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Research Article

Comparison of Different Methods to Isolate Urinary Extracellular Vesicles Useful as Possible Kidney Damage Biomarkers

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Abstract

Extracellular vesicles are lipid membrane-bound nanoparticles released from cells in different biological fluids. Particularly, urinary extracellular vesicles (UEVs) arising from the nephron cells, could be helpful as novel biomarkers for kidney allograft injury. This comparative study aims to identify the most efficient UEVs isolation method to detect novel kidney biomarkers. UEVs were isolated from urine samples using four commercial kits and purified by Izon qEV single SEC. The UEVs isolated by different methods showed high variability in both concentration and size besides a remarkable decrease between pre and post purified samples. Based on these results the most efficient and cheap method to isolate UEVs is the Izon qEV single SEC column.

Methods Summary: Four UEVs isolation techniques and Izon SEC purification were compared. Urine samples were collected and processed to discharge cellular debris. UEVs were isolated by Norgen, Exo Quick, QIAGEN, AMICON and Izon SEC methods. All samples were characterized in size and concentration by qNano instrument before and after protein purification. The protein concentration was evaluated by Pierce TM BCA assay. Based on samples concentration and protein contamination we identified the most efficient method to isolate extracellular vesicles in urine.

Keywords: Urinary Extracellular Vesicles; Biomarkers Isolation; Purification; Tunable Resistive Pulse Sensing Technology

Abbreviation

EVS: Extracellular Vesicles; UEVS: Urinary Extracellular Vesicles; MVS: Multivesicular; Mirna: Micro RNA; SEC: Size Exclusion Column; TRPS: Tunable Resistive Pulse Sensing Technology; PSD: Particle Size Distribution

Introduction

Recently, the clinical and scientific interest in the study of extracellular vesicles (EVs) has been increased due to their potential role as noninvasive biomarkers of pathophysiological health status in different medical areas. This small membrane bound vesicles are secreted from MVs-multivesicular body (exosome) in the extracellular space, or directly released from the cell through plasma membrane fusion and exocytosis (microvesicles) [1]. Their identity and characterization is not well defined, although there are many studies that focus on their lipidomic [2,3], proteomic [4,5], and transcriptomic profiling [6,7], to exploit their possible role as biomarkers in different human pathologies.

These nanoparticles are involved in intercellular communication [8] and in many different biological processes such as: regulation of immune response, tumor proliferation, inflammatory response and antigen presentation [9,10]. EVs are present in various type of body fluids (e.g. blood, serum, saliva, milk and urine) [11] and their concentration, cellular origin, composition, function is body health-(state) dependent [10]. Particularly, EVs content is useful to understand in which physiological regulation process they are involved. Many studies have shown that EVs carry different type of cargo, such as micro RNA (miRNA) and proteins. miRNA are short single-strand RNA molecules that are involved in several biological processes including cell death, proliferation, apoptosis, fat metabolism and oncogenesis [12]. Thus, measuring the concentration, cellular origin and content of EVs in body fluids has also a

potential to provide novel and non-invasive disease biomarkers. In the nephrological field the study of urinary extracellular vesicles (UEVs) have acquired a great importance, because they can directly reflect the healthy status of the kidney [13]. Therefore, UEVs are suitable for the prevention of kidney rejection in the transplanted population. In the last few years several methods and techniques for the isolation and characterization of UEVs have been developed, that are cheaper and more effective than the already known ultracentrifugation method. The choice of the best method to isolate and identify UEVs is essential to obtain good quality samples and optimal concentrations. Thus, in this study, we compared five different methods (four UEVs isolation techniques and Size Exclusion Column (SEC)) to clarify which among these is the most convenient. The evaluation of the five different procedures was done based on concentration, size and protein contamination of the isolated UEVs.

Material and Methods

Preparation of samples

Early morning fresh urine samples were collected from healthy volunteers and promptly processed as describe by Felix R., *et al* [14]. Informed consent was obtained from all individuals included in this study. The samples were used to isolate and characterize UEVs, whereas the samples excess was stored at - 80°C. The storage at -80°C compared with + 4 °C or - 20°C, better prevent urine nanoparticles degradation, although freshly processed urine is the most favorable approach [15].

UEVs were isolated from 5 ml of processed urine using five different methods: 1) Norgen Urine Exosome RNA Isolation Kit 2) Exo Quick-TC™ Exosome Precipitation Solution 3) QIAGEN exo Easy Maxi Kit, 4) Izon qEV single Size Exclusion Column and 5) the ultracentrifugation procedure (by Amicon® filter column).

Urine extracellular vesicles isolation methods

Method 1 (Norgen Urine Exosome RNA Isolation Kit; Norgen Biotek Corp, Ontario, CA, USA): According to Norgen protocol, described in Rachel E., *et al.* [16], this kit was adapted to isolate EVs from urine samples.

At the end of the procedure the supernatant, containing the eluted UEVs, was collected for further analysis.

Method 2 (ExoQuick-TCTM Exosome Precipitation Solution; System Biosciences, Mountain View, CA, USA): Urine samples were added Exo Quick-TC solution and processed as report in the protocol [17]. Afterwards, pellet was suspended in phosphate-buffered saline (PBS).

Method 3 (QIAGEN exo Easy Maxi Kit; QIAGEN, Leipzig GmbH, Hilden, GE): The QIAGEN protocol adopted in Daniel Enderle., *et al.* [18] was modified (as described in QIAGEN handbook) to isolate extracellular vesicles from urine samples. The same volume of XBP QIAGEN Buffer and urine sample were placed in a new tube and reversed for five or six times. The samples were left at room temperature for 15 minutes then transfer to a QIAGEN filter column and centrifuged at 500 g x 1 min. The flow-through was discarded and the filter column inserted in a new tube. We added XWP QIAGEN Buffer and centrifuged for 3000 g x 5 min to wash UEVs. Afterwards, the flow-through was discarded and vesicles were eluted with XE QIAGEN Buffer (centrifuged at 500 g and after at 3000 g x 5 min).

Method 4 (Ultracentrifugation procedure by Amicon® filter column; Billerica, Massachusetts, USA): The protocol described in Andrea Del Fattore., *et al.* [19] has been modified to ultrafiltration only (to maintain the integrity and functionality of the EVs) and applied to urine samples for vesicle isolation.

Method 5 (qEVsingle Size Exlusion Column; Izon Science, Burnside, Christchurch, NZ): Urine samples were mixed before processing. qEV columns (Analytical-scale max sample volume_100µL) were left at room temperature and the buffer was pipetted out above the top filter. 100 µl of urine sample were loaded and 200 µl of fraction were collected. The first five fractions are the "void volume" which does not contain vesicles. Immediately after the void volume, the following three fractions were collected. These are the fractions that contain UEVs and were processed further.

Quantification and characterization of UEVs

The UEVs extracted by the five methods were characterized by their quantity and diameter size. To obtain these data, the tunable resistive pulse sensing technology (TRPS) of the Izon qNanoTM (Izon Science, Burnside, Christchurch, NZ) was considered. The TRPS is the most appropriate and accurate technique to determine the particle size distribution (PSD) of extracellular vesicles [20].

Izon qNano measures continuously the electrical resistance of a small pore filled with conductive medium that is in contact with the solution containing the EVs [21,22]. Around the pore is applied an electric current needed to measure the resistance of the pore. Whereas the vesicles are moving through the pore, the pore resistance increase. This resistive pulse is revealed by Izon software as a decrease of current and it is detected as a particle with a definite size diameter [23]. To distinguish resistive pulses from noise, the threshold is set below the noise by the analysis software. The

maximum particles size that qNano can detect is directly correlated with nanopore size. Particles that are larger than pore size cause the vesicles flow blockage and consequently lead to an inadequate particles count. In the present study we adopted pore with an exclusion size of 150 nm, NP150. Quantification was performed based on calibrator CPC200 (Diameter 210 nm) with a particle rate concentration of 1 x 10^9. The instrument has been set with the following parameters: current flow between 110 -130 mA, pore stretch 47 mm, pressure 20 mbar and current voltage 0.58 V.

Results and Discussion

Results

Urinary extracellular vesicles are possible novel elective biomarkers of kidney health state. Indeed, UEVs carry different type of cargo such as protein and miRNA, that are suitable biomarkers released from renal cells. The isolation and detection of these vesicles may provide a useful diagnostic tool for early detection of kidney diseases. There are several methods for isolation and characterization of UEVs from urine samples collected from human patients, however up to now, a gold-standard technique for isolating UEVs in clinical practice has not be identified. Furthermore, many of the actual procedures to isolate vesicles from urine show protein contamination, that can interfere with particles analysis and quantification leading to a wrong assessment. In our study, we compared different methods for UEVs isolation and analyzed the resulting concentration, particle size pre and post purification, and protein contamination. Our aim was to describe in detail the most efficient and convenient method to isolate UEVs. The isolation methods adopted in this study are: QIAGEN exoR Neasy serum/plasma maxi kit, Norgen Exosome RNA Isolation kit, SDB ExoQuick-TC™, Amicon® Ultrafiltration column and Izon qEVsingle Size Exclusion Column. The samples isolated through the first four kits were also purified by Izon qEVsingle Size Exclusion Column to remove possible proteins contamination.

To detected the UEVs concentration and size we considered Tunable Resistive Pulse Sensing technology (TRPS) by Izon qNano. The instrument showed different pre purification values of UEVs' concentration with a range between 1.27 x 10^8 - 4.38 x 10^9 (Figure 1; Table 1). The highest score for the isolated particles was reached by Amicon® Ultrafiltration method whereas the QIAGEN was not quantifiable, probably due to the interference between QIAGEN reagents and the nanopore (NP150) used for qNano quantification. The same evaluation was performed also after the SEC purification. The highest amount of particles was shown by Izon qEVsingle Size Exclusion Column (6.47 x 108) in the directly purified urine samples. Although this data showed a great throughput for SEC purifier samples, the ultrafiltration followed by SEC purification

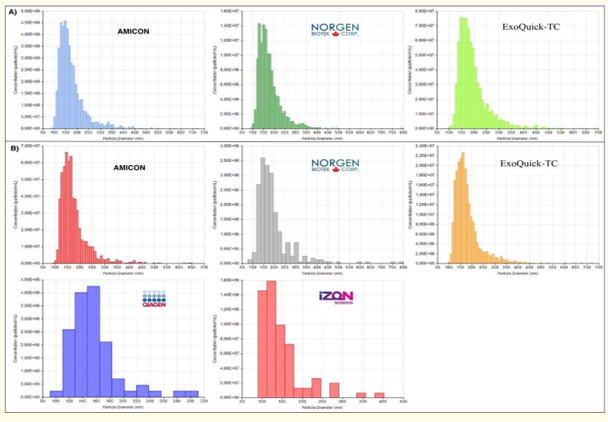

Figure 1: The size distribution of UEVs pre (A) and post (B) purification shows a similar distribution with the high concentration in the range of the microvesicles area.

resulted to be a satisfying method too, able to reach a concentration rank of 5.96 x 10^8. The size distribution of UEVs isolated by these different methods is within the macrovesicles size range (more than 100 nm) [24], both for pre and post purified samples. The particle diameter distribution was between 123 - 161 nm (Figure 1; Table 1). All pre and post purified samples were analyzed to reveal possible protein contamination, by Pierce ™ BCA Protein assay (Thermo Fisher scientific). The measurement showed a great variability in protein concentration by the different methods with the best performance obtained by Izon qEVsingle Size Exclusion Column. However, all aliquots treated by Izon columns showed a protein contamination between 0.2 - 0.35 mg/ml (Table 2).

Discussion

More than ten years have passed from the first reports on urinary vesicles [25] and currently a lot of methods for extracellular vesicles isolation are present, all based on different approaches such as: ultracentrifugation, density gradients, polymer-based precipitation, microfiltration and size-exclusion techniques. All of them sustain a high quality of their procedures considering vesicles concentration, size and purity [26]. Based on these considerations we tried to identify the best isolation method, among those listed, taking in account the criteria listed above and at the same time the reproducibility in laboratory. To our knowledge this is the first time that five different methodologies (four technique and direct SEC purification) are performed, at the same time, to identify the best approach for UEVs isolation considering both protein contamination and laboratory enforceability.

Our results showed differences in vesicles size and concentration (Figure 1; Table 1). At first glance, our study showed that the

Urine Volume	Isolation Technique	UEVs size diameter (Pre-Purification)	UEVs raw concentration (Pre-Purification)	UEVs size diameter (Post- Purification)	UEVs raw concentration (Post-Purification)
5 ml	QIAGEN exoRNeasy serum/ plasma maxi kit	Not detectable	Not detectable	156	1.53×10^7
5 ml	Norgen Exosome RNA Isolation kit	131	1.27×10^8	146	1.93×10^7
5 ml	SDB Exo Quick-$_{TC}$TM	143	7.51×10^8	161	2.16×10^8
5 ml	Amicon Ultrafiltration column	147	4.38×10^9	145	5.96×10^8
5 ml	Izon qEVsingle Size Exclusion Column			123	6.47×10^8

Table 1: UEVs concentrations and size distribution pre and post Izon SEC protein purification.

UEVs Isolation Technique	Protein concentration [mg/ml]_ Pre-purification	Protein concentration [mg/ml]_ Post-purification
QIAGEN exoRNeasy serum/plasma maxi kit	0.7	0.25
Norgen Exosome RNA Isolation kit	8.4	0.32
SDB ExoQuick-TCTM	23	0.31
Amicon Ultrafiltration column	11.47	0.35
Izon qEVsingle Size Exclusion Column		0.2

Table 2: UEVs protein contaminations pre and post Izon SEC purification.

best method to isolate urinary vesicles was Amicon® Ultrafiltration with a vesicles concentration of 4.38×10^9, whereas the one with the disputable performance was the Norgen Exosome RNA Isolation kit with a raw concentration of 1.27×10^8. In this first phase the QIAGEN kit did not give detectable results probably due to the interference of its reagents with the Izon qNano instrument. This first analysis did not take into account the possible protein contamination of isolated particles. However, their graphic distribution was homogeneous and their concentration-peak was within the area of interest of the extracellular vesicles (Figure 1A). In all case, the highest sample concentration after isolation was in a diameter size range between 131 and 147 nm that is within the range of macrovesicles size. Analyzing post-purified samples, we could observe that particles raw concentration dropped down, but it was still possible to quantify it. After purification also QIAGEN processed samples showed a good range of UEVs concentration although the best performance was observed for samples directly purified by Izon SEC column. The homogeneity and distribution of detected particles show the same kind of particles despite their spread distribution. Indeed, these vesicles belong to the same group, as shown in pre purified samples, that is the cluster of macrovesicles (Figure 1B). The presence of protein contamination in the different methods considered, confirm a great variability, especially between pre and post purification. The greatest protein contamination was detected in the samples processed by Exo Quick kit (23 mg/ml). Whereas, QIAGEN kit showed a low level of proteins contamination between pre and post purification (0.7-0.25 mg/ml). However, the best performance was observed for Izon SEC purification that show the lowest amount of protein among all the methods considered. It has to be pointed out that the difference in protein concentration in the post purified samples is negligible (about 0,15 mg/ml). The distribution of pre and post-purification particles is also homogeneous and is found in the microvesicles area.

This comparative study provides a starting point for a broader research on novel possible reliable and noninvasive kidney damage biomarkers. In our study Izon qEV single SEC resulted to be the most efficient method to isolate and purify urine extracellular vesicles, that are suitable for NGS and Proteomics analysis. This

method allows to have a greater quantity of traceable particles and without protein contamination, therefore the ideal condition for evaluating the real concentration of EVs in biological fluids.

Considering that urine is the unique biological fluid that shows a great variability (pH, concentration, composition and osmolarity of dissolved solutes) even in the same individual and in the different time of the day, it would be interesting to extend the samples analysis. Sampling urine at several hours of the day on healthy volunteers versus patients with various types of kidney disease will be useful to understand how the variation of functional and clinical data lead to a different expression of urinary extracellular vesicles.

Author Contributions

AC and SN conceived the study, collected samples and wrote the manuscript. AC performed UEVs isolation and purification. MG and AMT performed qNano analysis and BCA dosages. GF and DM collected samples. PDG, GP and LM contributed to the manuscript revision. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Financial and competing interests disclousure

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Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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Volume 3 Issue 11 November 2019