Reviews

A Method to Analyze Urinary Extracellular Vesicles

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Extracellular vesicles (EVs) play an important role in cell-to-cell communication by carrying molecular messages that reflect physiological and pathological conditions of the parent cells. EVs have been identified in all body fluids; and among them, urine stands out as a sample that is easy and inexpensive to obtain and can be collected over time to monitor changes. Various protocols have been established to study urinary extracellular vesicles (UEVs) and they have shown great potential as a biomarker source for clinical applications, not only for urological, but also non-urological diseases. Due to the high variability and low reproducibility of pre-analytical and analytical methods for UEVs, establishing a standardized protocol remains a challenge in the field of diagnosis. Here, we review UEV studies and present the techniques that are most commonly used, those that have been applied as new developments, and those that have the most potential for future applications. The workflow procedures from the sampling step to the qualitative and quantitative analysis steps are summarized along with advantages and disadvantages of the methodologies, in order to give consideration for choosing the most promising and suitable method to analyze human UEVs.

Keywords Urinary extracellular vesicles, cancer, biomarkers, isolation, characterization, analytical method, qualitative analysis, quantitative analysis

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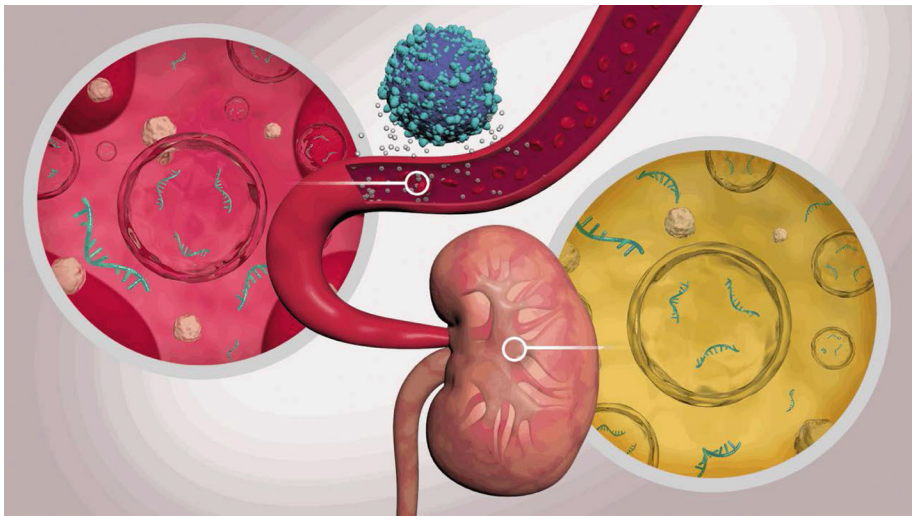

Fig. 1 Urinary extracellular vesicles derived from cells *via* blood vessels.

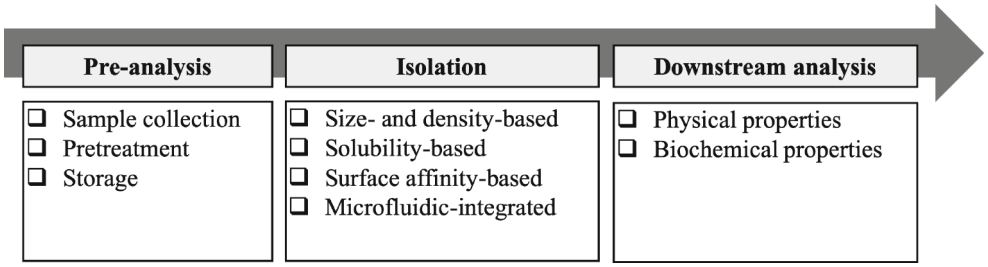

Fig. 2 Analytical workflow procedures of urinary extracellular vesicles.

1 Introduction

Extracellular vesicles (EVs) are spherically structured, cell-derived membrane vesicles and they play a role in cell-to-cell communication. The micro- and nano-sized vesicles carry molecular messages, including proteins, lipids, DNAs, RNAs, and metabolites. These molecules reflect the physiological and pathological conditions of parent cells and provide information used in clinical applications for early detection, diagnosis, and prognosis of diseases as well as for monitoring surgery and treatment responses.[1-10] EVs have been identified in many different biological fluids like whole blood, plasma, serum, urine, saliva, breast milk, *etc.* (Fig. 1).[11,12] Among these fluids, urine stands out as a non-invasive sample fluid that is easy and inexpensive to obtain and can be collected over time to monitor changes. The urinary extracellular vesicles (UEVs) were first isolated by Wiggins *et al.*[13] Consequently, the work of Pisitkun *et al.*[14] had a major impact of raising interest in UEVs by demonstrating proteomic analysis of urinary exosomes associated with renal diseases (kidney disorders). From that, the potential for clinical applications of UEVs became established. Recent reports have shown the presence of such molecules as RNAs, microRNAs, and proteins, which can be used potentially as biomarkers in renal diseases (*e.g.*, diabetic kidney disease),[15] urological malignancies (*e.g.*, prostate, bladder and kidney cancers),[16-19] non-urological malignancies (*e.g.*, lung, liver, pancreas cancers),[20] and arterial hypertension[21,22] in UEVs.

However, there is a concern regarding the application of UEVs to analysis due to the great variability among isolation techniques that have been used; this has resulted in it being difficult to apply the most effective protocol that is convenient and universal in a clinical setting. Thus, the lack of a standardized protocol remains a significant limitation in the field of UEV analysis, especially when all analytical validations of the assay have to be considered, including accuracy, precision, specificity, limits of detection and quantification. Comprehensive standardized guidelines for isolation of EVs[23] and overall analytical processes[24,25] have been provided that suggest all experimental details as well as critical evaluations in the EV analysis procedures. Some researchers have discussed the development of a variety of UEV isolation and interrogation approaches, and highlighted potentials and limitations.[26] Others have addressed the whole range of methods that are used for human UEV isolation and characterization.[27] In this review, we consolidate and select common protocols and up-to-date technologies that have been used specifically in UEV analysis. We present the overall workflow procedures from the sampling step to the output of qualitative and quantitative analysis results, in order to give some direction to researchers for choosing a method that is appropriate for meeting analysis needs.

2 Methodologies

The overall workflow procedures for studying UEVs consist of three main steps (Fig. 2): pretreatment, isolation, and

Table 1 Summary of isolation techniques for urinary extracellular vesicles

Isolation technique	Principle of technique	Advantages	Disadvantages	References
Ultracentrifugation (UC) or differential centrifugation (DC)	Size and density based separation by applied centrifugal force	Large sample capacity Large yield	High instrument cost Labor intensive Time consuming Low purity	45, 52
Ultrafiltration (UF)	Size based separation by ultra-fine pore size membrane	Low instrument cost Fast processing	Vesicles trapping Size overlapping Protein contaminants	48, 54 – 58
Size-exclusion chromatography (SEC)	Size and mobility based separation by packed column with porous materials	High purity Low protein contaminants High yield	Require additional concentration step Long run time	40, 47, 48, 59
Precipitation	Solubility based separation by substances in a solution	Fast processing High yield High quality for RNA profilling	Protein contaminants Chemical required	61 – 63
Immuno-based technique	Surface affinity basedseparation by antibody	High specificity High recovery	High antibody cost Low sample capacity	64 – 66
Microfluidics	Surface affinity based separation by various materials	Very fast High sensitivity High selectivity	Complexity of device High cost	67 – 70

downstream analysis. The key to successful determination of UEVs mostly relies on the sample preparation processes of pre-analysis and isolation since they directly determine the qualitative and quantitative information of UEVs from that sample. Here, we discuss the whole workflow. We selected the methodologies for discussion based on their frequency of use in current publications that focus on studying UEVs.

2·1 Techniques of pretreatment

Since the biggest advantage to using a urine sample is that it can be simply collected from an examinee into a sterilized container without any external instrumental needs, the ideal analysis methodology would isolate EVs directly from urine without any extensive pretreatment steps. Compared to other liquid biopsy samples (blood, plasma, breast milk), urine has a narrower spectrum of interference from matrix components and requires less pretreatment before subjecting it to further analytical processes.[28–30] Most pretreatment studies have used short low-speed centrifugation at $2000 \times g$ to remove large-sized components (cells, apoptotic bodies, and cell debris).[31–33] Some researchers have reported a unique pretreatment approach for increasing the secretion of UEVs and enrichment of prostatic markers using a digital rectal examination (DRE) to stimulate the prostate before urine collection.[34,35] For preventing the degradation of UEVs, it is recommended that the urine be pelletized and stored at below –80°C to get 100% recovery, and maintain uniform and unchanging morphology of the UEVs.[26] The functional properties of the UEVs, *e.g.* the vesicle membrane phospholipids, are not altered at that temperature.[36]

However, the pretreatment remains crucial for preparing the sample in order to prevent protein-UEV entrapment, by which researchers can gain the maximum information from their sample. The presence of excessive proteins, *e.g.* Tamm-Horsfall protein (THP), showed an affinity with UEVs resulting in lower efficiency with a decreasing number of UEVs. To improve the yield of total UEV particles and their purity, some chemical reagents were added to the urine sample to inhibit protein-UEV entrapment, such as dithiothreitol (DTT),[37–40] 3-((3-cholamido propyl)-dimethyl ammonio)-1-propane sulfonate (CHAPS),[41] sodium chloride (NaCl),[42] and zinc sulfate ($ZnSO_4$);[33] these treatments to inhibit protein-UEV entrapment are optional. It was found that the DTT treatment led to a relative increase in the number of UEVs, but it did not increase the yield of RNA.[43] Thus, the decision of whether or not to use protease inhibitors depends on the type of follow-up analyzes.

2·2 Techniques of isolation

Before carrying out any experiment, consideration must be given to what target information is expected to be obtained from UEVs in the downstream analysis, *e.g.* the morphology, variety of components, and proteomic and genomic information. The frozen urine pellets are used after thawing then placed in a 37°C water bath for 10 min,[44] and subsequently resuspended in phosphate-buffered saline (PBS) before the isolation process. The use of force in some techniques may lead to break-up or deformation of the UEV structure. There are many techniques that have been developed for UEV isolation. In this review, we focus on four principles that rely on specific properties of the UEVs, including size and density, solubility, and surface affinity, and that can be realized in an integrated microfluidic system. Each technique has its own key potential and limitations, as shown in Table 1. A number of recent studies have tried to combine two or more different techniques together to improve isolated matter purity, yield and richness of the biomolecule types, *e.g.* ultra-centrifugation with ultra-filtration (UC-UF),[45,46] ultra-centrifugation with size-exclusion chromatography (UC-SEC),[40,46] and ultra-filtration with size-exclusion chromatography (UF-SEC).[47,48] Still, all these techniques have intensive labor requirements.

A sample is subjected to a centrifugal force that sequentially separates the particulates according to their size and density.[49] The centrifugation-based techniques are the most commonly used to purify and isolate EVs from all sample sources because they are easy to use and widely available.[50,51] Centrifugation instruments are able to handle big volumes of urine; however, the work is both labor- and time-consuming. Due to the various types of UEVs, including microvesicles and exosomes, contained as a mixture in the sample, each component is separated step by step at different forces (g) with spinning ranging from $2000 \times g$ to $200000 \times g$.[45,52] This is known as differential centrifugation (DC) with ultra-centrifugation. As a result, some vesicles are lost during those serial-multiple cycles resulting in low yield. Increasing urine volume is the simplest way to overcome this drawback. However, the poor purity of EVs in this technique remains a problem.

Filtration is a well-established technique used to overcome non-robustness of differential centrifugation (DC) by requiring simpler processing and it can provide similar levels of recovery of UEVs and concentration of nucleic acids as DC.[53] Many types of polymer-based membranes have been developed with various ultra-fine pore sizes (microfilters and nanofilters) to separate EVs from a urine sample based on their diameter sizes.[48,54-56] One disadvantage of ultrafiltration (UF) using membranes is that some vesicles and non-dissolved protein might adhere to the filter, resulting in reduction of filter efficiency. Some researchers have developed hydrophilic filters to restrict protein binding such as polyvinylidene difluoride membrane showing the reduction of soluble proteins in urine sample.[57] Another disadvantage is contaminants from size-overlapping molecules and soluble proteins that also pass though the membrane pore.[58]

Size-exclusion chromatography (SEC) is an easy and potential method to purify molecules by size and mobility through a packed column with porous materials. This method allows to isolate EVs from urine with lower protein contaminants in downstream analysis.[47] However, sample loads and vesicle yields are limited by the SEC column sizes. The combination of additional concentration processes prior to SEC, *e.g.* UC and UF, were reported to improve isolation yield and purity.[40,47,48,59]

The precipitation techniques are based on the fact that UEVs change their solubility and/or aggregate with substances, *e.g.* polymers, protamine, and sodium acetate. ExoQuick is one of the widely used commercial reagents for exosome isolation.[60] The urine sample is incubated with precipitate-forming solution, then the mixture is centrifuged at low speed (1500 – 10000 × *g*), and finally pellets of exosomes are collected. ExoQuick offers a tool for quick and effective isolation with high quantity and quality of exosomes, and miRNA and mRNA molecules, suggesting that the protocol is suitable for RNA profiling.[61-63] One disadvantage of ExoQuick is that some proteins can also be co-precipitated.

Immuno-based techniques offer highly specific isolation using surface affinity between UEVs, surface proteins, and antibodies conjugated with magnetic beads or other materials.[64,65] However, these highly specific materials are high-cost. Recently, Wu *et al.*[66] developed a novel method called EVTRAP based on functionalized magnetic beads that have a unique affinity toward lipid-coated EVs for capture and isolation of EVs from urine samples. They found a significant improvement over current standard techniques with highly efficient capture giving more than 95% recovery yield.

Microfluidics techniques are an integrated platform that have been developed within the lab-on-a-chip concept promoting high robustness, sensitivity, and selectivity of UEV analysis. As noted earlier, the ideal analysis methodology is to detect EVs directly without any extensive pretreatment steps from urine, and numerous studies have tried to find out whether realizing this is possible. In 2017, we proposed a novel approach to UEV isolation microfluidics that enables EV collection at high efficiency and by *in situ* extractions of diverse miRNA molecules that significantly exceed the number of items being extracted by the conventional ultracentrifugation method and a commercially available kit.[67] More recently, Woo *et al.*[68] used a centrifugal microfluidics device integrated with six nanofiltration units, Exo-Hexa, to isolate UEVs and studied AR-V7 mRNA expression as a reliable biomarker in prostate cancer patients. Alternatively, a commercial on-chip device, ExoChip, has also allowed not only EV isolation from urine but also implementation of a downstream analysis system, which has been discussed in reviews of advanced microfluidics technology.[69,70]

2·3 Techniques of downstream analysis

The characterization of UEVs is primarily based on the variety of their biophysical and biochemical properties, which may be used to determine the molecular composition of vesicles for identifying markers of a disease. The qualitative analysis, quantitative analysis and characterization of isolated UEVs have been examined using several optical techniques, *e.g.* dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and flow cytometry. On the other hand, non-optical techniques include tunable resistive pulse sensing (TRPS), scanning ion occlusion sensing (SIOS), transmission electron microscopy (TEM), quantitative reverse transcriptase–PCR (qRT-PCR), microarrays, and next generation sequencing (NGS). The downstream analysis techniques for UEVs are summarized in Table 2. Each of these techniques provides different types of measurement information, *e.g.* morphology and biomolecular components, that could reflect different disease states. Importantly, the possible errors from the pre-analysis and isolation processes have to be considered to ensure accuracy in the downstream analysis.

Transmission electron microscopy (TEM) provides information on size, structure, and shape of UEVs. In the conventional sample preparation process for electron microscopy, sample fixation and negative staining are necessary. Those processes could lead to inaccurate measurement of vesicle size and shape because of dehydration.[71,72] Cryo-electron microscopic examinations have been applied to conserve the morphology of UEVs in the fully hydrated condition.[73-76] Cryo-transmission electron microscopy (Cryo-TEM) allows observation of an individual particle; however, sample preparation is complex and advanced instruments are required.[72]

Sample preparation and instrument operation for dynamic light scattering (DLS) are simple and the technique is capable of measuring the particle size distribution.[77] However, DLS does not measure each single particle. The technique is suitable for a monodispersed and homogeneous size distribution, but impurities from precipitated proteins in an isolated sample might affect the result. The nanoparticle tracking analysis (NTA) is a light scattering technique that was first established to study EVs in 2011.[78] Due to its high-throughput analysis of particle size distribution and concentration, currently NTA has become the most commonly used quantitative analysis for UEVs.[40,44,79,80] Some detergent such as Triton X is applied in NTA to gain more output for confirmation of EV-lipid moieties and specific particle fluorescent labeling.[81,82] However, similar to DLS, the impurities contained in isolated UEVs and dye aggregates might interfere and produce overestimation by NTA.

Tunable resistive pulse sensing (TRPS) and scanning ion occlusion sensing (SIOS) are non-optical, emerging, and potential techniques that have been used to determine individual EVs by measuring changes in electrical current when the particles pass through an adjustable nanopore. These methods provide high-throughput analysis of the vesicles, including size distribution, concentration, and surface charge (zeta-potential) of EVs without labelling.[83,84] This capability makes it a useful and applicable technique for UEV research.[76,85]

Flow cytometry (FCM) is a technique that allows the analysis of physical characteristics, such as the size of particles, using multiple-angle scattering light and also chemical properties of UEV components, such as proteins and nucleic acids, using fluorescent dyes. Due to its advantage of high-throughput measurements, flow cytometry has been applied to the reproducible analysis of clinical samples.[59,65,76,86] Notably, the overestimated scatter signals from high concentrations of EVs must be considered. The critical parameters necessary to

Table 2 Summary of downstream analysis techniques for urinary extracellular vesicles

Downstream analysis technique	Principle of technique	Advantages	Disadvantages	References
Transmission electron microscopy (TEM)	Electron microscopy (EM)	Physical properties: size, structure, shape	High instrument cost Complex sample preparation	71 – 76
Dynamic lightscattering (DLS)	Optical technique	Physical properties: size distribution, charge	Interference by similar size impurities *e.g.* protein aggregates	77
Nanoparticle tracking analysis (NTA)	Optical technique	Physical properties: size distribution, concentration	Interference by similar size impurities *e.g.* protein aggregates	40, 44, 79 – 82
Tunable resistive pulse sensing (TRPS) and scanning ion occlusion senseing (SIOS)	Electrical sensing	Physical properties: size distribution, concentration, charge	Liposomes for calibration required Interferences	76, 83 – 85
Flow cytometry (FCM)	Optical technique using fluorescence dyes	Physical properties: size distribution, Biohemical properties: vesicle components	Critical parameters variation required	59, 65, 76, 86
Liquid-chromatography–mass spectrometry/mass spectrometry (LC-MS/MS)	Chromatography and spectrometry	Biochemical properties: proteomics, phosphoproteomics	High instrument cost	66, 87 – 91
Western blot (WB)	Protein labelling using antibody	Biochemical properties: proteomics, phosphoproteomics	Labor intensive Non-quantitative	40, 45, 76
Quantitative reverse transcriptase–PCR (qT-PCR)	Polymerase chain reaction (PCR)	Biochemical properties: nucleic acids analysis, gene expression	Limited probe or primers Long run time Expensive	92 – 96
Microarray	Hybridization with fluorescence probes	Biochemical properties: nucleic acids analysis, gene expression	Limited probe or primers Long run time Expensive	67
Next generation sequencing (NGS)	Determining nucleic acid sequence	Biochemical properties: whole spectrum of nucleic acids	Many of technical challenges	97

increase UEV detection capacity by flow cytometry should be defined.

The pioneer publication on large-scale proteomics and phosphoproteomics of EVs in human urine appeared in 2009 and reported the use of liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS).[87] Recently, improvements in mass spectrometry technology have enabled high-throughput and large-scale quantitation and validation of candidate protein biomarkers in UEVs.[66,88-91] Western blot (WB) is another popular technique adopted to determine the protein composition that is suitable for confirming the correct isolation, despite being laborious and non-quantitative and sometimes yielding poor results, due to the quality of the antibodies used or to low exosome content in the sample.[40,45,76]

Quantitative reverse transcriptase–PCR (qRT-PCR) is a conventional detection and quantification approach for UEV-derived nucleic acids including RNA, microRNA and DNA in terms of gene expression and mutational profiles.[92-94] In cases that the concentration of nucleic acid isolated is too low to be directly detected, a pre-amplification protocol needs to be performed.[95] The normalization of the input to the volume of sample input is also recommended.[96] Moreover, the evaluation of this approach relies on the probe or primers used, which are limited. Microarray genomic analysis is another technology that offers a way to assess thousands of transcripts in UEVs using a gene expression array. This technique also relies on specific fluorophore probes and requires a costly instrument to interpret the fluorescent signal from hybridization of miNAs and mRNAs with the probes.[67] Next generation sequencing (NGS) is the latest fast-growing technology that has enabled characterization of the whole spectrum of nucleic acids in a given sample,[97] and it has been consistently applied to demonstrate the presence of the complex RNA molecules within the EV population released by various cells. A recent review by Turchinivich *et al.*[98] presented the state-of-the-art techniques in the field of EVs-associated RNA transcriptomes using next-generation sequencing.

3 Conclusions and Perspectives

Analysis of urinary extracellular vesicles (UEVs) to understand human health was built on long-practiced conventional methodologies. Different alternative protocols have certain advantages and limitations. Recent advanced technologies have been developed from various hypothesis to open up opportunities for realizing single-EV measurements exploiting their biochemical, electrical, mechanical, and optical properties and to overcome previous analytical problems. The ideal method for analysis of UEVs should be relatively simple and inexpensive and allow for isolation and characterization of UEVs from a large number of samples in order to promote assessible and user-friendly settings for clinical environments (Fig. 3). Microfluidics systems have high potential to become the next big evolutionary step in EV analysis platforms. Based on such systems, a possible long-term goal would be development of a universal method for UEV analysis.

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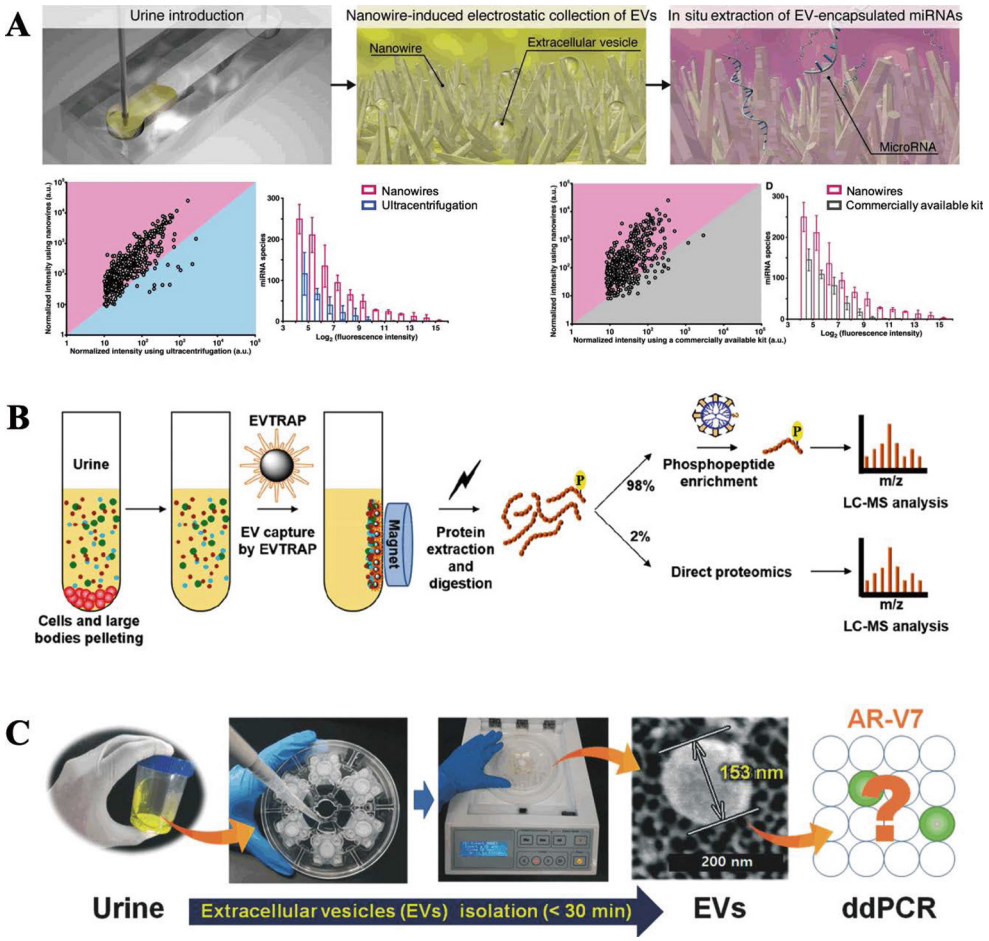

Fig. 3 Schematic demonstrating emerging techniques for urinary extracellular vesicles (UEVs) analysis and applications. (A) (adapted from Ref. 67) Nanowire-anchored microfluidics device used for microRNAs extraction from the collected UEVs to detect early-stage cancer. (B) (adapted from Ref. 66) Functionalized magnetic beads, EVTRAP, used to capture UEVs, and from which then were extracted the phosphorylated proteins for cancer detection. (C) (adapted from Ref. 68) A lab-on-a-disc, Exo-Hexa, consisting of nanofilters used to capture and purify UEVs for AR-V7 mRNA expression analysis in prostate cancer patients.

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