Extracellular vesicles isolation and their biomarker potential: are we ready for testing?

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In a recently published paper (Sci Rep 2016;6:33935), a group of Japanese investigators reported on a novel affinity-based method for the isolation of extracellular vesicles (EVs) from supernatants of cell lines or cells isolated from murine tissues (1). The paper calls attention to the growing interest of scientific and medical communities in EVs, their molecular and genetic characterization, their biomarker status and the role they play in human health and disease.

EVs are rapidly emerging as mediators of communication between cells (2). In multicellular organisms, the maintenance of cellular contact within tissues or between distant tissues is of critical importance for the development, organization and functional integrity of the whole organism. The presence in tissues and body fluids of soluble factors, including cytokines and chemokines, has been generally viewed as evidence for the existence of active cross-talk between cells communicating via cognate receptors expressed on their surface. More recently, EVs which are released by all cells and are ubiquitous in all body fluids, have assumed a predominant place as the highly efficient and biologically significant intercellular communication system (2). Cells release EVs of different types, and so, EVs found in body fluids are heterogeneous mixtures of membrane-bound vesicles originating from various cells and ranging in size from 30nm to >5,000 nm (3). The current nomenclature of EVs is based on size, and they have been arbitrarily divided into the smallest, exosomes, which are 30–150 nm in diameter, somewhat larger microvesicles (MVs, 200–1,000 nm) or large apoptotic bodies (1,000 to >5,000 nm). Each EV is bound by a lipid bilayer membrane containing numerous biologically-active transmembrane proteins. The vesicle lumen is filled with cytosolic proteins and nucleic acids derived from the EV-producing cell (4). EVs differ from one another not only by size but also by cellular mechanisms used for their secretion, the molecular content and functional properties (5). MVs are formed by "blebbing" or "pinching off" from the cellular membrane of the parent cell and contain parts of the cytosol more or less randomly enclosed in vesicular "blebs". Apoptotic bodies are remnants of dead parental cells. In contrast, the biogenesis of exosomes is unique: they originate from the endocytic compartment and their molecular content reflects, at least in part, that of the parental cell (5). For this reason, exosomes, serving as surrogates of their cells of origin, have been of the greatest interest among EVs as potential biomarkers and "liquid biopsies" (6). As communication vehicles, EVs transfer proteins, lipids and nucleic acids (mRNA, miRNA and DNA) from the parent to recipient cells, and this transfer of the molecular/genetic cargo is accompanied by re-programming of the recipient cell functions (6). Because the EV cargo determines cellular re-programming, efforts to isolate EVs from human body fluids and to characterize their molecular and genetic content have been intensively pursued.

The methodology for EV isolation was initially developed and used for their recovery from supernatants of cell lines. It involved a series of sequential differential centrifugation steps at increasing speeds (300 ×g,

2,000 ×g, 10,000 ×g) to remove cell debris and large EVs followed by ultrafiltration using 22 nm-pore filters and ultracentrifugation (UC) at 100,000 ×g for 2–3 h (7). The recovered pellets of small EVs or exosomes were then re-suspended in buffer and placed on a continuous sucrose density gradient for further exosome enrichment, taking advantage of the unique ability of exosomes to float at the density of ~1.15 g/mL of sucrose. This method for small EV isolation has been widely adopted as the prototype and is being used as the gold standard despite the fact that UC tends to aggregate EVs, is time consuming, requires special equipment and is not suitable for a high sample throughput. Purification of vesicles on sucrose gradients leads to a loss of aggregated vesicles. Thus, neither the EV morphologic integrity nor their recovery may be optimal with this procedure. Numerous other isolation methods utilizing various technologies such as polymer-based precipitation (e.g., total exosome isolation or TEI), microfluidic separation, affinity capture with antibodies coated on latex beads or size-exclusion chromatography have been introduced and are in use for EV isolation (8). Needless to say, the recovery, quality and molecular content of EVs obtained by these different methods vary. Many of the methods are commercially available. Often, these methods aim only at the isolation of nucleic acids, usually of miRNA or DNA, from EVs. Some methods do not discriminate small from large EVs, and few are concerned with EV integrity, purity and biological functions. EVs have the propensity for binding of exogenous molecules. Thus, EVs obtained from biological fluids such as plasma are always liberally coated with immunoglobulins (Igs) and albumin. The presence of these "contaminants", which stick to the surface of EV membranes but are not *bona fide* parts of the EV molecular content complicates subsequent molecular profiling and may interfere with biologic activities. To date, despite a wide choice of methods available for the isolation of EVs from various fluids, no single method guarantees their recovery for reliable qualitative and quantitative analyzes.

Since isolation of EVs based on size alone is not optimal, affinity-based capture has been considered as an option for improving recovery and purity of EVs. The success of this approach depends on the stable presence on the EV surface of a marker or markers that can strongly bind a detection reagent such as the marker-specific antibody (Ab). For example, Abs specific for the three tetraspanins classically used as exosome markers (CD9, CD63, CD81) have been used for immuno-affinity isolation of small EVs (9). The problems may arise, however, when levels of a given tetraspanin in the exosome cargo vary, as they often do, leading to a partial EV capture. Also, capturing Abs might interfere with downstream EV analyses, specifically mass spectrometry, and their removal following capture could impair vesicular integrity or functionality of EVs. Hence, utmost care has to be taken when selecting reagents for affinity-based EV isolation.

The paper by Nakai and colleagues describes a novel affinity-based isolation method for EVs which, in combination with initial differential centrifugation (the pre-clearing steps), yields highly purified small EVs and avoids some of the above mentioned pitfalls (1). The method uses the T cell immunoglobulin and mucin domain protein 4, Tim4, for EV capture. Tim4 is a transmembrane protein expressed on macrophages. It is a receptor for phosphatidylserine, and the receptor-ligand binding is Ca^{2+}-dependent (10). Phosphatidylserine decorates apoptotic bodies as well as MVs and exosomes. The authors used the extracellular domain of murine Tim4 fused to the Fc fragment of human IgG and immobilized Tim4-Fc protein on magnetic beads for EV capture in the presence of Ca^{2+}. The captured EVs were readily released from the beads by adding buffer containing a Ca^{2+} chelating agent (e.g., EDTA). The yield and purity of EVs isolated by this Tim4-Fc affinity capture were compared with those of EVs obtained by UC or TEI-based precipitation and were shown to be superior. Tim4-Fc affinity capture isolated small EVs or large EVs (if the pre-clearing steps were omitted), were largely free of protein contaminations, enriched in exosomal proteins and morphologically intact after their release from magnetic beads. *Ex vivo* functions of isolated EVs were not tested. The most important advantage of this isolation method was the relative absence of contaminating non-exosomal proteins, which enabled proteomics analysis of the "purified" EVs. The presence of contaminants in EVs isolated by the conventional methods listed above interferes with mass spectrometry and is a major disadvantage in exosome molecular profiling and biomarker studies.

While the new isolation method appears to be advantageous, it has been largely tested with supernatants of cultured cells and not with human plasma (although one example of human urine is presented). This is a significant problem that has plagued the exosome field from the start. Supernatants of cultured cells are a good source of EVs produced in large quantities by one cell type. In contrast, human plasma contains mixtures of exosome subsets derived from many different normal or abnormal cells. Isolation of "contamination-free" EVs from human plasma, especially

with specimens of patients with chronic diseases such as cancer, is proving to be a major challenge. So far, size exclusion chromatography of pre-cleared plasma which removes a bulk of exosome-associated plasma proteins, as recently described (11) appears to come the closest to yielding exosomes that have a low (but not absent) contaminating protein content, are morphologically intact and are functionally competent (11). It may be that the receptor-ligand-based isolation of EV, such as described by Nakai *et al*., will be successful in isolating "pure" EVs from human specimens. However, given the enormous heterogeneity of plasma EVs which likely have a broadly variable content of phosphatidylserine, the recovery as well as EV purity might be compromised. Also, there is no rational explanation for removal of contaminants by this type of capture, unless the step of EV release from beads using Ca^{2+} chelators contributes to overall final "purity".

Yet another aspect of EVs isolation from human body fluids that is in part related to EV purity and to their molecular profiling concerns separation of different EV subsets, specifically EV subsets produced by diseased cells, e.g., cancer cells. Affinity-based EV capture with Abs specific for a component of exosome cargo represents one way for exosome isolation and separation of from human body fluids. The best example of this approach was provided by Melo and colleagues, who used immune capture with Abs specific for glypican-1 to isolate pancreatic cancer-derived exosomes from patients' plasma and to study them as predictive biomarkers of the disease progression and outcome (12).

As disease biomarkers or "liquid biopsies", EV subsets in plasma that are derived from diseased cells and carrying the cargo partly derived from these cells are likely to be more specific and more reliable than total plasma EVs. The major barrier to isolation of such "disease specific" EVs has been the lack of capture reagents with sufficiently high avidity and specificity for unique markers that such EVs are expected to carry. The requirement for a two-step isolation method, first to isolate "pure" total plasma EVs and then to selectively capture the desired EV subset, further complicates the process. Using affinity capture directly from body fluids or after the preclearing steps but without removing contaminating proteins is less effective. Finally, the use of affinity capture for EV isolation means that the capturing agent will have to be removed and EVs safely recovered for downstream analyses, as done by Nakai *et al*. using Ca^{2+} chelation in their Ca^{2+}-dependent capture method. The upscaling of the procedure for high throughput EV capture necessary for human biomarker studies will require additional efforts. To date, selective isolation of "disease specific" EVs from body fluids remains an elusive goal. There are indications, however, that with a better understanding of the EV biology, it will be possible to achieve this goal perhaps by a combination of large-scale size-based fluidics with specific capture of desired EVs. In the context of this effort ongoing worldwide, methods for reliable, effective and uncomplicated isolation of "pure" total EVs such as described by Nakai *et al*. represent an important addition to the existing EV isolation repertoire.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

References

1. Nakai W, Yoshida T, Diez D, et al. A novel affinity-based method for the isolation of highly purified extracellular vesicles. Sci Rep 2016;6:33935.
2. Boyiadzis M, Whiteside TL. Information transfer by exosomes: A new frontier in hematologic malignancies. Blood Rev 2015;29:281-90.
3. van der Pol E, Böing AN, Harrison P, et al. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev 2012;64:676-705.
4. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200:373-83.
5. Cocucci E, Meldolesi J. Ectosomes and exosomes: shedding the confusion between extracellular vesicles. Trends Cell Biol 2015;25:364-72.
6. Abels ER, Breakefield XO. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. Cell Mol Neurobiol 2016;36:301-12.
7. Théry C, Amigorena S, Raposo G, et al. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006;Chapter 3:Unit 3.22.
8. Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. Methods 2015;87:3-10.

9. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One 2009;4:e5219.
10. Miyanishi M, Tada K, Koike M, et al. Identification of Tim4 as a phosphatidylserine receptor. Nature 2007;450:435-9.
11. Hong CS, Funk S, Muller L, et al. Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. J Extracell Vesicles 2016;5:29289.
12. Melo SA, Luecke LB, Kahlert C, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature 2015;523:177-82.

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