows the analysis of a dataset that contains several correlated independent variables. With PCA, important values can be extracted and converted to a set of principal components by an orthogonal transformation. The similarities and differences of data are displayed as points on a map, which can be further clustered based on their locations. The PCA plot shown in Figure 4 depicts that the glycosylation pattern of disease-free, Barrett's esophagus, and esophageal adenocarcinoma sera samples can be clearly discriminated. Adenocarcinoma samples were grouped distinctly, and Barrett's esophagus and disease-free samples can also be clustered with partial overlap. Additionally, in the group of adenocarcinoma data points, the distributions of CNPs data points (linked with black dashed lines), and GNs data points (linked with solid yellow lines) are closer than the other two conventional carbons, demonstrating the better reproducibility when using carbon nanomaterials. Also, as the results shown here, none of the carbon materials biased the grouping pattern, which further illustrates the applicability of these carbon materials in complex biological samples.

Volcano plots is a statistical scatter-plot that can quickly identify significant changes in a large dataset containing biological replicates. With the volcano plots, the most significant and largest changes can be visually observed and analyzed. Figure 5 depicts different volcano plots between disease samples and disease-free samples using different carbon materials. When comparing two carbon nanomaterials, CNP exhibited much better capacity than graphene in distinguishing glycomes between disease and disease sera, which is consistent with the aforementioned results. The use of CNP permitted the distinction of 13 glycan expressions between Barrett's esophagus sample and disease-free samples (Figure 5a) and 13 glycan changes in adenocarcinoma samples (Figure 5b) while only 5 differential expressions were observed using graphene in Barrett's esophagus (Figure 5c). Also, CNP achieved a higher significance in distinguishing glycans due to the less deviation it induced than graphene.

The function of two widely used conventional carbon materials, charcoal, and PGC were also investigated. For charcoal, 6 significant glycan expression alterations were observed in

Barrett's esophagus (Figure 5d), and 14 were observed in adenocarcinoma samples (Figure 5e). Another current carbon material PGC is widely used in glycan enrichment and separation. In this study, PGC allowed 8 differential expressions of glycan detected in Barrett's esophagus (Figure 5f) and 15 glycans in adenocarcinoma samples (Figure 5g) which is a little more than other carbon materials. Although charcoal and PGC obtained comparable numbers of differential glycan expressions when compared to CNP (Figure 5b, e, and g) between adenocarcinoma and disease-free samples, much less significant glycan expression changes were observed between Barrett's esophagus and disease-free samples - 6 as in Figure 5d and 8 as in Figure 5f - using charcoal and PGC, respectively. Thus, CNP demonstrated a superior performance relative to the other carbon materials in the investigation of glycol-biomarker discovery for Barrett's esophagus and adenocarcinoma diseases. This might be partially because CNP has a stronger and more stable interaction with permethylated glycans, leading a better enrichment and smaller deviation.

4 Conclusion

In this study, we compared the purification efficiencies of CNPs, GNs, activated charcoal, and PGC for permethylated N-glycans. Results showed that CNPs had the best performance among them since it allowed the most intensive signals with the highest signal-to-noise ratios. Also, more glycan structures were found in sera samples purified with CNPs than the other three carbons. Compared to liquid-liquid extraction [31], the conventional glycans purification method, our method avoids using a highly toxic solvent and has much less sample loss (less than 0.05%); compared to dialysis [33], which is time-consuming and not compatible with organic solution, our method is faster and overcomes the organic incompatibility issue. Overall, the CNPs based solid-phase purification method provided an alternative approach for permethylated N-glycan purification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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5 References

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Figure 1. Purification of permethylated glycan using different carbon materials (CNPs, GNs, charcoal, and PGC). a) Relative intensities of permethylated N-glycans derived from RNase B, purified with different carbon materials. b) Relative intensities of permethylated N-glycans derived from bovine fetuin, purified with different carbon.

Figure 2. MALDI-MS glycans profiles of human serum samples derived from a) disease-free, b) Barrett's esophagus, and c) esophageal adenocarcinoma, purified by using CNPs (blue), GNs (orange), charcoal (green) and PGC (purple).

Figure 3.
Ratios of the relative intensities of permethylated N-glycans derived from Barrett's esophagus/adenocarcinoma patient's serum samples to disease-free human serum, purified using different carbon materials (one-asterisk marks represent the data with a p -value lower than 0.05, two-asterisk marks represent data with a p -value lower than 0.01).

Figure 4. Principal component analysis (PCA) of the relative intensity of permethylated N-glycans of human serum samples derived from different esophageal diseases periods, purified with CNPs, GNs, charcoal, and PGC. Disease-free human serum (blue circle), Barrett's patients (green square), and esophageal adenocarcinoma patients (purple rhombus).

Figure 5.

Volcano plots of relative intensities of permethylated N-glycans derived from human serums with different esophageal diseases *versus* disease-free, a) Barrett's *vs.* disease-free with CNPs, b) adenocarcinoma *vs.* disease-free with CNPs, c) adenocarcinoma *vs.* disease-free with GNs, d) Barrett's *vs.* disease-free with charcoal, e) adenocarcinoma *vs.* disease-free with charcoal, f) Barrett's *vs.* disease-free with PGC, and g) adenocarcinoma *vs.* disease-free with PGC, purified with different carbon materials. Red dots represent the significant changes with a p -value less than 0.05.