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Review article

Chromatography and its hyphenation to mass spectrometry for extracellular vesicle analysis

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ABSTRACT

Extracellular vesicles (EVs), such as exosomes, microvesicles and apoptotic bodies are released by cells, both under physiological and pathological conditions. EVs can participate in a novel type of intercellular communication and deliver cargo of nucleic acids, proteins and lipids near or to distant host cells. EV research is proceeding at a fast pace; now they start to appear as promising therapeutic targets, diagnostic tools and drug delivery systems. Isolation and analysis of EVs are prerequisites for understanding their biological roles and for their clinical exploitation. In this process chromatography and mass spectrometry (MS)-based strategies are rapidly gaining importance; and are reviewed in the present communication.

Isolation and purification of EVs is mostly performed by ultracentrifugation at present. Chromatography-based strategies are gaining ground, among which affinity and size exclusion chromatography (SEC) are particularly strong contenders. Their major advantages are the relative simplicity, robustness and throughput. Affinity chromatography has the added advantage of separating EV subtypes based on molecular recognition of EV surface motifs. SEC has the advantage that isolated EVs may retain their biological activity.

EVs are typically isolated in small amounts, therefore high sensitivity is required for their analysis. Study of the molecular content of EVs (all compounds beside nucleic acids) is predominantly based on liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis. The chromatographic separation is mostly performed by reverse phase, nanoscale, ultra high performance LC technique. The MS analysis relying typically on nano-electrospray ionization MS/MS provides high sensitivity, selectivity and resolution, so that thousand(s) of proteins can be detected/identified/quantified in a EV sample. Beside protein identification, quantitation and characterization of protein post-translational modifications (PTMs), like glycosylation and phosphorylation are becoming feasible and increasingly important. Along with conventional LC–MS/MS, other chromatographic approaches hyphenated to MS are gaining importance for EV characterization. Hydrophilic interaction LC is used to characterize PTMs; LC–inductively coupled plasma/MS to identify metal containing molecules; while gas chromatography–MS to analyze some lipids and metabolites.

Contents

Abbreviations: 1-DE, one-dimensional gel electrophoresis; 2-DE, two-dimensional gel electrophoresis; AB, apoptotic body; SCX, strong cation exchange chromatography; CM, conditioned medium; DC, differential centrifugation; DGC, density gradient centrifugation; ESI, electrospray ionization; EV, extracellular vesicle; FBS, fetal bovine serum; GC, gas chromatography; HILIC, hydrophilic interaction liquid chromatography; ICP, inductively coupled plasma; LC, liquid chromatography; LOC, lab-on-a-chip; MF, microfiltration; MS, mass spectrometry; MV, microvesicle; MVB, multivesicular body; PTM, post-translational modification; RP, reverse phase; UF, ultrafiltration; UHPLC, ultra high performance liquid chromatography; SEC, size-exclusion chromatography; TFF, tangential flow filtration; PEG, polyethylene glycol; ZIC, Zwitterion chromatography.

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1. Introduction

In recent years, hyphenated techniques have received ever-increasing attention as the principal means to solve complex analytical problems. Hyphenated techniques combine a separation technique and one or more detection techniques to exploit the advantages of both. Extracellular vesicles (EVs) are intensively studied today because of their important roles in various biological and pathophysiological processes. In this review we have put in our best efforts to elaborate on the advantages and limitations of chromatography hyphenated to mass spectrometry (MS) for studying these complex vesicles.

1.1. Nomenclature, classification, biogenesis and biological roles of extracellular vesicles

A collective term called "extracellular vesicles" is used to describe all phospholipid bilayer-bound vesicles secreted by cells into the extracellular microenvironment, irrespective of differences in biogenesis, size and composition [1,2]. Their functions in various physiological and pathological processes have made them a hot topic for research. EVs are currently classified into different subgroups based on size, density, subcellular origin, function and molecular cargo [3]. Even a single cell can actively secrete different types of EVs which makes isolation and characterization a challenging task, as current methods of purification often result in a mixed population of vesicles. Furthermore, at present there are only a limited number of markers available to effectively distinguish all populations of cell-derived vesicles. The three best defined subgroups of EVs are exosomes, microvesicles (MVs), and apoptotic bodies (ABs) (Fig. 1) [3–6].

Exosomes are homogeneous membrane vesicles (30–100 nm in diameter) that originate through the endosomal trafficking pathway (Fig. 1) [7]. The first step in exosome biogenesis is the formation of early endosome. The main role of early endosome is to sort molecules for degradation or secretion. Early endosomes can interact with the Golgi apparatus and the endoplasmic reticulum. During the transformation of early endosome to late endosome the limiting membrane invaginates and pinches off forming intra-luminal vesicles. Late endosomes are also called as multivesicular bodies (MVBs) as these contain many intra-luminal vesicles within. These can either fuse with plasma membrane secreting the exosomes or fuse with lysosome and get degraded [4,8]. The formation of the MVBs as well as the secretion of exosomes are mediated through protein complexes called endosomal sorting complexes required for transport (ESCRT) [4,7,9,10].

The most abundant proteins of EV are membrane transport and fusion proteins (annexins, GTPases and flotillin), tetraspanins (CD9, CD63, CD81, CD82), heat shock proteins (Hsc70 and Hsp90), proteins involved in MVB biogenesis (Alix and Tsg101) as well as lipid related proteins and phospholipases [9,11,12]. The exosomal lipid bilayer comprises mainly of plasma membrane lipids including ganglioside GM3, phospholipids, sphingomyelin, and cholesterol [13,14]. The functional roles of exosomes may vary depending on their cells/tissues of origin [6]. Their role in antigen presentation, programmed cell death, angiogenesis, inflammation, differentiation, pregnancy and coagulation have been extensively studied [15]. Exosomes play a very important role in cell–cell communication both in regulation of cell and tissue homeostasis and during pathological conditions [4].

Microvesicles are heterogeneous membrane vesicles (100–1000 nm in diameter) that are formed by outward budding and fission of plasma membrane, which occurs as a result of dynamic interaction between phospholipid redistribution and cytoskeletal protein contraction (Fig. 1). The distribution of phospholipids is controlled by aminophospholipid translocases [4,15–17]. They are released under cellular stress or in pathological processes [4]. Like the exosomes MVs are able to transfer bioactive molecules to target cells. While the membrane composition of MVs is very similar, they appear to be enriched in some proteins, like B1 integrin receptors and vesicle-associated membrane protein 3, respect to that of the parent cell of origin.

Apoptotic bodies are large vesicles (1–5 μm in diameter) that are released only by cells undergoing apoptosis or programmed cell death (Fig. 1) [4,18]. ABs are characterized by the presence of cellular organelles and DNA. The clearance of ABs during normal development is done by macrophages. This clearance is mediated by specific interactions between recognition receptors on the phagocytes and specific changes in the membrane of apoptotic cells. Annexin V binds to the phosphatidylserine translocated to the outer leaflet of the lipid layer which is recognized by phagocytes [4]. The other well documented AB-related process involves the oxidation of surface molecules which creates sites for binding of thrombospondin or the complement protein C3b which are then recognized by phagocyte receptors [4,18–20].