

Extracellular vesicle isolation: present and future

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In recent years, extracellular vesicle (EV)-based biomarker discovery has received significant interest amongst scientist studying diverse disease conditions. Extracellular vesicles are released by variety of cells into the cellular microenvironment and have the natural ability of delivering different cargos and carry bioactive molecules such as non-coding RNA, genomic DNA, lipids, growth factors, and signaling molecules. EVs have been isolated from most biofluids including serum, plasma, serum, and saliva. It has been shown that EVs play substantial roles not only in the regulation of normal physiological processes but also in disease pathogenesis and their cargo reflects the status of parental cells at the time of secretion (1,2). Multiple studies found EVs enriched in lipid raft molecules (Flotillin) (3), membrane trafficking molecules (Annexins) and heat-shock proteins (HSP70, HSP90) (3,4). Based on mode of biogenesis, EVs can be divided into exosomes (30–100 nm), microvesicles (100–1,000 nm) and apoptotic bodies (>1,000 nm). The biogenesis and cellular pathways for generation of these different vesicle types, as well as their cargo, membrane composition and surface molecules are distinct with some overlapping features (5,6).

Although the field of EVs is rapidly growing, it has been hampered by challenges in EV isolation and characterization methods. Employing an efficient, rapid and reproducible isolation method is fundamental to analytical reproducibility.

Different studies employed various EV isolation techniques that can be broadly grouped into five distinct isolation methods: ultracentrifugation, density-gradient

separation, polymer-based precipitation, immuneselection, and microfluidic isolation methods (7).

These different methods can be used individually or combined for the isolation of extracellular vesicles from diverse biological sources. It is worth noting that each of these methods, while suitable for extracellular vesicle isolation, have some limitations. In this report we briefly describe some of the positive and negative attributes of each of these methods for EV isolation. We will also discuss a recently described new method of EV isolation published by Nakai *et al.* in *Scientific Reports* (8) by focusing on its novelty and how this method can complement or enhance the reliability of previously described methods for EV isolation.

Ultracentrifugation is generally regarded as the most commonly used method for isolating EVs and exosomes; however, different results from the literature demonstrate inconsistencies in reproducibility of this isolation technique. Such inconsistencies could be a result of different centrifugation time, speed, type of rotor or other technical factors (e.g., temperature) (9). Ultracentrifugation is time consuming, requires an ultracentrifuge and results in relatively low recovery of EVs (1). Another limiting factor is that isolation by ultracentrifugation causes non-vesicular macromolecule contamination (10) and aggregation of EVs that can lead to masking of surface antigens (11).

One of the limitations in using ultracentrifugation for isolation of EVs is coprecipitation of protein aggregates and nucleosome fragments which can lead to decreased sample purity and may affect downstream analysis. The density-

gradient separation, which employs ultracentrifugation combined with a sucrose gradient, can lead to increased sample purity as protein and protein-RNA aggregates can be efficiently separated from the exosomes. This method allows the separation of EVs based on their densities from low density exosomes to higher density microvesicles (12,13).

Polymer-based isolation is a widely used techniques based on poly-ethylene glycol based separation. This method allows for greater yield of the extracellular vesicles (14), but with a high portion of other contaminants (lipoproteins), especially from serum samples (15,16). Microfluidic techniques are based on trapping EVs in micro channels are a good option for low volume input of biofluids. There is lack of evidence about their efficacy and downstream clinical utility in the comparative settings (17).

Immuno-selection techniques use antibody-based separation methods targeting known surface markers on extracellular vesicles. Some of these markers include the well characterized tetraspanins (CD9, CD63, CD81) or immune-regulator molecules (MHC I&II) on the surface of the vesicles (18,19). This method is costly and is not suited for large sample volumes. Captured EVs may not retain desired functionality even if successfully eluted from the bead surface (13). However, the main limitation of this method, if is that not all microparticles can be isolated successfully (in a contrast with, e.g., polymer based techniques) since the isolation relies on surface markers and some of those might not be expressed on all types of extracellular vesicles. However, in specific designs in which enrichment of a subpopulation of EVs or isolation of viral particles from EVs is desired, this selectivity can serve as a distinct advantage (4).

Given the potential limitations with the above described methods, Nakai *et al.* published a novel affinity-based technique for the isolation of EVs, which uses Tim4 protein binding to phosphatidylserine, an enriched component of the EVs surface (8). The authors compared this new isolation method to conventional ultracentrifugation and polymer-based precipitation methods for powerful isolation of both small and large EVs. In their experiments, they used K562 cells and peritoneal macrophages (pMac). With Tim4 affinity-based isolation they demonstrate a robust enrichment of CD63, Flotillin2, CD9 and CD81 markers in the small EVs (sEVs) compared to ultracentrifugation and polymer-based precipitation. They provide Western blot analysis of the large EVs, demonstrating efficient isolation of LAMP2 positive microvesicles from the 10k pellet.

In further studies, they performed a proteomic analysis of sEVs, where they found an excellent recovery in the protein profile of sEVs (78.1%), especially compared to the sEVs isolated by polymer based precipitation (21.8%). They also provided a FACS-based quantification, where they found that exosome isolation by Tim4-conjugated beads was more efficient than that by CD63-cojugated beads, leading to 2 fold stronger signal-to-noise ratio.

While this new method described by Nakai *et al.* (8) provides a novel approach for EV isolation, the major limitation of their method is its inability to distinguish between microvesicles and exosomes since phosphatidylserine is expressed in different subpopulation of EVs including exosomes, microvesicles and apoptotic bodies. Therefore, Tim4 affinity-based isolation should not be used in studies assessing different subpopulations of EVs. Additional limitation of the Tim4-based isolation is that phosphatidylserine is also expressed on the apoptotic micro particles (EVs) and apoptotic bodies. Thus, a Tim4 based isolation can lead isolation of apoptotic bodies and not only EVs, which can significantly affect experimental conclusions and mislead EV focused investigations (1,20).

Moreover, the Nakai *et al.*'s (8) study is limited to the pMac and K562 human erythromyeloblastoid leukemia cell line model and the result should be replicated in other cells lines and different biofluids. Specifically, the effect of stimulation of cells with monensin which was used in this study should be corroborated. Monensin stimulation increases the cytosolic free Ca^{2+} and increases the release of EVs that harbor phosphatidylserine/Tim4. It is not clear that the EVs released in physiologic states or other pathologic/stress conditions harbor comparable amounts of phosphatidylserine.

Taken together, the Tim4-based isolation method is a novel and a potential powerful EV isolation method with excellent recovery. However, the inability of this method to distinguish between different populations of EVs based on Tim4 (namely exosomes), microvesicles and apoptotic bodies greatly limits its utility. The utility of Tim4 for affinity methods should be reproduced in other cell types and biofluids in physiological and pathological states. Despite these limitations, the newly discovered Tim4-based EV isolation method offers a promising new approach for future EV research.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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