Supporting Information

Comprehensive Analytical Approach toward Glycomic Characterization and Profiling in Urinary Exosomes

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The manuscript is accompanied with Supporting Information containing

Methods and Materials section

Figures S1, S2, S3, and S4

Materials and Methods

Chemicals and Reagents. Ammonia-borane complex, β-mercaptoethanol, sodium hydroxide beads, protease inhibitor cocktail solution or tablets, trypsin (TPCK treated, from bovine pancreas), fetuin (from fetal calf serum), methylamine hydrochloride, PyAOP (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate), NaN_3, triethanolamine, and methyl iodide were purchased from Sigma-Aldrich (St. Louis, MO). 2,5-Dihydroxybenzoic acid (2.5-DHB) and 4-methylmorpholine was received from Alfa Aesar (Ward Hill, MA). D,L-Dithiothreitol was received from BIO-RAD (Hercules, CA). N-Glycanase (PNGase F) was purchased from Prozyme (Hayward, CA). LC-MS grade water and acetonitrile were purchased from EMD Chemicals (Gibbstown, NJ). Sucrose, trifluoroacetic acid (TFA), glacial acetic acid, formic acid (FA), chloroform, dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) were products of Mallinckrodt Baker (Phillipsburg, NJ). Nonidet P-40 and pronase (from *Streptomyces griseus*) were received from Roche Diagnostics (Indianapolis, IN). Micro SpinColumn and Macro SpinColumn active charcoal, Micro SpinColumn C18, and Ultra-Micro SpinColumn amino columns were purchased from Harvard Apparatus (Hayward, CA), while bicinchoninic acid protein assay kit was obtained from Thermo Fisher (Waltham, MA). While exosome were in-house extracted from normal human urine, human blood serum (HBS) pooled from normal females was received from BioreclamationIVT (Baltimore, MD).

Sample Preparation for N-Glycan Measurements. The protein content of the isolated urinary exosomes was determined by bicinchoninic acid protein assay (Thermo Fisher BCA protein assay kit). Aliquots of exosome solutions containing 100 µg of protein were used, while the volume was adjusted to 100 µL with 0.2% SDS in 10 mM phosphate buffer (pH 7.4). One µL of 10% β-mercaptoethanol dissolved in H_2O was added, and the solution was subsequently

incubated at 60 ºC in water bath for 1 h. After the sample was cooled to room temperature, 10 µL of 10% Nonidet P-40 was added. An aliquot of 0.25 mU of peptide N-glycosidase F (Prozyme) was added and the solution was incubated at 37 °C for 21 h to release the N-linked glycans from the exosomes.

To the PNGase F-treated solution, 150 µL of 5% acetonitrile containing 0.1% TFA was added, and put on pre-equilibrated Micro-Spin C18 columns with three passages and washed with 400 µL of 5% acetonitrile containing 0.1% TFA. The eluents containing the released N-linked glycans were put on Micro-Spin charcoal columns, and washed with 400 µL of 5% acetonitrile containing 0.1% TFA. Subsequently, the N-linked glycans were eluted with 400 µL of 30% acetonitrile containing 0.1% TFA. The eluents were collected and dried using a CentriVap Centrifugal Vacuum Concentrator (Labconco, Kansas City, MO).

To the dried N-linked glycan samples, 10 µL of 10 mg/mL ammonia-borane complex in H_2O was added and incubated at 60 °C for 1 h to reduce the glycans to their alditol form. After cooling the solutions to room temperature, three 1 µL aliquots of glacial acetic acid were added to remove the excess reagent. The samples were then dried using the CentriVap Centrifugal Vacuum Concentrator. During the drying procedure, three aliquots of 100 µL methanol were added to remove the borate salts through forming volatile methyl borate esters.

The dried N-linked glycans were permethylated in solid phase according to our previously established procedure.[1,2] The samples were re-dissolved in the reaction solution, containing 65 µL of dimethylformamide, 5 µL of water, and 35 µL of methyl iodide. The solution was then applied onto the micro-spin column previously packed with sodium hydroxide beads, and incubated for 20 min. The samples were recovered by centrifugation, while a second

aliquot of 35 µL of methyl iodide was added, and the solution was incubated for additional 20 min before being collected by centrifugation. To recover permethylated glycans from the reaction mixture by liquid/liquid extraction, 400 µL chloroform was added and washed with 0.5 M NaCl and water. The chloroform layer containing the permethylated glycans was collected and dried on the CentriVap Centrifugal Vacuum Concentrator.

MALDI-TOF Mass Analysis. The dried samples were reconstituted in 5 µL of 50%/50% methanol/water solution. An aliquot of 0.5 µL of solution was spotted on a MALDI plate and allowed to dry. An aliquot of 0.5 µL of 10 mg/mL DHB in 50%/50% methanol/water with 1 mM sodium acetate as a matrix was added to the spots and dried under vacuum. Each spot was examined by an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer (ABSciex, LLC, Framinghan, MA) adjusted to the positive-ion reflector mode. The obtained MALDI spectra were processed through baseline correction and a noise filter using software Data Explorer® Software (ABSciex, LLC, Framinghan, MA). The intensity of each glycan was normalized as the percentage of total glycan intensities.

LC-MS of Permethylated Glycans.The reduced and permethylated glycans were analyzed with an LC-MS system consisting of a Model 1100 liquid chromatograph (Agilent Technologies), equipped with a nanopump, and connected to an LTQ ion trap (Thermo/Finnigan). A picofrit capillary with 0.1µm tip was used as a nanospray interface (New Objective, Woburn, MA). The glycans were separated on C18 columns packed in-house with 3 µm C18 particles (Magic C18AQ 200Å, 3U, Michrom Bioresources Inc., Auburn, CA) into 150 × 0.075 mm fused silica capillaries (Polymicro technologies, Phoenix, AZ). Prior to sample introduction into the LC-MS system, the permethylated glycans in 50% methanol were desalted on a C18 end-stage tip according to the manufacturer's instructions (Ziptip 0.6 µL, Millipore, Bradford, MA). Briefly,

the tips were conditioned and equilibrated with 60% acetonitrile/0.2% formic acid and HPLC grade H_2O/0.2% formic acid, respectively, prior to sample binding. The tips were washed with HPLC grade H_2O/0.2% formic acid and bound sample was eluted with 85% acetonitrile/0.2% formic acid and was subsequently lyophilized. Dried glycans were dissolved in 50% methanol, while approximately 5 µg protein equivalent samples were subjected to LC-MS analysis. The flow was 0.3 µL/min and the glycans were eluted with an increasing proportion of acetonitrile. The gradient was facilitated by mixing buffer A (LC-MS grade water with 100 µM sodium acetate at pH 4) and buffer B (LC-MS grade acetonitrile). Gradient settings: the gradient was set at 0-20 min 10 % B, 20-25 min 10-28 % B, 25-85 min 28-45 % B, 85-95 min 45 % B, 96-106 min 95% B and, finally, 107-117 min 10% B. Data were collected in the scan range of *m/z* 300-2000, while the top 3 ions were automatically selected for tandem MS CID (collision-induced dissociation) fragmentation. Dynamic exclusion was used after 5 spectra and released after 30 sec.

LC-ESI-MS and MS/MS Acquisition of Enzymatically Released N-Glycans from Urinary Exosomes. As described above, the urinary exosomal N-glycans were released by PNGase F. After being reduced, glycans equivalent to 16 µg protein were dissolved in water and separated on a 900 × 0.075 mm capillary column packed in-house with 3 µm porous graphitized carbon. The column efficiencies measured for standard glycans typically featured around 100,000 theoretical plates. Buffer A was 0.15% FA, adjusted to pH 3 with ammonia in water, while buffer B was acetonitrile with 0.15% FA. Using an Acquity M class UPLC instrument (Waters Corporation, Milford, MA), the gradient was set at 0-10 min 5 % B, 40 min 15 % B, 140 min 40 % B, 200 min 80 % B and 230 min 80 % B with a flow-rate of 0.3 µL/min. The glycan mixtures were analyzed with an Agilent LC/MSD ion trap XCT Plus mass spectrometer (Agilent

Technologies, Alpharetta, GA) with an on-line nanosource (G1982B), and the scan range set to *m/z* 600-2200 with a MS^n threshold of 2000 counts. The drying gas flow was set at 5 L/min with a drying temperature of 160°C. The capillary current was kept between 100-200 nA and the capillary plate offset at –500 V.

SDS-PAGE of human urinary exosomes

Urine was concentrated with 20kDa Ultrafiltration devices (Sartorius, Vivaspin 2). All samples were dissolved in loading buffer containing SDS and DTT and heated to 95°C for 10 min prior to being loaded on the gel.

LC-MS analysis of urinary exosomes

<u>Tryptic digest of urinary exosomes</u>

Ten µg aliquot of exosomes lysed in 0.2% SDS in PBS 0.01M was denaturated and reduced by the addition of DTT (SigmaAldrich, final conc. 5mM) and cleavable detergent Rapigest (Waters, final conc. 0.2%) dissolved in 50mM Ambic (SigmaAldrich) and heated in 60°C for 30 minutes. After cooling down, iodoacetamide (SigmaAldrich) was added to a final concentration of 15mM and incubated in darkness in RT for 30 min. 0.5µg trypsin was added (1:20 w/w) and incubated overnight at 37°C. The next day, Rapigest was cleaved with addition of formic acid, final concentration 0.5% (pH 3) and incubated at 37°C 45min. Sample was spinned in a centrifuge (13 000 × g, 10 min) and the supernatant was collected and lyophilized. The sample was dissolved in 1% acetonitrile 0.1% formic acid and desalted with two 10µL C18 ziptips (Millipore). Samples were lyophilized again and stored at -20°C.

NanoLC-MS analysis of tryptic peptides

The sample was dissolved in 10% acetonitrile/0.1% FA and 0.5µg was separated on a "NanoLC-ultra" liquid chromatography system (Eksigent Technologies, Dublin, CA)) with a reverse-phase C18 column. The samples were separated with a (Buffer A: H_20 0.1% formic acid; Buffer B: Acetonitrile 0.1% formic acid, 90-minute linear gradient of 10-30% B, followed by a 30 minute gradient of 30-40% B at a constant flow rate of 300 nL/min. An LTQ Orbitrap XL mass spectrometer (Thermo FisherScientific) was used for detection. After a FTMS full scan *m/z* 446-2000 (resolution 15,000), the top 6 ions were selected for CID fragmentation with 35% normalized energy.

LC-MS data analysis

The raw data were converted to MGF files with MSConvert and vendor peak-picking selected. The MASCOT (Matrix Science) search engine was used for protein identification with the following settings: Swissprot database 51.6 (15720 sequences), human taxonomy, trypsin 1 missed cleavage allowed, carbamidomethyl-fixed modification, variable modification oxidation of methionine, peptide mass tolerance 20ppm, fragment mass tolerance 0.6 Da. Out of 20,006 scans, **93** proteins with a MudPIT score of at least 50 were identified.

Isolation of Urinary Exosomes

Collect 40 mL urine and add 0.02% NaN_3 and protease inhibitor cocktail

Centrifuge at 17,000 X g at 20 C° for 30 min, keep the supernatant (SN1)

Resuspend the pellet in 0.5 mL of isolation solution with 200 mg/ml DTT , incubate at 37 C° for 45 min, centrifuge again at 17,000 X g at 20 C° for 7 min, and keep the supernatant (SN2)

Combine the supernatants SN1 and SN2 and centrifuge at 200,000 X g at 20 C° for 1 h

Resuspend the pellet in 0.5 mL of isolation solution with 200 mg/ml DTT, incubate at 37 C° for 45 min, centrifuge again at 17,000 X g for at 20 C° for 1 min, and keep the supernatant

Wash the pellet with 1 mL of isolation solution , centrifuge at 17,000 X g for 1 min, and keep the supernatant; Repeat this process two more times

Combine all the supernatants from last step (SN3), add 20 mL of isolation solution, and centrifuge at 200,000 X g at 20 C° for 1 h, and resuspend the pellet in 20 mL of PBS

Centrifuge at 200,000 X g at 20 C° for 1 h, keep the pellet for the following experiments

Figure S1. Procedure for the isolation of exosomes from urine.

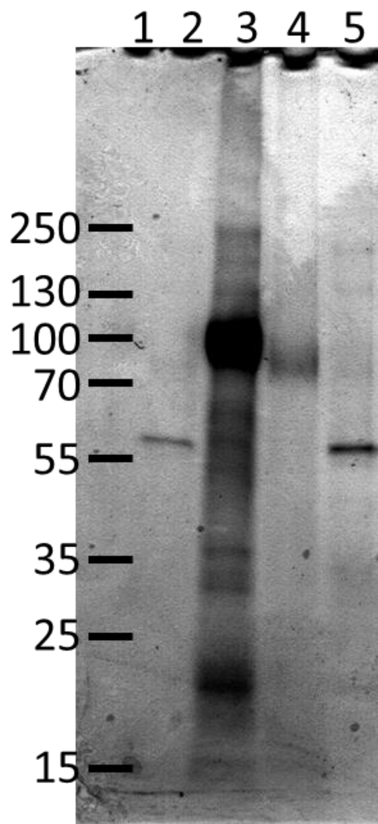

Figure S2. Characterization of human urinary exosomes purified with ultra-centrifugation and reduction of uromodulin. Urine (lane 2 and 5), exosomes (lane 3), cells from urine (lane 4) were separated on Tris-Glycin 4-20% SDS-PAGE gels stained with Coomassie blue R-250. Approximately 5 µg of exosomes and 2 µg of urine and cells were loaded on the gel. The urine sample stained weakly and strong bands were only observed between the 55 and 70kDa MW marker bands (PageRuler Plus), most likely indicating presence of Albumin (66kDa). The exosome sample stained stronger with many bands in the whole range of the gel with the strongest band co-migrating with the 100kDa MW marker band, which is similar to the 100kDa size of monomeric uromodulin protein. A band between 70 and 100kDa was seen in the lane with cells derived from urine.

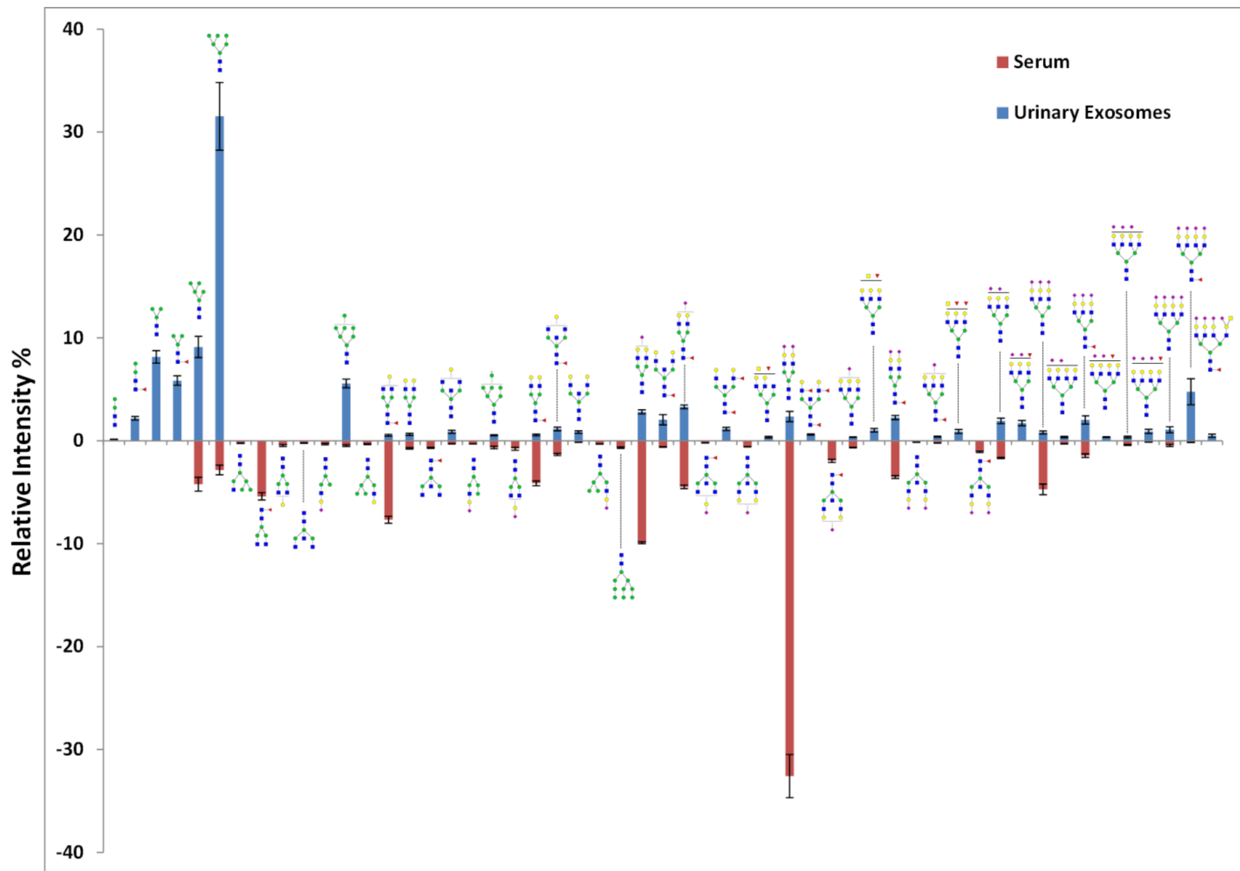

Figure S3. Relative abundance of the N-glycans identified from urinary exosomes (100 µg aliquot) compared to a typical blood serum extract (2.5 µL serum corresponds to roughly 150 µg protein).

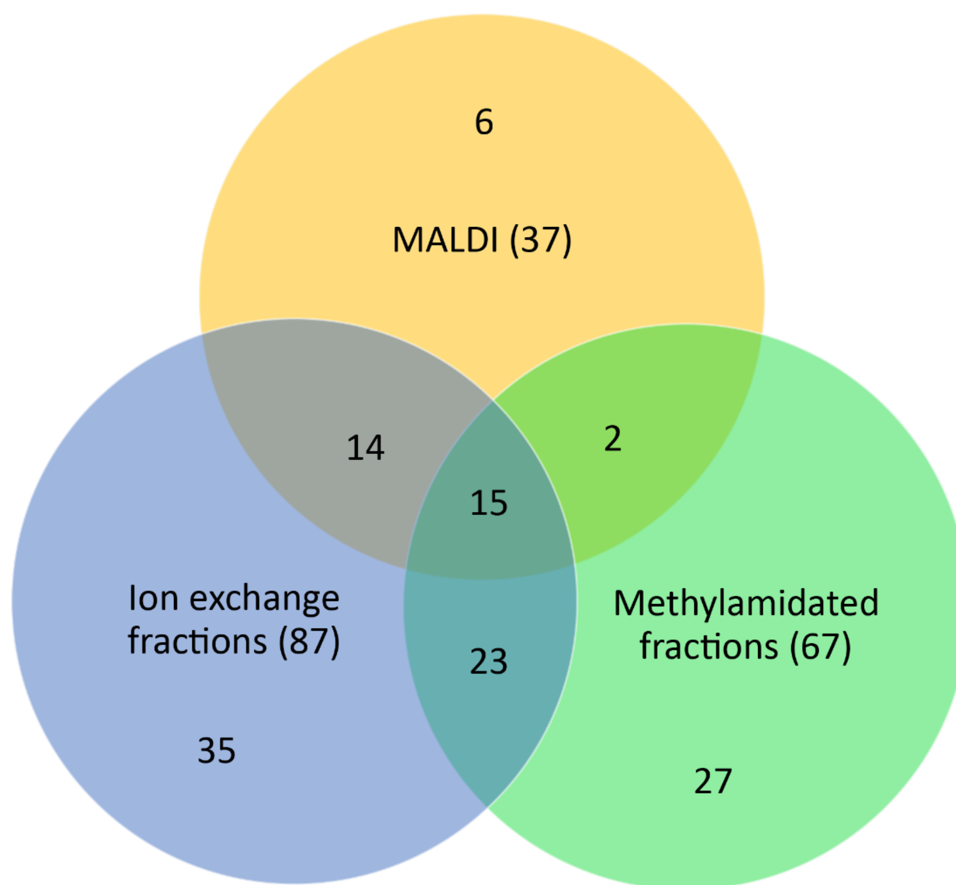

Figure S4. The Venn diagram displays the overlap of detected N-glycans between MALDI-TOF-MS, LC-MS (1-meter PGC column) with either reduced N-glycans or methylamidated reduced N-glycans. Since methylamidation only works on sialic acids, no neutral N-glycans are seen for the methylamidated glycans.

References for Supporting Data

(1) Alley, W. R., Jr.; Madera, M.; Mechref, Y.; Novotny, M. V. *Anal. Chem.* **2010**, *82*, 5095-5106.
(2) Kang, P.; Mechref, Y.; Klouckova, I.; Novotny, M. V. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3421-3428.