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REVIEW ARTICLE

# Uromodulin and the study of urinary extracellular vesicles

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**Abstract**

Urinary extracellular vesicles (uEVs) are a promising substrate for discovering new biomarkers. In order to investigate the origin of uEVs and the cargo they carry, some types of downstream analysis of uEVs may require concentration and enrichment as well as removal of contaminating substances. Co-isolation of the abundant urinary protein uromodulin with uEVs can be a problem, and may interfere with some techniques, in particular with proteomic analysis tools. Methods of separating out uromodulin and its removal have also not been standardized. This review highlights aspects of uromodulin structure that makes it recalcitrant to separation from uEVs, summarizes frequently used techniques for uEV enrichment and how they affect uromodulin separation, and specific methods for uromodulin removal during preparation of uEVs. The necessity of uromodulin removal for various study endpoints is also examined.

KEYWORDS
extracellular vesicles, Tamm–Horsfall protein, uEVs, urine, uromodulin

## 1 | INTRODUCTION

Urinary extracellular vesicles (uEVs) are biolipid membrane-bound particles released from cells that are excreted in urine. EVs in urine were first discovered by electron microscopy in 1986 (Wiggins et al., 1986). Subsequently, vesicles of tubular and podocyte origin were found (Pascual et al., 1994; Scherberich, 1989). Interest in uEVs greatly increased in 2004, when the pivotal work of Pisitkun et al. characterized uEV content by mass spectrometry, and found markers from tubular epithelial cells and the urothelium (Pisitkun et al., 2004). uEVs are heterogeneous in their origin, arising not only from cells of the urogenital tract but also distant anatomical sites and even resident bacteria. Likewise, uEVs are heterogeneous in structure, varying in size and composition, giving rise to a plethora of different names, including exosomes, microvesicles, apoptotic bodies, and so forth. In this review, we will default to the generic and inclusive term, uEV, as defined in the Minimal information for studies of extracellular vesicles 2023 guidelines and the position paper by the Urine Task Force of the International Society of Extracellular Vesicles (Erdbrügger et al., 2021; Welsh et al., 2024). uEVs can carry a variety of cargoes, including proteins, lipids, small metabolites, and nucleic acids. The realization that these cargoes reflect their cell of origin, as well as molecular and pathological processes ongoing in these cells, has expanded interest in the use of uEVs for biomarker discovery (Dhondt et al., 2020; Wu et al., 2021). The methodologies to investigate these cargo types vary, but a common starting point is often separation or enrichment of uEVs from urine and removing contaminating material (Dong et al., 2020; Liangsupree et al., 2021).

A recurring problem with the study of uEVs is the presence of the urinary protein uromodulin, due to its abundance and structure, and its propensity to co-isolate with uEVs. Highly abundant proteins, like uromodulin, can 'drown-out' the signal from less abundant proteins in proteomic studies by mass spectrometry (Abramowicz et al., 2016; Jain & Malik, 2024). Likewise, since uromodulin is glycosylated, it can obscure the signal from other glycosylated proteins from uEVs in mass spectrometry-based glycoproteomic analysis (Brown et al., 2020). Quantification of uEVs by nanoparticle tracking analysis (NTA) may also be compromised by the presence of uromodulin (Droste et al., 2021). A potential problem posed by uromodulin is the possible entrapment of uEVs in uromodulin filaments which might cause the loss of important uEV biomarkers from samples. Although the concept of uEV entrapment by uromodulin is frequently invoked in the literature, actual evidence of this mechanism is scant, which will be discussed further below.

There is no consensus on the best methods to separate uEVs from uromodulin, and a general lack of knowledge on how these purification methods influence loss or enhancement of uEV associated biomarkers. While most studies examining uEVs attempt to deal with uromodulin in some manner during enrichment and isolation, a minority of published papers have actually assessed the efficiency of uromodulin depletion, the effect these steps have on uEV yield or the endpoint of their characterization. This review will emphasize studies where the presence of uromodulin was examined during separation of uEVs, and how effective the methods used were in reducing interference from uromodulin. Finally, we will assess the necessity of uromodulin removal for various study endpoints.

## 1.1 | Uromodulin expression and structure

Several excellent reviews on uromodulin structure and its many functions, including protection from urinary tract infections, prevention of kidney stones, and roles in renal sodium, calcium and magnesium transport, have recently been published and will not be repeated here (Devuyst et al., 2017; LaFavers et al., 2022; Micanovic et al., 2020; Schaeffer et al., 2021). However, a brief summary of some uromodulin features is required for understanding the problem in the context of studying uEVs.

Uromodulin is the most abundant protein in urine under normal physiological conditions, with a range of excretion rates of 29–57 mg/day (Pruijm et al., 2016). It was first discovered in 1950 by Igor Tamm and Frank Horsfall as a mucoprotein capable of inhibiting viral hemagglutination (hence its other name, Tamm–Horsfall Protein or THP) (Tamm & Horsfall, 1950). Subsequently, it was independently discovered as a glycoprotein with immunomodulatory properties in pregnant women's urine and given the name uromodulin (Muchmore & Decker, 1985). We will use the Uniprot recommended name for the protein here, which is 'uromodulin' (UniProt Consortium, 2023). Only a few years later, it was shown that uromodulin and THP were the same protein (Pennica et al., 1987). Its structure and multiple functions remained enigmatic for many years but have begun to be elucidated.

Uromodulin is almost exclusively made by kidney, with about 90% coming from cells lining the Thick Ascending Limb (TAL) and the remaining 10% from the distal tubule (Schaeffer et al., 2021). It is translated as a 640 amino acid precursor protein with 48 cysteines, which form 24 intramolecular disulfide bonds. The importance of these disulfide bonds for protein processing and maturation is demonstrated by the frequent mutation of these cysteines in forms of autosomal dominant tubulointerstitial kidney disease (ADTKD–*UMOD*) (Eckardt et al., 2015). Loss of a cysteine in mutated uromodulin leads to a spare free cysteine, improper folding, and formation of aggregates in the endoplasmic reticulum (Schaeffer et al., 2021). Glycosylation occurs at eight asparagine-linked (N-linked) sites (Weiss et al., 2020). The domain architecture of uromodulin (depicted in Figure 1) consists of a signal sequence, three EGF-like repeats, a cysteine-rich domain (D10C), a fourth EGF-like repeat, a bipartite Zona Pellucida domain containing ZP-N and ZP-C subdomains with an Internal Hydrophobic Patch between them, a consensus cleavage site, external hydrophobic patch (EHP), and a C-terminal glycosyl-phosphatidyl-inositol (GPI) anchorage site. Until recently, a region contained within D10C, was known as the D8C domain, but new information on protein folding has redefined the borders of this domain (Stsiapanava et al., 2022).

The majority of uromodulin is secreted to the apical membrane of tubular epithelial cells where it is anchored by GPI, in an unpolymerized form due to association of the IHP with the EHP domain. However, it should be noted that a small amount of unpolymerized uromodulin is found in urine, blood and kidney interstitium, and this unpolymerized form retains the EHP domain (LaFavers et al., 2022; Micanovic et al., 2022). The EHP containing form of uromodulin found in urine has a C-terminus at F617, past the S614 GPI addition site, as opposed to EHP-retained isoform found in serum which terminates at K607 (LaFavers et al., 2022; Micanovic et al., 2022). This suggests the possibility that some unpolymerized uromodulin in urine could be anchored to uEVs via GPI. It is noteworthy that peptides corresponding to the region between S589 and S614 of uromodulin were detected in mass spectrometry analysis of purified uEVs (Musante et al., 2020). For the majority of uromodulin, cleavage by the protease hepsin at F587, causes the EHP to dissociate from the internal hydrophobic patch (IHP), making the uromodulin monomers competent for polymerization and excretion into the urine (Brunati et al., 2015). Polymerization occurs due to interaction between ZP-N and ZP-C subdomains from adjacent monomers, forming long polymerized filaments (Weiss et al., 2020; Stanisich et al., 2020; Stsiapanava et al., 2020).

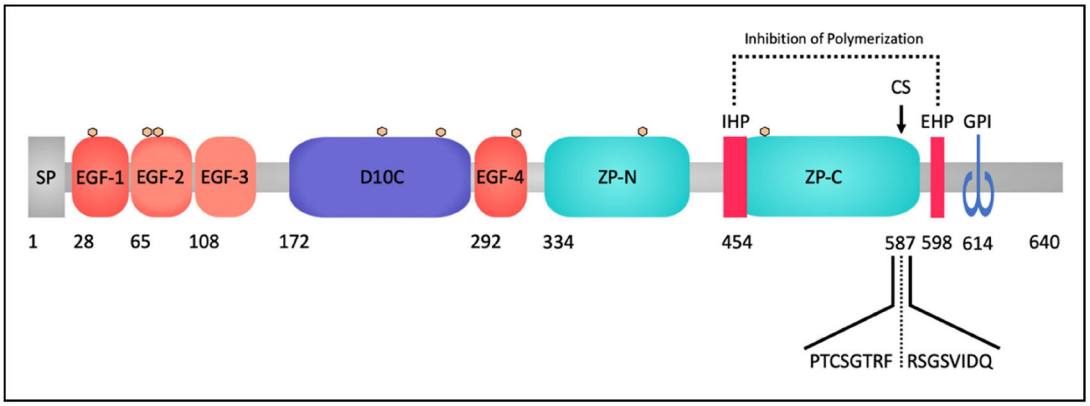


**FIGURE 1** Schematic presentation of the uromodulin domains. SP, signal peptide (leader peptide); CS, hepsin cleavage site; D10C, 10-cysteine domain; EGF, epidermal growth factor-like domains; EHP, External hydrophobic patch; GPI, glycosyl-phosphatidyl-inositol anchoring site; Hexagons, N-linked glycosylation site; IHP, internal hydrophobic patch; ZP-C, C-terminal fragment of the Zona Pellucida domain; ZP-N, N-terminal fragment of the Zona Pellucida domain (Rampoldi et al., 2011; UniProt Consortium, 2023; Wu et al., 2018).

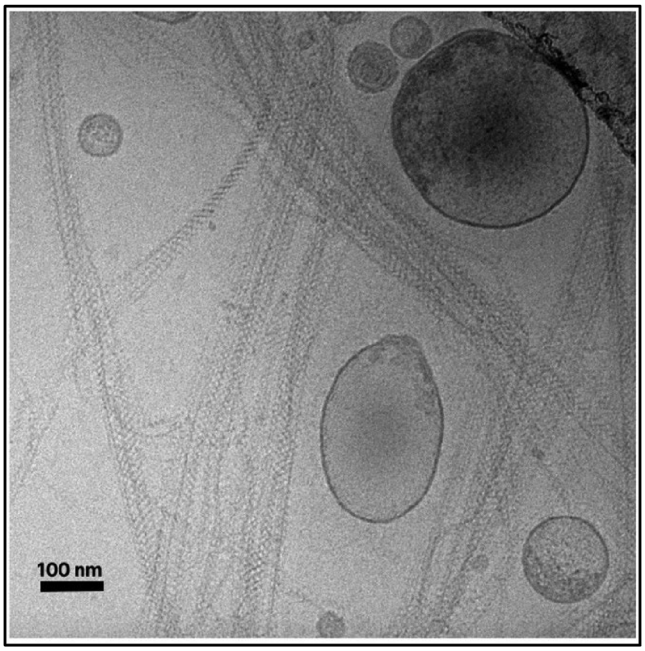


**FIGURE 2** Cryo-electron microscopy image of urinary extracellular vesicles (uEVs) showing uromodulin filaments. Uromodulin polymers form filamentous networks in urine that can co-isolate with uEVs, and possibly entrap them. Figure demonstrates that uEVs of different shapes and sizes co-isolate with uromodulin filaments.

There is also evidence from electron microscopy that these filaments can form higher order sheet-like structures or networks by lateral interactions, probably mediated by the protruding arms composed of the EGF I to III and D10C domains and associated N-linked glycosylation moieties (Stsiapanava et al., 2020; Weiss et al., 2020). A specific high-mannose glycosylation site, along with the N-terminal branch or arms, is known to be critical for binding entero-pathic *Escherichia coli* (Stsiapanava et al., 2022; Weiss et al., 2020). These uromodulin networks are thought to play a role in entrapping bacteria, thus inhibiting urinary tract infections. It is not known to what extent uEVs could become enmeshed in these filamentous networks in a similar fashion, but the association of uEVs with higher-order uromodulin structures seen by electron microscopy demonstrates the possibility (Gonzales et al., 2008; Wachalska et al., 2016) (Figure 2). The extent of uEV binding or entrapment by uromodulin versus co-isolation of uromodulin with uEVs deserves further investigation and is discussed below in Section 3.1.

Urine is a highly dynamic biofluid, whose solute composition and concentration varies widely based upon fluid intake, diet, time of day and disease status (Balhara et al., 2023). The amount of uromodulin normally excreted correlates with urine output, but its concentration has a large range (Pruijm et al., 2016). Uromodulin tends to polymerize and aggregate at high concentrations, low pH or in high ionic strength (especially Ca++ and Na+) solutions, even when these parameters are in the physiologic range

(McQueen & Engel, 1966; Stevenson et al., 1971). This variability in urine composition and the extent of uromodulin aggregation complicates separation of uEVs from uromodulin in disparate samples using physical methods like centrifugation.

## 2 | UEV ENRICHMENT METHODS AND EFFICACY OF UROMODULIN SEPARATION

Due to the greatly increased interest in EVs, a multitude of techniques for their separation from urine have been developed, which are summarized in several recent reviews (Barreiro & Holthofer, 2017; Konoshenko et al., 2018; Monguió-Tortajada et al., 2019; Sidhom et al., 2020). However, some of these methods have not been characterized for separation of EVs from urine or their ability to separate uEVs from uromodulin. Here, we will summarize the most frequently used uEV separation techniques, including differential centrifugation (dUC), density gradient ultracentrifugation, density cushion ultracentrifugation, size exclusion chromatography (SEC), ultrafiltration, hydrostatic filtration dialysis (HFD) and precipitation with polymers, and highlight their ability to remove uromodulin. Many studies employ a combination of these techniques to affect uEV enrichment and uromodulin depletion.

### 2.1 | Differential centrifugation

Differential centrifugation and/or differential ultracentrifugation (dUC) is the most commonly used EV separation technique according to a recent survey of the field, and this is also true for the study of urinary EVs according the EV-Track database (Royo et al., 2020; Van Deun et al., 2017). This method involves centrifuging urine at a series of increasing speeds to remove contaminants and concentrate uEVs: first low-speed (300–1000 g, some groups use slightly higher initial centrifugation in the 2000–5000 g range) to remove cells, intermediate speed (10,000–20,000 g) to remove cell debris, large protein aggregates and large or dense vesicles, and a final ultracentrifugation step (more than 100,000 g) to pellet uEVs (Nawaz et al., 2014). Most investigations have focused on uEVs pelleted in the final ultracentrifugation step, and thus ignore the uEV content of the low centrifugation pellets. It should be noted that there is considerable overlap in the populations of uEVs isolated at different speeds. Musante et al. have demonstrated that the population of uEVs pelleted at 21,000 g are heterogeneous in size (40–250 nm) and contain uEVs bearing the uEV markers TSG101 and CD9 (Musante et al., 2020). This population of uEVs is often discarded in studies utilizing differential centrifugation. In addition to losses during lower speed preparatory centrifugation, uEVs are lost in supernatants even after ultracentrifugation at 200,000 g (Barreiro & Holthofer, 2017; Musante et al., 2013; Musante et al., 2016). Although dUC is often combined with chemical reduction of uromodulin polymers (see discussion of dithiothreitol/DTT below) to make uromodulin more soluble and effect its removal during centrifugation, a significant amount remains in the uEV pellet after the final ultracentrifugation step (Hogan et al., 2014). Differential centrifugation is laborious and time consuming (the final ultracentrifugation step alone is often 2 h or more) and requires expensive ultracentrifuges and rotors.

### 2.2 | Density gradient and density cushion ultracentrifugation

Density gradient ultracentrifugation is a technique used to purify uEVs and even to fractionate different populations of uEVs based on density. Hogan et al. used continuous sucrose gradients made with deuterium oxide (D2O) to isolate populations of uEVs, enriched for markers arising from different nephron segments, and to effectively separate them from the bulk of uromodulin which was found at the bottom of the gradient (Hogan et al., 2009, 2014). Use of D2O to make the gradients allowed an increase in the overall density of the gradient, necessary for the separation of uromodulin from uEVs, without a corresponding increase of the solutions' osmolarity. Iodixanol (OptiprepTM) is a popular medium for gradient separation due to its ability to make iso-osmotic solutions at different densities (Van Deun et al., 2014). Effective separation of uEVs and depletion of uromodulin using iodixanol density gradients has been demonstrated (Dhondt et al., 2020; Mussack et al., 2019; Van Deun et al., 2014; Van Dorpe et al., 2023). Dhondt et al. compared Top-Down (TD) sample loading on discontinuous iodixanol gradients to Bottom-Up (buoyant density, BD) sample loading on discontinuous iodixanol gradients for separation of uEVs from contaminating proteins, including uromodulin (Dhondt et al., 2020). After ultracentrifugation, the gradients were fractionated by density ranges and characterized by NTA, western blotting and electron microscopy. The density fraction with peak particle counts by NTA also demonstrated the highest level of uEV marker proteins by western blotting using both TD and BU type gradients; however, separation of this uEV peak from uromodulin was superior in the BU loaded gradients. Dorpe et al. demonstrated that automatic liquid handling, compared to manual methods of preparation of discontinuous iodixanol density gradients and fraction collection, reduced the variability of results (Van Dorpe et al., 2023). This later study also demonstrated better separation of uEVs from uromodulin using Bottom-Up, as opposed to Top-Down sample loading.

Density gradient ultracentrifugation suffers from the same high-cost equipment disadvantages as dUC, while compounding the time of processing problem, as gradients are typically centrifuged for 18–24 h (Hogan et al., 2009, 2014; Mussack et al., 2019; Van Deun et al., 2014; Van Dorpe et al., 2023).

Density cushion ultracentrifugation is a simpler technique than density gradient ultracentrifugation but it also relies on buoyant density to concentrate and separate uEVs from contaminating material. Mitchell et al. used density cushions to separate uEVs from urine (Mitchell et al., 2009). In this study, urine was cleared of cells and debris by centrifugation at 330 g, and then at 2000 g. The supernatant was under-layered with a 30% sucrose/D2O cushion and centrifuged at 100,000 g for 2 h. After washing the uEVs captured in the cushion with PBS, the protein was ran on polyacrylamide gels and blotted for uEV markers. There was a marked reduction in uromodulin in the uEV samples when compared to the 2000 g supernatants and the supernatant above the cushion. The presence of uEV marker proteins was also greatly enriched in the uEVs from the cushion. In comparison, centrifugation of a 17,000 g supernatant at 200,000 g yielded more uromodulin contamination and weaker uEV marker bands in the 200,000 g pellet than the uEVs isolated using the density cushion. Raj et al. expanded on the density cushion ultracentrifugation technique by including a second, more-dense cushion below the first cushion (Raj et al., 2012). In this study, they first isolated uEVs by sequential centrifugation of urine at 400, 800 and 15,000 g, and pelleting of crude uEVs at 200,000 g for 1 h. The crude uEVs were then resuspended in either PBS (phosphate buffered saline) and under-layered with a 1 molar sucrose cushion prepared with PBS/D2O or resuspended in 20 millimolar tris pH8.6 buffer and under-layered with a first cushion of 1 molar sucrose in 20 millimolar tris pH8.6/D2O and a second cushion of 2 molar sucrose in 20 millimolar tris pH8.6/D2O. The cushions were then centrifuged at 110,00 g for 3 h. uEVs from each cushion layer were then washed twice with the respective suspension buffer (either PBS or 20 millimolar tris pH8.6). PAGE gel analysis showed significantly less uromodulin in both layers of the double cushion compared to the single cushion, which was attributed to solubilization of uromodulin in the low ionic strength tris buffer. They then performed western blotting for a panel of renal tubular/uEV proteins from the single cushion or each layer of the double cushion. Detection of uEV markers from uEVs separated from the single cushion or either layer of the double cushion was greater than in the crude uEV starting material but was greatest in the first cushion of the double cushion technique. Interestingly, mass spectrometry analysis of proteins isolated from the first and second cushion yielded some unique proteins in each population, perhaps indicating that some vesicles with specific origins have different densities. While this double density cushion technique yielded high quality uEVs with relatively little uromodulin contamination, it would be time-consuming due to the multiple ultracentrifugation steps involved, and the 1 L of starting urine necessitated pooling of individual samples, which reduces its utility in comparing patient samples.

## 2.3 | Size Exclusion Chromatography (SEC)

SEC is an increasingly popular technique for EV enrichment and purification (Monguió-Tortajada et al., 2019; Royo et al., 2020). The principle of SEC is that a mobile phase (i.e., biofluid containing EVs) is passed over a column containing a stationary phase composed of porous polymer beads (Sidhom et al., 2020). Large particles, like EVs, elute from the column first since they are excluded from the pores in the beads. Smaller components of the sample, like soluble proteins and small aggregates, transverse through the pores, delaying their elution. By collecting the eluate in fractions, it is possible to separate EVs from contaminating material. SEC has the capability of separating uEVs from contaminants while leaving them relatively unaltered and intact (Monguió-Tortajada et al., 2019; Sidhom et al., 2020). Several studies have shown the ability of SEC to separate uEVs from uromodulin (Cho et al., 2020; Dong et al., 2020; Droste et al., 2021; Karttunen et al., 2021; Rood et al., 2010). A limitation of SEC is the volume of starting material that can be loaded onto the column, necessitating concentration by other means, prior to loading. Karttunen et al. compared the efficiency of uEV separation and removal of uromodulin by SEC preceded by three different concentration methods, ultracentrifugation, ultrafiltration or a commercial polymer precipitation kit (Karttunen et al., 2021). They used NTA to quantify particle yields after SEC, and western blotted fractions for uEV marker proteins, TSG101, Alix and CD9 and also blotted for uromodulin contamination. SEC was able to produce uEVs positive for the markers and with greatly reduced amounts of uromodulin in the uEV containing fractions, regardless of the initial sample concentration method. Although ultracentrifugation followed by SEC produced the highest uEV counts, that method also had the greatest variability in yield between replicate preparations. Ultrafiltration followed by SEC had the least variability between replicate isolations measured by NTA of uEV counts. Dhondt et al. measured the recovery of uEVs by spiking in recombinant, GFP labelled EVs to the starting urine before concentration by centrifugal ultrafiltration followed by separation using SEC (Dhondt et al., 2020). They estimated approximately 60% recovery of labelled EVs after SEC. Therefore, the method of concentration can introduce variation and losses of uEVs into the workflow (Droste et al., 2021; Karttunen et al., 2021). Depending on the study endpoint, the eluted fractions from SEC which contain uEVs, may also need to be concentrated (Cho et al., 2020). Since uromodulin polymers could possibly interfere with uEV separation on SEC columns, and potentially co-elute with uEVs due to the large size of the polymers, many studies incorporate a denaturation step to diminish polymerization. Typically, chemical reduction using DTT has been used to reduce uromodulin to monomers or smaller aggregates before separation by SEC (Gheinani et al., 2018; Lozano-Ramos et al., 2015, 2018).

## 2.4 | Ultrafiltration

Ultrafiltration (UF) uses commercially available nanomembrane concentrators, with defined pore sizes or molecular weight cutoffs (MWCO), to enrich uEVs from urine. Cheruvanky et al. demonstrated the ability of polyethersulfone membrane concentrators with a MWCO of 100 kDa to capture uEVs, by western blotting for uEV markers, from as little as 0.5 mL of urine (Cheruvanky et al., 2007). However, there was a large amount of these markers retained on the membrane, indicating loss of uEVs. Alvarez et al. used the same type of concentrator as Cheruvanky et al. to isolate uEVs and compared it to various other methods of uEV purification (Alvarez et al., 2012). Ultrafiltration resulted in a large amount of co-isolated uromodulin and very low levels of the uEV markers ALIX and TSG101 in western blots of isolated uEV proteins compared to other methods, which included dUC and polymer precipitation (see below). In a study of uEV recovery after ultrafiltration on four different kinds of membranes and reconstituted cellulose membranes (RC) with two MWCO (10 kDa; 100 kDa), Vergauwen et al. found that RC concentrators with a 10 kDa MWCO yielded the best recovery of particles as judged by NTA and western blotting for the uEV marker syntenin-1 (Vergauwen et al., 2017). They also observed a large amount of uromodulin binding to every type of membrane tested. Using the previously mentioned method of spiking urine with GFP-labelled EVs, Dhondt et al. obtained approximately 60% recovery of uEVs after filtration with a 10 kDa MWCO centrifugal filter device. Ultrafiltration is a rapid, easily implemented uEV enrichment technique, amenable to processing large numbers of clinical samples. However, it suffers from significant co-isolation of protein impurities, and potential loss of uEVs.

A new type of ultrafiltration separation of uEVs is microfluidics or tangential flow filtration. Although these techniques have not been widely used, Dong et al. have evaluated the use of a microfluidic tangential flow filtration device, Exodisc, to isolate uEVs (Dong et al., 2020). The Exodisc demonstrated good recovery of uEVs, measured by western blotting for CD63 and CD81 uEV marker proteins, with little uromodulin contamination. A downside of microfluidic separation of uEVs is the requirement for very specialized equipment.

## 2.5 | Hydrostatic Filtration Dialysis (HFD)

Another type of filtration enrichment of uEVs is HFD, developed by Musante et al (Musante et al., 2014). It uses a low-speed centrifugation (2000 g) step to clear urine of debris, followed by sample placement in a dialysis membrane with a high MWCO (1000 kDa). Hydrostatic pressure of the sample allows retention of analytes larger than the MWCO, while solutes and particles smaller than the MWCO can pass through (Barreiro & Holthofer, 2017). It is particularly suited to concentration of EVs from large, dilute volumes like those encountered with urine. An advantage of HFD is that it allows washing of the uEVs during the process and equalization of buffer conditions between samples. It was shown that 67% of uromodulin in the initial, first morning void urine was removed in the low-speed centrifugation step, and only an estimated 4% of uromodulin was retained with the uEV isolates after HFD; however, it remains the most abundant protein in the uEV fraction (Musante et al., 2014). Furthermore, loss of uEVs on the dialysis membrane was only 18%, as measured by western blotting for TSG101. HFD has proven to be a simple, low-cost and efficient means for harvesting uEVs for several downstream applications, including proteomics and RNA analysis (Barreiro et al., 2020; Xu et al., 2019).

## 2.6 | uEV precipitation with polymers

Precipitation of uEVs with hydrophilic polymers, like polyethylene glycol (PEG), is another widely used method for concentrating uEVs (Royo et al., 2020). The hydrophilic polymer works by volume exclusion, forcing the uEVs out of solution, so that they can be collected by low-speed centrifugation. Polymer based precipitation is also the method used by several commercially made kits for EV isolation, namely, Exoquick (System Biosciences), Total Exosome Isolation Reagent (ThermoFisher), ExoPrep (Exom Biopharma) and miRCURY Exosome Isolation Kit (Qiagen) (Konoshenko et al., 2018). Alverez et al. developed a modified Exoquick based protocol to isolate uEVs and compared its performance to the standard Exoquick protocol, ultracentrifugation, and ultrafiltration using total protein gel electrophoresis, western blotting for uEV markers, and RNA yield (Alvarez et al., 2012). The modified Exoquick protocol yielded the highest amount of Alix and TSG101 positive EVs, and the highest RNA amounts, but the resulting uEV pellet still had a significant amount of contaminating proteins including uromodulin. Royo et al. compared the Exoquick and Total Exosome Isolation kits to ultracentrifugation by western blotting the samples for several uEV marker proteins and uromodulin (Royo et al., 2016). They found more co-isolation of uromodulin and uEVs with ultracentrifugation than with either precipitation kit, but there was significantly less signal for several uEV marker proteins for samples isolated with these two kits. While polymer-based precipitation is a simple method suited to study a large number of samples, the large amount of impurities isolated with uEVs is problematic for many study end-points. In addition to co-precipitation of impurities

in the sample, contamination of uEV samples with PEG or similar polymers is incompatible with mass spectrometry analysis (Abramowicz et al., 2016).

### 2.7 | Affinity capture

While various affinity capture techniques, including lectin binding and immunoprecipitation, have been used in the isolation of uEVs, their performance regarding uromodulin contamination has not been extensively evaluated. The presence of glycosylated proteins on the surface of uEVs allows their binding by specific carbohydrate-binding lectins coupled to biotin, followed by capture using streptavidin-coupled magnetic beads (reviewed by Svenningsen et al., 2020) (Svenningsen et al., 2020). Svenningsen et al. also demonstrated the selective isolation of uEVs from the proximal tubule using lotus tetragonolobus lectin (LTL) and from principal collecting duct cells with Dolichos Biflorus Agglutinin (DBA), by western blotting eluates for cell type specific markers (Svenningsen et al., 2020). Isaksson et al. used this technique to investigate the presence of activated complement proteins in uEVs collected from kidney transplant recipients after PEG precipitation. They found significantly less uromodulin contamination in the uEV eluates after LTL binding than with DBA binding, suggesting greater affinity of glycosylated uromodulin for the DBA lectin (Isaksson et al., 2022). Droste et al. evaluated the performance of a commercial kit that utilizes immunoaffinity capture of CD9, CD63 and CD81 tetraspanin positive uEVs on magnetic beads (Miltenyi Biotec exosome isolation pan kit) (Droste et al., 2021). After concentration of urine by ultrafiltration and isolation of uEVs by the kit, samples were western blotted for uEV marker proteins and uromodulin. Immunoaffinity isolated samples were positive for TSG101 and CD9 but contained barely detectable amounts of uromodulin. However, the authors noted that samples isolated with this kit could not be analysed by flow cytometry or NTA due to the presence of 'EV-microbead aggregates'.

Other affinity capture-based techniques, notably the commercial kit from Norgen that uses a proprietary resin to bind uEVs, have been studied for their ability to separate uEVs from uromodulin. In the previously mentioned study by Royo et al., uEVs isolation by the Norgen kit was compared to ultracentrifugation as well as the two precipitation-based kits (Royo et al., 2016). While the Norgen kit yielded good recovery of most uEV marker proteins in western blots, the contamination with uromodulin was the highest of any of the methods tested. This is in contrast to the results from Barreiro et al. that found small amounts of uromodulin co-isolating with uEVs using the Norgen kit, but also very low levels of uEV marker proteins when compared to uEVs isolated by ultracentrifugation or HFD (Barreiro et al., 2020).

## 3 | REMOVAL OF UROMODULIN DURING ISOLATION OF UEVS

The ability of uromodulin to form high molecular weight polymers and aggregates makes it recalcitrant to removal from uEV preparations by the methods described above. While these physical separation techniques can reduce the amount of contaminating uromodulin, they cannot fully achieve separation. However, various combinations of the aforementioned techniques used in conjunction with methods to de-polymerize uromodulin or break-up uromodulin aggregates, have proven effective at minimizing co-isolation of uromodulin while also improving uEV yield. The methods used for de-polymerizing or preventing uromodulin aggregation include reducing agents, increasing pH and decreasing the ionic strength of the media (i.e., dilution), lowering of calcium and sodium concentration, and the use of detergents and chaotropic agents. An alternative strategy is to decrease the solubility and increase aggregation of uromodulin by adding salt to the urine sample, enabling removal of uromodulin by an initial low-speed centrifugation step. Table 1 summarizes various methods for removing uromodulin in the preparation of uEVs.

### 3.1 | Reducing agents

The first paper to molecularly characterize the contents of uEVs used the strong reducing agent DTT to depolymerize uromodulin, in conjunction with a two-step dUC protocol, to allow the analysis of uEV proteins by a gel-based mass spectrometry method (Pisitkun et al., 2004). In this study, and a subsequent paper from the same Knepper group (Gonzales et al., 2009), urine was cleared of debris by an initial 17,000 g centrifugation followed by centrifugation at 200,000 g to pellet the uEVs. The pelleted uEVs were resuspended in a solution containing 200 mg/mL DTT and heated at 95°C for 2 min. It was posited that DTT treatment allowed removal of de-polymerized uromodulin, after a second high speed, 200,000 g centrifugation (Gonzales et al., 2009; Pisitkun et al., 2004). Although, no data was shown in either of these studies demonstrating depolymerization or removal of uromodulin from the uEV preparations. In a follow-up study, Fernandez–Llama et al. treated an initial 17,000 g pellet with DTT, then combined the treated pellet with the initial 17,000 g supernatant and re-centrifuged at 17,000 g (Fernández-Llama et al., 2010). The amount of uromodulin in the second 17,000 g pellet was greatly reduced; however, the amount of uromodulin pelleted after a subsequent 200,000 g ultracentrifugation was greatly increased. In this later study it was empirically determined

**TABLE 1** Uromodulin removal methods.

| Removal method | Mechanism | Advantages | Disadvantages/Limitations |
|---|---|---|---|
| **Reducing agents**<br>**Dithiothreitol (DTT)** (Fernández-Llama et al., 2010; Gonzales et al., 2009; Musante et al., 2012; Pisitkun et al., 2004)<br>**Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl)** (Musante et al., 2020) | -DTT = Strong reducing agent which breaks disulfide bonds<br>-Depolymerizes and denatures uromodulin<br>-Milder reducing agent than DTT<br>-Depolymerizes uromodulin | -Reduces co-sedimentation of uromodulin with uEVs<br>-Can increase uEV yield in dUC isolation<br>-Useful for uEV proteomics<br>-Reduces co-isolation of uromodulin with uEVs at 10 nM concentration | -May damage uEV structure<br>-Not selective for uromodulin, likely to alter the dimerization, folding and structure of uEV proteins<br>-Could impact the activity of resident receptors and enzymes → undesirable for functional studies<br>-May increase contamination of uEV isolates with non-uEV derived protein/RNA complexes<br>-Not selective for uromodulin, likely to alter the dimerization, folding and structure of some uEV proteins<br>-IGFBP-7, TIMP-2 seem to be differentially affected by TCEP reduction<br>-Undesirable for some functional studies |
| **pH, ionic strength (e.g., low sodium and calcium) and dilution** (Puhka et al., 2017; Raj et al., 2012) | -Minimizes hydrophobic interactions that cause the formation of large uromodulin aggregates<br>-Can be used for repeated 'washing' of uEV pellets to remove uromodulin | -Keeps uromodulin in soluble fraction upon centrifugation<br>-Significant increase in uEV recovery | -Does not result in uromodulin depolymerization with some filamentous uromodulin remaining in EM images of uEV prep<br>-Possible loss of vesicles upon washing steps |
| **Detergent agents**<br>**3- [(3-cholamidopropyl) dimethylammonio]-1-propane sulfonic (CHAPS)** (Musante et al., 2012) | -CHAPS is a non-denaturating zwitterionic detergent<br>-Disrupts non-specific protein interactions, and increases the solubility of uromodulin to allow separation from uEVs | -Reduction of sedimentation of uromodulin upon centrifugation<br>-Possible choice for uEV proteomics and RNA content analysis<br>-CHAPS facilitates removal of uromodulin while maintaining normal uEV structure<br>-Retention of the enzymatic activity of two uEV integral membrane proteases, indicating some preserved function of uEVs. | -Risks some damage to uEV structure and functions |

(Continues)

**TABLE 1** (Continued)

| Removal method | Mechanism | Advantages | Disadvantages/Limitations |
|---|---|---|---|
| **Denaturing agents/Chaotropes** (Bielopolski et al., 2024; Xu et al., 2019)<br>**Urea or guanidine hydrochloride** | -Denatures uromodulin, preventing large aggregates and increasing its solubility<br>-Removal of denaturing agents and solubilized uromodulin by passage through a filtration/dialysis membrane | -Preservation of uEV specific markers<br>-Urea treated preps were suitable for proteomic analysis by mass spectroscopy<br>-Urea treatment does not destroy uEV architecture to an extent, that would interfere with downstream separation and analysis.<br>−6M guanidine hydrochloride dissolved in acidic citrate buffer, depolymerizes uromodulin sufficient for SEC | -Not suitable for functional studies<br>-Non-specific denaturation would likely alter folding, and interactions of resident uEV proteins, therefore effecting their biological activity |
| **Salt precipitation of uromodulin or ZnSO4 treatment**<br>**Salt (NaCl) precipitation** (Kosanović & Janković, 2014)<br>**Zinc sulphate ($ZnSO_4$)** (Liu et al., 2018) | -Addition of 0.58 M NaCl to urine decreases solubility of uromodulin so that it can be removed by low-speed centrifugation<br>-Mechanism not fully understood<br>-Removes uromodulin by addition of ZnSO4 prior to vesicle isolation by differential centrifugation | -Little loss of CD63 uEV specific marker in NaCl treated, low-speed pellet<br>-Simple, effective removal of uromodulin from final uEV isolate<br>-Some preservation of structure/function as seen by uEV glycan binding to lectins<br>-Reduces uromodulin interference in mass spectroscopy analysis of uEV proteome<br>-No effect on size, number and concentration of the vesicle | -Needs further evaluation for loss of uEVs by co-precipitation with uromodulin<br>-High salt treatment may denature some uEV proteins, resulting in loss of function<br>-Efficiency of uEV recovery was not evaluated<br>-More investigation is required |

that pretreatment with 200 mg/mL (1.3 molar) of DTT was necessary for removal of uromodulin from the 17,000 g pellet. This concentration of DTT has been used to depolymerize and denature uromodulin in numerous uEV studies, and has become a standard, although few reports demonstrate that this concentration of DTT is appropriate for the samples being studied or that it affects the removal of uromodulin from the ultimate uEV isolates (i.e., uEVs pelleted by ultracentrifugation) (Bielopolski et al., 2024).

Fernandez–Llama et al. observed increased recovery of uEVs in the 200,000 g pellet after pretreatment of the 17,000 g pellet with DTT, as determined by western blotting for uEV markers (Fernández-Llama et al., 2010). They attributed this to entrapment of uEVs by uromodulin pelleted in the first 17,000 g centrifugation, and release from this entrapment by reduction of uromodulin with DTT. Some other studies discussed below have addressed the extent of uEV entrapment by uromodulin (Correll et al., 2022; Musante et al., 2014).

Musante et al. used a differential centrifugation scheme where urine was first centrifuged at 2000 g. This first 2000 g pellet was then treated with DTT according to Fernández–Llama et al. (Fernández-Llama et al., 2010), and recentrifuged at 2000, 17,000 and 200,000 g (Musante et al., 2014). Only traces of the uEV marker TSG101 was recovered in the initial 2000 g pellet and even less after higher speed centrifugation of the sample from the DTT-treated 2000 g pellet. Moreover, it was estimated that nearly 67% of uromodulin was recovered in the initial 2000 g pellet. If uromodulin polymers and aggregates significantly entrap uEVs, then a majority of uEVs would be expected to be lost in the first 2000 g pellet, which was not observed.

Correll et al. sought to directly measure the proportion of uEVs that are potentially trapped by uromodulin and which could be recovered by DTT treatment (Correll et al., 2022). In this experiment, a sample of urine with Expressed Prostatic Secretions (EPS-urine, urine sample obtained after digital rectal examination), was centrifuged initially at 20,000 g. The 20,000 g supernatant was then centrifuged at 175,000 g and the pellet washed with an alkaline wash solution, filtered through a 0.22 μm membrane and uEVs recovered by another centrifugation at 175,000 g. The 20,000 g pellet was treated with DTT, similar to the procedure described by Fernández-Llama (Fernández-Llama et al., 2010), and centrifuged again at 20,000 g. This second, DTT-treated 20,000 g supernatant was then centrifuged at 175,000 g and the pellet washed in parallel with the first sample. NTA was used to obtain a size profile and number of particles recovered from the DTT-treated and untreated samples. The size distribution of particles recovered from the two samples closely matched, but the particle yield from the DTT-treated sample was 5-fold less than the particle number from the untreated sample. This led the authors to conclude that reduction of uromodulin by DTT could increase uEV yield by about 20%. However, the identification of particles recovered by DTT treatment as true uEVs was not confirmed in this experiment.

Musante et al. have shown that the reducing agent tris (2-carboxyethyl) phosphine hydrochloride (TCEP) can efficiently remove uromodulin from uEVs isolated by a relatively low-speed (21,000 g) centrifugation (Musante et al., 2020). In this study, high density uEVs were recovered from a 21,000 g pellet after initial clearing of the urine by centrifugation at 4600 g. The 21,000 g pellet was resuspended in buffer containing 10 millimolar TCEP, pH 7 incubated at room temperature for 15 min, diluted approximately 10-fold in 4 millimolar TCEP buffer, pH 8.8 and recentrifuged at 21,000 g. The protocol enhanced recovery of uEV-associated protein markers in the 21,000 g pellets, but it did not prevent co-sedimentation of uromodulin upon higher speed ultracentrifugation. It is noteworthy that TCEP differentially affected the recovery of GFBP-7 and TIMP-2 from uEVs. This observation emphasizes the need for optimization of uromodulin removal techniques for specific experimental targets.

The use of reducing agents in uEV isolation may potentially increase co-isolation of some types of contaminants. Wachalska et al. compared a two-step dUC isolation, with and without DTT treatment of an initial 15,000 g pellet containing uromodulin, by profiling miRNAs (microRNAs) found in the EVs obtained upon final ultracentrifugation (Wachalska et al., 2016). They found an approximate 10-fold increase in RNA in the DTT-treated compared to the untreated samples. This was in contrast to the relatively small amount of uEVs they estimated were trapped by uromodulin in the 15,000 g pellet. The conclusion was that miRNAs are complexed with non-uEV proteins which co-sediment with uromodulin, and that DTT treatment releases these RNA/protein complexes, increasing their co-isolation with uEVs upon ultracentrifugation. Although it is also possible that inactivation of RNases by DTT may have contributed to the increased miRNA yield seen in the DTT-treated uEV samples. Therefore, measurements of uEV RNA content should be treated with caution if DTT treatment is used during isolation.

Treatments with reducing agents are likely to alter the dimerization and structure of uEV proteins that depend on disulfide bonds. These alterations could impact the activity of receptors and enzymes in uEVs, making these techniques undesirable for functional studies. Although reduction of uromodulin may modestly decrease uEV loss during relatively low-speed centrifugation and isolation, it will not prevent the co-sedimentation of uromodulin with uEVs upon ultracentrifugation. Reduction of uromodulin with DTT is a widely used method in studies that use gel-based separation of proteins prior to mass spectrometry for proteomics. However, uromodulin is still an abundant protein detected in many uEV proteomics studies that utilize reduction by DTT during isolation (Gonzales et al., 2009; Musante et al., 2012; Pisitkun et al., 2004).

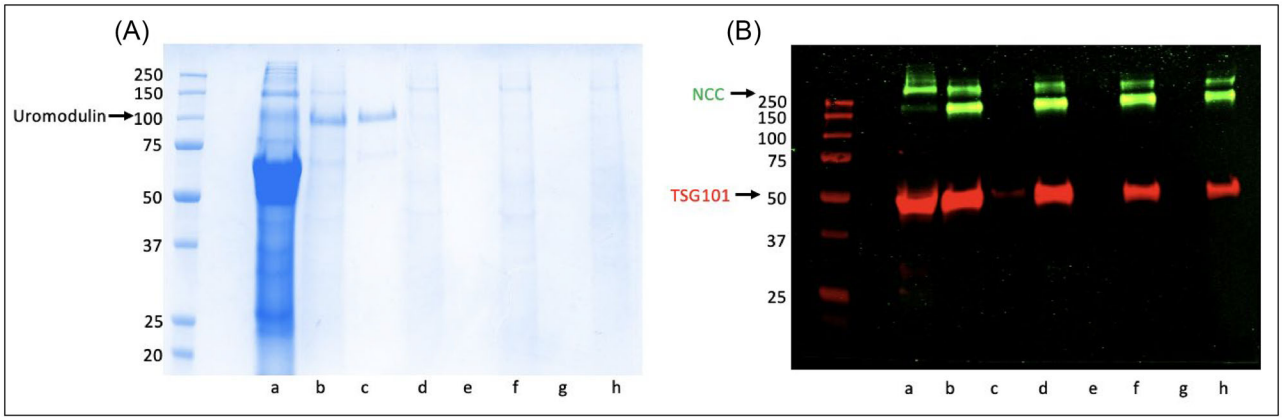


**FIGURE 3** Image of coomassie blue stained gel (A) and immunoblot (B). Reducing the presence of uromodulin by washing with a low ionic strength buffer three times followed by centrifugation at 20,000 g for 30 min. Uromodulin is significantly reduced in (a) without losing NCC and TSG101 signal in (b). (a) Supernatant before washing. (b) Pellet before washing. (c) Supernatant after first wash. (d) Pellet after first wash. (e) Supernatant after second wash. (f) Pellet after second wash. (g) Supernatant after third wash. (h) Pellet after third wash.

## 3.2 | pH, ionic strength and dilution

Conditions that solubilize uromodulin were identified in a paper by Kobayashi et al (Kobayashi & Fukuoka, 2001). Use of alkaline pH, low ionic strength (e.g., low sodium and calcium) and the addition of Triton X-100 was shown to maintain uromodulin in the soluble fraction upon low-speed centrifugation (14,000 g). The authors concluded that hydrophobic interactions likely mediate aggregation of uromodulin filaments and minimizing these interactions increases solubility. The solubilization was achieved by diluting purified or urinary uromodulin in a low ionic strength buffer containing Triton X-100 and EDTA at pH of 7.5 (TEA buffer). Youhanna et al. found that diluting urine with water had a similar effect to the low ionic strength TEA buffer on increasing the solubility of uromodulin (*Youhanna et al.*, 2014). Although, neither Kobayashi et al. nor Youhanna et al. addressed the study of uEVs, they did describe properties of uromodulin that have been exploited by extracellular vesicle researchers to enhance its removal during physical enrichment of uEVs.

A simple means of minimizing uromodulin co-isolation during uEV enrichment is urine dilution, as demonstrated by Pukha et al. in a protocol termed 'KeepEX' (Puhka et al., 2017). They found that Tris-HCl buffer, pH 8.6 mixed with urine at 4:1 ratio increased by several fold the uEVs recovery by differential centrifugation, followed by ultracentrifugation at 100,000 g. The authors optimized the protocol by addition of EDTA, a chelator of metal ions, to the buffer and increasing the pH to 9.0. The Tris-EDTA buffer, pH 9.0 mixed with urine at 4:1 ratio (buffer:urine) improved particle counts by NTA and increased the uEV markers by western blotting. Uromodulin polymers were still observed by Electron Microscopy (EM) in the uEV preparations using the dilution protocol. The authors hypothesized that urine dilution 'loosened' uromodulin aggregations or decreased uromodulin binding to uEVs but did not result in uromodulin depolymerization. Interestingly, they did not observe a corresponding increase in uEV associated RNA content, which they hypothesized could be due to loss of RNA bound to the uEV surface, which is offset by the increase in uEV numbers isolated with the KeepEX protocol. One of the main disadvantages is to further dilute already dilute urine samples, necessitating the processing of large volumes.

It is worth noting that diluting urine with low ionic strength buffers, removing calcium and sodium, and using alkaline pH can be done at different steps of uEV isolation. We have found that serial suspension and washing of uEVs after centrifugation at 20,000 g in a buffer consisting of 10 millimolar HEPES, pH 7.4 and 50 millimolar EDTA can remove most of the uromodulin from the uEVs isolated by this relatively low-speed centrifugation. Figure 3 shows uromodulin by gel electrophoresis, and uEV specific markers by western blotting, of the pellets and supernatants during a series of washes leading to reduction of uromodulin (Bielopolski et al., 2024). Likewise, isolation of uEVs by Hydrostatic Filter Dialysis (HFD) entails 'washing' of retained uEVs with water (Musante et al., 2014; Barreiro et al., 2020). Although not all uromodulin is removed, improved uEV recovery and diminished uromodulin co-isolation after HFD suggests that their association is minimized by the process.

Correll et al. compared washing uEVs with an alkaline solution or with PBS for removal of uromodulin (Correll et al., 2022). They also compared the effects of reduction with DTT prior to the washing steps. Urine samples containing Expressed Prostatic Secretions (EPS-urine) were first centrifuged at 20,000 g. In one arm of the experiment, the pellets were treated with DTT, and centrifuged again at 20,000 g. The supernatants from the first and second 20,000 g spins were pooled and then centrifuged at 175,000 g. The 175,000 g pellets were then washed with either alkaline solution (150 millimolar $Na_2CO_3$ pH 11.0) or with PBS, filtered through a 0.22 μm filter, and uEVs recovered by centrifugation at 175,000 g. In the other arm of the experiment, treatment with DTT was omitted and the first 20,000 g supernatants were processed in the same manner. uEV recovery was assessed by

EM and western blotting for EV markers, CD9 and TSG101, and for uromodulin. EM showed isolation of intact uEVs from all the samples. The amount of uromodulin was greatly diminished in the DTT-treated and alkaline washed sample compared to the DTT-treated, PBS washed sample and either of the non-DTT treated samples. Interestingly, the DTT-treated and alkaline washed sample also had the highest levels of CD9 and TSG101. However, mass spectrometry analysis suggested that proteins associated with the uEV surface or 'protein corona', may have been lost in the alkaline washed uEVs.

### 3.3 | Detergents

Detergents have been shown to increase the solubility of uromodulin and separation from uEVs. The addition of triton X-100 in the TEA buffer discussed above, greatly reduced the sedimentation of uromodulin upon centrifugation (Kobayashi & Fukuoka, 2001). However, the use of strong detergents risks damaging the uEV structure, which precludes their use in the isolation of uEVs. Musante et al. introduced a treatment with CHAPS, a non-denaturing zwitterionic detergent, during uEV isolation by differential centrifugation (Musante et al., 2012). CHAPS facilitated removal of uromodulin without affecting the uEVs structure, determined by EM. While the protein content of CHAPS treated uEVs was similar to DTT treated uEVs, CHAPS treated uEVs retained the enzymatic activity of integral membrane proteases better than DTT treated uEVs. With both treatments, when the 17,000 g supernatants were ultracentrifuged without mixing with the first supernatant (according to Fernández-Llama et al. (Fernández-Llama et al., 2010)), it was clear that the high speed was responsible of the sedimentation of uromodulin, even in presence of CHAPS or DTT.

### 3.4 | Denaturing agents

Strong protein denaturing agents or chaotropes have also been used to denature uromodulin, facilitating its separation from uEVs. Xu et al. tested chaotropes, detergents, and reducing agents to remove uromodulin from uEVs isolated by HFD (Xu et al., 2019). After treatment, the denaturing agents and solubilized uromodulin were removed by repeating the HFD. The authors found that citrate buffer, pH 3.5, containing 6 molar urea and 10 millimolar arginine removed uromodulin sufficiently for mass spectroscopy analysis of the uEV proteome, detection of several uEV marker proteins by western blot, and retention of uEV architecture by EM. Protein denaturing agents have utility when studying uEV protein content, with diminished uromodulin contamination, but are not suitable for uEV functional studies.

### 3.5 | Salt precipitation of uromodulin and zinc sulphate

In an adaptation of a technique previously used to isolate uromodulin, salt precipitation has been used to remove uromodulin from urine, followed by differential centrifugation to isolate uEVs (Kosanović & Janković, 2014). Kosanovic et al. added sodium chloride to urine (final concentration of 0.58 molar) before pelleting uromodulin by low-speed centrifugation at 3000 g. uEVs were then enriched from this supernatant by a conventional dUC protocol of centrifugation at 17,000 g followed by ultracentrifugation at 100,000 g. They then assessed uromodulin removal by polyacrylamide gel electrophoresis of proteins from the various fractions. The majority of uromodulin appeared in the 3000 g pellet, with little detectable uromodulin in the final uEV pellet after ultracentrifugation. The basic uEV architecture, assessed by EM, was preserved in the salt-treated samples. Immunoblotting showed very little loss of the uEV-specific marker CD63 in the 3000 or 17,000 g pellets when compared to the 100,000 g pellet. Furthermore, uEV surface glycans were still able to bind plant lectins in a specific manner, indicating some possible preservation of function.

Another, analogous method demonstrated to decrease uromodulin co-isolation with uEVs, is inclusion of zinc sulphate (ZnSO4) during centrifugation (Liu et al., 2018). Liu et al. could not attribute a mechanism to this ZnSO4 effect but speculated that it could either promote uromodulin aggregation/polymerization, resulting in removal of uromodulin by initial low-speed centrifugation, or increase the solubility of uromodulin in the subsequent ultra-centrifugation. Although ZnSO4 treatment was able to effectively reduce uromodulin interference in analysis of the uEV proteome by mass spectroscopy in this study, the efficiency of uEV recovery was not evaluated. Further investigation is required to determine if ZnSO4 treatment promotes or inhibits co-isolation of some uEVs with uromodulin.

## 4 | CONCLUSIONS

The extent to which uromodulin interferes with the study of uEVs, greatly depends on the experimental endpoints (Table 2). Furthermore, it is not known whether specific subclasses of uEVs preferentially associate with uromodulin, and this lack of

**TABLE 2** Utility of uromodulin removal for different experimental endpoints.

| Method | Necessity of uromodulin removal (Yes/No) | Reason |
|---|---|---|
| **NTA enumeration** (Droste et al., 2021; Musante et al., 2020) | Yes | Uromodulin polymers or aggregates can be counted as particles |
| **Western Blot** (Droste et al., 2021; Musante et al., 2020) | No | For majority of target proteins, uromodulin removal is not needed, because gels are ran with denaturing (SDS) and reducing (DTT) conditions<br>Abundant uromodulin contamination could distort bands that run at same size and possibly cause background antibody binding |
| **Proteomics/MS analysis** (Gonzales et al., 2009; Pisitkun et al., 2004) | Yes | Uromodulin can obscure presence of similarly sized protein |
| **Lipidomics** (Bourderioux et al., 2015) | No | Except for possible entrapment of subclasses of uEVs by uromodulin could alter results<br>Analysis of uEV lipid composition by high-performance thin-layer chromatography (HPTLC) does not require removal of uromodulin |
| **Functional studies** | Yes | Testing for true EV effect |
| **Targeted or total RNA** (Barreiro et al., 2020; Cheng et al., 2014; Wachalska et al., 2016) | Yes/No | Removal of uromodulin by agents like DTT can increase uEV RNA yield in some studies but not others<br>DTT treatment risks co-isolating protein-RNA complexes with uEVs |
| **Flow cytometry** (Coumans et al., 2017; Musante et al., 2020; Rikkert et al., 2020) | No | Single uEV analysis of minimally processed samples<br>Possible entrapment of subclasses of uEVs by uromodulin could alter results |
| **Single-particle interferometric reflectance imaging** (Breitwieser et al., 2022) | No | Single uEV analysis of minimally processed samples<br>Possible entrapment of subclasses of uEVs by uromodulin could alter results |
| **Cryogenic electron microscopy imaging** (Fernández-Llama et al., 2010; Musante et al., 2020; Royo et al., 2016) | Yes/No | Possible entrapment of subclasses of uEVs by uromodulin could alter results |

knowledge complicates the decision on which uEV enrichment and uromodulin removal scheme to use. It is known that large amounts of contaminating uromodulin in uEV samples can interfere with some types of analysis. For example, large uromodulin polymers and aggregates can be counted as particles by NTA, invalidating the true uEV counts within a sample. An abundance of uromodulin can obscure less abundant, similarly sized proteins, in mass spectroscopy analysis of the uEV proteome. Also, in functional studies, it is necessary to remove uromodulin to ensure that the resultant phenotypes are mediated solely by uEVs. However, the methods used to separate uEVs from uromodulin can also introduce problems with some types of analysis, such as profiling uEV RNA content. The potential increase of non-uEV protein/RNA complexes co-isolating with uEVs upon DTT-reduction of uromodulin polymers discussed above, is one such example. Clearly, more rigorous documentation of the fate of uromodulin using various uEV isolation schemes needs to be done, being mindful of the possibility of inadvertently isolating subsets of uEVs.

Some of the challenges of uEV isolation and uromodulin depletion may be overcome with techniques that analyse single uEVs from minimally processed samples. Conventional flow cytometry analysis of uEVs is complicated by their small size, limiting detection by light scatter and fluorescent antibody binding due to the small number of epitopes per particle. However, using specialized optimization techniques and newly developed instruments with greater sensitivity, flow cytometry can be a powerful tool for the analysis of single uEVs (Coumans et al., 2017; Erdbrügger & Lannigan, 2016; Rikkert et al., 2020). Imaging and spectral flow cytometry is also being used more often for uEV analysis (Droste et al., 2021; Musante et al., 2020).

New platforms that couple antibody-based capture with fluorescent detection and imaging of single uEVs in a population, hold the promise of detailed uEV surface protein measurement and co-localization, with minimal sample processing. An example is single-particle interferometric reflectance imaging which can detect uEVs without further enrichment (Breitwieser et al., 2022). However, interference by uromodulin entrapment of specific subclasses of uEVs has not been fully evaluated with these single uEV analysis techniques.

Although the presence of uromodulin in uEV preparations is usually treated as a contaminant, it's ubiquitous presence, at some level, in even the most rigorous isolation techniques, suggests that it could also be a legitimate uEV cargo protein carried by a subset of uEVs. More detailed investigation of its association and interactions with uEVs needs to be done. This would also suggest that its complete removal during isolation of uEVs may not always be achievable or even desirable, based on the focus of the study.

## AUTHOR CONTRIBUTIONS

**Michael A. Harding**: Data curation (equal); investigation (equal); Methodology (equal); writing—original draft (lead). **Hayrettin Yavuz**: Data curation (equal); investigation (equal); methodology (equal); writing—original draft (equal). **Annika Gathmann**: Data curation; investigation; visualization. **Samantha Upson**: Data curation; investigation; visualization. **Agnieszka Swiatecka-Urban**: writing—review and editing. **Uta Erdbrügger**: Data curation (equal); investigation (equal); project administration (lead); supervision (lead); writing—review & editing (lead).

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## CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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