Isolation of urinary extracellular vesicles from Tamm-Horsfall protein–depleted urine and their application in the development of a lectin-exosome-binding assay

Maja Kosanović and Miroslava Janković
University of Belgrade, Institute for the Application of Nuclear Energy, INEP, Belgrade, Serbia

BioTechniques 57:143-149 (September 2014) doi 10.2144/000114208
Keywords: extracellular vesicles; exosomes; isolation; urine; THP; lectin-exosome-binding assay

Urine is a readily available source of relatively large quantities of extracellular vesicles (EVs). However, the isolation of urinary EVs (uEVs) is complicated by the presence of Tamm-Horsfall protein (THP), which polymerizes and co-precipitates as a contaminant. This may make glycan analysis of uEVs difficult since THP is heavily glycosylated. To facilitate glycosylation analysis and address the need for elimination of non-uEV glycans, we present a modification of the uEV isolation procedure and use the isolated uEVs in the development of a lectin-exosome binding assay. Salt precipitation was employed to remove THP under conditions originally described for its separation from urine, followed by differential centrifugation. The quality of the isolated uEVs was examined by electron microscopy, SDS-PAGE, and immunoblotting. The uEVs were subsequently immobilized on solid phase and probed with labeled plant lectins using the lectin-exosome binding assay. Our results indicate that the isolated uEVs had preserved structural integrity and reacted with labeled plant lectins in a selective, carbohydrate-dependent manner. The basic lectin binding pattern of uEVs obtained by our method can be used as a reference for assessing the composition of their surface glycans in different physiological and pathological conditions.

Extracellular vesicles (EVs), also referred to as exosomes or microvesicles, are membranous vesicles released by cells into the extracellular space (1,2). EVs originate from multivesicular bodies or bud from the plasma membrane and represent an important communication tool because, among other functions, they transport different molecules to target cells during various physiological and pathological processes (3–6). EVs also have potential as biomarkers because they carry a specific subset of parent cell molecules (7–10).

Urine is a readily available source of relatively large quantities of EVs originating from urinary tract cells (11,12). Urinary EVs (uEVs) can be used to diagnose or monitor the progress of different urological diseases or to monitor the response to therapy in urinary tract pathologies. However, the isolation of uEVs encounters a problem imposed by the presence of Tamm-Horsfall protein (THP; also known as uromodulin) in urine. THP polymerizes into a high molecular weight network (13) that co-precipitates as a contaminant (14–16). This may lead to difficulty in downstream analysis, such as the masking of low abundance proteins by THP in mass spectrometry analysis (17), or interference with glycan analysis since THP is a highly glycosylated protein.

To overcome this problem, THP depolymerization by DTT (dithiothreitol) treatment (18) or uEV isolation by sucrose gradient centrifugation (15,16,19,20) have been routinely used, but both approaches have disadvantages (12,16,21,22). Even when DTT is used, THP can still be present in the isolated uEVs (15,21,23,24), whereas separation on sucrose gradients or cushions (15,19,20) is time-consuming, taking up to 24 hours (15,16,19,20). In addition, treatment with DTT may lead to deleterious effects on the folding and biological activity of particular proteins in uEV preparations (24). Thus, preparations of uEVs obtained in this ways may not be suitable for functional studies. Therefore, further strategies for eliminating THP are needed (25).

To facilitate glycan analysis of uEVs and address the need for elimination of contamination with non-uEV-glycans such as THP, we present a modification of the classical isolation procedure that bypasses the use of either DTT treatment or sucrose gradients and is also takes considerably less time. Our approach is based on the initial removal of THP from urine by salt precipitation under conditions originally described for the isolation of THP from urine (26). This resulted in a uEV preparation of high quality and sufficient yield.

METHOD SUMMARY

Initial removal of Tamm-Horsfall protein (THP) by salt precipitation was introduced as a modification to the classical isolation procedure for urinary extracellular vesicles (uEVs) based on differential centrifugation and ultrafiltration. Isolated uEVs were immobilized on a solid phase and probed with labeled plant lectins to establish a lectin-exosome binding assay as a tool for assessment of the composition of their surface glycans. Our approach is time-saving, efficient, and easy to perform in any laboratory for analyses of EVs of different origins.
that was then used in the development of a lectin-exosome binding assay optimized for analysis of uEV surface glycans. Since complex carbohydrates are important determinants in recognition processes, (27–29), analysis of EV glycans is a topic of general interest for understanding their function and may also have applications in biomedicine.

Materials and methods

Reagents

Anti-CD63 monoclonal antibody (clone TS63) and the BCA Protein Quantification Kit were purchased from Abcam (Cambridge, UK). Goat anti-human galectin-3 (gal-3) antibody was obtained from R&D Systems (Minneapolis, MN). Biotinylated plant lectins [concanavalin A (Con A), Lens culinaris agglutinin (LCA), peanut agglutinin (PNA), Sambucus nigra agglutinin (SNA), Maackia amurensis lectin II (MAL II), wheat germ agglutinin (WGA), Ricinus communis agglutinin I (RCA I), Vicia villosa lectin (VVL), and Dolichos biflorus agglutinin (DBA)], VECTASTAIN ABC Kit, biotinylated goat anti-mouse IgG, and biotinylated rabbit anti-goat IgG were purchased from Vector Laboratories (Burlingame, CA). Methyl α-D-mannopyranoside, fucose (Fuc), lactose (Lac), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), BSA, poly-L-lysine solution, 3,3′,5,5′-tetramethylbenzidine (TMB), and glutaraldehyde were obtained from Sigma (St. Louis, MO). Galactose (Gal) and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). Galactose (Gal) and dithiothreitol (DTT) were purchased from ICN Biochemicals (Cleveland, OH). Silver stain kit and calibrated with broad range molecular mass standards (6 kDa—200 kDa). Proteins were stained using a Bio-Rad silver stain kit and calibrated with broad range molecular mass standards (6 kDa—200 kDa).

Isolation of extracellular vesicles

Extracellular vesicles were isolated from THP-depleted urine according to Pisitkun et al. and Zhou et al., with slight modifications (21,30). THP-depleted urine was centrifuged at 17,000 × g for 20 min at 20°C on an Optima L-90K ultracentrifuge using a Ti 50.2 rotor (Beckman Coulter, Fullerton, CA) and filtered through a 0.22 μm filter. The resulting supernatant was ultracentrifuged at 100,000 × g for 2 h at 20°C (k factor = 157.7). The pellet containing uEVs was resuspended in 0.05 M phosphate buffered saline (PBS), pH 7.2, and washed with 6 volumes of PBS in 100 kDa cut-off concentrators (Millipore, Billerica, MA). The uEV suspension was stored at -80°C. Protein concentration was determined using a BCA Protein Quantification Kit.

In parallel, uEVs were isolated from the same urine sample by serial centrifugation at 17,000 × g and 100,000 × g, as described.

SDS-PAGE

SDS-PAGE was performed on a 6% separating gel and a 3.75% stacking gel (31). Samples were prepared in Laemmli buffer (1:1) or Laemmli buffer supplemented with 200 mg/mL DTT and analyzed as isolated. Proteins were stained using a Bio-Rad silver stain kit and calibrated with broad range molecular mass standards (6 kDa—200 kDa).

Immunoblot

Proteins were transferred onto nitrocellulose membranes by semi-dry blotting using a Trans-blot SD (Bio-Rad). The conditions were as follows: transfer buffer 0.025 M Tris containing 0.0192 M glycine and 20% methanol (pH 8.3) with a current density of 1.2 mA/cm² for 45 min. The membrane was blocked with 1% casein in 0.05 M PBS (pH 7.2) for 1 h at RT, washed 3 times for 5 min with PBS, and incubated with the corresponding antibodies (anti-CD63 at 20 μg/mL, or anti-gal-3 at 0.5 μg/mL) overnight at 4°C. After washing, the appropriate antibody (biotinylated goat anti-mouse IgG or biotinylated rabbit anti-goat IgG) was added, followed by
incubation for 30 min at RT. The membrane was rinsed, and the avidin/biotinylated horse-radish peroxidase (HRPO) mixture from the Elite Vectastain ABC kit was added, followed by incubation for 30 min at RT. After another wash, the blots were visualized using Pierce ECL Western blotting substrate according to the manufacturer’s instructions.

The corresponding negative controls (omitting the primary antibody) were also included and gave no visible reactions.

Densitometry analysis of the immunoblots was performed using TotalLab Software, version 2.00 (Amersham Biosciences, Buckinghamshire, UK).

**Transmission electron microscopy (TEM)**

Extracellular vesicles (10 μL) were applied to Formvar-coated copper grids by floating the grids on a droplet of sample for 10 min at RT. After washing twice with PBS, the uEVs were dehydrated through a series of ethanol standards (MWSt) in kDa. Following removal of excess fluid, each grid was transferred to a droplet of 2.5% glutaraldehyde and incubated for 2 h. The grids were blocked with 50 μL 1% BSA for 1.5 h, and then washed again. Anti-CD63 monoclonal antibody was added (50 μL, 20 μg/mL), and allowed to react with the immobilized uEVs for 1 h. After the washing step, biotinylated goat anti-mouse IgG (50 μL, 0.75 μg/mL) was added and incubated for 30 min. Unbound conjugate was removed by washing followed by addition of 50 μL of avidin/biotin-HRPO complex (VECTASTAIN ABC Kit, prepared according to the manufacturer’s instructions). After incubation for 30 min, the plates were rinsed and developed using 50 μL TMB/H₂O₂ solution. The reaction was stopped with 50 μL 2 N sulfuric acid. Optical density was read at 450 nm using a Wallac 1420 Multilabel counter (Perkin Elmer, Waltham, MA). All probes were done in duplicate.

**Lectin binding assay**

Extracellular vesicles were probed for binding to the following plant lectins: Con A, WGA, RCA I, SNA, MAL II, PHA-E, PHA-L, LCA, PNA, VVL, and DBA. Biotinylated plant lectins (50 μL, 0.5 μg/mL) were added to solid phase-immobilized uEVs and allowed to react for 30 min at RT. After washing with 0.05 M PBS (pH 7.2; 3 x 300 μL), 50 μL of avidin/biotin-HRPO complex (VECTASTAIN ABC Kit, prepared according to manufacturer’s instructions) was added followed by incubation for 30 min, at RT. The plates were further processed as described above.

In parallel, the specificity of binding was assayed with lectins pre-incubated for 30 min with the corresponding inhibiting sugars: 0.2 M methyl α-D-mannopyranoside for Con A and LCA; 0.2 M lactose for SNA and RCA I; 0.1 M GlcNAc for WGA; 0.2 M galactose for PHA-E, PHA-L, and PNA; 0.2 M GalNAc for VVL and DBA; and 1 μg/mL fetuin for MAL II.

**Results and discussion**

Given the importance of preserving molecular structure while eliminating interference by THP, we performed a salt precipitation to deplete THP as the initial step for isolation of urinary EVs, preceding differential- and ultra-centrifugation. Electrophoretic profiles of the salting out pellet and subsequent pellets, as well as the corresponding supernatants, revealed effective removal of THP (Figure 1A). In contrast, matching fractions from the same

---

**Figure 1. THP in fractions separated during isolation of urinary EVs.** (A) Representative SDS-PAGE of pellets (P) and supernatants (S) separated by salt precipitation of urine at 3000 x g (P3000, S3000), 17,000 x g (P17000, S17000), and 100,000 x g (P100000, S100000). The broad strong band at 110 kDa corresponding to THP monomer was detected for the 3000 x g pellet (P3000), whereas a weak band at the same position was detected for the 17,000 x g pellet (P17000). (B) Matching fractions separated from the same urine sample without salt precipitation had different protein compositions, and the THP band was evident in the uEV preparation (Figure 1B). The gels were silver-stained. Numbers indicate the position of molecular mass standards (MWSt) in kDa. (C) Representative immunoblot of the 100,000 x g P containing pure uEVs. uEV proteins were transferred onto a membrane and probed with monoclonal anti-CD63 antibody and polyclonal anti-gal-3 antibodies. (D) Transmission electron micrographs of uEVs isolated from THP-depleted urine by salt precipitation. Isolated uEVs were of typical size and shape and did not differ between male (a) and female (b) donors.
Figure 2. CD63-immunoreactivity in fractions separated during isolation of urinary EVs. Representative immunoblots of pellets (P) separated from the same urine sample with (A) or without salt precipitation (C) at 3000 × g (P3000), 17,000 × g (P17000), and 100,000 × g (P100000). (B and D) Densitometry analysis: signal intensities of CD63 immunoreactive bands are expressed relative to that of the 100,000 × g pellet. Numbers indicate the position of molecular mass standards (kDa).

Figure 3. Solid and fluid-phase binding assay for urinary EVs. (A) uEVs were adsorbed on a poly-lysine-coated surface and immobilization was monitored using anti-CD63 antibody as the marker (a). Dose-dependent interaction was observed in the concentration range tested. Con A binding was probed in parallel as a reference for detection of uEV glycans (b). Carbohydrate-dependent interaction was confirmed by inhibition with methyl α-D-mannopyranoside (c). (B) Con A binding to uEVs in suspension resulted in the formation of aggregates as seen by SEM. Magnification was 50,000×.
urine sample without salt precipitation, had different protein compositions, and the THP band was evident in the uEV preparation (Figure 1B).

Thus, the results indicate that THP migrating as a very prominent band at 110 kDa was fully recovered in the 3000 × g pellet (Figure 1A, P3000), whereas a faint THP band can be observed in the 17,000 × g pellet (Figure 1A, P17000), and no band was observed in the 100,000 × g pellet (Figure 1A, P100000). The latter pellet represents the isolated preparation of uEVs, which exhibited a typical pattern of protein bands including the characteristic EVs markers CD63 and gal-3, as shown by immunoblot (Figure 1C). The final yield was 105 µg of total protein in the 100,000 × g pellet / 100 mL of urine. TEM micrographs demonstrated that uEVs isolated in this way had a spherical shape with diameters ranging from 30 to 150 nm. There was no significant difference is uEVs isolated from urine from healthy men (Figure 1D-a) compared to women (Figure 1D-b).

To monitor the loss of uEVs in different urine fractions separated by salt precipitation and centrifugation, immunoreactivity to CD63 was analyzed as a marker of EVs (Figure 2A). Faint/but detectable CD63 in the 3000 × g pellet (Figure 2A, P3000), and low but consistent CD63 in the 17,000 × g pellet (Figure 2A, P17000) were observed. Thus, the results indicate that CD63 was recovered in the corresponding pellets independent of the presence or absence of THP, in agreement with available experimental data (24). When the signal intensity of CD63-immunoreactive bands in the 3000 × g pellet and the 17,000 × g pellet were expressed relative to that detected in the 100,000 × g pellet, uEV loss was assessed as 0.9% and 3.4%, respectively (Figure 2B). uEV loss during isolation without salt precipitation was 10% (Figure 2C and D), but the pattern of CD63 was slightly different.

In immunoblots, the signal intensity and pattern of EV markers in particular urine fractions were found to be highly variable among different subjects and different markers (18). In addition, the yield of uEVs, reported as concentration of total protein, can vary significantly between different isolation protocols due to contamination with non-exosomal proteins (23). The influence of salt precipitation on the efficiency of pelleting uEVs was not examined, but dialysis with water to lower the density of the urine might improve the yield.

In comparison to current protocols, the approach used here is simple and rapid, omitting additional time-consuming washing steps and efficiently preventing contamination by THP. Moreover, the isolated uEVs retained their native properties in respect to size, shape, and the presence of the common markers CD63 and gal-3, in accordance with the literature (15,16,18,19,24). All this supports
Several studies of uEVs dealing with the methods of isolation, proteomic profiling, and clinical diagnostic value have been conducted (9,15,22,23,32). We opted to use uEVs for development of a lectin-exosome binding assay, aiming at its application for the analysis of surface glycans on EVs. These glycans could have significant biomedical importance, but data in the available literature are very scarce (27–29).

Initially, the possibility of detecting glycans in uEVs in suspension or on solid phase after immobilization was examined using the lectin Con A. Using anti-CD63 binding as a reference, isolated uEVs were efficiently immobilized on poly-L-lysine-coated surfaces, and they could be recognized by Con A in a dose-dependent manner (Figure 3A). This lectin binding was inhibited by a specific sugar (mannose), pointing to carbohydrate-based recognition. In solution, Con A binding resulted in aggregation of uEVs (Figure 3B), confirming the accessibility and integrity of their glycans.

Under defined experimental conditions, immobilized uEVs were subsequently probed with selected plant lectins having distinct carbohydrate specificities as tools to recognize glycans of importance as possible biomarkers. Lectin binding to immobilized uEVs from male and female donors was similar (Figure 4). Among the lectins tested, Con A showed the highest reactivity. Considerable binding was also observed with PHA-E, RCA, WGA, and SNA. In addition, VVL, PNA, and DBL exhibited moderate binding, whereas MAA, PHA-L, and LCA showed negligible reactions.

Thus, the basic pattern of glycans from uEVs from healthy subjects was obtained using the lectin-exosome binding assay described here. It suggested abundant N-linked glycans of the high mannose or complex type, related to biantennary chains without core fucosylation (according to Con A and LCA reactivities). Regarding complex type N-linked glycans, modification of core mannose by addition of bieactected GlcNAc was shown (according to PHA-E reactivity), whereas glycans of higher antennarity were substantially less frequent (according to PHA-L reactivity). Sialic acid in α2,6 linkage was dominant over sialic acid in α2,3 linkage (according to SNA and MAA reactivities). Terminal hexosamine/hexose/lactosamine residues, assessed by the binding profiles of VVL, DBA, PNA, RCA, and WGA, indicated that lactosamine structures also contributed to the binding profile observed and that they could be associated with both N-linked glycans and O-linked glycans. Their presence was also suggested by the detection of specific carbohydrate epitope(s): Tn antigen, T antigen, and blood group A antigen.

The results obtained using our lectin binding assay on native uEVs isolated from THP-depleted urine could be correlated with published data on urinary exovesicles isolated from whole urine either by the classical protocol (14) or using sucrose gradients (19) suggesting that these methods preserve general glycan structure. Thus, mass spectrometry analysis of enzymatically released glycans (surface and cytosolic glycans) from urinary EVs from healthy subjects indicated prevalent high mannose-type glycans (80%) and also biantennary complex-type glycans without core fucosylation (30%) (14). The latter comprised minor subtypes having terminal hexosamine and glycans of higher antennarity. In addition, flow cytometry and microarray analysis of uEV glycans using the same lectins or different ones, but with comparable specificity to those used in this study, revealed considerable binding of PHA-E, RCA-I, GSL-I-B4, jacalin, PNA, and WFA, with detectable binding of AAA, MAA, and SNA-I (19). Glycan analysis
of EVs from other sources, indicated similarities without regard to their origin as evidenced by a conserved glycan signature (33). This could be related to protein sorting to specific membranes, based on carbohydrate-recognition by a specific set of lectins comprising galectins (14, 34). Our results show, for the first time, gal-3 association with uEVs, which has demonstrated a new method for examining surface glycans on uEVs. Until now, glycosylation of uEVs was analyzed only in respect to epithelial membrane N-linked glycans found in healthy subjects and patients with classical galactosemia (14), and in autosomal dominant polycystic kidney disease (19). The lectin-exosome binding assay for characterization of uEVs is of general interest and can be easily used to evaluate glycosylation differences in EVs from diverse origins.

**Author contributions**

M.K. and M.J. equally contributed to the conception and development of this work as well as interpretation of the results and writing the paper.

**Acknowledgments**

This work is supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, project code 173010.1.

**Competing interests**

The authors declare no competing interests.

**References**