Management of Tamm–Horsfall Protein for Reliable Urinary Analytics

Xiaomeng Xu, Karina Barreiro, Luca Musante, Oliver Kretz, Hanfei Lin, Hequn Zou,* Tobias B. Huber, and Harry Holthofer*

Purpose: Urinary extracellular vesicles (uEVs) are a novel source of biomarkers. However, urinary Tamm–Horsfall Protein (THP; uromodulin) interferes with all vesicle isolation attempts, precipitates with normal urinary proteins, thus, representing an unwanted "contaminant" in urinary assays. Thus, the aim is to develop a simple method to manage THP efficiently.
Experimental design: The uEVs are isolated by hydrostatic filtration dialysis (HFD) and treated with a defined solution of urea to optimize release of uEVs from sample. Presence of uEVs is confirmed by transmission electron microscopy, Western blotting, and proteomic profiling in MS.
Results: Using HFD with urea treatment for uEV isolation reduces sample complexity to a great extent. The novel simplified uEV isolation protocol allows comprehensive vesicle proteomics analysis and should be part of any urine analytics to release all sample constituents from THP trap.
Conclusions and clinical relevance: The method brings a quick and easy protocol for THP management during uEV isolation, providing major benefits for comprehensive sample analytics.

1. Introduction

Urinary extracellular vesicles (uEVs) are small size (30–400 nm in diameter) phospholipid bilayered structures actively secreted by all cell types facing the urinary space.[1] Originating either from the endosomal compartment or directly from evagination of cellular plasma membrane, diversity of extracellular vesicles (EV) found in urine can be considered as a snapshot of physiopathological activity upstream in the kidney and entire body as reflected by EVs filtered from circulation through the kidney.[2] Thus, uEV profiling represents a lucrative platform for novel biomarkers to provide dynamic molecular information for early disease mechanisms, prediction of outcome and response to therapy.[3]

uEVs carry a wide variety of molecules including specialized proteins, lipids, and (deoxy) ribonucleic acids uniquely protected from deleterious actions of ubiquitous proteases, ribonucleases, and lipases present abundantly in crude urine.[4] The precise functions of uEVs are not fully understood. Interestingly, they may be shuttled from upstream cell types to more distant target cells allowing a novel way of cell-to-cell communication.[5] Despite the exploding interest to EV biology, there are still many elementary challenges related, e.g., to overlapping nomenclature of EV categories and, especially, to the methods applied to isolate uEVs efficiently for downstream applications. Notably, the

X. Xu, H. Lin, Dr. H. Zou
Institute of Nephrology and Urology
The Third Affiliated Hospital of Southern Medical University
Guangzhou, China
E-mail: hequnzou@hotmail.com
K. Barreiro, Dr. H. Holthofer
Institute for Molecular Medicine Finland (FIMM)
University of Helsinki
Helsinki, Finland

Dr. L. Musante
Division of Nephrology
University of Virginia
Charlottesville, USA
O. Kretz, Dr. T. B. Huber, Dr. H. Holthofer
III. Department of Medicine
University Medical Center Hamburg-Eppendorf
Hamburg, Germany
X. Xu
Guangdong Shunde Southern Medical University Science Park
Dr. H. Holthofer
Centre for Bioanalytical Sciences (CBAS)
Dublin City University
Dublin, Ireland
E-mail: harry.holthofer@dcu.ie

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traditional uEV isolation methods have been challenging, labor-intensive, costly, and time consuming. Furthermore, they have not met the inherent challenges of urine: wide ionic concentration and pH range, intra- and interindividual variability, artifacts caused by naturally occurring urinary pigments and proteins as well as protease effects all complicating their isolation and downstream analytical processes. Particularly, Tamm–Horsfall protein (THP; uromodulin), the most abundant and glycan-rich glycoprotein in urine[6] can naturally entrap a large portion of uEVs to form filamentous networks.[7] Hence, THP represents a significant challenge as it distorts the spectrum of uEV classes recovered. For MS analysis, overabundant contaminant proteins take up a significant total mass fraction and may bind low abundance peptides to cause serious interference.[8]

Several methods have been proposed to eliminate THP contamination. These include, e.g., use of detergents such as dithiothreitol (DTT) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS),[7,9,10] salt precipitation followed by differential centrifugation,[11] and a dilution protocol using alkaline pH and low ionic concentration called KeepEX.[12] Although these methods can be successful in reducing THP level in uEV preparations, they have obvious shortcomings. DTT reduces disulfide bridges, which affects original protein structure. This directly influences proteomic analysis of EVs. CHAPS reasonably preserve protein conformation and enzymatic activities but its use is more time consuming than the DTT method. KeepEX substantially increases the volume of sample thus increasing the processing time.

In this study, we used the Hydrostatic Filtration Dialysis in combination with various chaotropic reagents to yield an optimized protocol for THP denaturation, depolymerization, and subsequent EV release and characterized the uEVs freed from the bulk of THP.

2. Experimental Section

Majority of reagents were purchased from Sigma–Aldrich (St. Louis, MI) unless specified otherwise in the text.

2.1. Urine Samples

First morning void urine samples were collected from healthy laboratory staff, aged 20–44 ($N = 4$), during three consecutive days. First morning void urine was processed within 3 h without adding protease inhibitors. Urine was anonymously labeled and pooled together. The urine collection and isolation of vesicles were done in Dublin City University. The study design and sample collection were approved by the Research Ethics Committee of Dublin City University (DCU REC/2014/222). All the experiments were performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants.

2.2. Vesicle Purification

uEVs were enriched by HFD according to our previous protocol[13] with minor modifications.[14] Briefly, urine samples

Statement of clinical relevance

Urinary extracellular vesicles (uEVs) are a hot research topic to establish disease biomarkers. However, a robust, standardized isolation protocol for uEVs is still missing. Tamm–Horsfall protein (THP) is the most abundant protein in normal urine and, at the same time, is known to interfere with most downstream biochemical urine analyses and, notably, uEV isolation. For this reason, we optimized a simple and robust method suitable for uEV isolation and applicable to crude urine analytics as well to rid urine samples of THP interference. The method includes standardized treatment of sample with urea solution to release most of uEVs from THP meshwork. This method meets the needs of a clinical research laboratory and allows quick and easy sample process before downstream analytics. Management of THP can be easily integrated into EV isolation process done by any method and provides major benefits for comprehensive sample analytics.

were centrifuged at $2000 \times g$ for 30 min at room temperature (RT). Before storing at –80 °C, 50 mL of a stock solution of 1 M sodium citrate and 5 mM sodium EDTA disodium salt was added to 950 mL supernatant 2000 g (SN 2000). A total of 0.1 mg silver colloid and 4.5 mg sodium dichloroisocyanurate were mixed with the sample before HFD was performed. Then 5 mL concentrated SN2000 was left in the membrane, 200 mL treated Milli-Q water (0.1 mg silver colloid and 4.5 mg sodium dichloroisocyanurate L^{-1}) was filled to wash the membrane. Concentrated SN2000 named as HFDa was collected.

2.3. Tamm–Horsfall Denaturation

A summary of the uEV isolation and THP elimination protocol is shown in **Figure 1A** and Figure S1, Supporting Information. One milligram of HFDa was treated with 5 mL (final volume) of a solution made of:

a) 200 mM dithiothreitol (DTT) in 10 mM triethanolamine.[7]
b) 1% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic (CHAPS).[10]
c) 6 M Urea in 50 mM citrate buffer, pH 3.5, and 10 mM arginine;
d) 200 mM DTT in10 mM triethanolamine plus 6 M urea,
e) 1% CHAPS and 200 mM DTT in10 mM triethanolamine.

All samples were mixed in end-over-end agitation (40 revolutions min^{-1}) for 1 h at RT and placed again in the HFD system using a 1000 kDa molecular weight cut-off (MWCO) dialysis membrane. Fifty milliliters of the same buffer followed by 200 mL of deionized (DI) water was used to remove all the proteins below the MWCO (HFDb).

2.4. Transmission Electron Microscopy, Protein assay, Gel Electrophoresis, and Western Blotting

A full description of the methods is provided in Supporting Information (see Supporting Information Methods)
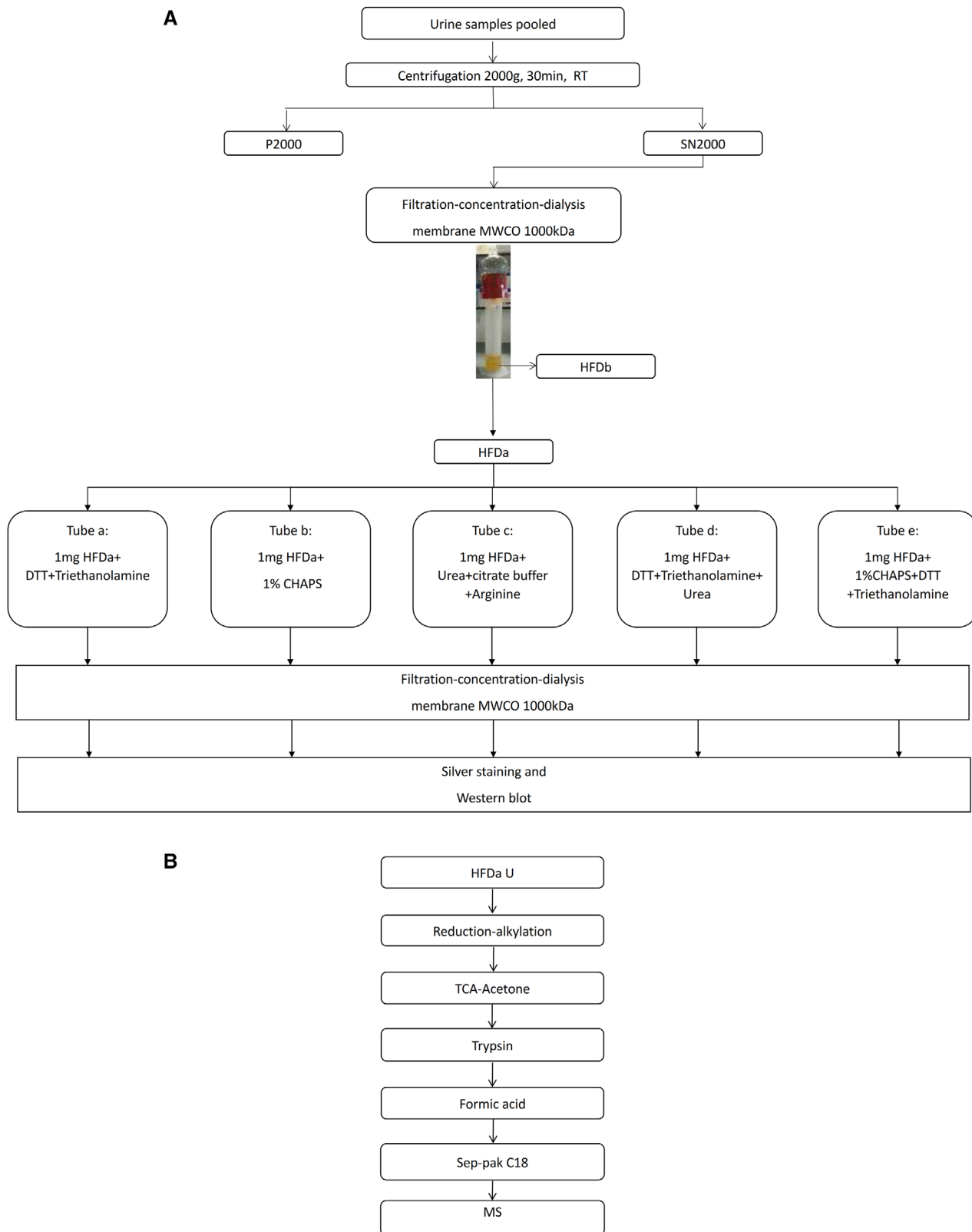
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A

Urine samples pooled

Centrifugation 2000g, 30min, RT

P2000

SN2000

Filtration-concentration-dialysis
membrane MWCO 1000kDa

HFDb

HFDa

| Tube a:
1mg HFDa+
DTT+Triethanolamine | Tube b:
1mg HFDa+
1% CHAPS | Tube c:
1mg HFDa+
Urea+citrate buffer
+Arginine | Tube d:
1mg HFDa+
DTT+Triethanolamine+
Urea | Tube e:
1mg HFDa+
1%CHAPS+DTT
+Triethanolamine |

Filtration-concentration-dialysis
membrane MWCO 1000kDa

Silver staining and
Western blot

B

HFDa U

Reduction-alkylation

TCA-Acetone

Trypsin

Formic acid

Sep-pak C18

MS

Figure 1. Workflow of the uEV isolation and THP elimination (A) and sample preparation for MS analysis (B).

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2.5. Tunable Resistive Pulse Sensing

Tunable Resistive Pulse Sensing (TRPS) measurements were performed with qNano instrument (Izon Ltd, Christchurch, New Zealand) according to manufacturer's instructions. Polyurethane nanopore membrane NP150 (analysis range 85–300 nm) and NP300 (analysis range 150–900 nm; Izon Ltd) were utilized, membranes were stretched at 46 mm (NP150) and 44.5 mm (NP300); the voltage was set at 0.46 V (NP150) and 0.34 V (NP300), Multipressure was set at 2, 5, and 8 mBar (NP150) and at 3, 6, and 9 mBar (NP300), respectively, to determine particle concentration. Electrolyte solution was made of PBS and 0.05% (v/v) Triton X100 filtered with a Millipore Millex GS 0.22 µm syringe filter (Merck Millipore). Current pulse signals were collected using Izon Control Suite 3.1.2.268 software (Izon Ltd). Blockade counts setting in this study was fixed at minimum of 1000 events. Calibration was made using standard polystyrene particles of 100, 200, and 330 nm (CPC100B; CPC200B, and CPC400B; Izon Ltd.). uEV fractions were sonicated for 5 min and before the analysis with the NP150 samples were filtered with a Millipore Millex GS 0.45 µm syringe filter (Merck Millipore). A full description of the method is provided in Supporting Information (see Supporting Information Methods).

2.6. MS Analysis

One hundred microgram as dry pellet of HFDa U (Figure 1B) was resolubilized and reduced in 100 µL of 10 mm Tris (carboxyethyl) phosphine (TCEP), 8 m urea, 0.1 mm EDTA, and 0.2 m Tris buffer PH8.8 in dark for 1 h at RT, followed by alkylation with 20 mm iodoacetamide (IAA) in dark for 2 h. Finally, 40 mm N-acetyl cysteine (NAC) was used to quench the excess of IAA. The sample was then precipitated by TCA-acetone to eliminate the excess of salts. Samples were digested by sequencing grade trypsin in presence of 1% w/v sodium deoxycholate (DOC) in 50 mm Tris-HCl pH 7.8 in a ratio of 1 µg of trypsin for 50 µg of total protein, at +37 °C overnight. After the digestion samples were acidified with 1% v/v formic acid (FA) to precipitate DOC, the supernatant, along with the external proteome, was cleaned up by Sep-Pak C18 cartridge (Waters Associates, Milford, Ma) according to manufacturer's instructions.

Cleaned up samples were dissolved in 0.1% v/v TFA and 2% v/v acetonitrile (ACN) solution, directly loaded onto an RP analytical column (300 µm id × 5 mm, packed with Acclaim PepMap RSLC C18, 5 µm, 100 Å, nanoViper). The gradient was composed of an increase from 5% to 50% solvent B (0.1% FA in 80% ACN) over 65 min, and climbing to 90% in 4 min, then holding at 90% for 6 min. All was done at a constant flow rate of 300 nL min^{-1}. The MS analysis was performed on Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific).

The peptides were subjected to NSI source followed by MS/MS in Q ExactiveTM (Thermo Fisher) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70 000. Peptides were selected for MS/MS using NCE setting as 27; ion fragments were detected in the Orbitrap at a resolution of 17 500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 1E5 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800 m/z. Fixed first mass was set as 100 m/z. Protein identification were performed with Protein Pilot 5.0 (AB Sciex) software by searching Uniprot-Human (http://www.uniprot.org/proteomes/UP000005640).

3. Results

3.1. THP Denaturation

DTT, urea, CHAPS, and a combination of urea-DTT and CHAPS-DTT were used to compare THP polymer denaturation and release THP complexing with uEVs and then harvested with a second HFD step.

Figure 2 shows the partitioning of THP, the large band at 100 kDa (Figure 2A,B), TSG101 as a general vesicle marker (Figure 2C,D), and human serum albumin (HSA) as a soluble control protein (Figure 2E,F).

Among the chaotropic reagents and their combinations tested, only urea in acidic buffer, urea, and DTT at pH 7.6 were effective to remove the bulk of THP from the vesicles fraction (Figure 2A, lanes 3a and 4a). This fraction was flushed in the flow-through (Figure 2B, lanes 3b and 4b) without affecting the integrity of vesicles as indirectly evidenced by presence of TSG101 in the retentate (Figure 2C, lanes 3a and 4a) but not in the flow-through (Figure 2D, lanes 3b and 4b). Notably, while both treatments successfully removed THP, they were practically ineffective to strip vesicles from HSA as only a small fraction of HSA was recovered in the flow-through with the DTT–urea treatment. A minimum of 6 m urea (Figure S2, Supporting Information) in acidic pH (Figure S3, Supporting Information) was necessary to depolymerize THP filaments at a molecular weight smaller than the MWCO of the dialysis membrane (1000 kDa).

3.2. Transmission Electron Microscopy

Electron microscopy analysis confirmed the presence of a heterogeneous population of vesicles in respect to size and morphology (Figure S4, Supporting Information). This shows there is no apparent change in vesicle class proportions. Notably, THP significantly entrapped uEV into its filamentous meshwork in HFDa samples (Figure S4A, Supporting Information), but in HFDa after urea treatment (HFDa U) samples this THP protein meshwork was not seen (Figure S4B, Supporting Information).

3.3. TRPS for Vesicle Size Distribution

With the particle size distribution (PSD) method used for PSD analysis, it was evident that their number and size were closely similar for both HFDa and HFDaU (Table S1, Supporting
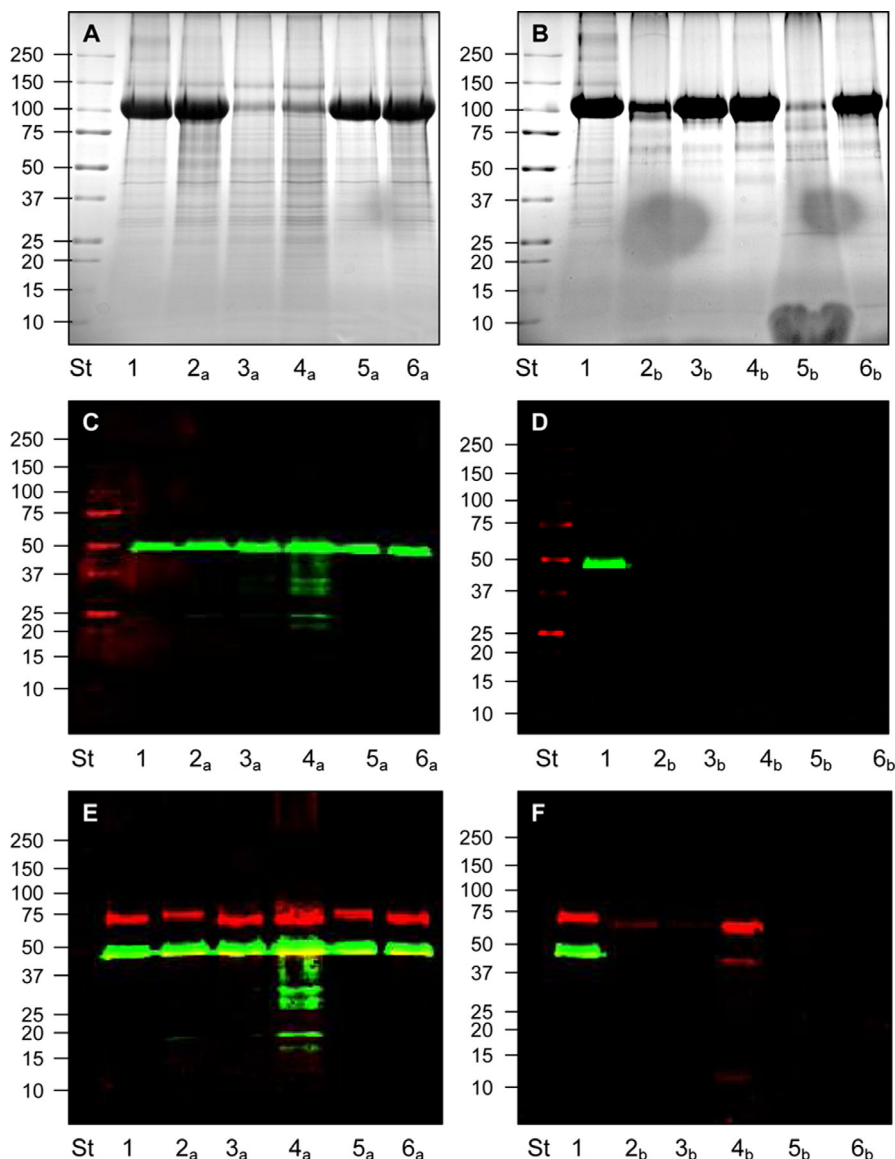
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Figure 2. Colloidal Coomassie staining and Western blotting of the fraction after the chaotropic treatments. A) Retentate from the dialysis tube (a: above the membrane molecular cut-off). B) Flow through (b: below the membrane molecular cut -off). Ten micrograms of total protein per lane was loaded. C and D) Detection of TSG101; E and F) Detection of human serum albumin and TSG101. Lane 1, HFDa; Lane 2a and 2b, DTT; Lane 3a and 3b, urea pH 3.5; Lane 4a and 4b, urea + DTT pH7.6; Lane 5a and 5b, CHAPS; Lane 6a and 6b, CHAPS + DTT

Information) uEVs. This result directly shows the good recovery of uEVs after urea treatment without effect to any vesicle categories.

3.4. Western Blot Analysis of uEV Markers Before (HFDa) and After (HFDaU) Urea Treatment

Analyzed by Western blotting (**Figure 3**), we observed that the level of nine EV markers (six membrane proteins and three internal EV content markers) was not affected by urea treatment. This finding shows that integrity of uEVs after urea treatment remains intact and urea is not affecting stability of vesicles. In addition, EV markers used were not filtered through the dialy-

sis membrane, nor present in HFDb urea, which would occur if they were not protected within EV. Some of the non-EV associated proteins present in the HFDa were not significantly affected by urea treatment. Accordingly, albumin, IgA α-chain, and IgM μ-chain remained intact while minor reduction in level of IgG γ-chain and α-1-antitrypsin inhibitor was seen in HFDaU.

3.5. Mass Spectrometry

Protein profile of uEVs recovered by the HFDaU was done by in-solution trypsin digestion proteomic analysis.[15] Accordingly, we found 1171 (HFDaU1), 1176 (HFDaU2), and 1021 (HFDaU3) proteins identified by at least two peptides with 95% of
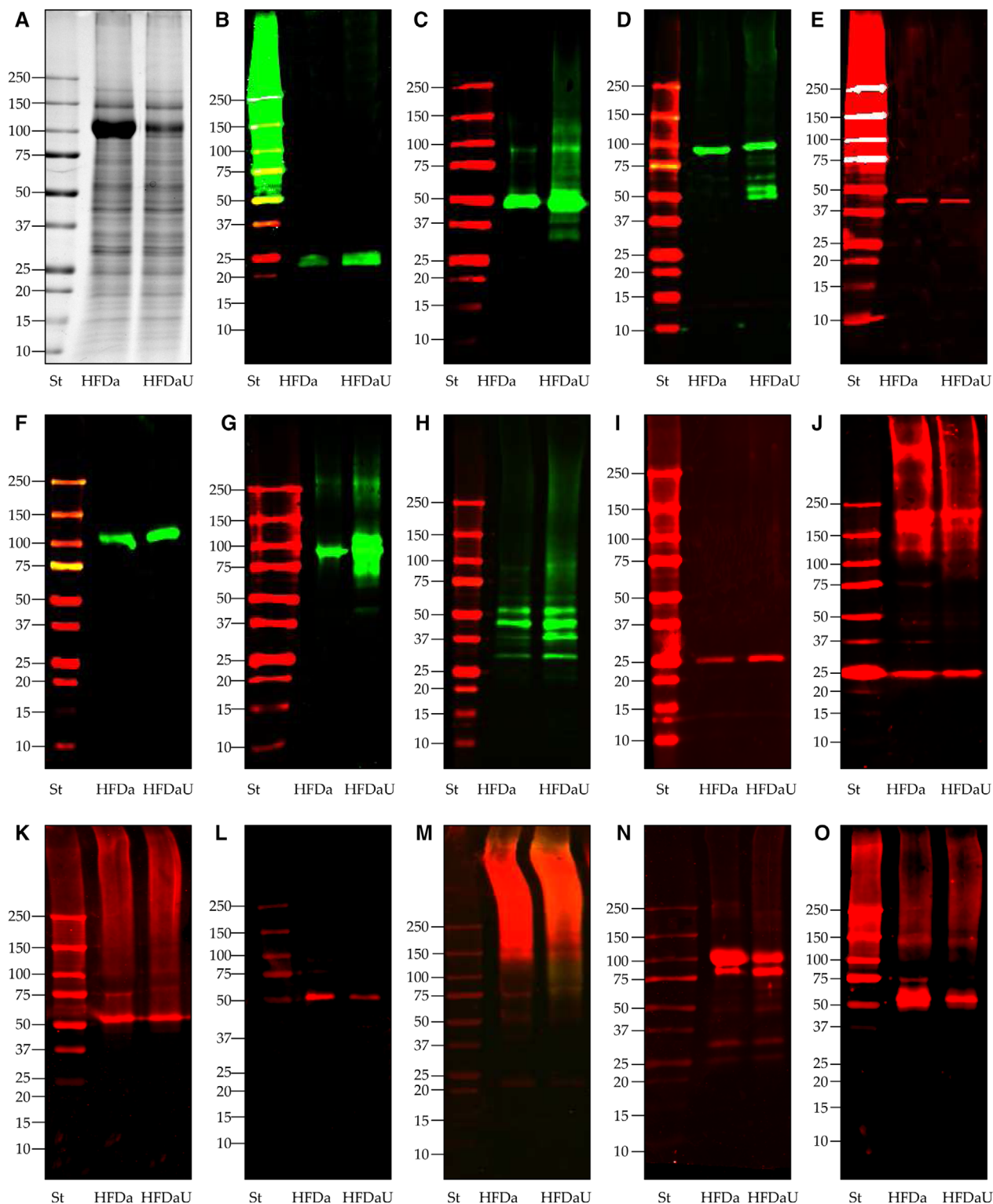
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Figure 3. Western blot analysis of uEV markers (B–J) and non EV-associated proteins (K–O) before (HFDa) and after (HFDaU) urea treatment. A) Coomassie Staining; B) CD9; C) TSG101; D) ALIX; E) β Actin; F) dipeptidyl peptidase 4 DPP4; G) Nephrilysin NEP; H) Podocin; I) Calbindin (CALB); J) Rab5a; K) Human serum albumin (HSA); L) IgG γ chain; M) IgA α chain, N) IgM μ chain; O) α1 antitrypsin inhibitor.

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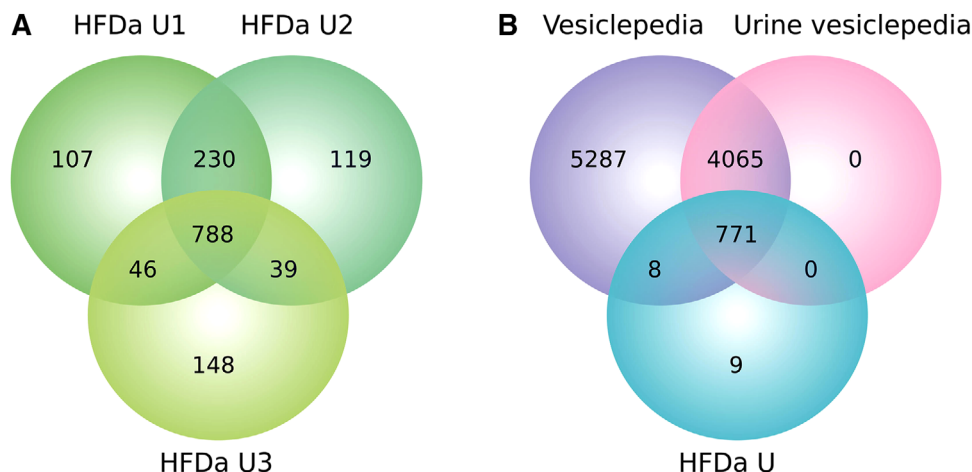www.clinical.proteomics-journal.com

Figure 4. Venn diagram of the protein identification: A) Common proteins in HFDa U1, HFDa U2, and HFDa U3; B) Common and unique proteins in HFDa U (788), vesiclepedia (10130) and urine vesiclepedia (4835).

confidence, respectively, for the three replicas of same preparations. Out of 1477 non-redundant protein identification (HFDa U1+U2+U3), 788 proteins were common in all three datasets (**Figure 4A**). Interestingly, 771 of our protein yield were found in the Vesiclepedia repository (Figure 4B; www.microvesicles.org), which collects all published information EV findings, while nine proteins have not been reported in this repository before.

Gene ontology classification (http://www.pantherdb.org/; large-scale gene function analysis with the PANTHER classification system; **Table 1A**) of those 788 proteins showed that 20 of the proteins identified in HFDaU belong to the endosomal sorting complex required for vesicle transport (ESCRT) pathway itself.[16] This shows that our urea treatment does not appear to damage vesicles. Altogether around 69% of all ESCRT machinery components were detected from HFDaU in our study, showing that exosomes remain abundant in the uEV fraction after urea treatment.

Tetraspanin-enriched microdomains are yet another class of known proteins involved in the intracellular sorting machinery to yield microvesicles and exosomes.[17] Notably, 11, eight, and four out of the 33 human tetraspanin proteins were identified in HFDaU1, -U2, and -U3, respectively (see Table 1B). This directly shows that urea treatment is delicate enough not to destroy the fragile protein components of microvesicles and exosome biogenesis.

Using the GO biological process analysis approach, we could show that additional ten of our found HFDaU proteins are involved in cytoskeletal trafficking of vesicles (Tables S2 and S3, Supporting Information).

In summary our results show that urea treatment is delicate enough and can release uEV proteins when THP contaminant is carefully managed.

4. Discussion

Tamm–Horsfall glycoprotein is the most abundant "normal" physiological protein found in urine, with tendency to aggregate and form filamentous meshworks to actively bind urinary par-ticles, apoptotic cells[18] and interact directly with uropathogenic bacteria.[19] Accordingly, THP appears to protect against urinary tract infections but also prevents, e.g., calcium crystal formation and kidney stones.[20] Moreover, it regulates innate immunity by binding to immunoglobulins and cytokines[21] evidencing the multitude of its actions in the urine. Beyond these data, the full physiological role of THP still remains incompletely understood.

Notably, THP level in urine appears to be a valuable indicator of functional kidney mass[22] and the respective UMOD gene is associated with chronic kidney disease and, especially, diabetic kidney damage[23] by mechanisms to be fully elucidated.

Here, we show how THP directly affects recovery of uEV harvest and distorts its downstream protein analysis. Many known artifacts in urinary analytics are caused by THP aggregation, which harvests free proteins, reduces the number of urinary cells, as well as distorts extracellular vesicle yield.[14] As shown in this study, better management of THP in urine adds new proteins to those reported in earlier uEV studies (see www.microvesicles.org). Notably, artifacts caused by THP similarly distort analytics directly from crude urines.

Several approaches have been used earlier to manage the known THP effects on uEVs. These have attempted to eliminate the bulk of urinary THP. In some approaches, reagents including dithiothreitol (DTT)[7] or deuterated water to create sucrose cushion or gradient[24] have been used. Other methods to rid THP include size exclusion chromatography[25] or serial combination of DTT, centrifugation, centrifugal ultrafiltration,[26] and size exclusion chromatography.[27] Yet another alternative to eliminate THP artifacts is to induce THP polymerization with diatomatous earth filter.[28] Each of these approaches may have some benefits but in general they are inefficient, tedious, time consuming, and costly. According to our hypothesis the interference with THP occurs due to aggregation. Solubilization of these aggregates could also reduce contamination of uEV. For this, we tested three chaotropic agents, DTT, CHAPS, and urea. DTT disrupts the polymeric network presumably by reduction of disulfide bonds linking monomers.[7] CHAPS is a non-denaturing zwitterionic detergent suited for the disruption of nonspecific protein interactions, while also protecting conformation of protein(s).[10]

Table 1. List of proteins identified in HFDa Urea belonging to ESCRT machinery (A) and tetraspanin family (B).

A

Complex	Accession number	Gene name	Protein name	Number of ESCRT proteins	
				HFDa U	database
ESCRT-0	O14964	HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate	1	1
ESCRT-I	Q99816	TSG101	Tumor susceptibility gene 101 protein	7	9
	Q9UK41	VPS28	Vacuolar protein sorting-associated protein 28 homolog		
	Q9H9H4	VPS37B	Vacuolar protein sorting-associated protein 37B		
	A5D8V6	VPS37C	Vacuolar protein sorting-associated protein 37C		
	Q86XT2	VPS37D	Vacuolar protein sorting-associated protein 37D		
	Q96EY5	MVB12A	Multivesicular body subunit 12A		
	Q9H7P6	MVB12B	Multivesicular body subunit 12B		
ESCRT-II	Q9BRG1	VPS25	Vacuolar protein-sorting-associated protein 25	2	3
	Q86VN1	VPS36	Vacuolar protein-sorting-associated protein 36		
ESCRT-III	O43633	CHMP2A	Charged multivesicular body protein 2a	6	11
	Q9UQN3	CHMP2B	Charged multivesicular body protein 2b		
	Q96FZ7	CHMP6	Charged multivesicular body protein 6		
	Q9H444	CHMP4B	Charged multivesicular body protein 4b		
	Q9NZZ3	CHMP5	Charged multivesicular body protein 5		
	Q7LBR1	CHMP1B	Charged multivesicular body protein 1b		
Vps4-Vta1	P53990	IST1	IST1 homolog (hIST1) (Putative MAPK-activating protein PM28)	4	4
	Q9UN37	VPS4A	Vacuolar protein sorting-associated protein 4A		
	O75351	VPS4B	Vacuolar protein sorting-associated protein 4B		
	Q9NP79	VTA1	Vacuolar protein sorting-associated protein VTA1 homolog		

B

Protein accession	Gene name	Protein name	HFDa U1	HFDa U2	HFDa U3
O60637	TSPAN3	Tetraspanin-3	√	√	√
O43657	TSPAN6	Tetraspanin-6	√	√	√
P19075	TSPAN8	Tetraspanin-8	√	√	√
O00322	UPK1A	Tetraspanin-21	√	√	√
O60635	TSPAN1	TSPAN1	√	√	
O14817	TSPAN4	TSPAN4	√	√	
P60033	CD81	TSPAN28	√	√	
P08962	CD63	TSPAN30	√	√	
Q86UF1	TSPAN33	TSPAN33	√		
P48509	CD151	TSPAN24	√		
P27701	CD82	TSPAN27	√		

Urea disrupts noncovalent bonds in proteins, thus working as protein denaturant, and it can be used to increase solubility of proteins.[29] In addition, arginine was experimented as a co-solvent with urea, as it is known to increase solubility and contribute to protein folding.[29]

The principles behind urea effects on proteins have been actively discussed for decades but still remain unknown. Urea may exert its effect directly, by binding to the protein and disrupting van der Waals interactions resulting in denaturation. Alternatively, urea acts as a chaotrope, altering the hydrogen-bond structure of water thus promoting the solvation of hydrophobic groups. Urea can also bind to the amino acid backbone to compete with native interactions in the unfolding process. Why pH 3.5 is optimum for urea action can be due to ionizable groups like tyrosine (pK_a = 10.07), cysteine (pK_a = 8.33), histidine (pK_a = 5.97), aspartic (pK_a = 3.65), and glutamic acid (pK_a = 4.25), which could lose the negative charge, respectively. In general low pH incudes very low electrostatic interactions among charged groups while the protein backbone remains positive. The pH effect on protein–glycan moieties may also play a role.

We have shown earlier that failure to manage THP properly may lead to up to 40% loss of uEVs, thus loosing

unselectively important uEVs of different classes.[30] Resulting lower uEV yields may very potentially loose valuable biomarkers. Furthermore, most of the earlier approaches have been limited by urine volumes: if up to 40% of uEVs are lost in THP precipitation, bigger starting volumes are needed to compensate losses in harvesting. Elimination of THP typically also adds one extra step to EVs isolation protocols. As we demonstrate here, management of THP can be efficiently integrated into EVs isolation process with Hydrostatic Filtration Dialysis with major benefits of vastly simplified and less time consuming procedure.

Existence of valuable biological material entrapped within vesicles of variable sizes has been reported since late 1980s.[31] The seminal reports of EVs found also in urine and, especially, of their valuable contents of structural and functional proteins, lipids, DNA, and a variety of RNA species have created a fundamentally new opportunity for simplified biomarker identification. Notably, EVs appear to consist qualitatively 4–10× more of potential biomarkers compared to use of crude urine and should thus be preferred. Furthermore, the protein and RNA diversity of uEVs appears well protected against, e.g., free urinary ribonuclease and protease activities.

With the recent development of a new approach called HFD with its optimization by urea treatment many known caveats of conventional EV isolation techniques can be avoided as shown here. HFD is simple, inexpensive, and easy to adopt in any laboratory without costly machinery or ultracentrifugation device. It also easily handles big volumes of urine rapidly, by itself equals urinary electrolytes and can easily bypass known artifacts caused by urinary pigments.

In summary, we provide here an easy method optimized to manage challenges with urinary THP caused artifacts in all urinary analytics.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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