Novel bifunctional material coupled to PASEF enables sensitive extracellular vesicles phosphoproteomic analysis of urine

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Introduction

Extracellular vesicles (EVs) have emerged as important carriers for intercellular communication and biological sources for diagnosis and therapeutics. Low efficiency in EV isolation from biofluids, however, severely restricts their down-stream characterization and analysis. Here, we introduced a novel bifunctionalized magnetic beads (BiMBs) coupled trapped ion mobility (TIMS) and PASEF for phosphoproteomic analysis of urine samples. We demonstrated its efficient isolation of EVs from urine samples with low contamination, high recovery (>80%), and short separation time (within 1 h), resulting in the identification of 36262 unique EV peptides corresponding to 3302 unique proteins and 3233 unique phosphopeptides representing 1098 unique phosphoproteins using only 100 µL and 5 mL urine samples, respectively. Furthermore, quantitative phosphoproteomics of urine samples from prostate cancer patients and healthy individuals revealed 121 upregulated phosphoproteins in cancer patients in contrast to the healthy group.

Methods

Urine EVs were isolated by functionalized magnetic beads and the sample was followed by the additional phosphopeptide enrichment using polyMAC, prior to LC-MS/MS analyses. These peptides were separated on a home packed C18 column (25 cm, 75 µm i.d., 2.2 µm) using a nanoElute nano LC (Bruker Daltonics) coupled to a trapped ion mobility equipped Q-TOF mass spectrometer (timsTOF Pro). Samples were separated using a 100-minute gradient ranging from 2% to 37% acetonitrile (0.1% FA) at 300 nL/min at column temperature of 50°C. The raw files were searched directly against the Uniprot database version downloaded November 2019 with no redundant entries, using PEAKS Studio X+ software. The false discovery rates (FDRs) of proteins, peptides, and phosphosites were all set to 1% (-10lgP ≥ 20 ≥1 unique peptide for proteins).

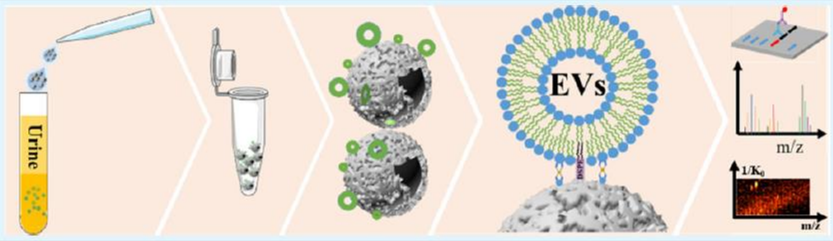

Figure 1: Workflow of extracellular vesicles phosphoproteomic analysis. Urine EVs were isolated by BiMBs and the sample was followed by the additional phosphopeptide enrichment using polyMAC. The resulting peptides then were analyzed on the timsTOF Pro and data were processed by PEAKS.

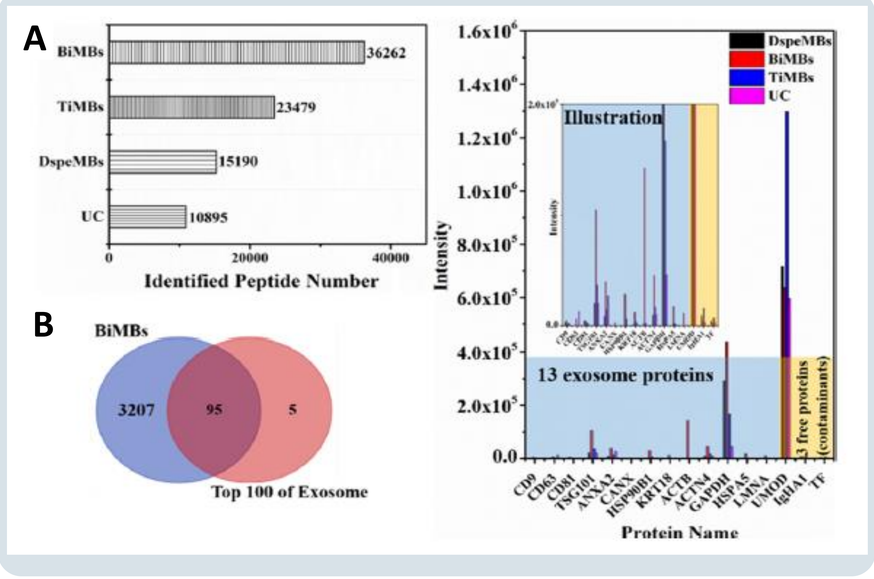

Figure 2: Proteome analysis of EVs isolated by different methods. (A) Full proteome isolated by BiMBs and (B) overlapping with top 100 proteins of exosome. (C) Quantitation of 13 common exosome proteins and 3 free urine proteins isolated by different methods. Zoomed view was illustrated as the insert.

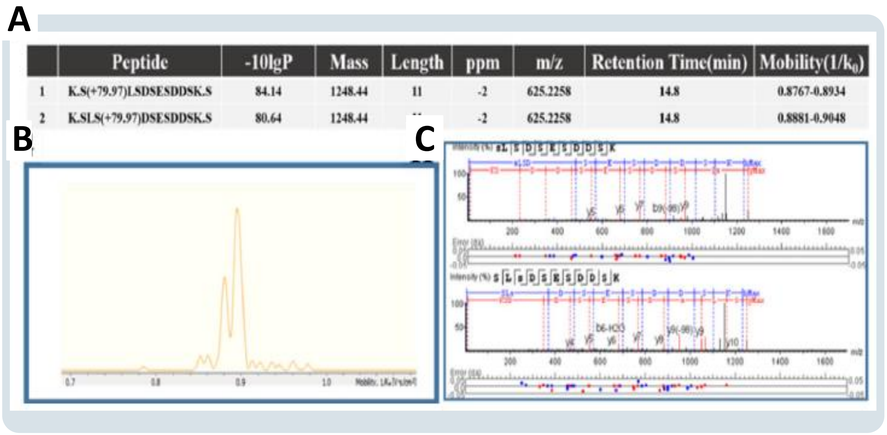

Figure 4: The results of prostate cancer-related phosphorylated peptide isomers. (A) Detection of two phosphorylated isoforms from Chromobox protein homolog 3. (B) Extraction ion mobilogram of the two phosphopeptides co-eluting from the LC but having clear difference in mobility (CCS). (C) MS/MS spectra matching peptides.

References

(1) Jie Sun, et. al., Synergistically Bifunctional Paramagnetic Separation Enables Efficient Isolation of Urine Extracellular Vesicles and Downstream Phosphoproteomic Analysis. ACS Applied Materials & Interfaces 2021, 13, 3, 3622-3630.

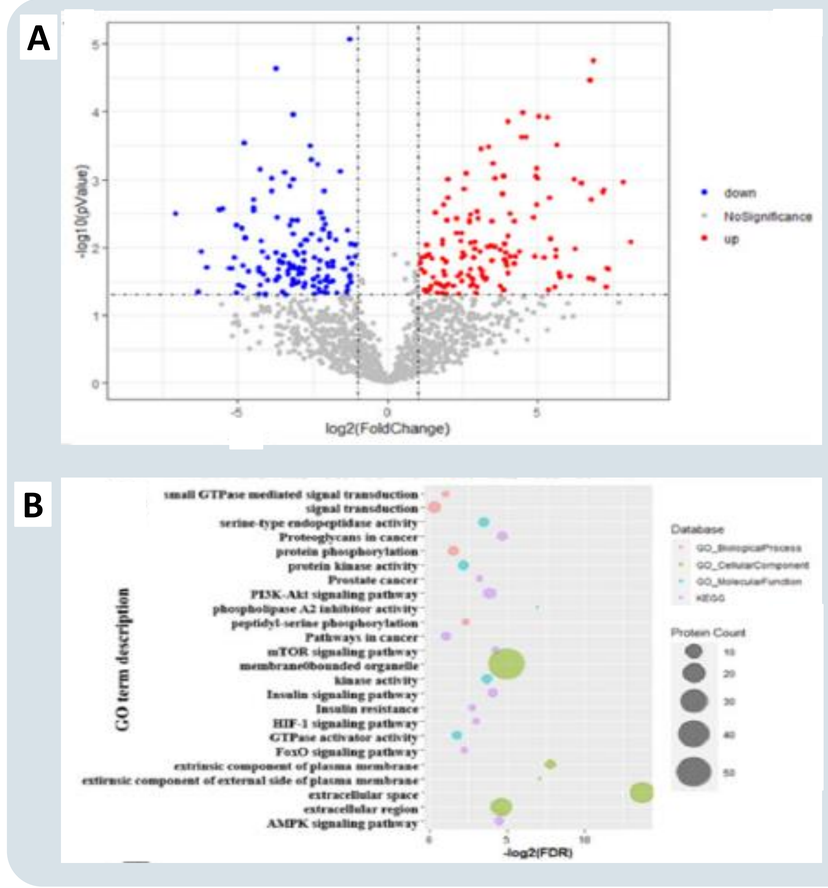

Figure 3: Quantitative phosphoproteomics of urine EVs from prostate cancer patients and healthy controls. (A) Volcano plot comparison of the up-regulated phosphoproteins. (B) The GO and KEGG pathway analysis of these up-regulated phosphoproteins.

Results

Here, we introduced a novel bifunctionalized magnetic beads (BiMBs) coupled trapped ion mobility (TIMS) and PASEF for phosphoproteomic analysis of urine samples (Fig. 1). Benefiting from the highly efficient beads together with highly sensitive timsTOF Pro, we can identify 36262 unique EV peptides corresponding to 3302 unique proteins and 3233 unique phosphopeptides representing 1098 unique phosphoproteins using only 100 µL and 5 mL urine samples, respectively. The EV proteome from BiMBs overlapped with 95 out of 100 top exosome marker proteins (Fig. 2). Furthermore, quantitative phosphoproteomics of urine samples from prostate cancer patients and healthy individuals revealed 121 upregulated phosphoproteins in cancer patients in contrast to the healthy group (Fig. 3). In addition, TIMS provides one more dimension of separation that enables to distinguish the isobaric peptides with different modified positions (Fig. 4).

Conclusions

- Highly efficient beads together with highly sensitive timsTOF Pro can achieve depth of proteome coverage even with low amount urine sample
- Many upregulated phosphoproteins associate with prostate cancer according to quantitative phosphoproteomics analysis
- TIMS provides additional dimension of information on ions with specific CCS values, which may lead to broader applications with PTM analyses.

timsTOF Pro