Talanta 117 (2013) 1–7

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Graphene based soft nanoreactors for facile "one-step" glycan enrichment and derivatization for MALDI-TOF-MS analysis

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ARTICLE INFO

Article history:
Received 21 May 2013
Received in revised form
14 August 2013
Accepted 17 August 2013
Available online 28 August 2013

Keywords:
Reduced graphene oxide
Glycan
Enrichment
Derivatization
Desalting
1-Pyrenebutyric hydrazide

ABSTRACT

Protein glycosylation is involved in the control of many important biological processes and structural alterations of the N-linked glycans are correlated with various kinds of disease. High-throughput N-glycan profiling is a key technique for elucidating the functions of glycans in biological process and disease development as well as discovering new diagnostic biomarkers. However, the low abundance of glycans existing in living organism, the competition/suppression effect of other highly abundant biological molecules and the inherent lack of alkalinity and hydrophobicity of glycans leads to particularly poor detection sensitivity in MS analysis. Here, we demonstrated the first "one-step" approach for highly efficient glycan enrichment and derivatization using reduced graphene oxide as nanoreactors and 1-pyrenebutyric hydrazide for glycan capture and derivatization, which resulted in a 33-fold increase in the glycan detection sensitivity in MALDI-TOF-MS and the identification of 48N-glycoforms from human plasma.

1. Introduction

Graphene oxide (GO) and reduced graphene oxide (rGO) are rapidly becoming some of the most widely studied materials and have been used to develop biological sensors [1–5], MALDI-MS matrices [6,7], imaging/sensing reagents [8,9], energy storage materials [10] and composite materials [11–14] due to their unique physical and chemical properties. The extremely large specific surface area, strong hydrophobicity and π-electron rich structure of rGO make it particularly attractive for the enrichment of trace amounts of biomolecules. However, the potential of this material has not been fully exploited because the immobilization of rGO on solid supporting materials leads to a large reduction in the specific surface area and flexibility of rGO [15–18].

As one of the most important low abundant biomolecules, glycans of glycoproteins are involved in the control of many crucial biological processes, such as protein folding and stability and cell–cell recognition and interactions [19–21]. It has been demonstrated that structural alterations of glycans are correlated with various kinds of disease, including cancer, diabetes and immune disorders [22,23]. Currently, mass spectrometry (MS) based high-throughput glycan profiling is the key technique for discovering new diagnostic biomarkers [24–26]. However, MS has limited detection sensitivity for the trace amounts of glycans obtained from complex biological samples due to the competition/suppression effect of other highly abundant biomolecules such as proteins and peptides. Furthermore, the inherent lack of hydrophobicity and proton affinity of glycans leads to particularly low ionization efficiency in MS analysis. Although various enrichment methods, including lectin or hydrophilic interaction liquid chromatography (HILIC) enrichment have been developed [27–30], the relatively low affinity/selectivity of lectin or HILIC for glycans results in low enrichment efficiency and coelution with highly abundant impurities. In addition to enrichment, chemical derivatization by coupling hydrophobic/alkaline reagents to the reducing end of glycans is another way to increase the MS detection sensitivity of glycans [31–35]. Unfortunately, chemical derivatization which involves repeated sample purification is extremely tedious and time-consuming and inevitably results in excessive sample losses.

In this work, we demonstrated the first application of free rGO as soft nanoreactors for facile "one-step" glycan enrichment and derivatization with high specificity and efficiency for MALDI-TOF-MS analysis. In this approach, glycan enrichment and derivatization

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http://dx.doi.org/10.1016/j.talanta.2013.08.031

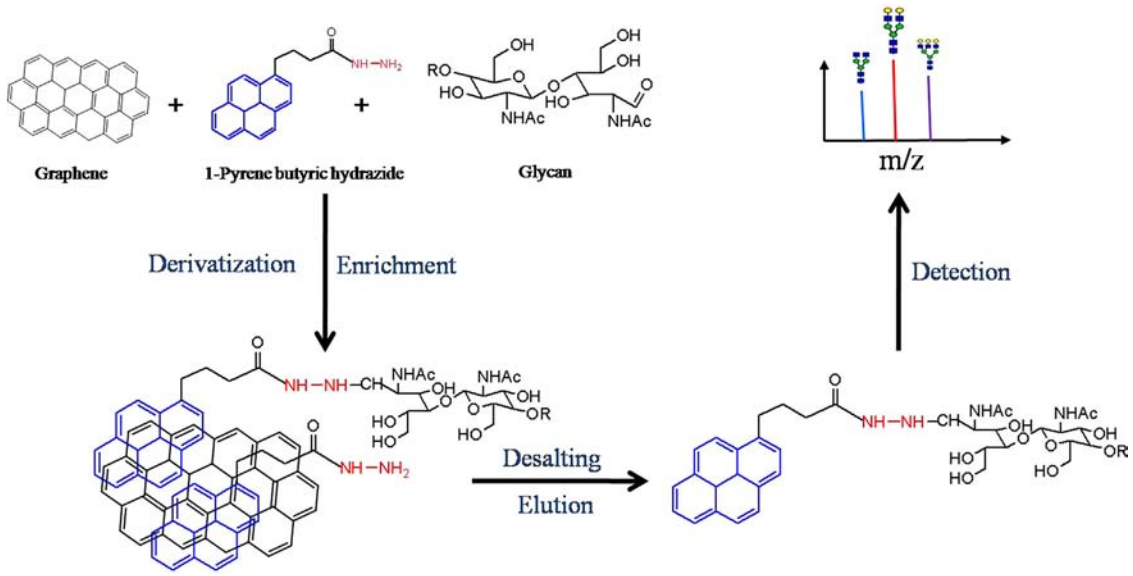

Fig. 1. Schematic overview of free rGO based "one-step" method for glycan enrichment, derivatization and MS analysis.

are realized by exploiting the strong affinity of rGO for planar polycyclic aromatic hydrocarbons [36–38] such as 1-pyrenebutyric hydrazide (PBH), combined with the highly specific hydrazide–aldehyde coupling of PBH to glycans (Fig. 1). PBH functions as a bifunctional coupling reagent that efficiently adsorbs on rGO via π–π stacking and hydrophobic interactions and also selectively reacts with the aldehyde group at the reducing end of a glycan, therefore "one-step" glycan enrichment and derivatization is achieved. Finally, the captured and derivatized glycans were eluted for MS analysis. Using well dispersed free rGO as soft nanoreactors for glycan enrichment and derivatization has three distinct advantages. First, the high specific surface area and flexibility of free rGO and the specific interactions among rGO, PBH and glycan lead to obvious improvements in the enrichment selectivity, efficiency and derivatization completeness. Second, the coelution of impurities with PBH–glycan conjugates is not observed. Due to this selective elution, sample desalting can be skipped without causing signal suppression in MS analysis by impurities such as excess PBH, protein residues or salts. Third, the facile combination of glycan enrichment, derivatization and desalting into a "one-step" procedure reduces both the number of processing steps required and sample loss and therefore leads to a significant improvement in the MS detection sensitivity of glycans.

2. Experiments

2.1. Material and reagents

Maltoheptaose (DP7), 1-pyrenebutyric hydrazide (PBH), 2,5-dihydroxybenzoic acid, N,N-dimethylformamide (DMF), trifluoroacetic acid (TFA), graphite flakes and asialofetuin were purchased from Sigma (St. Louis, MO, USA). Analytical grade methanol, ethanol, ammonium bicarbonate, sodium nitrate, sulfuric acid (H_2SO_4), sodium borohydride, hydrochloric acid, potassium permanganate, sodium carbonate and hydrogen peroxide (H_2O_2) were obtained from Acros (Belgium). Peptide N-glycosidase (PNGase F) was acquired from New England Biolabs (Ipswich, MA, USA). Deionized water (with resistance $>18\ M\Omega/cm$) was prepared by using Millipore purification system (Billerica, MA, USA) and used throughout this work.

2.2. Synthesis of reduced graphene oxide

Reduced graphene oxide (rGO) was synthesized from graphite flakes using reported methods [39,40]. First, graphite flakes were oxidized and exfoliated using the modified Hummer's method to obtain GO. Specifically, 0.5 g of graphite flakes and 0.5 g Na_2NO_3 were added into 23 mL pre-cooled H_2SO_4 with 4 g $KMnO_4$. The mixture was stirred in an ice bath for 10 min and under 35 °C for 1 h. After that, 40 mL deionized water was added to the mixture and heated at 90 °C for 1 h. After adding 100 mL deionized water and 3 mL H_2O_2, the resultant mixture was stirred at room temperature for 2 h. The obtained mixture was washed with 1000 mL deionized water containing 5 mL HCl (37%) and 1000 mL deionized water, sequentially. Finally, the acquired mixture was dried in a vacuum oven at 50 °C for 24 h. Next, the obtained GO was reduced by $NaBH_4$. Typically, 20 mL $NaBH_4$ solution (1 M) was added to 100 mg GO dispersed in water under stirring. After adjusting pH to 10 by adding 10 wt% Na_2CO_3 solution, the mixture was heated at 90 °C for 1 h. Finally, the mixture was washed several times with deionized water and ethanol and then dried in a vacuum oven to obtain rGO.

2.3. rGO based one-step approach for maltoheptaose (DP7) enrichment and derivatization

Mixture of 1.15 μg DP7 and 57.5 μg BSA (1:50, w/w) was mixed with 5 mg rGO and 15.1 μg PBH (DP7:PBH=1:50, molar ratio) dispersed in 1 mL methanol and allowed to react at 90 °C for 60 min in the presence of 0.5% acetic acid. Next, the rGO was collected by centrifuging at 9600*g* for 10 min. After repeated washing the rGO with water and methanol, the derivatized DP7 was eluted by DMF.

2.4. N-glycans releasing from glycoproteins

Asialofetuin (1 mg/mL) was dissolved in 50 mM NH_4HCO_3 (pH=7.8) and denatured by boiling at 95 °C for 10 min. After cooling to room temperature, PNGase F was added to the denatured asialofetuin solution (1 U enzyme for 10 μg glycoprotein) and incubated at 37 °C for 16 h for N-glycan releasing. Human plasma was centrifuged at 9600*g* for 30 min to remove lipids.

Solution at the bottom was taken out and diluted with 50 mM NH_4HCO_3 to a final protein concentration of 1 mg/mL. Next, N-glycans were released using the same protocol as the one used in N-glycan releasing from asialofetuin.

2.5. rGO based one-step enrichment and derivatization of glycans released from asialofetuin or human plasma proteins

10 μL raw N-glycans sample released from asialofetuin or 100 μL human plasma proteins (1 mg/mL) were mixed with 15 μg PBH and 5 mg rGO dispersed in methanol and allowed to react at 90 °C for 60 min in the presence of 0.5% acetic acid for glycan enrichment and derivatization. Next, the rGO was collected by centrifuging at 9600*g* for 10 min. After repeated washing the rGO with water and methanol, the derivatized N-glycans were eluted by DMF.

2.6. Characterization of rGO

The morphology of rGO was characterized by atomic force microscopy (AFM) and transmission electron microscopy (TEM). Tapping mode AFM characterization was conducted on a Nanoscope IIIa (Veeco Instruments) scanning probe microscope. TEM images were obtained by a JEOL JEM-2000 EX transmission electron microscope (JEOL, Tokyo, Japan) operated with acceleration voltage of 80 kV. The surface elemental composition of rGO before and after adsorption of PBH was analyzed by X-ray photoelectron spectroscopy (XPS) using a Kratos AMICUS system (Shimadzu, Japan) with Mg KR radiation ($h\nu = 12$ kV) at a power of 180 W.

2.7. MALDI-TOF-MS analysis

Glycan samples were mixed with SDHB matrix made from 9 mg 2,5-dihydroxybenzoic acid and 1 mg 5-methyl salicylic acid dissolved in 1 mL 50% ACN containing 0.1% TFA. 1 μL sample and SDHB matrix mixture (1:1) was dropped on a target plate and allowed to air dry. MALDI-TOF-MS analysis were carried out using a 4800 MALDI TOF/TOF analyzer (AB SCIEX, MA, USA) equipped with a pulsed Nd:YAG laser at excitation wavelength of 355 nm. All mass spectra (1000 laser shots for every spectrum) were analyzed by Data Explorer 4.5 software. GlycoWorkbench software was applied for mass spectrometric data interpretation and glycoform analysis.

2.8. Liquid chromatography analysis

Liquid chromatography analyses were performed on an Elite P230 liquid chromatography (Dalian, China) equipped with an ultraviolet detector and a Supelco Discovery C_{18} analytical column (4.6 mm × 250 mm, 5 μm, St. Louis, MO, USA). The detection wavelength was set as 242 nm for detection of PBH. ACN and deionized water (8:2, v/v) delivering at a flow rate of 1 mL/min was used as the mobile phase.

3. Results and discussion

3.1. Preparation and characterization of rGO

In our study, rGO was obtained using the modified Hummer's method, followed by reduction with $NaBH_4$. AFM and TEM characterizations were carried out to demonstrate that rGO was successfully synthesized. The cross-sectional analysis of rGO by AFM (Fig. 2) indicates that the average thickness of rGO was 0.97 ± 0.02 nm ($n = 4$), which is consistent with the literature

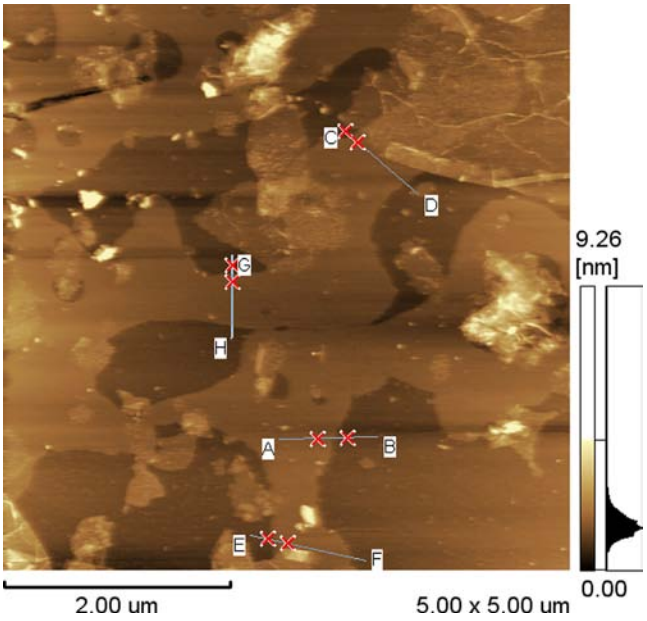

Fig. 2. AFM characterization of rGO conducted on a Nanoscope IIIa scanning probe microscope.

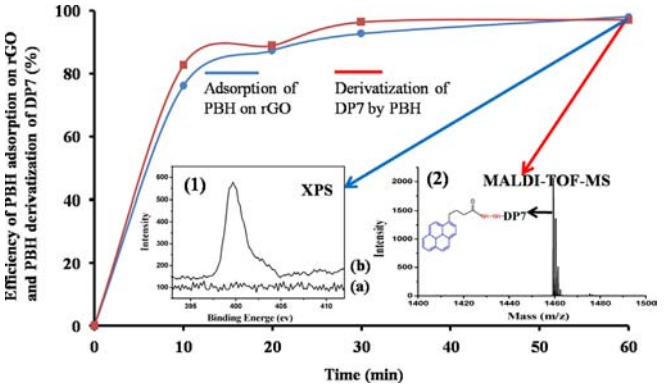

Fig. 3. Efficiency of PBH adsorption on rGO demonstrated by liquid chromatography based method and PBH derivatization of DP7 at 90 °C in a 50:1 ratio of PBH to DP7 for 60 min. Inset (1): XPS characterization of rGO before (a) and after (b) 60 min PBH adsorption. Inset (2): MS spectrum of DP7 after 60 min derivatization by PBH.

reported data [39,40]. Since the rGO prepared by liquid exfoliation and $NaBH_4$ reduction usually has some defects and un-reduced hydroxyl, carboxyl and epoxy groups on the surface, its thickness is always higher than the corresponding theoretical value of a single layered graphene (0.35 nm). The successful synthesis of rGO with good reproducibility was further demonstrated by the observation of semi-transparent membranes resembling wrinkled silk veilin TEM (Fig. S1, Supplementary material), which is the typical view of rGO.

3.2. Adsorption efficiency, maximum loading capacity of PBH on rGO and conversion rate of glycan derivatization with PBH

Before using rGO and PBH for glycan enrichment and derivatization, the adsorption efficiency, maximum loading capacity of PBH on rGO and conversion rate of glycan derivatization with PBH were determined. A total of 15 nmol PBH was mixed with 1 mg rGO in methanol and heated at 90 °C. The supernatant was taken out to quantify the PBH residue after heating for 10, 20, 30 and 60 min. As shown in Fig. 3, the adsorption of PBH on free rGO

is highly efficient with ~95% of PBH adsorbed within 60 min. The resulting PBH–rGO complex was further characterized by XPS to confirm the successful adsorption of PBH on rGO. As shown in the inset (1) of Fig. 3, PBH adsorption on rGO was clearly demonstrated by the strong N1s absorption peak at 399.6 eV in the XPS spectrum. In contrast, no corresponding peak was observed for the untreated rGO. The maximum loading capacity of PBH on rGO was determined by exposing rGO to excess of PBH and was found to be 229 ± 35 μg/mg ($n=3$), which is about two times higher than that of the micrometre-sized HILIC packing materials commonly used for glycan enrichment, thus indicating that rGO has good potential for glycans enrichment [41].

The conversion rate of the glycan derivatization with PBH was studied using a standard oligosaccharide. DP7 was reacted with a 50-fold excess of PBH in methanol at 90 °C for 10, 20, 30 and 60 min and analyzed by MALDI-TOF-MS. The conversion rates (Fig. 3) show a clear rising trend with increasing reaction time and the reaction goes to completion after 60 min (Fig. 3, inset (2)). Close inspection of Fig. 3 reveals very similar increasing trends for PBH adsorption on rGO and DP7 derivatization with PBH with increasing reaction time, making the combination of glycan enrichment and derivatization in "one step" possible.

3.3. The free-rGO-based "one-step" approach for glycan enrichment and derivatization

After evaluated the adsorption efficiency and conversion rate, the feasibility of using the free-rGO-based "one-step" approach for low-abundant glycan enrichment and derivatization was evaluated using the mixture of DP7 and BSA (1:50, w/w) as a model sample. The sample was mixed with 5 mg free rGO and a 50-fold excess PBH dispersed in methanol. After heating at 90 °C for 60 min, rGO was collected by centrifugation and the PBH derivatized DP7 was eluted by DMF for MALDI-TOF-MS analysis. Using excess derivatization reagents is crucial for achieving complete derivatization of glycans and had been demonstrated by previous work [31]. As shown in Fig. 4a, the signal of untreated DP7 is hardly identifiable due to the low ionization efficiency of hydrophilic glycans in MS and the signal suppression effect of BSA. In contrast, the signal intensity of the same amount of DP7 increases by 33 times after rGO–PBH enrichment and derivatization (Fig. 4b). Interestingly, further analysis of the eluate from rGO using MALDI-TOF-MS (Fig. 5) and HPLC (Fig. 6) shows that almost no BSA or PBH could be detected, indicating no coelute of BSA or PBH with PBH–DP7 conjugates. This selective elution of derivatized glycans by leaving excess PBH and residue BSA adsorbed on rGO is particularly beneficial to reduce the interference of excess derivatization reagents with the crystallization of glycans and resulting a poor MS signal. Meanwhile, residue proteins may compete with glycans for ionization and also suppress glycans' MS signal. We attribute the separation of PBH–glycan conjugates from PBH and proteins to their different affinities with rGO. Coupling PBH with glycans might disrupt the strong π–π and hydrophobic interactions between PBH and rGO, which causes the specific elution of PBH–glycan by DMF.

3.4. Comparison of the free-rGO-based "one-step" method with commonly adopted enrichment and derivatization methods

The advantages of using this free-rGO-based "one-step" approach were further demonstrated by comparing this method with two other commonly used enrichment/derivatization methods for analyzing N-glycans released from asialofetuin. Fig. 7 shows the MS spectra peak areas of untreated, HILIC-enriched, 2-Hydrazinopyridine (2-HP) derivatized and rGO–PBH-treated N-glycans. Clearly, the peak area of the rGO–PBH treated N-glycans is the largest for each glycoform. In contrast to HILIC enrichment or 2-HP derivatization that only leads to minor increase of the MS signal, obvious signal enhancement is achieved for all four glycoforms after rGO–PBH enrichment and derivatization. This improvement in glycan identification can be attributed to the unbiased affinity of rGO for PBH–glycans with different glycoforms and the large surface area of well-dispersed free rGO, which allows for a considerable increase in the number of collisions between N-glycans and rGO.

3.5. Human plasma N-glycan analysis using the free-rGO-based "one-step" method

The structural changes of N-glycans of glycoproteins in human plasma have long been considered to be associated with various types of cancer, such as pancreatic, liver and lung cancer. Therefore,

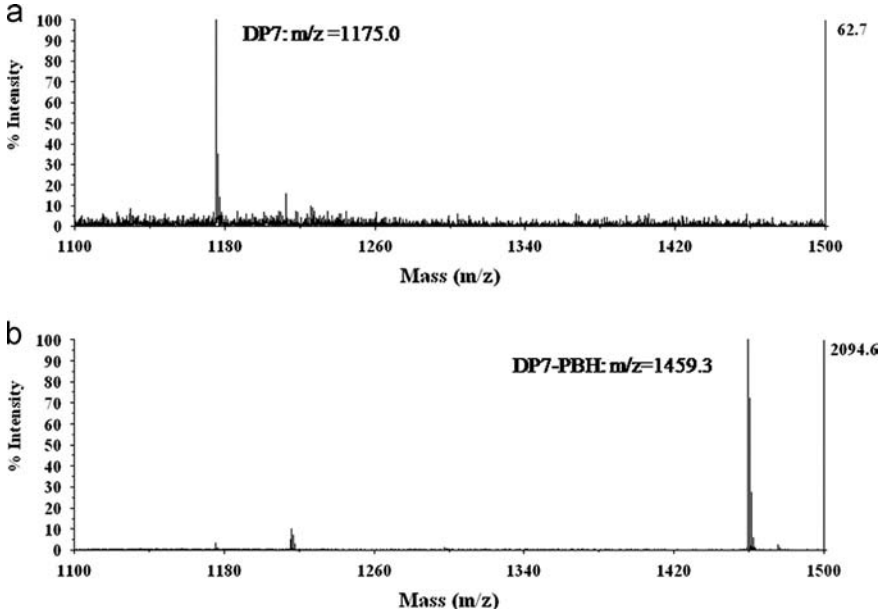

Fig. 4. MALDI-TOF-MS spectra of mixture of DP7 and BSA before (a, $[M+Na]^+=1175.0$) and after (b, $[M+Na]^+=1459.3$) rGO–PBH enrichment-derivatization.

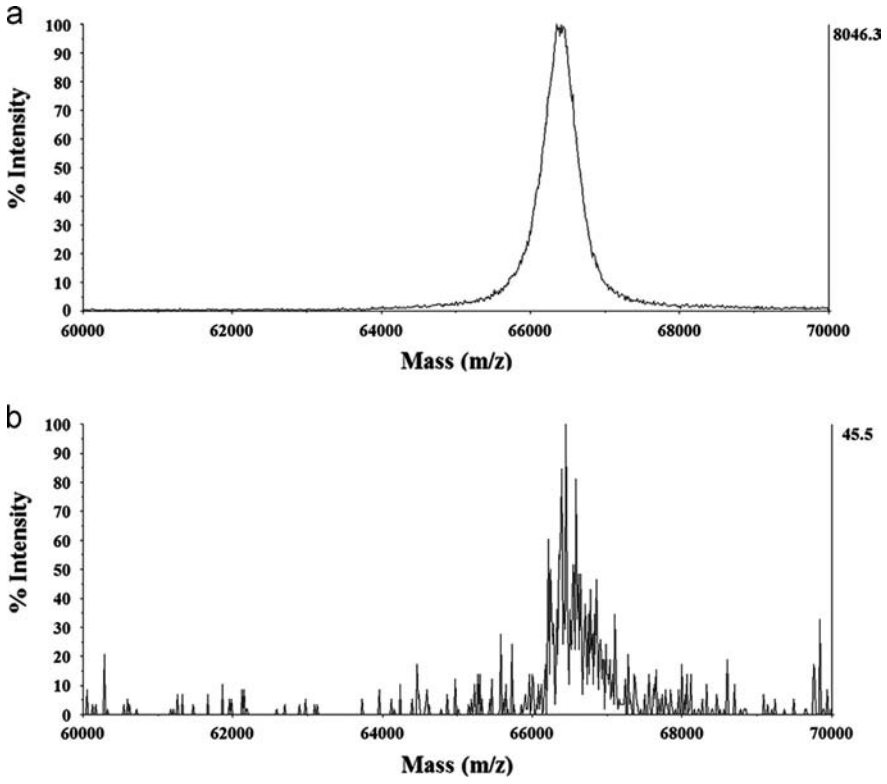

Fig. 5. MALDI-TOF-MS spectra of mixture of DP7 and BSA before rGO–PBH enrichment-derivatization (a) and eluate from rGO after enrichment-derivatization (b).

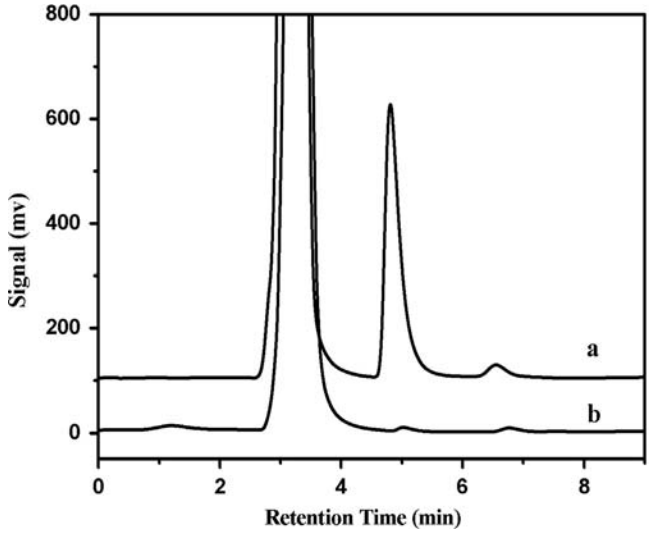

Fig. 6. Liquid chromatography spectra of (a) 0.15 mM PBH in DMF and (b) DMF eluate of 0.15 mM PBH treated rGO.

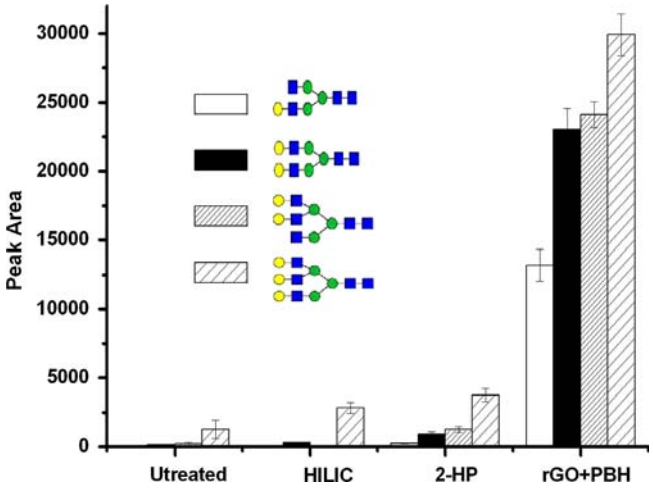

Fig. 7. MS spectra peak area comparison of untreated, HILIC enriched, 2-Hydrazinopyridine derivatized and rGO–PBH treated N-glycans released from asialofetuin.

plasma N-glycan analysis is highly useful for identifying biomarkers for cancer diagnosis. Using only 10 μL human plasma, the free-rGO based "one-step" approach results in the identification of 48 possible N-glycoforms (Fig. 8), which covers all three types of N-glycoforms (high mannose, complex and hybrid types). This result indicates that this efficient and unbiased enrichment and derivatization method can be successfully employed for highly complex samples. Another possible explanation for the improved plasma glycan identification is the efficient removal of large amounts of plasma proteins, as revealed by MALDI-TOF-MS analysis of the high molecular weight range (data not shown). Furthermore, the glycan recovery of this one step method towards plasma sample was also evaluated using DP7 as a model glycan and was found to be $72.5 \pm 5.1\%$ ($n=3$). Though the recovery is lower than that obtained using BSA as background (~100% recovery for DP7), considering the much more complex nature of plasma sample, we think this recovery should be acceptable for biomarker identification.

4. Conclusion

In conclusion, we have established a highly efficient, selective and convenient "one-step" method for glycan enrichment, derivatization and desalting based on the strong π–π interactions between rGO and PBH as well as the specific hydrazide–aldehyde coupling between PBH and glycans. As a result, sample losses are remarkably minimized and improved glycan detection sensitivity

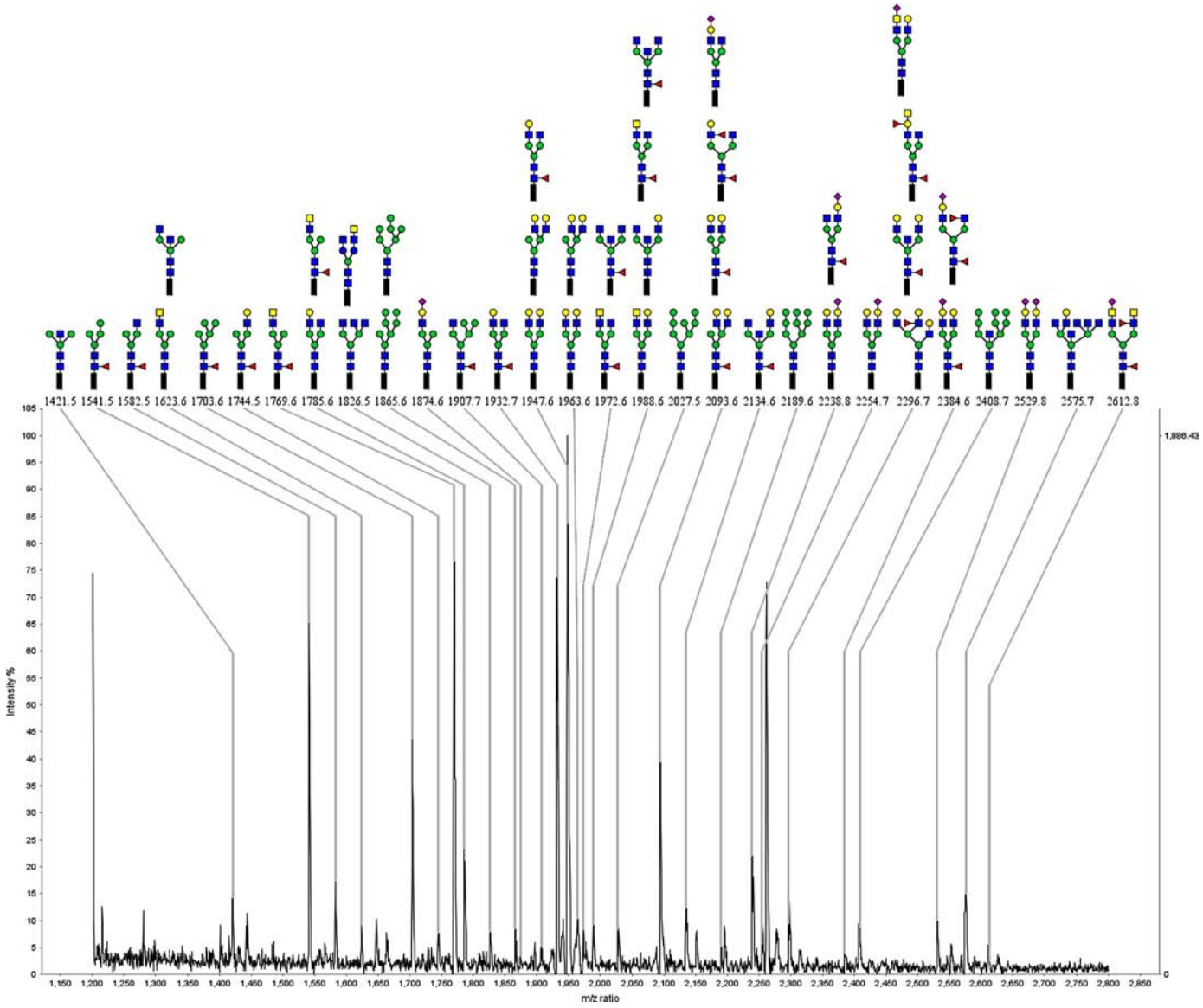

Fig. 8. MALDI-TOF-MS spectrum of rGO–PBH enriched and derivatized N-glycans of human plasma proteins.

is achieved. Hence, this free rGO based "one-step" method offers a promising way for sensitive glycans variation screening in complex biological and clinical samples.

Acknowledgments

This work was supported by National Key Program for Basic Research of China (Nos. 2013CB911204, 2011CB910603), National Key Scientific Instrument Development Program of China (2011YQ09000504), National High-Tech Research and Development Program (2012AA020200), International Scientific Cooperation Project of China (No. 2011DFB30370) and National Natural Science Foundation of China (No. 21275005, 21235001).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.08.031.

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