Opportunities and challenges in studying the extracellular vesicle corona

The extracellular vesicle (EV) surface corona is emerging as a crucial mediator of EV functions. This Comment discusses the roles and biogenesis of the EV corona, as well as the importance of controls to determine whether a biological effect is attributable to the internal EV cargo or to the corona associated with the EV exofacial surface.

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Extracellular vesicles (EVs) are released by all cells and have critical roles in homeostatic processes and in intercellular communication. A defining feature of EVs is a phospholipid bilayer. The lipid composition of EVs varies between different types of the released vesicle, and their lipid shell contains rafts and transmembrane proteins, such as tetraspanins[1]. For decades, researchers in the EV field devoted substantial efforts to separating EVs from contaminating co-isolated proteins and lipoproteins. However, none of these efforts or separation platforms have led to pure EV preparations: purifying EVs has proved to be extremely challenging and only partially successful — in particular, when separating EVs from protein-rich matrices such as blood plasma[1].

Detection and characterization of corona-coated EVs

The EV field is witnessing a paradigm shift as recent independent studies detected the spontaneous formation of a protein corona around EVs[2–5] — molecules associating with the outsides of EVs once the EVs are released by the producing cell and immersed in the extracellular milieu. This corona contains molecules that were previously considered to be contaminants of EV preparations. Importantly, a corona is formed not only around EVs in bodily fluids, but also during EV biogenesis within cells, and both the intracellularly and extracellularly formed types of corona may be equally important functionally[6]. That particles can acquire a protein corona in the extracellular space has been long known in the field of synthetic nanoparticles. Identifying the protein corona around EVs is substantially more difficult than detecting coronas around synthetic nanoparticles or viruses because both EV proteins and the corona components are from the same host organism. However, these challenges could be overcome by incubating nascent EVs (such as EVs released by cells in serum-free condition) in bodily fluids[2]. The lack of serum in the tissue culture medium used for nascent EV production ensures that when nascent EVs are incubated with biological fluid samples and subjected to extensive washing steps, an extracellularly acquired corona is formed and can be analysed. Once corona-coated EVs are separated and washed, several downstream analytical tools can be used to characterize the corona, including immunoblotting, mass spectrometry, nanoparticle tracking analysis (NTA) or tuneable resistive pulse sensing, confocal and super-resolution microscopy, transmission and immune electron microscopy, high-resolution flow cytometry, ELISA and single-particle interferometric reflectance imaging sensor detection. Functional assays can be performed to assess the significance of the corona, including analyses of cellular uptake, proliferation, viability or the induction of cell death, gene expression, autophagy, senescence, metabolism, migration, wound healing and angiogenesis.

The EV corona includes components shared with the coronas formed around synthetic nanoparticles or viruses[2]. These 'universal' corona proteins include highly abundant plasma proteins such as apolipoproteins and immunoglobulins. Apolipoproteins are amphipathic molecules and have the ability to surround lipids. The mechanism by which immunoglobulins associate with the surface of EVs remains to be established. A major difference, however, between the coronas of synthetic nanoparticles and EVs is that specific interactions mediated by membrane receptors, and other interactions between plasma membrane glycoproteins or glycolipids and carbohydrate-binding proteins, typically only occur in the case of EVs.

Recent evidence supports the idea that although certain corona proteins are evenly distributed along the exofacial surface of EVs, forming a 'patchy corona', larger aggregates and protein complexes are also found attached to the EV surface[2]. Analysing EVs derived from red blood cells by dynamic light scattering, NTA and non-optical methods such as microfluidic resistive pulse sensing and very-small-angle neutron scattering, one study found that the protein corona averages 5.3 ± 0.3 nm in thickness and is surrounded by a hydration shell 10–20 nm thick[7].

Formation of the corona

Components of the protein corona are peripheral membrane proteins that establish interactions with the membrane of EVs. Thiol interactions are considered to be involved in the binding of albumin, uromodulin and fibrinogen to EV membranes, as these proteins contain 35, 48 and 58 cysteine residues, respectively[8]. These proteins are present in the corona formed around EVs in body fluids such as blood plasma[2] or urine[8]. Electrostatic interactions may also be involved in the association of basic proteins with the negatively charged, externalized phosphatidylserine molecules on the surface of EVs or with glycoproteins, glycolipids and proteoglycans. Vesicle surface proteoglycans are of particular interest in regard to EV corona formation. Proteoglycans identified in association with certain EVs include syndecan-1 (SDC-1), glypican 1 (GPC1) and betaglycan[9]. The carboxylate groups and *O*- and *N*-sulfates give polyanionic character to the proteoglycans and enable them to interact with ~400 different heparin-binding proteins[10]. These proteins include growth factors (such as acidic fibroblast growth factor, basic fibroblast growth factor, hepatocyte growth factor, vascular endothelial growth factor and platelet-derived growth factor), cytokines or chemokines (such as CXCL8 (also known as IL-8), TGFβ, CXCL12 and CCL5), enzymes and enzyme inhibitors (such as heparinases 1 and 2 and thrombin), lipoproteins (such as low-density lipoprotein (LDL)) and other proteins, including annexin A5 and

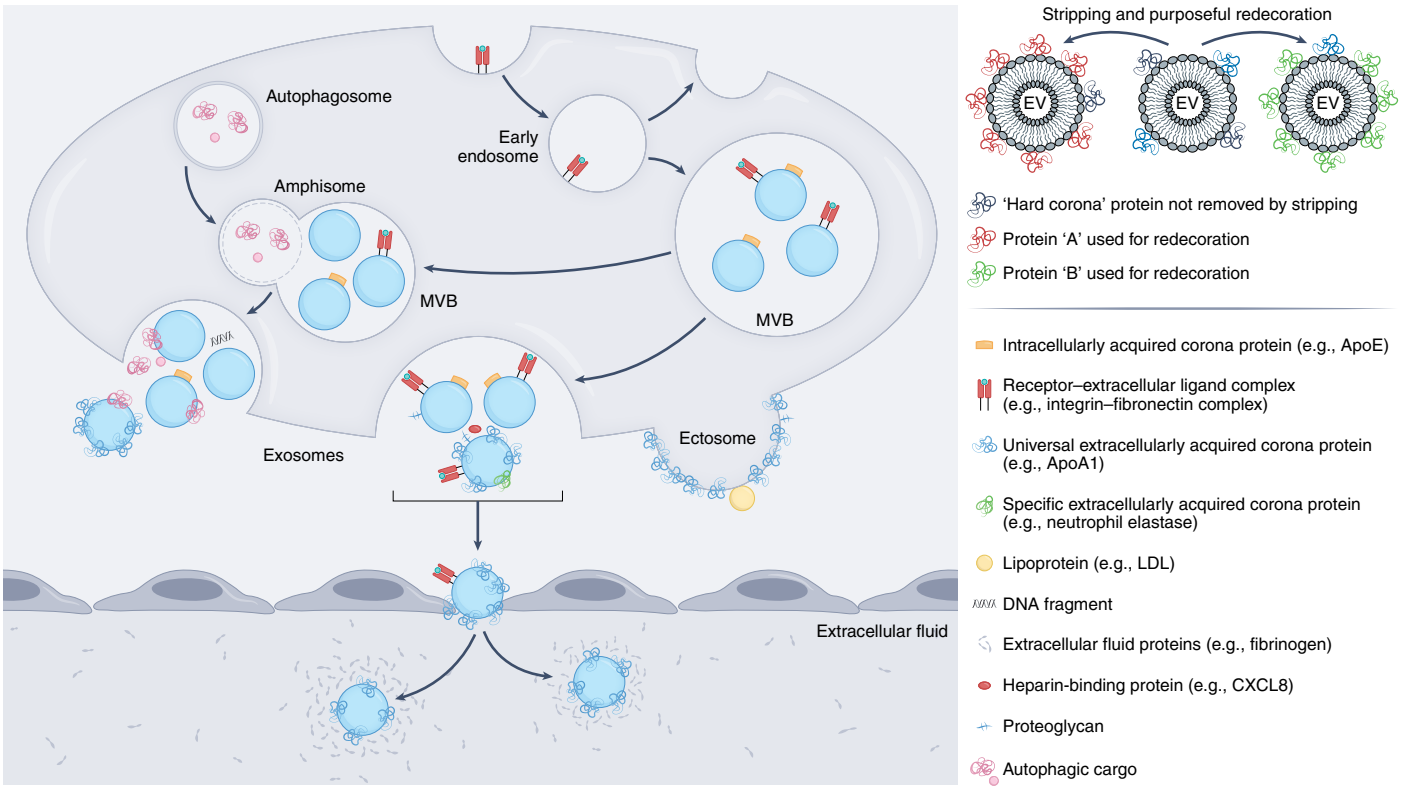

Fig. 1 | Formation of a biomolecular corona around EVs. Proteins, protein complexes, nucleic acids and lipoproteins associate with the surface of EVs. Corona molecules can associate with the EV surface either intracellularly, in multivesicular bodies (MVBs) or amphisomes, or in extracellular fluid, such as blood. EVs released from cells by budding from the plasma membrane, referred to as ectosomes, immediately become exposed to the extracellular milieu and therefore are always associated with corona proteins. Endosome-derived EVs, referred to as exosomes, acquire a corona once they are released by the cells, or within cells during EV biogenesis and secretion. Some corona components ('universal' components) are ubiquitous, present in all protein coronas, while others are specific to the releasing cell. The biomolecular corona of nascent EVs (that is, released by cells under serum-free conditions) or EVs partially stripped by ultracentrifugation or size-exclusion chromatography can be purposefully decorated with other functional corona coats.

annexin A2. The electrostatic interaction between LDL and proteoglycans is known to be mediated by the basic amino acids in apoB-100 clustered into a surface patch of the protein[11].

Although numerous proteins adsorb to the surface of EVs by nonspecific mechanisms to form the 'universal corona', some acquired EV corona components are specific ligands of EV surface receptors, such as the TNF receptor or integrin receptors[9]. A cell-specific association has been described between TGFβ and $CD63^+$ EVs derived from highly malignant osteosarcoma cells. EVs from these tumours carry membrane-associated TGFβ, which binds to mesenchymal stem cell (MSC) surface receptors and initiates signalling[12]. Importantly, the effect of EV-associated TGFβ was not observed in the presence of soluble TGFβ, indicating that it depends either on a particular conformation acquired by TGFβ on the EV surface or on the effects of other EV-associated molecules in addition to TGFβ[12].

Neutrophil-derived $CD63^+$ $CD66b^+$ EVs represent another example of a cell-specific, extracellularly created EV corona. During neutrophil degranulation, EVs associate with the secreted neutrophil elastase through electrostatic interaction, and the EV-bound enzyme becomes resistant to αl-antitrypsin. Upon transfer from human to mouse, these enzyme-decorated EVs recapitulate hallmarks of chronic obstructive pulmonary disease such as structural changes of the alveoli, increased airway resistance and ventricular hypertrophy. Of note, protamine sulfate dissociates the enzyme from the EV surface, rendering it susceptible to endogenous αl-antitrypsin[13].

Among the components of the plasma-induced corona, an unexpectedly extensive protein–protein interaction network has been identified using the STRING database[2]. This suggests that EVs trigger the formation of a network composed of many interactive proteins around the EV surface[2]. Interestingly, heparin or heparan-sulfate-binding proteins also form interactive protein–protein networks that share several components with the EV protein corona (including ApoB, ApoA1, ApoA2, ApoC3, ALB, ApoH and FGB)[10].

Nucleic acids in the corona

An emerging question is whether the EV corona comprises only proteins. A growing body of evidence suggests that it also contains DNA, RNA and lipids[4,14] and thus should be referred to as a biomolecular corona, rather than a protein corona. The release of DNA in association with EVs appears to be inducible by genotoxic stress[14].

A subpopulation of small EVs carry DNA fragments spanning all chromosomes and mitochondrial DNA, which are predominantly found in association with the surface of EVs[15]. DNA-binding proteins (such as histones and mitochondrial-DNA-binding proteins) are also detected in association with EV surface. A high-salt wash can remove DNA from the EV surface[14], suggesting that electrostatic

interactions — possibly between the EV membrane and basic histone proteins — are at work. Exosomes carrying DNA fragments may help maintain cellular homeostasis by removing harmful DNA from cells.

Although RNA is also reported to associate with the surface of EVs[4], the mechanism for this is not clear. Protein coronas do not typically contain RNA-binding proteins[2]. However, EV-associated lipoproteins may mediate RNA association with the surface of EVs[16].

Distinguishing corona components from the internal EV cargo

To determine whether a biological effect is due to EVs or to other components of the biological sample requires assessing a dose-dependent effect of the complete biological fluid, the fluid of the EV-depleted biological sample and the separated EVs[1]. The topology of a protein can be determined by digestions with proteinase K or trypsin and Lys-C, which can be combined with biotinylation of proteins. Identification of the intracellularly acquired 'innate corona' proteins can be based on their association with nascent EVs, their topology (surface association) and their lack of a transmembrane domain.

Although EV-surface-associated nucleic acids can be removed by DNase I or RNase digestion, the internal cargo molecules are protected from degradation by the vesicle membrane. The internal cargo of EVs can be made accessible with detergents (such as SDS or Triton X-100). Liposomes, non-EV nanoparticles (such as exomeres and supermeres) and protein aggregates can be used as additional controls. Glycosidases and sulfatases can be used to remove glycans and sulfate groups, respectively, from the EV surface, and high-salt washing can disrupt electrostatic interactions. EDTA reduces Ca^{2+}-dependent interactions, and Tween-20 can be applied to reduce protein–protein interactions. Reducing agents (such as β-mercaptoethanol) can be used to break disulfide bonds[8]. Furthermore, the shear force during high-speed centrifugation or ultracentrifugation strips off certain corona proteins[5] (soft corona), and thus EVs that are and are not subjected to ultracentrifugation can be compared. Detergent-resistant protein aggregates resemble EVs (especially those with protein aggregates in their corona) in size and proteomic composition. However, EVs are detergent sensitive and can thereby be distinguished from EV-mimicking detergent-resistant protein aggregates[2].

There is a need for rigorous, consensus protocols in the community to avoid misinterpretation of data — such as the attribution of a function to EV internal cargo as opposed to EV corona components. To address whether EVs also exist in a 'nude' form — that is, without a corona coating on the exofacial EV membrane — in biological systems, one has to consider that plasma-membrane-shed EVs (referred to as ectosomes) are in continuous contact with the extracellular milieu throughout their formation by budding. Therefore, they are probably always associated with corona proteins. EVs released from the endolysosomal system are referred to as exosomes[1]. They acquire a protein corona once they are released by the cells and also during their biogenesis in intracellular compartments. Such intracellular EV–protein associations occur inside multivesicular bodies (MVBs), for example in the case of ApoE[17] or fibronectin[6]. The mechanisms involved in the formation of the 'intracellular corona' or 'innate corona' may include protein–lipid or integrin–fibronectin interactions. For instance, fibronectin is endocytosed in association with integrins, targeted to MVBs and re-secreted on the surface of exosomes[6]. It can be hypothesized that the acidic pH within MVBs may induce conformational changes of proteins that might facilitate protein aggregation around the surface of the intraluminal vesicles. Besides the attachment of corona components to EVs in MVBs, intracellular decoration of EV membranes may occur during secretory autophagy. Upon fusion of autophagosomes with MVBs, amphisomes are formed in which autophagic material can mix with intraluminal vesicles and may be released on the surface of EVs[18].

Do EVs acquire the same corona in different biological fluids? Because urine contains only small amounts of protein, it is conceivable that the complexity of the protein corona of urine EVs may substantially differ from that of the corona formed around EVs in blood plasma. Proteomic analysis of EVs separated from cerebrospinal fluid (CSF) reveals numerous proteins also identified in the plasma-induced corona of EVs (such as ApoE, ApoA1, fibrinogen, fibronectin, C3 and C4). This suggests that in the CSF, a protein corona may form around EVs with a composition that at least partially overlaps with the corona of EVs in blood plasma[19]. Although we still have limited information on the impact of EV type on the corona, differences have been detected between the coronas formed around EVs derived from the human monocytic THP1 cell line and human platelet EVs[2]. Considering the known variation in the surface glycosylation of EVs of different sources, the type and extent of EV surface glycosylation may impact the zeta potential (the potential difference between the surface of EVs in a conducting liquid and the bulk of the liquid) and the surface interactome of EVs.

Biological significance of corona formation

There is an increasing recognition that proteins interacting with the surface of various nanoparticles either facilitate the cellular uptake of the nanoparticles, acting as opsonins (for example, LDL, IgG and C3b), or decrease the uptake by acting as dysopsonins (for example, albumin, ApoA4, ApoC3 and clusterin)[20]. Important evidence for the functional significance of EV-associated corona proteins comes from a study in which plasma EVs from naive mice were transferred into mice with active experimental allergic encephalomyelitis (EAE). These EVs induced a spontaneous relapsing–remitting $CD8^+$-T-cell-mediated EAE only if fibrinogen was associated with EVs[21]. Another study found that removing the EV corona via size-exclusion chromatography or ultracentrifugation of EVs abolished their functional effects in angiogenesis, skin regeneration and immunomodulation; generating a new corona with a cocktail of growth factors restored the functional effect of EVs[5]. Another interesting study reported that EVs released by tumour cells of brain metastases (but not by primary tumour cells) bound to LDL and induced EV–LDL aggregation; EV–LDL aggregation then facilitated the uptake of EVs by monocytes, cells that have a key role in the brain metastatic niche[22].

An important example of the biological significance of an intracellularly acquired corona involves fibroblast-derived exosomes. Once these EVs are internalized by breast cancer cells, they acquire surface-associated WNT11 and induce autocrine Wnt–planar cell polarity signalling in the cells, facilitating their invasive behaviour[23].

Lastly, α-synuclein associates with vesicles both intracellularly and extracellularly, with secreted EVs. The α-synuclein–EV interaction facilitates α-synuclein misfolding and results in the formation of large fibrils, suggesting that this EV surface interaction may have a critical role in synucleinopathy disorders[24]. These recent studies offer a strong indication that the exofacial surface of EVs may play important cell biological roles that require further dissection and may be relevant to our understanding of the roles of EVs in disease and therapy.

Outlook

Can the information about the potentially exchangeable corona coat of EVs be

exploited for therapeutic applications? It is feasible to decorate nascent EVs[2] or stripped EVs[5] with a 'coat' of proteins. In a recent study, synthetic EV mimetics deocrated with angiopep-2 peptide were found to bind to the lipoprotein-receptor-related protein 1 (LRP1) receptor and improve blood–brain barrier penetration ability. The presence of angiopep-2 on the surface of EV-mimetic nanoparticles led to a reduction in serum protein corona formation[25]. Purposefully decorating EVs with growth factors, cytokines or enzymes, or engineering and loading them with various drugs, may endow EVs with unique therapeutic potential.

In conclusion, accumulating data point to the formation of a complex and dynamic biocorona around EVs. Although the presence of this corona adds a layer of complexity to the EV structure, it also opens up opportunities for EV tailoring and targeting (Fig. 1). ❐

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Competing interests

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