Published in final edited form as:

J Proteome Res. 2018 September 07; 17(9): 3308–3316. doi:10.1021/acs.jproteome.8b00459.

Highly Efficient Phosphoproteome Capture and Analysis from Urinary Extracellular Vesicles

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Abstract

Analysis of protein phosphorylation in extracellular vesicles (EVs) offers an unprecedented potential for understanding cancer signaling AND early stage disease diagnosis. However, prior to the phosphoproteome analysis step, the isolation of EVs from biofluids remains a challenging issue to overcome due to the low yield and impurity from current isolation methods. Here, we carry out an extensive assessment of several EV isolation methods including a novel rapid isolation method EVTRAP for highly efficient capture of extracellular vesicles from human urine sample. We demonstrate that over 95% recovery yield can be consistently achieved by EVTRAP, a significant improvement over current standard techniques. We then applied EVTRAP to identify over 16 000 unique peptides representing 2000 unique EV proteins from 200 μ L urine sample, including all known EV markers with substantially increased recovery levels over ultracentrifugation. Most importantly, close to 2000 unique phosphopeptides were identified from more than 860 unique phosphoproteins using 10 mL of urine. The data demonstrated that EVTRAP is a highly effective and potentially widely implementable clinical isolation method for analysis of EV protein phosphorylation.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00459.

Tables S1–S12 with proteome and phosphoproteome identification and quantitation of LC–MS data (ZIP) Table S13 (XLSX)

Figures showing more Western Blot examples of EVTRAP and UC comparison, EVTRAP reproducibility testing, and comparison between EVTRAP and commercial exosome isolation methods (PDF)

The authors declare the following competing financial interest(s): W.A.T. and A.I. are co-founders of Tymora Analytical Operations.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD010480 and 10.6019/PXD010480.

Keywords

extracellular vesicles; EVs; exosomes; urine; phosphoproteomics; EVTRAP

INTRODUCTION

Protein phosphorylation is a key control mechanism for cellular regulatory pathways and one that is often targeted by drug developers to create inhibitors that block signaling pathways involved in cancer and other diseases. Phosphorylation analysis, in particular the quantitative measurement of changes in phosphorylation, is vital to understanding how signaling networks interact and function and how they are misregulated in disease states.^{1,2} Multiple groups have already shown with some success that the development of cancer profiling and monitoring assays based on phosphorylation signaling events has high diagnostic potential.³⁻⁵ However, the profiling is usually done on a tumor biopsy, an invasive and painful procedure, and one that certainly is impractical for early disease diagnosis and continuous monitoring. “Liquid biopsies”, analysis of biofluids such as plasma, serum, urine, tears, or saliva, have recently gained momentum as a better source of diagnostic biomarkers. Liquid biopsies offer numerous advantages for a clinical analysis including noninvasive nature, a suitable sample source for longitudinal disease monitoring, better

screenshot of tumor heterogeneity, higher stability and sample volumes, faster processing times, and lower rejection rates and cost compared to their tissue counterpart. However, as far as liquid biopsy is concerned, phosphoproteins have been virtually ignored due to their instability and low abundance levels in biofluids, leading to low reported identification numbers. For example, only 31, 105, and 64 phosphoproteins were identified in three separate studies.^{6–8}

To help overcome these challenges, a new source of biological information has generated a lot of interest over the past few years: cell-secreted extracellular vesicles (EVs). These generally include smaller size exosomes derived from multivesicular endosome-based secretions and microvesicles (MVs) derived from the plasma membrane.^{9,10} EVs provide an effective and ubiquitous method for intercellular communication, stimulation of immune system, removal of harmful materials, and many other functions.^{11–13} As these particles are shed into virtually every biological fluid and embody a good representation of their parent cell, analysis of the EV cargo has great potential for biomarker discovery and disease diagnosis.^{14,15} Notably, researchers have also found many differentiating characteristics of the cancer cell-derived cargo including driver mutations, molecular subtypes, active miRNA, and proteins, which possess metastatic properties and have been shown to prepare the premetastatic niche.^{16–23} Particularly promising are the findings that these EV-based disease markers can be identified well before the onset of symptoms or physiological detection of a tumor, making them promising candidates for early stage cancer and other disease detection.^{24,25} In addition, EVs are membrane-covered nanoparticles, which protect the inside contents from external proteases, phosphatases, and other enzymes.^{26–28}

As noted before, because of active phosphatases and complex environment, there have been very few detectable phosphoproteins reported in urine or other biofluids. Because EVs are membrane-covered nanoparticles, which protect the inside contents from external proteases and phosphatases, there is a strong potential for phosphoproteome identification. As a confirmation, a number of reports have shown the presence of a few active phosphoproteins in EVs during the study of cancer or neurological diseases.^{28–30} It has been even demonstrated that inhibition of tumor cells using kinase inhibitors resulted in an emission burst of exosomes containing active phosphoproteins of the inhibited kinase.³¹ Despite the potential promise, to our knowledge there have been only one published study of urinary EV phosphoproteins, identifying 14 phosphoproteins from 400 mL of urine.³² We reason that such low identification number is likely due to low yield in EV recovery and suboptimal phosphoproteome extraction conditions.

Given the immaturity of EV analysis, a standardized method for collecting and processing EVs has not yet been developed.³³ Differential centrifugation with ultracentrifugation (UC) as the final step has been typically used for EV isolation (particularly for exosome purification). However, this approach is time-consuming (typically 6–22 h), requires expensive equipment, is low-throughput, and is overall not suitable for a clinical setting due to poor reproducibility.^{20,34–36} In addition, multiple studies have shown that the exosome recovery rate after ultracentrifugation is only 5–25%.^{36–38} Several other groups have published and commercialized new methods for EV isolation, which include polymer-induced precipitation,^{39,40} antibody-based affinity capture of outer membrane proteins,^{41,42}

affinity filtration,⁴³ size-exclusion chromatography,^{38,44} etc. However, each one has its own limitations.^{34,35,45} For example, precipitation based on polymers such as polyethylene glycol (PEG) typically results in low specificity and coprecipitation of a large number of high-abundant contaminating non-EV proteins.^{35,46} This issue may not be a major concern for RNA/DNA analysis, but it presents a challenge for proteome or phosphoproteome explorations. Additionally, the precipitating polymer is not fully compatible with subsequent MS analysis. Affinity-based exosome capture approach does offer improved specificity but is constrained to small volumes, results in relatively low recovery yield, and can be significantly biased due to tumor heterogeneity and target antigen changes over time^{15,35,47–49}.

The majority of the new methods that have been developed so far aim at reducing the time and cost associated with ultracentrifugation. Many have certainly succeeded in this goal. They also typically have higher reproducibility and demonstrate exosome recovery rates that are usually similar or slightly worse than ultracentrifugation.^{36,38,43,50–53} These can certainly be judged as a significant improvement over UC and can be used as good, fast alternatives for EV isolation. However, at 5–25% published yields, the efficiency of isolation still leaves much room for improvement.

Here, we compared several EV isolation methods and in particular examined a novel method EVTRAP based on functionalized magnetic beads for fast and reproducible capture and isolation of EVs from urine samples. EVTRAP (extracellular vesicles total recovery and purification) enables the capture of EVs onto beads modified with a combination of hydrophilic and lipophilic groups that have a unique affinity toward lipid-coated EVs. We find that EVTRAP is superior and capable of complete capture of EVs onto beads. Using the EVTRAP capture and an improved protein extraction method,⁵⁴ we were routinely able to detect >16 000 unique peptides representing ~2000 unique proteins from only 200 μL of urine in a single 90 min liquid chromatography–mass spectrometry (LC–MS) run. We also compared this method to standard EV isolation techniques and found that all detected markers and exosome-enriched proteins were captured at significantly higher levels by EVTRAP.

We have applied the EVTRAP-based isolation method to EV phosphoproteome analysis (general workflow illustrated in Figure 1). The capture approach, followed by optimized membrane proteome extraction and polyMAC-based phosphopeptide enrichment, allowed identification of close to 2000 unique phosphopeptides representing over 860 unique phosphoproteins from 10 mL of urine in a single 60 min LC–MS run. By comparison, exosomes isolated by 100 K ultracentrifugation followed by the same subsequent protocol produced 165 phosphopeptides from 104 phosphoproteins.

EXPERIMENTAL SECTION

Experimental details on materials, ultracentrifugation, EV-TRAP-based isolation, Western Blotting, silver staining, LC–MS analysis, and data processing are included in the Supporting Information.

Midmorning urine was collected from healthy individuals without DRE after informed consent and centrifuged within 1 h of collection at $2500 \times g$ for 10 min to remove cell debris and large apoptotic bodies. The supernatant was collected and stored in 10 mL aliquots at -80°C . These urine samples were considered as direct unfiltered urine for the experiments. To generate the filtered urine samples, 10 mL of the frozen urine was thawed and added into 100 000 Da filter (Amicon Ultra-15, Ultracel-100 K Centrifugal Filters) to filter out a portion of free molecules with small molecular mass, including proteins and RNA, according to the manufacturer's instructions. The samples were washed once with $1\times$ PBS and diluted with 1 mL of $1\times$ PBS to obtain the $10\times$ concentration of the EVs. The filtered urine was either used directly or stored at -80°C .

LC-MS Sample Preparation

The digestion was performed using a phase-transfer surfactant aided procedure. The lysis solution was added to the 100 K UC pellet or directly to the EVTRAP beads (Tymora Analytical). EVs were isolated based on the manufacturer's instruction with some modifications. First, EVs were solubilized in the lysis solution containing 12 mM sodium deoxycholate, 12 mM sodium lauroyl sarcosinate, 10 mM TCEP, 40 mM CAA, and phosphatase inhibitor cocktail (Millipore-Sigma) in 50 mM Tris-HCl, pH 8.5 by incubating 10 min at 95°C . This step also denatured, reduced, and alkylated the proteins. The samples were diluted five-fold with 50 mM triethylammonium bicarbonate and digested with Lys-C (Wako) at 1:100 (w/w) enzyme-to-protein ratio for 3 h at 37°C . Trypsin was added to a final 1:50 (w/w) enzyme-to-protein ratio for overnight digestion at 37°C . To the digested peptides, ethyl acetate solution was added at 1:1 ratio and the samples were acidified with trifluoroacetic acid (TFA) to a final concentration of 1% TFA. The mixture was vortexed for 2 min and then centrifuged at $20\,000 \times g$ for 2 min to obtain aqueous and organic phases. The organic phase (top layer) was removed, and the aqueous phase was collected, dried down to $\sim 20\%$ original volume in a vacuum centrifuge, and desalted using 100-mg Sep-Pak C18 columns (Waters) according to manufacturer's instructions. Each sample was split into 98% and 2% aliquots for phosphoproteomic and proteomic experiments, respectively. The samples were dried completely in a vacuum centrifuge and stored at -80°C . For phosphoproteome analysis, the 98% portion of each sample was subjected to phosphopeptide enrichment using PolyMAC Phosphopeptide Enrichment kit (Tymora Analytical) according to manufacturer's instructions, and the eluted phosphopeptides dried completely in a vacuum centrifuge.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD010480 and 10.6019/PXD010480.

RESULTS AND DISCUSSION

Comparison of EV Capture Efficiency

Total EV or exosome capture and isolation has been the focus of many recent studies, with particular consideration toward simple and easy protocol. The goal to replace

ultracentrifugation (UC) has yet to be achieved due to significant limitations of other technologies. Here, we assess and compare EV isolation methods including EVTRAP or UC.

For the initial comparison and method optimization, we removed cells and apoptotic bodies through low speed centrifugation and filtered and concentrated urine using a 100 kDa filtration step, as demonstrated before.⁵¹ This allows for the sample volumes to be more manageable through 10× concentration. More importantly, this step removes the majority of the free proteins and small molecules while leaving EV population intact. Thus, we were able to run a portion of the filtered sample directly on a gel to serve as the direct control of the complete EV population. After the ultracentrifugation step (100 K UC), the pellet was used directly or washed with PBS once or twice and centrifuged again. Then a pellet portion corresponding to 50 μ L of concentrated urine (500 μ L original urine) was loaded on the gel after protein extraction by boiling with LDS loading buffer. The supernatant from 100 K UC step was dried and loaded on the gel in the same proportion (equivalent to 50 μ L concentrated urine). The same volume of 50 μ L of concentrated urine was captured by the EVTRAP beads. After 1-h incubation, the supernatant (unbound fraction) was collected, dried, and loaded on the gel. In two variations of the EVTRAP experiment, the captured EVs were eluted by incubation for 10 min with triethylamine or by boiling in LDS buffer directly to determine whether elution was complete. All samples were loaded on the same gel and detected by Western Blot using a primary antibody for CD9, a common exosome marker. This experiment was carried out five times separately. A representative blot is shown in Figure 2A, and the quantitative values for each CD9 band signal are listed in the bar graph in Figure 2B. More representative blots are included in Supplementary Figure S1. As the results show, ultracentrifugation step indeed captured only a portion of the exosomes, 14% on average in our case, a recovery rate similar to other studies.^{36,37} Washing of the UC pellet, as is a commonplace procedure, further decreased the yield (also shown by others³⁶). Detection of the UC supernatant further confirmed the incomplete capture, as it is expected to see a large percentage of EVs remaining in the supernatant.⁵⁵ In contrast, EVTRAP method resulted in no detectable CD9-containing exosomes remaining in the supernatant (unbound fraction), with vast majority of the exosomes being captured and eluted off (almost 99% for TEA elution and >86% for LDS elution compared to the original control). We also found that TEA-based elution from the EVTRAP beads provided the most consistent and reproducible results. To demonstrate this, we ran 20 separate EVTRAP experiments (eluted by TEA or LDS) and carried out Western Blot comparison with the control urine sample. As shown in Supplementary Figure S2 and quantified in Figure 2B, the recovery and reproducibility are outstanding, resulting in standard deviation of 3.9% for TEA-based elution.

Comparison of EV Isolation Directly from Unfiltered Urine Samples

In the initial experiments, it was convenient to use filtered 10× concentrated urine for comparison so that the control urine sample can be analyzed as well. However, true measure of success for such a method would be the direct isolation from urine without any extensive pretreatment steps. After a short low-speed centrifugation for cells and apoptotic bodies removal, urine samples (1 mL each) were subjected to EVTRAP capture (for MVs +

exosomes collection), along with 10 000g centrifugation (10K pellet for MVs collection) and 100 000g centrifugation with or without PBS wash (100 K UC pellet for exosomes collection) for comparison. While both MVs and exosomes are considered EVs, they have unique formation mechanisms, pellet at different speeds and have varying buoyancy. The majority of the previous publications have looked at exosomes exclusively and often even generalized them as EVs. However, it has been previously shown that microvesicles (MVs) can be as valuable for diagnostic purposes as exosomes, and therefore, their capture by EVTRAP would also be desirable^{9,13,54,56–58}. To examine the exosomes and MVs capture efficiency, we detected CD9 signal (exosome marker) and mitofilin signal (MV marker⁵⁹) simultaneously (Figure 2C; signal quantitation in Figure 2D). As expected, the majority of mitofilin signal was detected in the 10K pellet, while the majority of the CD9 signal was found in the 100 K UC fraction. By comparison, EVTRAP enabled a much more complete capture of exosomes than 100 K ultracentrifugation (4-fold increase; > 20-fold increase if the pellet was washed once) while still allowing for the complete simultaneous capture of the MVs.

Despite EV capture analysis, another important feature is the purity of the captured EVs. To examine the amount of contamination by urine proteins present in each sample, we used 50 μL of direct urine for each experimental treatment and detected with silver stain for total protein analysis (Figure 2E). As expected, 100 K UC sample had very little contamination when compared to direct urine control, and even less after the PBS wash. However, when analyzing the 10K pellet, we saw a significant contamination even after the PBS wash. From this result, it is obvious that differential centrifugation does not work well for microvesicle capture due to high amount of urine protein pelleting. By comparison, EVTRAP isolation showed the vast majority of the contaminating proteins present in the unbound supernatant fraction, with only few contaminants eluting together with EVs. Compared to the complete EV capture by differential ultracentrifugation (10K pellet +100 K pellet), EVTRAP exhibited much lower amount of contamination.

Besides ultracentrifugation, we also sought to compare EVTRAP method to other frequently used approaches. We used three common commercially available methods including membrane affinity spin method,⁴³ size-based filtration tube, and polymer-based EV precipitation.³⁹ Direct urine was used in each case, and 500 μL of urine equivalent was run after capture on two different gels and detected by anti-CD9 antibody (Supplementary Figure S3A) or silver stain for purity assessment (Supplementary Figure S3B). For 100 K UC control, 500 μL of direct urine or 50 μL of filtered 10 $\times$ concentrated urine was used for a more complete evaluation. As the results in Supplementary Figure S3A demonstrate, the alternative methods produced somewhat similar exosome recovery signal compared to 100 K ultracentrifugation, matching the previously published results for these methods.^{36,38,43,51,52} When compared to 100 K UC pellet from unfiltered urine, the polymer-based EV precipitation even produced higher exosome yield, although the contamination level was also much higher (Supplementary Figure S3B). Nonetheless, EVTRAP still produced the highest exosome recovery yield compared to any other approach (Supplementary Figure S3A) with lower level of contamination.

Full Proteome Comparison of Captured EVs

While Western Blot-based detection does allow simple analysis of EV markers, it usually works well only for a few targets at a time and only those with good antibodies available. On the other hand, mass spectrometry analysis enables the detection and quantitation of hundreds or thousands of proteins in a single experiment, while uncovering previously unknown targets. For the LC–MS proteome analysis, we used 10 mL of urine for each treatment as the starting material and filtered/concentrated it down to 1 mL each. Some of the samples were centrifuged at 10 000g to remove microvesicles and the supernatant used for exosome analysis. As a control, 100 000g centrifugation was carried out for 2 h and the pellet used directly for protein extraction with no washing step. The supernatant from the 100 K UC sample was then captured on EVTRAP beads to analyze the exosomes left after the ultracentrifugation step. For the sample comparison, 10K supernatant and 1 mL of concentrated urine were also captured by EVTRAP. We have previously developed a highly effective protocol for EV protein extraction using phase-transfer surfactant and applied it for plasma EV analysis.⁵⁴ In this project, we used this protocol for EV lysis and extraction followed by on-beads digestion and LC–MS analysis.

While we used 1 mL of the 10x concentrated urine as the starting material, only 2% of each sample was loaded onto the LC column for direct LC–MS analysis (equivalent to only 200 μ L of starting urine amount). The rest of each sample was used for phosphoproteomic analysis. Using a single 90 min LC–MS gradient, we were able to identify >16 000 unique peptides from ~2000 unique proteins from the EVTRAP experiment (Supplementary Table S1). By comparison, 100 K UC method produced >7200 unique peptides from ~1100 unique proteins (Supplementary Table S2). EVTRAP capture of the 100 K supernatant again showed that the majority of the exosomes were not recovered by the ultracentrifugation but could then be captured by EVTRAP (Supplementary Table S3; EVTRAP of 10K supernatant data shown in Supplementary Table S4).

Although the data appear promising, the comparison based on total peptide and protein identification numbers may not be fully accurate. Therefore, we utilized label-free quantitation to compare all of the proteins identified by each method (Supplementary Table S5). ExoCarta is a web-based exosome data compendium and a useful tool that collects a large number of EV studies.^{60–62} They produced a list of 100 top exosome markers and exosome-enriched proteins that were found by most published studies. In our experiment, we have identified 94 of these exosome proteins (including common markers like CD9, CD63, CD81, TSG101), with all of them showing a significant increase after EVTRAP capture. This is significant because many other studies have shown that different methods are able to enrich different exosome populations with various success rates.^{52,63} The EVTRAP appears to have captured an unbiased and complete EV profile. As an example of the data, we have listed a few common exosome markers in a bar chart in Figure 3A. The average fold increase of all detected exosome markers compared to the 100 K UC sample is shown in Figure 3B. When using the 10K supernatant (exosomes only fraction) for EVTRAP experiment (EV3 data point), the average increase in marker signal is ~9-fold, which matches very well with the Western Blot data. However, when the complete urine is

used without MV removal (EV4 data point), the increase over the control is almost 17-fold (Supplementary Table S5).

As has been previously suggested for minimal EV study requirements,³³ we also attempted to measure the amount of contaminating urine proteins in the samples. We found 42 common urinary proteins⁶⁴ in our data not known to associate with EVs (including the ubiquitous uromodulin), five of which were shown in the same bar chart (Figure 3A). The average increase in contaminant level for all 42 proteins is also provided in Figure 3B (complete data in Supplementary Table S5). Overall, the contamination level correlates generally with the marker quantitation level, demonstrating similar ratio of exosome markers to the contaminating proteins as in the UC sample. There has been evidence of some urine proteins being captured together with exosomes^{65,66} partially due to non-specific binding to exosome surface proteins and exosomes themselves. Therefore, it is expected that some small portion of the free proteins be captured together with EVs. This is likely the reason why the increase in EV recovery was observed along with the increased the free protein recovery. Nonetheless, the vast majority of the free protein contaminants have been removed during the procedure.

EV phosphoproteome analysis of urine sample

Our ultimate goal was to be capable of routinely detecting phosphorylated proteins from urinary EVs. Because phosphoproteins are typically present at substoichiometric levels, it requires high recovery rate during sample preparation steps for high signal in LC–MS/MS analyses. We believe poor recovery rate is the reason for small number of EV phosphoproteins in previous studies, where 400 mL of urine was used to identify 14 phosphoproteins from exosomes.³²

For our phosphoproteomic analyses, we used the same samples as detailed above in the proteome analysis section. After we took out 2% of each sample for direct proteomic experiments, the remaining 98% of each sample was used for phosphopeptide enrichment by PolyMAC and analyzed by a 60 min LC–MS run (as shown in Figure 1). Figure 4A and B illustrate the EV phosphoproteome data (Supplementary Tables S6–S9). Our 100 K UC sample produced 165 unique phosphopeptides from 105 unique phosphoproteins (Supplementary Table S6), which to our knowledge is already one of the highest reported urine EV phosphoprotein numbers. However, when EVTRAP was used for capture, we saw a significant increase in the coverage of EV phosphoproteome. We identified almost 2000 unique phosphopeptides from >860 unique phosphoproteins using only 10 mL of urine in a single 60 min LC–MS run (Supplementary Table S9). The list includes almost all of the phosphoproteins detected after UC (Figure 4B). Figure 4C lists the total increase of the identified phosphoproteins compared to UC (Supplementary Table S10) with label-free quantitative measurement. While the largest 41-fold increase came from the urine sample containing complete EV profile (EV4 data point), even 10K supernatant captured by EVTRAP showed a 19-fold increase in the phosphoproteome level (EV3 data point).

Though the 100 kDa filtration of urine provides a relatively clean starting material for processing, the filtration step itself adds an extra 20–25 min to the protocol and reduces the throughput capacity. To determine if phosphoproteomic experiments can be carried out with

untreated clinically relevant samples, we used EVTRAP to capture 10 mL of unfiltered urine directly. Following the same procedure as before, we identified almost 1500 unique phosphopeptides from 775 unique phosphoproteins (Supplementary Table S11). The average increase of phosphoproteome signal was >31-fold higher than that after ultracentrifugation (Supplementary Table S12). As in the previous experiment, we also took out 2% of the sample for direct proteome analysis and compared the result to 100 K UC pellet of 10 mL unfiltered urine (60 min LC gradient for each run). We found that the average level for the exosome markers from the EVTRAP sample was increased 20-fold compared to 100 K UC, while the level of contaminating proteins increased 11-fold (Supplementary Table S13). These data demonstrate that EVTRAP can be applied to directly process unfiltered urine, which would be highly useful for routine clinical analysis. With 10 mL of urine being sufficient to easily identify hundreds of phosphoproteins, the volume is also convenient for everyday analysis.

CONCLUSION

We have carried out a comprehensive assessment and comparison of several EV isolation methods for urinary phosphoproteomic studies. The EVTRAP method enables the recovery of >95% of exosomes, with relatively low contamination level. All exosome markers were captured at higher levels than by the common ultracentrifugation approach. While we used on-beads digestion for all of the presented LC–MS data in the study, we also discovered that triethylamine elution followed by digestion in solution resulted in similar EV recovery (MS data not shown). Thus, the isolated vesicles can be used for different types of follow-up analyses, not just LC–MS or Western Blotting. We expect researchers equipped with EVTRAP will be able to uncover more low-abundant urinary biomarkers such as EV proteins with post-translational modifications (PTMs) such as phosphorylation. We hypothesize that, with simple and efficient method like EVTRAP, urine EV phosphoproteins can one day be used for early stage cancer detection, longitudinal monitoring, and as companion diagnostics for targeted cancer treatments, particularly those involving kinase inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study was supported in part by NIH Grant Nos. 1R01GM111788 and 1R41CA210772 and by National Science Foundation Grant No. 1506752. Additional support was provided by the Purdue University Center for Cancer Research (NIH Grant No. P30 CA023168).

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Figure 1.
Workflow illustration of urinary EV phosphoproteome analysis using EVTRAP capture method.

Figure 2.

Comparison between ultracentrifugation (UC) and EVTRAP for exosome capture. (A) Detection of CD9 exosome marker using Western Blot. (B) Quantitation of WB data in panel A as a percent recovery from the control sample (exosome control = 100%). Each point represents average and standard deviation of a minimum five separate experiments on the same or different blots. (C) Capture of total EV population from direct unfiltered urine and detection of CD9 (exosome marker) and mitofilin (MV marker) using Western Blot. (D) Quantitation of the Western Blot signal in panel C. (E) Silver stain total protein detection of EV capture samples.

Figure 3.

LC-MS total proteome analysis of 100 K UC and EVTRAP samples. (A) Quantitation of 13 common exosome proteins and 5 free urine proteins. (B) Fold increase in total proteome intensity of known exosomal proteins and free urine proteins from LC-MS data compared to UC sample (EV1 = 100 K UC pellet; EV2 = EVTRAP of 100 K UC supernatant; EV3 = EVTRAP of 10K supernatant; EV4 = EVTRAP of urine).

Figure 4.

LC-MS phosphoproteomic analysis of 100 K UC and EVTRAP samples. (A) Total number of unique phosphopeptides identified. (B) Total number of unique phosphoproteins identified and ID overlap between 100 K UC and EVTRAP samples. (C) Fold increase in total phosphoproteome intensity from LC-MS data compared to UC sample. (EV1 = 100 K UC pellet; EV2 = EVTRAP of 100 K UC supernatant; EV3 = EVTRAP of 10K supernatant; EV4 = EVTRAP of urine).