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Sulfonyl hydrazine-functionalized polymer as a specific capturer of reducing glycans from complex samples for high-throughput analysis by electrospray ionization mass spectrometry†

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Qualitative and quantitative studies of glycosylation patterns of various biologically important proteins represent a key field for the understanding of their complex structure–function relationships. However, the analysis of glycoprotein glycans is usually undermined by tedious sample processing steps prior to detection, including deproteination, desalting and removal of some other non-glycan impurities, which results in considerable sample loss and increased difficulty of quantitative analysis. Herein we report a facile and versatile method for the quantitative isolation of reducing glycans from complex samples using sulfonyl hydrazine-functionalized polystyrene (SHPS) beads, namely the SHPS-based glycan capturing procedure. This method allows the chemoselective and efficient condensation of the aldehyde group of reducing glycans with the active sulfonyl hydrazine group of SHPS beads under anhydrous conditions, resulting in the formation of sulfonyl hydrazone conjugates. The non-glycan components in samples, such as proteins, salts and some other impurities, can be completely removed by washing the sulfonyl hydrazone conjugates. Regeneration of the reducing glycans can be performed *via* mild hydrolysis of the washed sulfonyl hydrazone conjugates. This procedure is compatible with almost all the current techniques for the derivatization or detection of reducing glycans. We have obtained essential data for this method, including optimized reaction conditions, linearity and reproducibility for glycan quantitation, as well as a final glycan recovery ratio. Moreover, mass spectrometric analysis of the glycans from some complex biological samples, including milk, human blood plasma and fetal bovine serum, was achieved, demonstrating good applicability of this novel procedure.

1 Introduction

Glycosylation is a common post-translational covalent modification that confers altered physicochemical properties and functional activities on nascent protein chains.[1] It is estimated that more than half of all naturally occurring proteins and over 70% of all human proteins bear carbohydrate chains (glycans).[2,3] A series of recent advances have revealed the pivotal roles of glycans in many important biological processes, such as cell proliferation and differentiation, cell adhesion, immune response, fertilization and signal transduction.[4–9] Moreover, a number of diseases such as cancer, Walker–Warburg Syndrome and congenital disorders of glycosylation (CDG) have also proved to be associated with abnormal expression of glycoprotein glycans.[10] Therefore, qualitative and quantitative analysis of structurally diverse glycans from various biological samples represents an essential field for the investigation of their complex structure–function correlations (glycomics) and for the understanding of the pathogenesis of many diseases.

In glycomic studies, researchers require high-quality and high-throughput glycan analysis, which relies largely on fast and efficient isolation of target glycans from various complex samples. In general, almost all glycan samples must undergo several tedious purification steps prior to analysis, such as deproteination, desalting, and removal of some other impurities, because most non-glycan components cause severe interference in glycan analysis. Traditional solid phase extraction (SPE) techniques, using for example, cellulose columns,[11] silica gel columns,[12] C18 SPE columns[13,14] and graphitized carbon columns,[15,16] are based on the like-dissolves-like physical extraction processes of target molecules by solid materials from liquid phase, and have been broadly utilized to isolate

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† Electronic supplementary information (ESI) available: RP-HPLC chromatograms of the AEC derivatives of lactose and maltodextrin treated using the SHPS-based glycan capturing procedure, RP-HPLC chromatograms of the AEC derivatives of lactose treated using different functional materials under the optimal conditions of the SHPS-based method, and the data to illustrate the reproducibility of the SHPS-based glycan capturing procedure obtained from the analysis of maltodextrin. See DOI: 10.1039/c3an00931a

glycans from complex mixtures, enabling the recovery of high-purity glycans for high-quality analysis. However, these techniques suffer from some non-negligible defects. Firstly, these SPE approaches usually cause the fractionation of different types of glycans, which results in a prominent loss of some glycans and changes their relative abundance remarkably. Secondly, a single SPE method cannot achieve the complete removal of all types of impurities, this requires a combination of different SPE procedures, resulting in considerable sample loss and a more complex operation. Thirdly, most of these methods do not result in the stable and quantitative recovery of various types of glycans, increasing the difficulties of quantitative analysis. Immobilized lectin affinity columns permit the rapid and highly-selective enrichment of some glycans, but are only applicable to certain types of glycans, limiting their extensive application. Thus, the fast and quantitative isolation of various glycans from complex mixtures remains a challenge in glycomic studies.

Recently, some researchers have introduced several reversible chemical reactions into the purification of glycans and have developed some chemoselective glycan isolation methods, such as those based on hydrazide-functionalized beads,[17,18] oxime-functionalized beads,[19] aldehyde-functionalized polymers,[20,21] and boronic acid-functionalized beads.[22,23] These methods are generally composed of three operation steps, including covalent conjugation of glycans with certain types of solid functional materials, removal of impurities by washing the glycan-attached solid materials, and regeneration of free glycans using reverse reactions. Due to the highly selective and intense combination of glycans with solid functional materials, these approaches allow the rapid isolation and efficient purification of different types of glycans and promise great advances in glycomic studies. However, most of these methods have some problems, such as complex preparation processes for functional polymers due to their commercial unavailability and the inability to achieve stable and rigorous quantitative isolation of glycans, which results in inaccurate quantitative analysis of glycans and greatly restricts the scope of their applications.

Herein, we present a facile and versatile procedure, in pursuit of high-throughput quantitative isolation of reducing glycans from complex mixtures using a commercially available functional material, to assist in the development of the chemoselective glycan extraction methods and their applications in glycomic studies. In this study, a novel type of functional polymer, sulfonyl hydrazine-functionalized polystyrene (SHPS), is introduced into the quantitative extraction and purification of reducing glycans, without any complex preparation steps for functional materials. This method features efficient and quantitative covalent conjugation of the aldehyde group of reducing glycans with the active sulfonyl hydrazine group of the SHPS beads under anhydrous conditions, resulting in the formation of a sulfonyl hydrazone bond. The unreactive non-glycan components in the samples, such as proteins, salts and some other impurities, can be completely removed by repeatedly washing the sulfonyl hydrazone conjugate beads. The quantitative regeneration of the free reducing glycans can be performed *via* a mild hydrolysis of the washed sulfonyl hydrazone conjugates. The suggested procedure is compatible with almost all the current techniques for the derivatization or detection of reducing glycans. We have obtained fundamental data for this method using maltodextrin and lactose as model glycans, including the optimized reaction conditions, the linearity and reproducibility for glycan quantitation, and the glycan recovery. Moreover, this method was successfully applied to the efficient isolation and analysis of the glycans from some glycoproteins, including bovine ribonuclease B (RNase B) and hen egg albumin (ovalbumin), as well as a series of complex biological samples, such as milk, human blood plasma and fetal bovine serum (FBS), demonstrating the excellent applicability of this novel procedure.

2 Experimental

2.1 Chemicals and materials

Lactose was purchased from Beijing Aoboxing Biotech Co. Ltd (Beijing, China). Macroporous sulfonyl hydrazine-functionalized polystyrene (SHPS) beads, maltodextrin, dimethyl sulfoxide (DMSO), 3-amino-9-ethylcarbazole (AEC), sodium cyanoborohydride ($NaBH_3CN$), ovalbumin (hen egg white albumin), β-cyclodextrin and ribonuclease B (RNase B) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrazide gel was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Polymer-supported tris(2-aminoethyl)amine was purchased from Alfa-Aesar (Ward Hill, MA, USA). Hydrazide magnetic beads were provided by Prof. Yali Cui (College of Life Science, Northwest University). Analytical grade *N*,*N*-dimethylformamide (DMF) was a product of Tianjin North Fine Chemical Co., Ltd. Analytical grade glacial acetic acid and aqueous ammonia (26–28%, v/v) were purchased from Xi'an Sanpu Chemical Reagent Co. Ltd (Xi'an, Shaanxi, China), and analytical grade methylene dichloride and ammonium acetate from Tianli Chemical Reagent Co. Ltd (Tianjin, China). Peptide *N*-glycosidase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA, USA). Non-porous graphitized carbon (Carbograph) solid phase extraction (SPE) columns (150 mg/4 mL) were purchased from Alltech Associates (Deerfield, IL, USA). Sodium dodecyl sulfate (SDS), DL-dithiothreitol (DTT), and Nonidet P-40 (NP-40) were purchased from Aladdin Industrial Inc. (Shanghai, China). Analytical grade sodium phosphate was from Shantou Xilong Chemical Factory (Guangdong, China). HPLC grade acetonitrile (ACN) and methanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). Water used was purified *via* a Milli-Q ultrapure water purification system from Millipore (Burlington, MA, USA). Human milk was provided by a healthy volunteer (at 20 days after parturition) who attended the First Affiliated Hospital of Medical College (Xi'an Jiao Tong University, China). Porcine milk was collected from healthy pigs at a farm in the Enping Trial Center of Premix Inve Nutrition (Shenzhen) Co., Ltd (Guangdong Province, China). Bovine milk and ovine milk were collected from a healthy cow and a healthy goat, respectively, in Lantian Pasture (Shaanxi Province, China). Fetal bovine serum (FBS) was from Beijing Dingguo Changsheng Biotech. Co. Ltd (Beijing, China). O-Type human plasma (Rh^+) from a healthy volunteer was provided by the Second

Affiliated Hospital of Xi'an Jiao Tong University. MD34 (8000–14 000) dialysis membrane was the product of Union Carbide Co. (Danbury, CT, USA).

2.2 Pretreatment of blood samples

2 mL of human plasma or FBS was dialyzed against 1 L of Milli-Q water at 4 °C for 72 hours. During this process, the water was refreshed once every 12 hours. The dialyzed blood samples were finally lyophilized and stored at −20 °C for further use.

2.3 Release of *N*-glycans using PNGase F

This experiment was performed based on a previously reported procedure.[24] Concisely, 900 μL of protein denaturation solution containing 0.4 M DTT and 5% SDS was added to a screw-capped tube containing 1–2 mg of glycoprotein or 5–8 mg of lyophilized serum sample, prior to heating at 100 °C for 10 minutes. The sample was cooled down, and then 90 μL of 1 M sodium phosphate buffer (pH 7.5), 90 μL of 10% aqueous NP-40 (v/v) and 2 μL of PNGase F solution (1000 unit) were successively added. After incubation at 37 °C for 24 hours, the sample was heated in boiling water for 5 minutes and lyophilized.

2.4 Isolation of reducing glycans using SHPS beads

The SHPS beads were heated in DMF at 90 °C for 2 hours, ultrasonically washed with methanol at 45 °C for 2 hours, dried at 80 °C for 1 hour in an oven, and stored at −20 °C till use. To capture reducing glycans, 10 mg of the pre-processed SHPS beads, 212.5 μL of DMF and 37.5 μL of glacial acetic acid were added to a screw-capped tube containing lyophilized glycan sample to be purified. The tube was vigorously shaken using a vortex shaker for 3 minutes, and then heated at 90 °C for 4 hours. In this glycan capturing process, the sample was vigorously vibrated once every 30 minutes. After the conjugation reaction was completed, the DMF–acetic acid solution and many small solid protein particles in the sample were removed using a Transferpettor, and the remaining glycan-covered SHPS beads in the sample were ultrasonically washed several times with 50% (v/v) methanol in aqueous ammonia and Milli-Q water, successively. For the regeneration of the reducing glycans, 200 μL of 1% (v/v) aqueous acetic acid solution was added to the washed glycan-covered SHPS beads, and then the sample was incubated at 70 °C for 1 hour. The resulting aqueous glycan solution was finally separated from the SHPS beads using a Transferpettor.

2.5 Labeling of reducing glycans with AEC

This procedure was based on a previously reported method.[25,26] Briefly, 200 μL of 0.5 M AEC in methanol or DMF, 50 μL of 2 M aqueous $NaBH_3CN$ solution, 50 μL of acetic acid and 200 μL of aqueous glycan solution were successively added to a screw-capped tube and mixed well by shaking. Then the tube was heated at 70 °C for 1 hour. After the reaction was completed, 20 μL of aqueous ammonia was added to neutralize the acid in the sample solution. The neutralized sample solution was extracted with 1 mL of methylene dichloride to remove excess AEC. After centrifugation of the sample at 12 000 r min^{-1} for 3 minutes, the aqueous layer was taken out and diluted with Milli-Q water for chromatographic and MS analysis.

2.6 HPLC separation

The HPLC analysis was performed on a Waters Alliance 2695 separation module coupling with an in-line Waters 2996 photodiode array (PDA) detector, using a 4.6 mm × 250 mm SinoChrom C8 column (5 μm) (Dalian Elite Analytical Instruments Co., Ltd., China) at room temperature. The detection wavelength was set at 254 nm, and data were collected and processed using Waters Empower software. The sample injection volume was 20 μL. Solvent A was ACN. Solvent B was 100 mM ammonium acetate, titrated to pH 4.5 with glacial acetic acid, in Milli-Q water. Solvent C was Milli-Q water. The sample separation gradient was as follows: time = 0 min ($t = 0$), 10% A, 90% B (1 mL min^{-1}); $t = 90$, 25% A, 75% B (1 mL min^{-1}). An alternative sample separation gradient was as follows: $t = 0$, 22% A, 78% B (0.8 mL min^{-1}); $t = 30$, 28% A, 72% B (0.8 mL min^{-1}). The column regeneration gradient was as follows: $t = 0$, 25% A, 75% C (1 mL min^{-1}); $t = 30$, 95% A, 5% C (1 mL min^{-1}); $t = 45$, 95% A, 5% C (1 mL min^{-1}).

2.7 ESI-MS analysis

The MS analysis was performed with an LTQ XL linear ion trap electrospray ionization mass spectrometer coupled with a high-performance liquid chromatography system (Thermo Scientific, USA) using a set of unoptimized experimental conditions. The samples were infused *via* a 2 μL Rheodyne loop and brought into the electrospray ion source by a stream of 50% methanol at a flow rate of 200 μL min^{-1}. The spray voltage was set at 4 kV, with a sheath gas (nitrogen gas) flow rate of 30 arb., an auxiliary gas (nitrogen gas) flow rate of 5.0 arb., a capillary voltage of 37 V, a tube lens voltage of 250 V, and a capillary temperature of 375 °C. The MS data were recorded using LTQ Tune software.

2.8 On-line LC–MS and MS/MS analysis

The glycan samples were separated using a 2.1 mm × 150 mm XBridge C18 column (3.5 μm) (Waters, USA) at room temperature. The sample injection volume was 20 μL. Solvent A was ACN, and solvent B was 10 mM ammonium acetate (pH 4.5). The separation gradient was as follows: time = 0 min ($t = 0$), 10% A, 90% B (200 μL min^{-1}); $t = 90$, 25% A, 75% B (200 μL min^{-1}). MS/MS analysis was carried out using helium (He) as the collision gas, a normalized collision energy degree of 57%, and an isotope width of m/z 1.00. The MS data were recorded using Xcalibur software. The other conditions were the same as those described in above section.

3 Results and discussion

3.1 Principle of the SHPS-based glycan capturing procedure

The novel method, termed the SHPS-based glycan capturing procedure, involves reversible reactions between the aldehyde groups of reducing glycans and the sulfonyl hydrazine groups of a solid functional material, SHPS beads. Aldehydes react

selectively with amine- or hydrazine-containing molecules catalyzed by acids, resulting in the formation of Schiff's bases or hydrazones.[27] However, the produced Schiff's bases or hydrazones are unstable and are easily hydrolyzed in aqueous solutions of acids, resulting in the regeneration of aldehydes as well as amines or hydrazides. Moreover, the directions of the reversible reactions between aldehydes and amines or hydrazides can be controlled *via* a change of the reaction conditions.[28,29] Thus, the formation reaction and hydrolysis reaction of hydrazone or Schiff's base can be combined and utilized to efficiently isolate aldehydes from complex mixtures using solid materials bearing amine or hydrazine groups.[17–19] The chemical reactivity of amine or hydrazine groups to aldehyde groups rather than other types of chemical groups guarantees the perfect isolation specificity of this method for aldehydes.[27] Obviously, our SHPS-based glycan capturing procedure follows this principle.

The SHPS-based procedure consists of three operation steps (Fig. 1). (i) Coupling: the reducing glycans react with sulfonyl hydrazine groups immobilized on polystyrene (PS) beads, under the catalysis of organic acids in anhydrous solvents, to form sulfonyl hydrazone bonds. (ii) Washing: the produced solid sulfonyl hydrazone conjugates are repeatedly washed with different types of solvents to remove all the unreacted non-glycan components, such as salts, proteins, peptides, amino acids, lipids and some other small molecules. (iii) Regeneration: the washed sulfonyl hydrazone conjugates hydrolyze in mild aqueous acids, to regenerate free reducing glycans for glycomic studies and active SHPS beads for further use. These operation steps are very simple and theoretically suitable for highly selective and high-throughput isolation of reducing glycans from various complex biological samples, such as glycoproteins, body fluids, cells, tissues and organisms. More importantly, the obtained reducing glycans are identical to those before isolation in structure and can be derivatized using almost all the extensively used glycan derivatization methods for structural identification and quantitative analysis by various techniques, such as high-performance liquid chromatography (HPLC), gas chromatography coupling with mass spectrometry (GC–MS), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), electrospray ionization mass spectrometry (ESI-MS), liquid chromatography coupling with mass spectrometry (LC–MS), tandem mass spectrometry (MS/MS and MS^n) and glycan microarrays.[30,31]

3.2 Optimization of reaction conditions

The two sets of reaction conditions of the SHPS-based procedure for glycan coupling and regeneration, respectively, were optimized in a stepwise manner, using maltodextrin and lactose as model glycans, to seek efficient and quantitative isolation of reducing glycans. On the one hand, with reference to previously reported related reaction conditions for the formation of hydrazones,[32] the conjugation conditions including the type of glycan solvent, reaction temperature, acetic acid content and reaction time were successively varied and optimized, based on the quantitative analysis of the residual glycans as AEC derivatives after conjugation reactions by reversed-phase HPLC

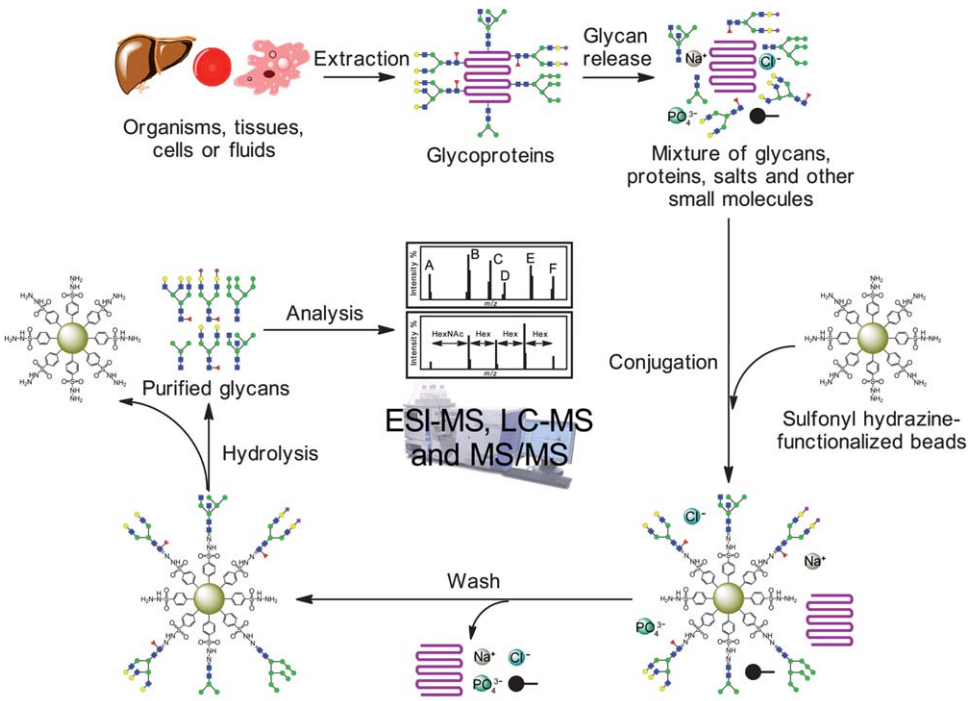


Fig. 1 Schematic diagram of the SHPS-based glycan capturing strategy. This strategy consists of several operation steps, including extraction of glycoproteins, release of glycans from glycoproteins, sample drying and reconditioning in anhydrous solvent, chemoselective covalent conjugation of released reducing glycans with SHPS beads, removal of non-glycan impurities by washing the glycan-covered beads and regeneration of reducing glycans by hydrolysis.

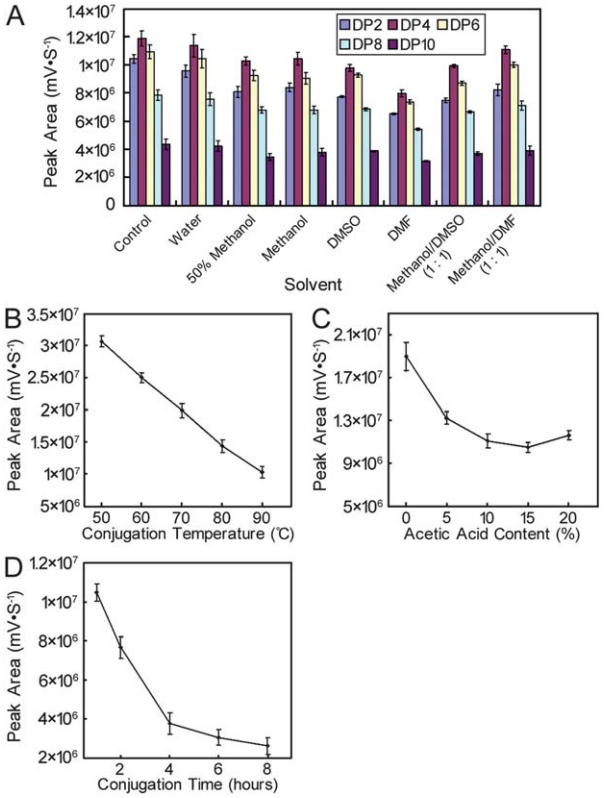


Fig. 2 Optimization of reaction conditions for the conjugation of reducing glycans with SHPS beads. The optimized conditions were obtained according to the RP-HPLC peak area of AEC derivatives of residual glycans obtained after the conjugation reaction. (A) Optimization of the type of glycan solvent using maltodextrin as model glycans. (B) Optimization of reaction temperature using lactose as a model glycan. (C) Optimization of the content of acetic acid in the solvent using lactose as a model glycan. (D) Optimization of conjugation time using lactose as a model glycan. DP, degrees of polymerization of glycans.

(RP-HPLC) (see Fig. S1† and 2). The results indicated a set of optimal reaction conditions for the conjugation of reducing glycans with SHPS beads, including DMF as the reaction solvent, a reaction temperature of 90 °C, acetic acid content of 15% and a reaction time of 4 hours. Under these conditions, the reaction solvent contributes much to the conjugation rate of reducing glycans with SHPS beads. As shown in Fig. 2A, water causes the lowest glycan conjugation rate, indicating the conspicuous influence of the water content of solvents on the directions of the reactions between reducing glycans and SHPS beads. In contrast, DMF gives the highest conjugation rate, suggesting the good solubility of glycans with different degrees of polymerization (DP) in DMF, as well as a certain catalytic activity of DMF in the conjugation reaction.

On the other hand, with reference to previously reported related reaction conditions for the hydrolysis of hydrazones,[17] the glycan regeneration conditions including acetic acid content, reaction temperature and reaction time were successively varied and optimized, based on the quantitative analysis of the regenerated glycans as AEC derivatives after hydrolysis reactions by RP-HPLC (see Fig. S1† and 3). The results indicated a set of optimal reaction conditions for the hydrolysis of the

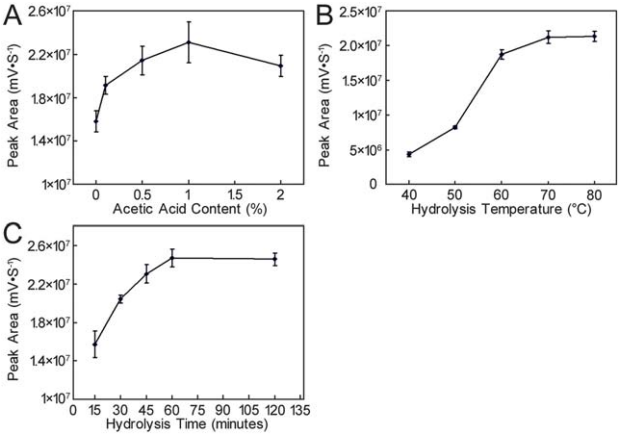


Fig. 3 Optimization of reaction conditions for the release of glycans from SHPS beads. The optimized conditions were obtained according to the RP-HPLC peak area of AEC derivatives of lactose obtained after the hydrolysis reaction. (A) Optimization of the content of acetic acid in water. (B) Optimization of reaction temperature. (C) Optimization of reaction time.

sulfonyl hydrazone conjugates, including an acetic acid content of 1% in water, a reaction temperature of 70 °C and a reaction time of 60 minutes. Importantly, no by-products such as glucose and galactose were observed in the RP-HPLC profile of the regenerated lactose, indicating clearly the structural integrity of the glycans isolated with the SHPS-based method (Fig. S1†).

3.3 Evaluation of the SHPS-based glycan capturing procedure using model glycans

The quantitative capability, isolation efficiency and stability of our novel method were investigated. Firstly, the quantitative linearity was confirmed using various levels of concentrations of lactose in DMF containing 15% acetic acid (ranging from 0.04 mM to 120 mM). Each sample was treated with the SHPS-based glycan capturing method under the optimal conditions

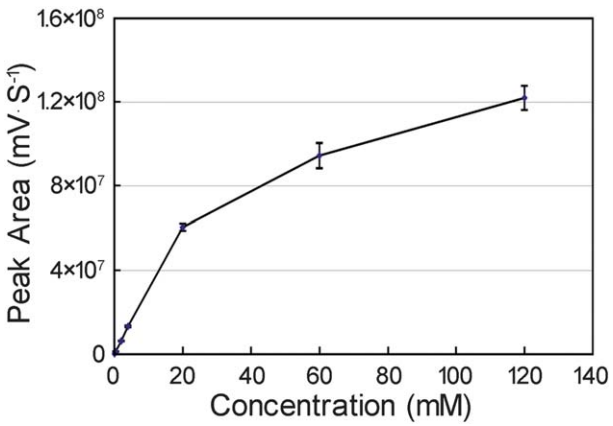


Fig. 4 Verification of the correlation between peak area and concentration of lactose. The vertical axis represents the total RP-HPLC peak area of AEC derivatives of lactose obtained using the SHPS-based capturing procedure, and the horizontal axis represents the concentration of lactose loaded before its conjugation to SHPS beads.

described above, and the regenerated lactose was derivatized with AEC and analyzed by RP-HPLC. The correlation between the peak area and the concentration of lactose was verified. As shown in Fig. 4, the peak area of the regenerated lactose as an AEC derivative increases linearly with the rise of the concentration of lactose from 0.04 mM to 20 mM, demonstrating the invariability of the glycan recovery and the good quantitative capability of our method over a wide glycan concentration range, as well as the large glycan-carrying capacity of the SHPS beads. Meanwhile, the glycan recovery gradually decreases when the concentration of lactose increases above 20 mM, due to the fact that the amount of glycans is close to the amount of sulfonyl hydrazine groups on the SHPS beads. However, this does not interfere with the general conclusion that the SHPS-based procedure can achieve quantitative recovery of reducing oligosaccharides in a broad concentration range.

Secondly, the glycan recovery of the SHPS-based procedure was evaluated using lactose as a model glycan. A 20 mM lactose sample dissolved in DMF containing 15% acetic acid was treated with the SHPS-based glycan capturing method, and the glycan recovery was calculated based on the quantitative analysis of the original and regenerated lactose as an AEC derivative by RP-HPLC. As a result, the glycan recovery of the SHPS-based procedure was determined to be 63.9%, showing the acceptable isolation efficiency of the method. In addition, several other types of commercially available amine- or hydrazide-containing functional materials were also examined under the reaction conditions developed merely on the basis of the SHPS beads as described above. As shown in Fig. S2† and 5, these amine- or hydrazide-containing functional materials achieve far lower glycan recoveries than the SHPS beads. This might be caused by the fact that the testing reaction conditions are not optimal for these amine- or hydrazide-containing functional materials. These results indicate that only the SHPS beads can achieve a high glycan recovery under the newly developed reaction conditions.

Finally, the stability and reliability of the SHPS-based glycan capturing procedure was verified. As shown in Fig. S1B†, the obtained RP-HPLC profiles show a high correspondence between the original and regenerated oligosaccharides in relative abundance. The coefficients of variation (CV) for the peak areas of different types of oligosaccharides vary from 0.7% to 3.3% ($n = 3$), showing the good reproducibility of the novel method (see Table S1†). Quantitative investigations of the procedure on purified *N*-glycans using β-cyclodextrin as an internal standard (see Fig. S3†) also achieved similar glycan recoveries, without conspicuous variance in relative abundance of different types of glycans. These results indicate that the suggested procedure causes no fractionation of different types of oligosaccharides. In contrast, lower recoveries were obtained when the method was tested on monosaccharides. We found that the recoveries of *N*-acetylglucosamine and *N*-acetylgalactosamine were around two thirds of those obtained for lactose, and glucose, recoveries for galactose, mannose and fucose were around one fifth of that of lactose, with larger CV values than those of oligosaccharides. This indicates that the

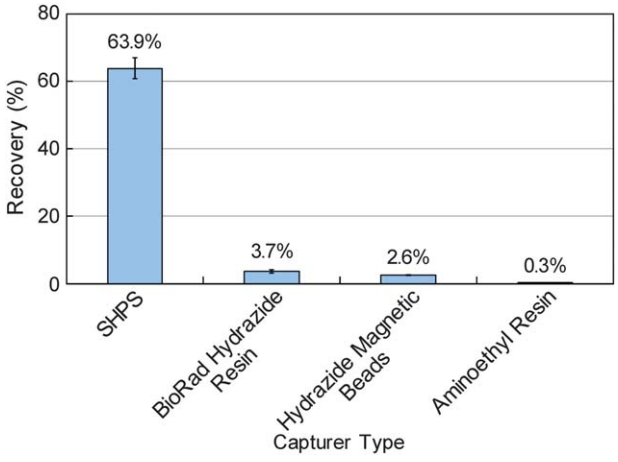


Fig. 5 Recovery ratios of lactose obtained using different functional materials under the reaction conditions developed merely on the basis of SHPS beads. The recovery ratio was calculated according to the RP-HPLC peak area of AEC derivatives of lactose at a loading concentration of 20 mM before the conjugation reaction.

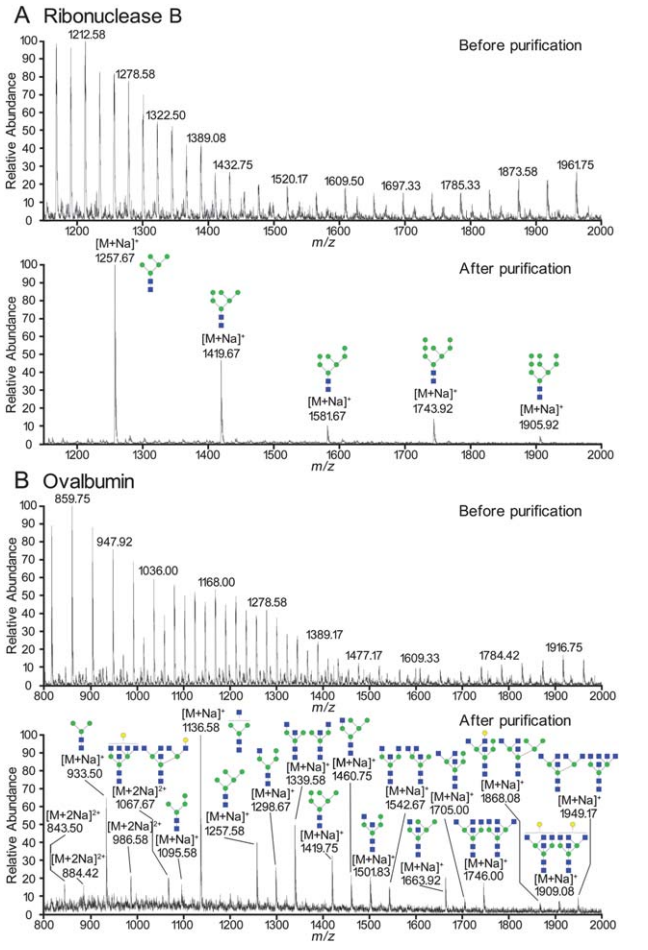


Fig. 6 Verification of the isolation selectivity of the SHPS-based glycan capturing procedure using model glycoproteins. (A) ESI-MS spectra of *N*-glycans released from ribonuclease B before and after purification. (B) ESI-MS spectra of *N*-glycans released from ovalbumin before and after purification. Structural formulae: blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose.

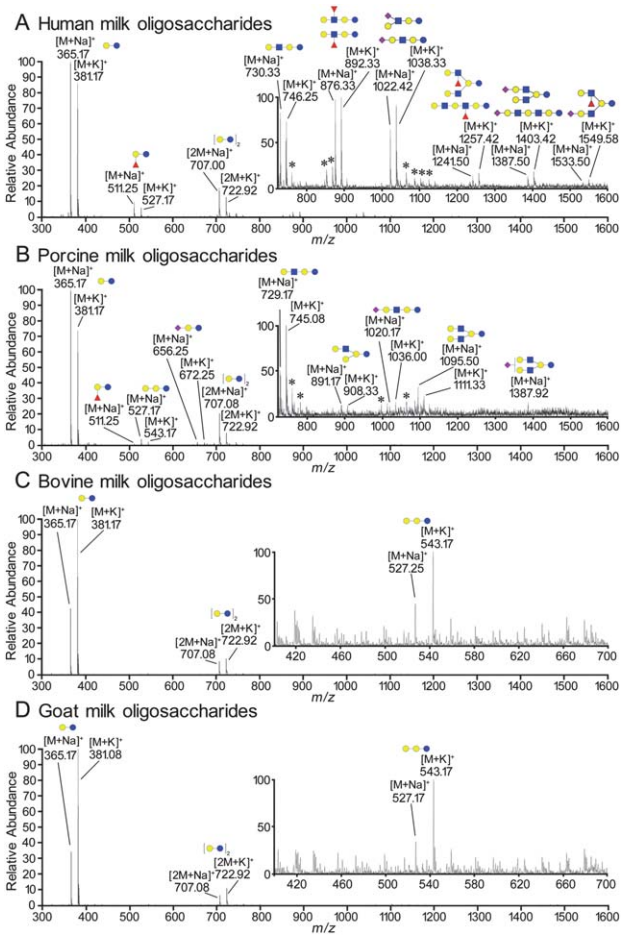


Fig. 7 ESI-MS spectra of free reducing oligosaccharides isolated from different milk samples using the SHPS-based glycan capturing procedure. (A) Human milk oligosaccharides. (B) Porcine milk oligosaccharides. (C) Bovine milk oligosaccharides. (D) Goat milk oligosaccharides. Structural formulas: blue circle, glucose; yellow circle, galactose; blue square, *N*-acetylglucosamine; red triangle, fucose; purple diamond, *N*-acetylneuraminic acid. "*" denotes unknown peaks.

SHPS-based procedure is not suitable for the quantitative analysis of monosaccharides under the reaction conditions recommended here. However, this does not interfere with the compatibility of the SHPS-based procedure with the quantitative analysis of oligosaccharides. Therefore, our SHPS-based procedure possesses good isolation stability and reliability for different types of oligosaccharides.

3.4 Isolation selectivity of the SHPS-based glycan capturing procedure

The SHPS-based glycan capturing method was applied to the analysis of *N*-glycans released from some model glycoproteins and the free reducing oligosaccharides of some milk samples, to investigate the feasibility and glycan isolation selectivity of the method. First of all, two purified glycoproteins, bovine RNase B and hen ovalbumin, were digested with PNGase F and lyophilized, prior to glycan isolation using the SHPS-based glycan capturing procedure as described above and analysis by ESI-MS and MS/MS. As shown in Fig. 6, the ESI-MS profiles of the two samples both exhibit a mass of complex ion peaks of peptides before purification. After purification using our SHPS-based procedure, however, the two samples give a series of sodiated molecular ion peaks of typical neutral *N*-glycans. The *N*-glycans released from RNase B are all of a high-mannose type, while those from ovalbumin are a mixture of high-mannose, complex and hybrid type, in accordance with previous literature reports.[31–34] The MS profiles of the two purified glycan samples both had high signal-to-noise ratios, without any conspicuous signals of peptides, degraded glycans or other types of impurities, indicating definitely the feasibility and excellent glycan isolation selectivity of the novel method.

Moreover, four types of biological samples, including human milk, porcine milk, bovine milk and goat milk, were also purified using the SHPS-based glycan capturing procedure, and the obtained free reducing oligosaccharides were analyzed by ESI-MS and MS/MS. As a result, eight human milk oligosaccharides, nine porcine milk oligosaccharides, two bovine milk oligosaccharides and two goat milk oligosaccharides were successfully detected (see Fig. 7), in accordance with previous literature reports.[35–38] It was noted that three sialylated human milk oligosaccharides and three sialylated porcine milk

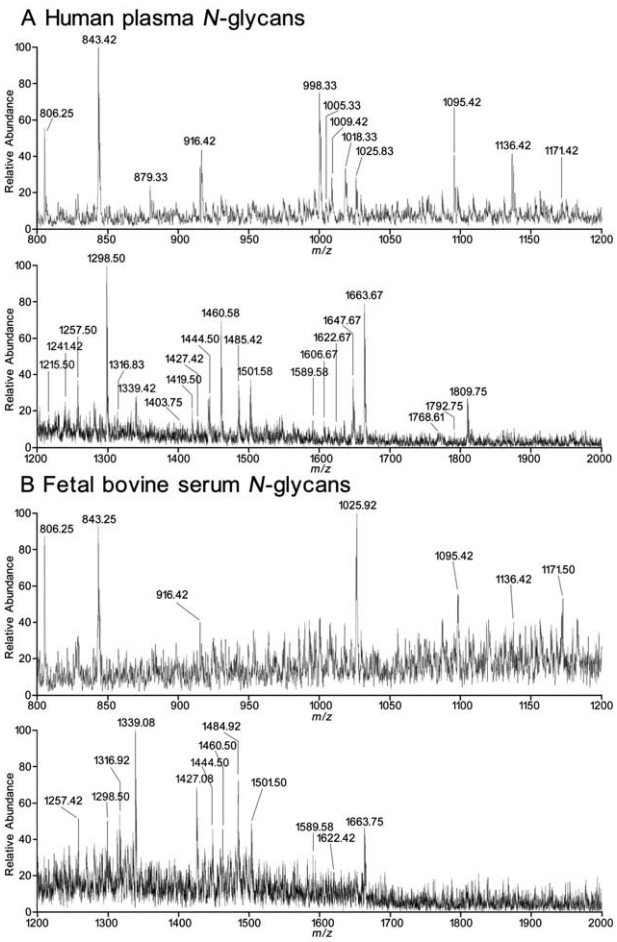


Fig. 8 ESI-MS spectra of *N*-glycans released from complex biological samples purified using the SHPS-based glycan capturing procedure. (A) Human plasma *N*-glycans. (B) FBS *N*-glycans.

Table 1 Composition and structure of SHPS-captured *N*-glycans from human plasma and FBS identified by ESI-MS and on-line LC–MS/MS[a]

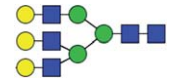
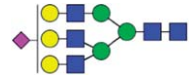
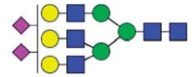
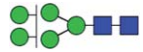
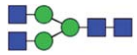
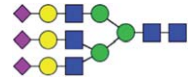
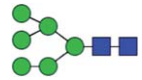
Human plasma			Fetal bovine serum				
Observed *m/z* (native/AEC-derivatized)	Charge state (native/AEC-derivatized)	Fraction number in LC-MS TIC profile	Observed *m/z* (native/AEC-derivatized)	Charge state (native/AEC-derivatized)	Fraction number in LC-MS TIC profile	Monosaccharide composition	Proposed main structure
1025.83/1111.83	$[M + 2Na]^{2+}/[M + H + Na]^{2+}$	H4	1025.92/1111.83	$[M + 2Na]^{2+}/$ $[M + H + Na]^{2+}$	F5	H_6N_5	
1171.42/1248.33, 1257.75	$[M + 2Na]^{2+}/[M + 2H]^{2+}$, $[M + H + Na]^{2+}$	H4, H6, H7	1171.50/1248.42, 1257.83	$[M + 2Na]^{2+}/[M + 2H]^{2+}$, $[M + H + Na]^{2+}$	F5, F7, F8	$A_1H_6N_5$	
1095.42/1290.42	$[M + Na]^{+}/[M + Na]^{+}$	H9, H16	1095.42/1290.50	$[M + Na]^{+}/[M + Na]^{+}$	F9, F16	H_4N_2	
1136.42/1308.25	$[M + Na]^{+}/[M + H]^{+}$	H2, H4	1136.42/1308.33	$[M + Na]^{+}/[M + H]^{+}$	F4, F6	H_3N_3	
1316.83/1402.75	$[M + 2Na]^{2+}/[M + H + Na]^{2+}$	H1, H4	1316.92/1402.92	$[M + 2Na]^{2+}/[M + H + Na]^{2+}$	F5	$A_2H_6N_5$	
1257.50/1429.42, 1451.33	$[M + Na]^{+}/[M + H]^{+}$, $[M + Na]^{+}$	H16, H20	1257.42/1429.42, 1452.42	$[M + Na]^{+}/[M + H]^{+}$, $[M + Na]^{+}$	F15, F17	H_5N_2	
1241.42/1436.50	$[M + Na]^{+}/[M + Na]^{+}$	H18, H24	—/1436.42	—/$[M + Na]^{+}$	F21	$F_1H_4N_2$	
1298.50/1470.42, 1492.33	$[M + Na]^{+}/[M + H]^{+}$, $[M + Na]^{+}$	H8, H10	1298.50/1470.42, 1492.33	$[M + Na]^{+}/[M + H]^{+}$, $[M + Na]^{+}$	F9, F11	H_4N_3	
1339.42/1511.25	$[M + Na]^{+}/[M + H]^{+}$	H26	1339.08/—	$[M + Na]^{+}$/—	—	H_3N_4	
—/1548.83	—/$[M + H + Na]^{2+}$	H4	—/1548.42	—/$[M + H + Na]^{2+}$	F5	$A_3H_6N_5$	
1419.50/1591.33, 1614.42	$[M + Na]^{+}/[M + H]^{+}$, $[M + Na]^{+}$	H12, H22	—/1591.33	—/$[M + H]^{+}$	F13	H_6N_2	
1427.42/1599.42	$[M + Na]^{+}/[M + H]^{+}$	H1, H4	1427.08/1599.42	$[M + Na]^{+}/[M + H]^{+}$	F3, F5	$A_1H_3N_3$	
1444.50/1639.58	$[M + Na]^{+}/[M + Na]^{+}$	H25	1444.50/1639.50	$[M + Na]^{+}/[M + Na]^{+}$	F23	$F_1H_4N_3$	
1460.58/1632.33, 1654.33	$[M + Na]^{+}/[M + H]^{+}$, $[M + Na]^{+}$	H13	1460.50/1654.50	$[M + Na]^{+}/[M + Na]^{+}$	F10, F12	H_5N_3	

Table 1 (*Contd.*)

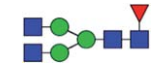
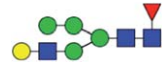
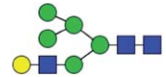
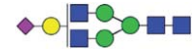
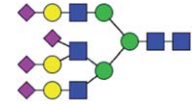
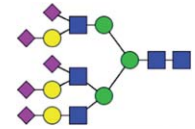
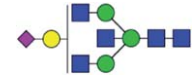
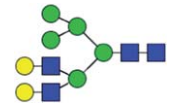
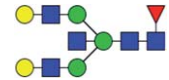
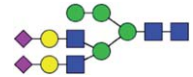
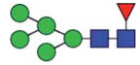
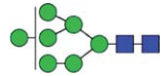
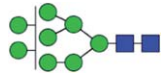
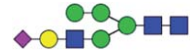
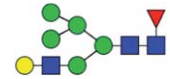
Human plasma			Fetal bovine serum				
Observed *m/z* (native/AEC-derivatized)	Charge state (native/AEC-derivatized)	Fraction number in LC-MS TIC profile	Observed *m/z* (native/AEC-derivatized)	Charge state (native/AEC-derivatized)	Fraction number in LC-MS TIC profile	Monosaccharide composition	Proposed main structure
1485.42/1657.50, 1679.33	$[M + Na]^+/[M + H]^+$, $[M + Na]^+$	H21	1484.92/1657.42	$[M + Na]^+/[M + H]^+$	F20	$F_1H_3N_4$	
1501.58/1673.42	$[M + Na]^+/[M + H]^+$	H5, H14	1501.50/1673.42	$[M + Na]^+/[M + H]^+$	F7	H_4N_4	
806.25, 1589.58/1761.42	$[M + 2Na]^{2+}$, $[M + Na]^+/[M + H]^+$	H8	806.25, 1589.58/1761.42	$[M + 2Na]^{2+}$, $[M + Na]^+/[M + H]^+$	F9	$A_1H_4N_3$	
1606.67/1801.58	$[M + Na]^+/[M + Na]^+$	H17	—/1801.42	—/$[M + Na]^+$	F22	$F_1H_5N_3$	
1622.67/1817.58	$[M + Na]^+/[M + Na]^+$	H20	1622.42/1817.58	$[M + Na]^+/[M + Na]^+$	F15	H_6N_3	
1647.67/1819.58	$[M + Na]^+/[M + H]^+$	H19	—/1819.42	—/$[M + H]^+$	F19	$F_1H_4N_4$	
843.42, 1663.67/1835.58, 1857.50	$[M + 2Na]^{2+}$, $[M + Na]^+/[M + H]^+$, $[M + Na]^+$	H13	843.25, 1663.75/1835.50, 1857.58	$[M + 2Na]^{2+}$, $[M + Na]^+/[M + H]^+$, $[M + Na]^+$	F14	H_5N_4	
1792.75/1964.67	$[M + Na]^+/[M + H]^+$	H4	—/1964.58	—/$[M + H]^+$	F5	$A_1H_4N_4$	
916.42, 1809.75/1981.50	$[M + 2Na]^{2+}$, $[M + Na]^+/[M + H]^+$	H18	916.42/1981.50	$[M + 2Na]^{2+}/[M + H]^+$	F18	$F_1H_5N_4$	
—	—	—	—/1693.92	—/$[M + H + Na]^{2+}$	F2	$A_4H_6N_5$	
—	—	—	—/1828.50	—/$[M + 2H]^{2+}$	F1	$A_5H_6N_5$	
998.33, 1009.42/1094.67	$[M + H + Na]^{2+}$, $[M + 2Na]^{2+}/[M + H + Na]^{2+}$	H17	—	—	—	$A_1H_4N_5$	

Table 1 (*Contd.*)

Human plasma			Fetal bovine serum				
Observed *m/z* (native/AEC-derivatized)	Charge state (native/AEC-derivatized)	Fraction number in LC-MS TIC profile	Observed *m/z* (native/AEC-derivatized)	Charge state (native/AEC-derivatized)	Fraction number in LC-MS TIC profile	Monosaccharide composition	Proposed main structure
1005.33/1102.83	$[M + 2Na]^{2+}/[M + 2Na]^{2+}$	H3, H7	—	—	—	H_7N_4	
1018.33/-	$[M + 2Na]^{2+}$/—	—	—	—	—	$F_1H_5N_5$	
1215.50/1146.25	$[M + 2Na]^{2+}/[M + 2H]^{2+}$	H1	—	—	—	$A_2H_6N_4$	
1403.75/1598.42	$[M + Na]^{+}/[M + Na]^{+}$	H23	—	—	—	$F_1H_5N_2$	
—/1753.42	—/$[M + H]^{+}$	H12	—	—	—	H_7N_2	
879.33/1907.50	$[M + 2Na]^{2+}/[M + H]^{+}$	H15, H22	—	—	—	$F_1A_1H_4N_3$	
—/1915.50	—/$[M + H]^{+}$	H10, H11, H23	—	—	—	H_8N_2	
—/1946.42	—/$[M + Na]^{+}$	H3	—	—	—	$A_1H_5N_3$	
1768.61/1963.58	$[M + Na]^{+}/[M + Na]^{+}$	H4	—	—	—	$F_1H_6N_3$	

[a] Structural formulas: F, deoxyhexose; A, *N*-acetylneuraminic acid; H, hexose; N, *N*-acetylhexosamine; blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; red triangle, fucose; purple diamond, *N*-acetylneuraminic acid. "—" denotes no detected information.

oligosaccharides were found, indicating clearly the applicability of the SHPS-based method to sialylated reducing glycans. Meanwhile, some unknown ion signals in low relative abundance were also observed. They might be some complex pseudo molecular ions of unknown glycans. However, this does not interfere with the general conclusion that the SHPS-based glycan capturing method allows selective and efficient isolation of reducing glycans from biological samples.

3.5 Application of the SHPS-based method to the *N*-glycan analysis of human blood plasma and fetal bovine serum

In order to illustrate the applicability of the SHPS-based glycan capturing procedure to more challenging complex biological samples as well as its compatibility with mass spectrometric techniques, the *N*-glycans derived from human blood plasma and FBS were isolated and analyzed by ESI-MS, on-line LC–MS and MS/MS. Dialyzed lyophilized blood samples were digested by PNGase F as described above. The enzymatically digested samples were lyophilized and treated using the SHPS-based glycan capturing procedure to obtain purified *N*-glycans. These purified *N*-glycans were directly profiled by ESI-MS or derivatized with AEC for analysis by on-line LC–MS and MS/MS.

As a result, the obtained ESI-MS profiles of the two samples (shown in Fig. 8) both present a lot of pseudo molecular ion signals of *N*-glycans, which are assigned in Table 1. This confirms the applicability of the described novel method to complex biological samples, though there are some unassigned unknown minimal ion peaks. Moreover, as shown in Fig. 9, on-line LC–MS and MS/MS analysis provide rich structural information of these *N*-glycans, including the isomer number, hydrophobicity (determined as elution time in the RP-HPLC profiles) and sequence of each glycan. The obtained analytical data of these *N*-glycans are presented in Table 1. In total, thirty three human plasma *N*-glycans have been detected, including five high-mannose type, nineteen complex type, nine hybrid

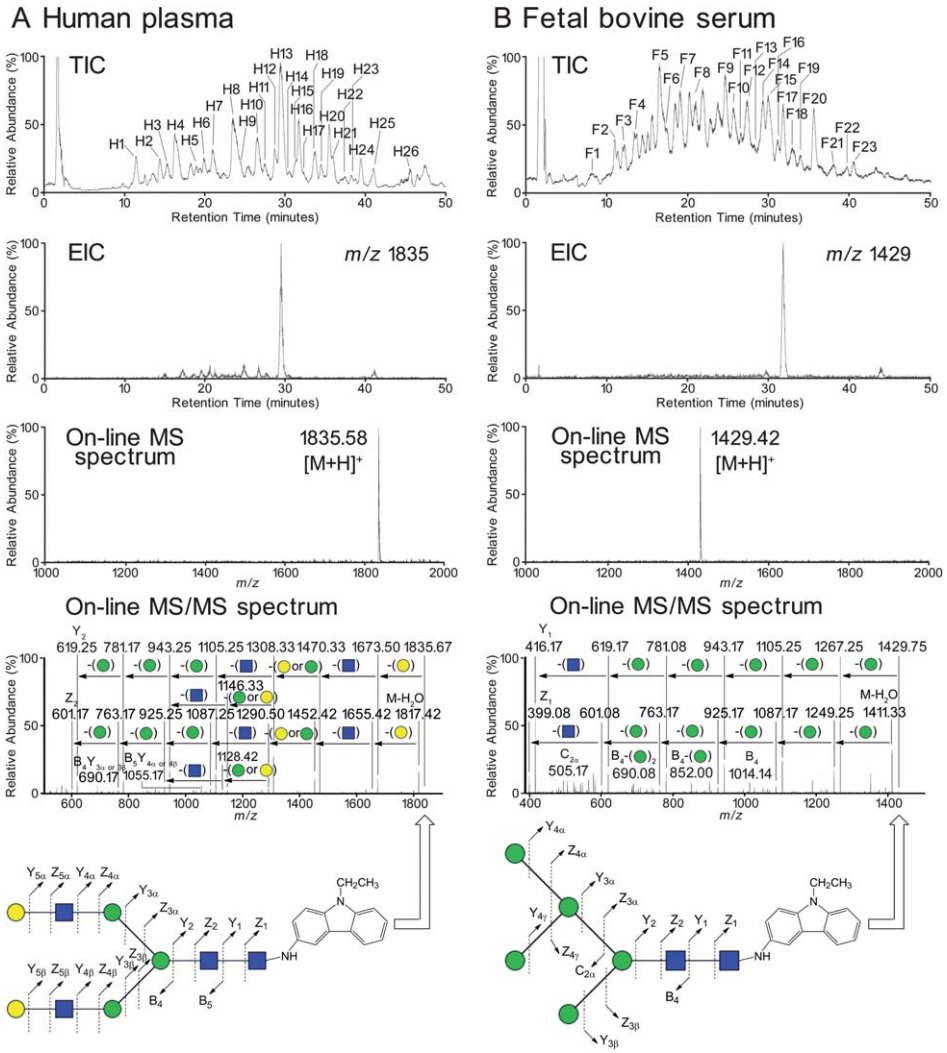


Fig. 9 On-line HPLC–MS and MS/MS analysis of *N*-glycans released from complex biological samples purified using the SHPS-based glycan capturing procedure. (A) Total ion chromatogram (TIC), extracted ion chromatogram (EIC), on-line MS spectrum and on-line MS/MS spectrum of *N*-glycans derived from human plasma. (B) TIC, EIC, on-line MS spectrum and on-line MS/MS spectrum of *N*-glycans derived from FBS. Structural formulae: blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose. The MS/MS fragment ions were assigned using GlycoWorkbench.[39]

type and ten sialylated glycans. Of these human plasma *N*-glycans, twenty eight were observed in both ESI-MS and on-line LC–S profiles, while only four were observed in the on-line LC–MS profile and only one in the ESI-MS profile. This is attributed to the fact that AEC labeling can improve the ionization efficiency and MS detection sensitivity of many glycans.[26] In contrast, twenty five fetal bovine serum *N*-glycans have been obtained, including three high-mannose type, eighteen complex type, four hybrid type and eight sialylated ones. Of these FBS *N*-glycans, sixteen were observed in both ESI-MS and on-line LC–MS profiles, while one only in the ESI-MS profile and only eight in the on-line LC–MS profile. It is noted that twenty three *N*-glycans were found in both human plasma and FBS, indicating the partial glycomic similarity of the two samples. Importantly, all of these *N*-glycan data are in accordance with previous literature reports.[14,38–44] Therefore, the SHPS-based glycan capturing procedure is compatible with the ESI-MS, LC–MS and MS/MS techniques and can be used as the special method for the preparation and purification of reducing glycans derived from complex biological samples for mass spectrometric analysis.

4 Conclusions

A facile and versatile method has been developed, based on the reversible reactions between aldehydes and sulfonyl hydrazine-functionalized polymer, for the quantitative and high-throughput isolation of reducing glycans from complex sample mixtures. We have optimized the reaction conditions of the method and investigated its quantitative features using maltodextrin and lactose as model glycans, demonstrating DMF as the optimal sample solvent for conjugation reactions, a linear range of glycan concentration from 0.04 mM to 20 mM, a general glycan recovery of 63.9% and good quantitative reproducibility characterized with CV values below 3.3%. The feasibility and isolation selectivity of the method were confirmed by the isolation and mass spectrometric profiling of the *N*-glycans released from bovine RNase B and hen ovalbumin, as well as the free reducing oligosaccharides of some milk samples. Furthermore, the *N*-glycans derived from human blood plasma and FBS were successfully isolated and analyzed by ESI-MS, on-line LC–MS and MS/MS, showing the good applicability of the novel method to complex biological samples as well as its compatibility with mass spectrometric techniques. In comparison with most of the glycan purification methods reported previously, our SHPS-based glycan capturing procedure has some conspicuous advantages, which are described as follows: (i) the SHPS-based method can achieve convenient isolation and purification of both neutral and sialylated reducing glycans from various complex biological samples; (ii) the SHPS-based method can achieve quantitative isolation of various reducing glycans and is applicable to quantitative glycomics; (iii) the SHPS-based method is developed using commercially available SHPS polymer and is suitable for extensive application in glycomic studies, without any complex preparation processes for functional materials. Besides SHPS beads, some other types of sulfonyl hydrazine-functionalized materials can also be utilized in this method. Although this study is focused on the analysis of some model glycans, free oligosaccharides and glycoprotein *N*-glycans, the SHPS-based procedure is directly applicable to other types of reducing glycans, such as reducing *O*-glycans released from glycoproteins and reducing glycans derived from glycolipids. Therefore, we demonstrate that the SHPS-based glycan capturing procedure represents a convenient and promising sample preparation method for glycomic studies.

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