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REVIEW ARTICLE

The functional role of soluble proteins acquired by extracellular vesicles

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Abstract

Extracellular vesicles (EVs) are lipid bilayer-enclosed nanosized particles released by all cell types during physiological as well as pathophysiological processes to carry out diverse biological functions, including acting as sources of cellular dumping, signalosomes and mineralisation nanoreactors. The ability of EVs to perform specific biological functions is due to their biochemical machinery. Among the components of the EVs' biochemical machinery, surface proteins are of critical functional significance as they mediate the interactions of EVs with components of the extracellular milieu, the extracellular matrix and neighbouring cells. Surface proteins are thought to be native, that is, pre-assembled on the EVs' surface by the parent cells before the vesicles are released. However, numerous pieces of evidence have suggested that soluble proteins are acquired by the EVs' surface from the extracellular milieu and further modulate the biological functions of EVs during innate and adaptive immune responses, autoimmune disorders, complement activation, coagulation, viral infection and biomineralisation. Herein, we will describe the methods currently used to identify the EVs' surface proteins and discuss recent knowledge on the functional relevance of the soluble proteins acquired by EVs.

KEYWORDS
extracellular milieu, extracellular vesicles, matrix vesicles, soluble proteins, surface proteins

1 | INTRODUCTION

Extracellular vesicles (EVs) are lipid bilayer-enclosed nanosized particles that are released by all types of cells in the extracellular milieu during both physiologic and pathologic processes, and cannot replicate, that is, are devoid of a functional nucleus (EL Andaloussi et al., 2013). EVs have been commonly divided into three main classes according to their biogenesis mechanism as follows: (a) exosomes originating by an endo-lysosomal pathway starting from early endosome, late endosome and continuing through the inward budding of multivesicular bodies and their fusion with the cell membrane; (b) microvesicles, often referred to as microparticles, originating by the outward budding of the cell membrane; and (c) apoptotic bodies originating by the outward blebbing of apoptotic cell membranes (EL Andaloussi et al., 2013). However, assigning an EV to a specific biogenesis mechanism is difficult unless the release of the vesicle is followed through live imaging approaches. Additionally, referring to membrane-derived EVs as 'microparticles' may create confusion since the term is commonly used in the field of material science to describe synthetic (e.g., metallic, polymeric, lipid-based) particles with a diametre higher than 1 μm. In 2014, the International Society for Extracellular Vesicles (ISEV) proposed the Minimal Information for Studies of Extracellular Vesicles (MISEV2014) guidelines to suggest the 'minimal experimental requirements for definition of EVs and their functions' (Lötvall et al., 2014). The MISEV

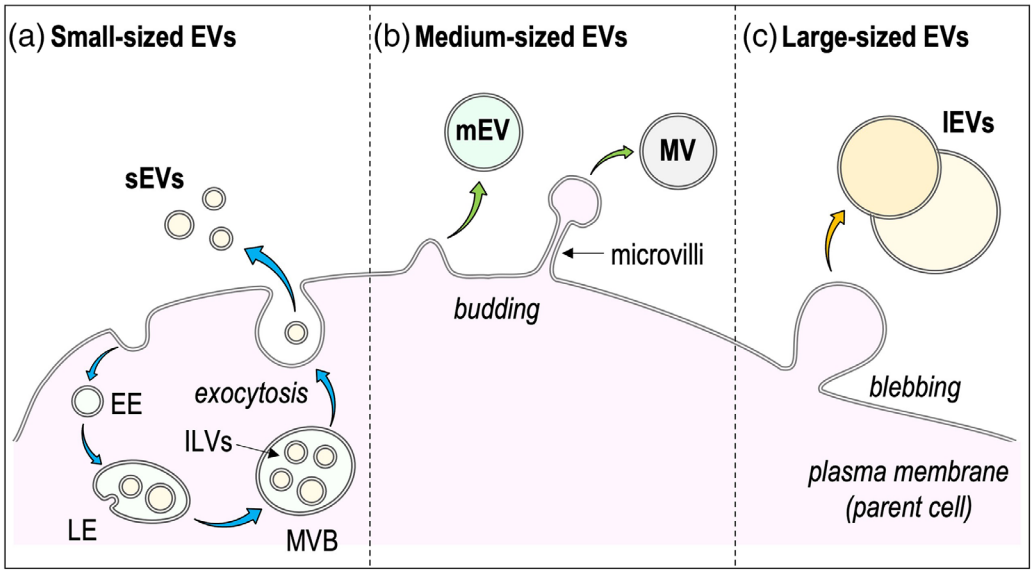

FIGURE 1 Classes of extracellular vesicles. Extracellular vesicles have been commonly divided into three main classes based on their biogenesis mechanism. Exosomes (small-sized EVs, 30–150 nm diametre) are released through an endolysosomal pathway ending with the exocytosis of multivesicular bodies generated by internal budding of late endosomes and formation of intraluminal vesicles (a). Microvesicles (medium-sized EVs, from 150 nm to 1 μm) are released through the outward budding of the parent cells' plasma membrane. Matrix vesicles (100–300 nm diametre) are a special class of medium-sized EVs released from the apical side of microvilli-like membranes of mineralization-competent cells (b). Apoptotic bodies (large-sized EVs, up to 5 μm) are released thought the outward blebbing of apoptotic membranes (c). In this review, we will use the designations small-sized, medium-size and large-sized extracellular vesicles instead of, respectively, exosomes, microvesicle and apoptotic bodies

Abbreviations: AB, apoptotic body; EE, early endosome; EV, extracellular vesicles; ILV, intraluminal vesicle; LE, late endosome; MV, matrix vesicle; MVB, multivesicular body; lEV, large-sized extracellular vesicle; mEV, medium-sized extracellular vesicle; sEV, small-sized extracellular vesicle.

was updated in 2018 (MISEV2018) (Théry et al., 2018). Regarding the EV nomenclature, the MISEV suggested the authors to classify the EVs according to operational terms such as a physical property [e.g., size ('small-sized EVs', 'medium-sized EVs' and 'large-sized EVs') or density ('low-density EVs', 'middle-density EVs' and 'high-density EVs')], the biochemical composition (e.g., $CD63^+$ EVs, $CD9^+$ (EVs), or the description of biological processes or parent cells (e.g., tolerosomes, oncosomes, apoptotic bodies). Herein, we will follow the nomenclature suggested by the MISEV2018 and use the designations small-sized (<150 nm), medium-sized (>150 nm and <1μm) and large-sized (>1μm) EVs to refer to the exosomes, microvesicles (microparticles) and apoptotic bodies, respectively, described in the reviewed publications (Figure 1). However, we will keep the designation 'matrix vesicles' to refer to the sub-class of medium-sized EVs bound to collagen fibrils and contributing to the mineralisation of the extracellular matrix by producing apatitic mineral (see section 3.6).

EVs are released by cells to carry out diverse functions (EL Andaloussi et al., 2013). For instance, EVs are exploited by cells to remove unnecessary or unwanted molecules from the cytosol and the plasma membrane (EL Andaloussi et al., 2013). EVs also act as signalosomes, by either interacting directly with cell surface receptors or delivering information cargoes (proteins, nucleic acids and lipids) into target cells, to participate in various processes, including cell–cell communication, cell maintenance, immune responses and tumour progression (EL Andaloussi et al., 2013). EVs do not act only as sources of cellular dumping or signalosomes. For example, matrix vesicles are a sub-class of medium-sized EVs released by outward budding from the apical microvilli of mineralisation-competent cells, including hypertrophic chondrocytes, osteoblasts and odontoblasts, to initiate bone and dentin calcification by forming apatite minerals in their lumen (Bottini et al., 2018). Matrix vesicles are not the only class of mineralizing EVs released by cells during calcification processes (Bottini et al., 2018). For instance, studies have suggested that vascular smooth muscle cells and M1 macrophages contribute to vascular calcifications by releasing mineralizing EVs at the sites of medial vascular calcification and in the plaques' microenvironment, respectively (Bottini et al., 2018).

EVs harbour the complete biochemical machinery needed to perform their biological functions (EL Andaloussi et al., 2013). Among the constituents, surface proteins are of critical functional significance as they mediate the interactions of EVs with components of the extracellular milieu, the extracellular matrix and neighbouring cells (Buzás et al., 2018). Cells are thought to release fully active EVs equipped with the complete set of functional surface proteins (*native* surface proteins). However, numerous pieces of evidence have suggested that cells also release 'precursor', rather than fully active, EVs whose functions are further modulated by the adsorption of soluble proteins on the vesicles' surface in the extracellular milieu (*acquired* surface proteins) (Buzás et al., 2018). Here, we will describe the methods currently used to identify the EVs' surface proteins. Next, we review current knowledge of the functional relevance of the EVs' acquired surface proteins in biologic processes, including innate and adaptive immune responses, autoimmune disorders, complement activation, coagulation, viral infection and biomineralisation.

2 | METHODS TO IDENTIFY THE EVS' SURFACE PROTEINS

EVs can be isolated based on their size or density by means of ultracentrifugation steps, with or without a gradient of sucrose, ficoll or iodixanol. EVs with a diametre smaller (or bigger) than a certain value can be isolated by size exclusion chromatography or ultrafiltration [e.g., direct flow filtration (DFF) or tangential flow filtration (TFF)]. Alternatively, EVs displaying a specific biomacromolecule (protein or lipid) can be purified by affinity capture methods based on beads functionalised with antibodies against the biomacromolecule of interest (we suggest the reader the ref. (Konoshenko et al., 2018) for a comprehensive description of EV isolation techniques). In general, the method used to isolate the EVs shall follow the MISEV2018 guidelines and be clearly delineated to enable the transfer of protocols and standardisation of results among laboratories (Théry et al., 2018; Torres Crigna et al., 2021). Quantitative differences in EV yield may be related to equipment- and operator-dependent technical variability in the EV isolation (Torres Crigna et al., 2021).

After isolation, mass spectrometry and western blotting are commonly used to characterise the proteomic profile of the vesicles (Purushothaman et al., 2016); however, a limitation of these methods is the difficulty to assess the topology of the EVs' surface proteins, which is important for function. The MISEV2018 guidelines suggest determining the topology of putative functional proteins 'by performing digestions, permeabilisation, or antibody studies' (Théry et al., 2018). Digestion and permeabilisation steps followed by an appropriate analytical method (mass spectrometry, western blotting, polymerase chain reaction, etc.) have enabled to assess if the proteins bound to the EVs' membrane have a 'conventional' or 'reversed' topology compared to what it would be expected by considering the biogenesis process. For instance, Cvjetkovic et al. isolated EVs from human mast cell line HMC-1 cells and treated the vesicles either with proteinase K or with trypsin and endoproteinase Lys/C followed by biotin tagging (Cvjetkovic et al., 2016). By analyzing nontreated, proteinase K-treated and biotinylated samples with liquid chromatography-tandem mass spectrometry and western blotting, the authors assessed the topology of several EV membrane proteins and found that four proteins (secretory carrier-associated membrane protein 3, solute carrier family 2-facilitated glucose transporter member 3, leukosialin and solute carrier family 12 member 6) have an inside-out topology. The authors hypothesised that the presence of 'inside-out proteins' could be a consequence of changes in the lipid composition of the vesicle's membrane during the biogenesis process. Another possible explanation could be that the digestion with proteases compromised the stability of some membrane proteins in such a way to induce a change in their orientation.

Alternatively, the topology of proteins on the EVs' membrane can be identified by labelling the vesicles' surface with probes (e.g., fluorescent, or nanoparticle-labelled antibodies) to tag the proteins of interest and identifying them by spectroscopic and microscopic methods (Hartjes et al., 2019). A detailed description of these methods will be the topic of the next two sections.

2.1 | Spectroscopic methods

Fluorescence-activated cell sorting (FACS) is one of the most used spectroscopic techniques to assess the EVs' molecular fingerprinting (Campos-Silva et al., 2019; Cloutier et al., 2013; Zecher et al., 2014). Cloutier et al. identified and quantified the complexes of platelet-derived medium-sized EVs with immunoglobulins in the synovial fluid of patients with rheumatoid arthritis by labelling the complexes with antibodies against CD41 (integrin α2b, a platelet marker) and immunoglobulin G (IgG) and measuring the percentage of complexes positive for both CD41 and immunoglobulin G ($CD41^+$ IgG^+) by FACS (Cloutier et al., 2013). FACS has also been used to assess the binding of soluble proteins to EVs. For instance, by incubating the samples with an anti-iC3b antibody and using FACS, Zecher et al. showed that erythrocyte-derived medium-sized EVs became cloaked by C3 fragments after incubation with plasma (Zecher et al., 2014). Of note, the resolution power of FACS is ~300 nm, thus it can detect medium-sized and large-sized EVs but not small-sized vesicles. The detection of small-sized EVs by FACS requires the use of microbeads. For example, Campos-Silva et al. identified tetraspanins (CD9, CD63 and CD81, markers of small-EVs released through an endolysosomal pathway) on the surface of urine small-sized EVs by immune-capturing $CD3^+$ or $CD63^+$ vesicles on antibody-modified magnetic fluorescent microbeads and analysing the captured vesicles by using fluorescent-labelled anti-tetraspanin antibodies (Campos-Silva et al., 2019). Along with FACS, nanoparticle tracking analysis in fluorescence mode (NTA-FM) has been also used to identify and quantitate sub-populations of EVs. For instance, Desgeorges et al. used NTA-FM to count and phenotype the sub-populations of EVs released from human umbilical cord-derived mesenchymal stromal cells (Desgeorges et al., 2020). They found that tetraspanins were strongly exposed on the EVs' surface. The EVs were also positive for additional markers, including CD29, CD44 and CD49e, and devoid of platelet-specific markers (CD41, CD42, CD62p) (Desgeorges et al., 2020).

Both FACS and NTA-FM have been exploited to assess the molecular fingerprinting (native or acquired) of EVs in solution (Torres Crigna et al., 2021). Alternatively, EVs are captured by biomolecules immobilised on the surface of a sensor and the event detected by the changes in the physical properties of a signal interacting with the sensor (Han et al., 2021; Németh et al., 2017; Rupert et al., 2016; Suthar et al., 2020). By exploiting this approach, the expression of tetraspanins on EVs has been detected by using both a surface plasmon resonance (SPR) system and a quartz crystal microbalance with dissipation monitoring (QCM-D)

equipped with anti-tetraspanin antibody-coated sensors (Rupert et al., 2016; Suthar et al., 2020). Similarly, how exofacial DNA affects the binding of fibronectin to small-sized EVs released by ciprofloxacin-treated Jurkat cells has been assessed by means of a resonant waveguide grating-based label-free optical biosensor coated with fibronectin (Németh et al., 2017). In a slightly different approach, biotinylated EVs were captured o an avidin-modified glass cover, tagged with fluorescent-labelled antibodies, and imaged by means of a total internal reflection fluorescence (TIRF) microscope equipped with multiple excitation lasers and a multi-channel simultaneous-imaging system (Han et al., 2021). This approach enabled the characterisation of the molecular fingerprinting of EVs from different cell lines (HEK293, MCF-7 and B16BL6) as well as EVs isolated by three frequently used methods (density gradient, buoyant density gradient and size exclusion chromatography) by tetraspanin co-localisation (Han et al., 2021). While the described approaches (i.e., FACS, NTA-FM, SPR, QCM-D and TIRF) enable the molecular fingerprinting of particles, they might not distinguish between EVs and other types of particles (e.g., protein aggregates), which may remain after isolation, thus data analysis might be ambiguous. A possible solution to this issue would be to use microscopic methods, including super-resolution optical techniques, transmission electron microscopy (TEM), cryo-TEM and/or atomic force microscopy (AFM), in addition to spectroscopic ones, to assess the purity, morphology and integrity of the isolated EVs.

2.2 | Microscopic methods

Fluorescence microscopy has been traditionally used to localise proteins on the surface of EVs. However, conventional optical microscopy has a resolution of several hundreds of nanometres, which hampers the accurate molecular fingerprinting of individual EVs. Recently, a series of novel super-resolution optical techniques with lateral imaging resolutions smaller than 50 nm, including high-resolution confocal microscopy, photoactivated localisation microscopy (PALM), and stochastic optical reconstruction microscopy (STORM), have been developed and enabled the visualisation of ultrasmall biological structures by tagging the vesicles with fluorophore-labelled antibodies (Chen et al., 2016; Treps et al., 2017). For instance, Treps et al. used a super-resolution confocal microscope to image $CD63^+$ EVs released from patient-derived glioblastoma stem-like cells (Treps et al., 2017). They found that the vesicles were formed by both small-sized and medium-sized EVs, and both the subpopulations expressed the vascular endothelial growth factor-A (VEGF-A). Recently, Chen et al. used PALM/STORM to image CD63 and human epidermal growth factor receptor-2 (HER2) on individual small-sized EVs released by human breast cancer cells (SKBR3 cells), thus demonstrating that these techniques can be exploited to achieve simultaneous super-resolution imaging of multiple proteins on EVs with sizes below the optical diffraction limit (Chen et al., 2016).

TEM has been used to assess the presence of proteins on EVs by tagging the vesicles with nanoparticle-labelled antibodies. By using this approach, Romancino et al. identified ALG-2 interacting protein X [Alix, a conventional marker of small-sized EVs (Hoshino et al., 2020)] on EVs released from 3D cultures of C2C12 myoblast cells (Romancino et al., 2013), while Radons et al. identified heat shock protein 70 [Hsp70, abundantly expressed by cancer cells, (Chanteloup et al., 2020)] on EVs released from tumour cells (Radons & Multhoff, 2005). However, the process of dehydration, embedding and labelling used to prepare TEM samples can lead to structural artefacts. These limitations can be bypassed by using cryo-TEM and AFM. In cryo-TEM, the sample is directly visualised in a frozen-hydrated state, which enables preservation of the sample's native morphology. By means of cryo-TEM and tagging the EVs with annexin A5 conjugated to 4 nm-in-diametre gold nanoparticles, Coultier et al. identified the presence of phosphatidylserine on the membrane's outer leaflet of EVs isolated from the synovial fluid from patients with rheumatoid arthritis, whereas the presence of immunoglobulins on the EVs' surface was determined using larger gold nanoparticles (10 nm) conjugated to protein A (Cloutier et al., 2013). The identification of phosphatidylserine on the surface biological membranes has been implicated in several biologic processes. Phosphatidylserine externalisation from the inner to the outer membrane leaflet has been described to trigger the immune-suppressive actions of cells and small-sized EVs as well as to trigger the uptake of EVs by cells (Vaupel & Multhoff, 2018). Additionally, an increased blood level of small-sized EVs with surface exposed phosphatidylserine is a specific indicator of cancer and may serve as an earlier marker of malignancy (Sharma et al., 2017). Finally, phosphatidylserine exposed on the surface of small-sized EVs released from vascular smooth muscle cells (Kapustin et al., 2011) and of small-sized EVs released from M1 macrophages (New et al., 2013) have been described to promote pathologic calcification by forming complexes with annexins.

AFM belongs to the family of scanning probes microscopes, where morphological as well as physical and chemical properties of the samples can be acquired by the interaction of the microscope's probe with the sample (Dufrêne et al., 2017). Since it can work in liquid, AFM has also been used to assess the biochemical and biophysical properties of EVs in physiological conditions (Bairamukov et al., 2020; Plaut et al., 2019). In AFM-based single molecule force spectroscopy (AFM-SMFS) analyses, the AFM probe's tip is functionalised with antibodies and its interactions with EVs immobilised on a surface are recorded by the detector and analysed to assess the vesicles' molecular fingerprinting (Dufrêne et al., 2017). By using AFM probes functionalised with anti-CD63 antibodies, Sharma et al. showed that EVs from the saliva of oral cancer patients exhibited significantly increased CD63 surface densities than EVs from healthy volunteers (Sharma et al., 2011). Recently, Paul et al. has assessed the presence of CD9 and hyaluronan on colon cancer HCT 116 and normal colon epithelial CCD-18CO cell-derived EVs by using AFM-SMFS (Paul et al., 2020). The authors found that, while the expression of CD9 was slightly greater on colon cancer EVs than normal

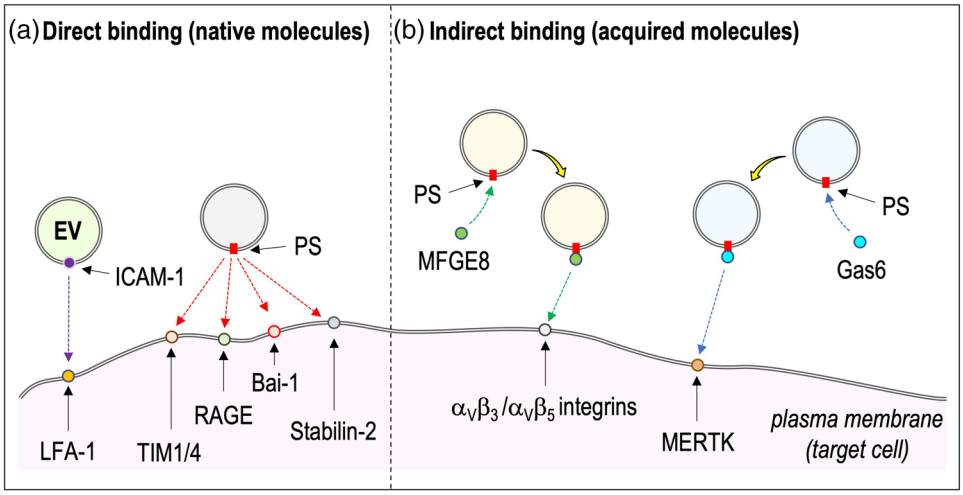

FIGURE 2 Binding of extracellular vesicles to target cells in immune response. Extracellular vesicles contribute to immune response by binding to target cells exploiting both native surface biomolecules (direct binding) (a) and acquired surface biomolecules (indirect binding) (b) (Graham et al., 2014; Hanayama et al., 2002; He et al., 2011; Kobayashi et al., 2007; Park et al., 2007; Park et al., 2008; Segura et al., 2005)
Abbreviations: Bai-1, brain-specific angiogenesis inhibitor 1; EV, extracellular vesicle; Gas6, growth arrest-specific protein 6; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function associated antigen 1; MERTK, MER proto-oncogene tyrosine kinase; MFGE8, milk fat globule epidermal growth factor factor 8; PS, phosphatidylserine; RAGE, receptor for advanced glycation end products; TIM1/4, T-cell immunoglobulin mucin 1/4.

EVs, the expression of hyaluronan, a non-sulphated glycosaminoglycan that has been linked to malignant tumour growth (Rilla et al., 2014), was significantly greater on cancer EVs than normal vesicles.

3 | ROLE OF THE SOLUBLE PROTEINS ACQUIRED BY EVS IN BIOLOGIC PROCESSES

3.1 | Innate and adaptive immune response

EVs from antigen presenting cells (APCs), such as B cells and dendritic cells, are thought to have crucial roles in immune response due to their peculiar surface composition (Robbins & Morelli, 2014). Segura et al. found that major histocompatibility complex (MHC) class II, co-stimulatory molecule B7.2/CD86, and intercellular adhesion molecule 1 (ICAM-1)/CD54 were strongly increased in small-sized EVs from lipopolysaccharide-treated (mature) dendritic cells as compared with the plasma membrane of mature cells and small-sized EVs from immature cells (Segura et al., 2005). Mature small-sized EVs had the capacity to stimulate T cells more efficiently than immature small-sized EVs. This phenomenon could be due to the higher expression of ICAM-1 on mature small-sized EVs, which increases their binding of the vesicles to lymphocyte function associated antigen 1 (LFA-1/CD11a) on APCs and ability to present the antigen-MHC complexes to T cells (Figure 2a) (Montecalvo et al., 2008; Morelli et al., 2004; Utsugi-Kobukai et al., 2003).

Studies have suggested that other biomolecules, along with ICAM-1, could mediate the binding of small-sized EVs to APCs (Morelli et al., 2004). Hanayama et al. showed that secreted glycoprotein milk fat globule epidermal growth factor 8 (MFGE8; also known as lactadherin) can bind to phosphatidylserine on lipid membranes via its two epidermal growth factor domains and act as a bridge between phosphatidylserine-rich lipid membranes and APCs by binding to cell $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins via its RGD domain (Figure 2b) (Hanayama et al., 2002). This suggests that MFGE8 could bind to secreted small-sized EVs and mediate their function in immune response; however, a validation of MFGE8 binding to small-sized EVs in conditioned media as well as in biological fluids is still missing. Of note, Oshima et al. described that MFGE8 is also secreted as MFGE8-EV complexes and induction of MFGE8 expression in mammary epithelial cell line COMMA-1D increased the secretion of the complexes, suggesting its possible role in EV biogenesis (Oshima et al., 2002).

Two independent studies from Segura et al. and Véron et al. showed that EVs released from MFGE8-deficient dendritic cells can still present their antigen–MHC complexes to immune cells, thus suggesting a redundancy in the mechanisms through which EVs bind to target cells (Segura et al., 2005; Véron et al., 2005). Along with ICAM-1, phosphatidylserine on the outer leaflet of the EVs' membrane can mediate vesicle binding to immune cells' receptors both directly and indirectly (Robbins & Morelli, 2014) (Figure 2). Direct EV binding involves the recognition of phosphatidylserine by T-cell immunoglobulin mucin 1 (TIM1) and 4 (TIM4) (Kobayashi et al., 2007), receptor for advanced glycation end products (RAGE) (He et al., 2011), brain-specific angiogenesis inhibitor 1 (Bai-1) (Park et al., 2007) and stabilin-2 (Park et al., 2008) on the target cells' plasma membrane

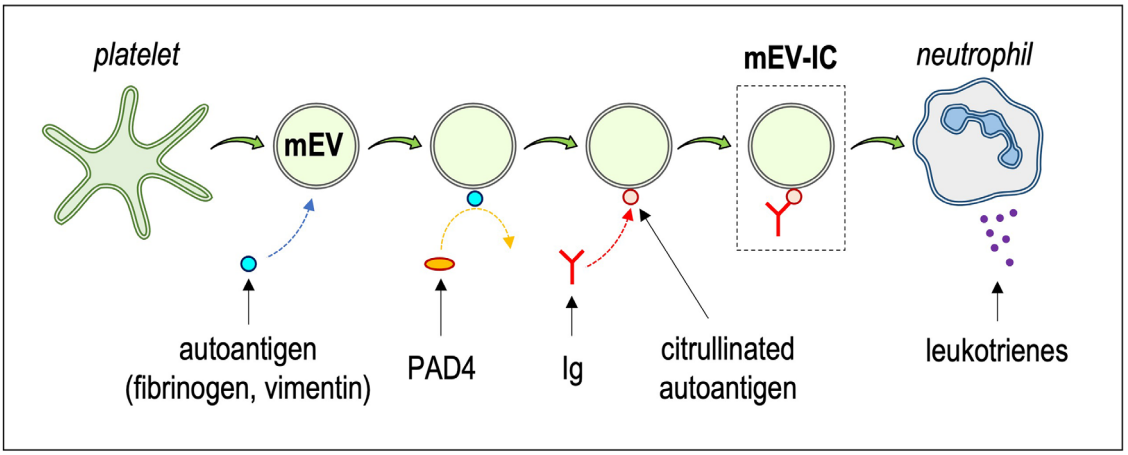

FIGURE 3 The functional role of soluble proteins acquired by extracellular vesicles in rheumatoid arthritis. Medium-sized extracellular vesicles are released by platelets in the synovial cavity of inflamed articulations and are cloaked by autoantigens that, following citrullination by peptidyl arginine deiminase 4, contribute to the formation of pro-inflammatory medium-sized extracellular vesicle-containing immune complexes (Cloutier et al., 2013) Abbreviations: Ig, immunoglobulin; mEV, medium-sized extracellular vesicle; mEV-IC, medium-sized extracellular vesicle-containing immune complex; PAD4, peptidyl arginine deiminase 4.

(Figure 2a). Regarding the mechanisms of indirect binding of EVs to immune cells, along with MFGE8, growth arrest-specific protein 6 (Gas6) could act as a bridge between phosphatidylserine on large-sized EVs and MER proto-oncogene tyrosine kinase (MERTK) of the TAM family of receptor tyrosine kinases (RTKs) on macrophages, inducing the engulfment of large-sized EVs and subsequent anti-inflammatory macrophage polarisation (Figure 2b) (Graham et al., 2014).

3.2 | Autoimmune disorders

EVs may have a role in mediating inflammatory autoimmune disorders with a significant type III hypersensitivity component, including rheumatoid arthritis and systemic lupus erythematous, by contributing to the formation of antigen-immunoglobulin complexes (immune complexes) (Buzas et al., 2014; Mazzariol et al., 2021). Cloutier et al. assessed the role of medium-sized EVs in the formation of immune complexes in rheumatoid arthritis, by using EVs isolated from the synovial fluid from patients with rheumatoid arthritis and EVs released by cultured platelets (Cloutier et al., 2013). They found that platelet-derived ($CD41^+$) medium-sized EVs from the synovial fluid of patients with rheumatoid arthritis contained autoantigens, including vimentin and fibrinogen, and form immune complexes (Cloutier et al., 2013). *In vitro* tests showed that fibrinogen was able to adsorb onto platelet-derived medium-sized EVs, which suggested that autoantigens are not only pre-assembled onto the platelets' surface before the medium-sized EVs are released but they can also adsorb onto released vesicles (Cloutier et al., 2013). The authors also found that medium-sized EVs in the synovial fluid from patients with rheumatoid arthritis contained citrullinated epitopes (Cloutier et al., 2013). In vitro tests showed that peptidyl arginine deiminase 4 (PAD4) carried out the citrullination of autoantigens on the medium-sized EVs' surface, and citrullinated neoepitopes were recognised by immunoglobulins, leading to the formation of medium-sized EV-containing immune complexes (Cloutier et al., 2013). Finally, the authors found that medium-sized EV-containing immune complexes isolated from the synovial fluid of patients with rheumatoid arthritis stimulated neutrophils to produce leukotrienes (Cloutier et al., 2013). Taken together, these results suggested the model that medium-sized EVs released by platelets in the synovial cavity of inflamed articulations can undergo a multi-step surface modification – adsorption of autoantigens, autoantigen citrullination by PAD4 and deposition of immunoglobulins against citrullinated autoantigens – and support the formation of pathological immune complexes (Figure 3).

Soluble proteins also adsorb onto medium-sized EVs in the bloodstream. Adsorption of soluble proteins on THP-1 cell-derived medium-sized EVs incubated with the blood plasma from healthy individuals and from patients with rheumatoid arthritis has been recently described by Tóth et al. (Tóth et al., 2021). The authors found slight differences in the two sets of adsorbed proteins. Interestingly, they found that among the proteins common to the two sets of adsorbed proteins, nine proteins were opsonins (apolipoproteins, complement and clotting factors and immunoglobulins) that have been found to adsorb onto the surface of other types of synthetic (e.g., liposomes and carbon nanotubes) and natural (e.g., viruses) nanoparticles in the bloodstream (Brückner et al., 2020; Di Santo et al., 2020; Papafilippou et al., 2020; Sacchetti et al., 2013; Tóth et al., 2021). The adsorption of soluble proteins onto synthetic nanoparticles entering the bloodstream has been described to drive their biologic functions (Farshbaf et al., 2022). A similar paradigm has been recently hypothesised to be also valid for the viruses (Gao et al., 2021).

However, the results of Tóth et al. did not show a significant correlation between the set of adsorbed proteins and the effects of the vesicles on monocyte-derived dendritic cells.

The plasma level of immunoglobulin-coated medium-sized EVs is higher in patients with systemic lupus erythematous than in healthy individuals, supporting the model that medium-sized EVs also contribute to the development of the disease by forming pathological immune complexes (Nielsen et al., 2012). Fortin et al. found an association between the cellular origin of medium-sized EV-containing immune complexes and the aetiology and clinical progression of systemic lupus erythematous (Fortin et al., 2016). To identify distinct populations of EVs, specific fluorescence markers were employed (Fortin et al., 2016). An annexin A5-conjugated fluorescence probe was used to identify EVs with exposed phosphatidylserine (annexin $A5^+$ EVs) or unexposed phosphatidylserine EVs (annexin $A5^-$ EVs). A phycoerythrin-conjugated anti-CD41 was used to reveal the presence of CD41 in EVs ($CD41^+$ EVs, which can derive from platelets) or its absence ($CD41^-$EVs, which can derive from non-platelets) (Fortin et al., 2016). Immunoglobulin G complexes harboured in annexin $A5^+$ $CD41^-$ EVs and the presence of such complexes correlated with arterial thickening of the intima-media (Fortin et al., 2016). This result suggested that cell lineages different from platelets can release medium-sized EVs that undergo surface modification by immunoglobulin cloaking and get involved in the progression of systemic lupus erythematous. These cell lineages might include endothelial cells since they are $CD41^-$ and have been described to release medium-sized EVs in patients with systemic lupus erythematous (Parker et al., 2014). However, a validation of the adsorption of autoantigens and/or immunoglobulins on medium-sized EVs in the synovial fluid of patients with rheumatoid arthritis as well as in the plasma of patients with systemic lupus erythematous is still missing. It is worth noting that soluble antigen-immunoglobulin complexes overlap in biophysical properties (lateral size, sedimentation and light scattering) with medium-sized EVs, which may affect the quantification of medium-sized EV-containing immune complexes by flow cytometry as well as their isolation by differential centrifugation (György et al., 2011). Thus, the quantitation and isolation of medium-sized EV-containing immune complexes from the biological milieu should be accompanied by microscopic analyses to validate the nature of the complexes.

Autoantigens and immunoglobulins are not the solely soluble proteins that associate with EVs during the development of autoimmune diseases (Buzas et al., 2014; Cloutier et al., 2013; Fortin et al., 2016; Nielsen et al., 2012). Peroxiredoxin 1 (Prdx 1) has a role in inflammation and autoimmune processes (Szabó et al., 2009). It is present in the blood plasma both as a soluble protein and associated with EVs (Mullen et al., 2015). Recently, Szabó-Taylor et al. found that monocytes from patients with rheumatoid arthritis displayed increased surface thiol levels (Szabó-Taylor et al., 2017). They also found that the concentration of $Prdx1^+$ EVs was higher in individuals with rheumatoid arthritis than in healthy ones. Enzymatically inactive, overoxidised Prdx1 was detected in EVs from both patients with rheumatoid arthritis and healthy subjects. These results suggested that monocytes released $Prdx1^+$ EVs to remove oxidised molecules from plasma membrane. Alternatively, EVs containing Prdx1 either derived from parent cells or adsorbed in the extracellular milieu, may act as a danger signal. Further experiments are warranted to validate the adsorption of Prdx1 on EVs and the biological role of $Prdx1^+$ EVs in rheumatoid arthritis.

3.3 | Complement activation

During the development of local and systemic inflammation, both the concentration of EVs and complement activation increase (Curry et al., 2014; Dunkelberger & Song, 2010). The co-presence of EVs and products of the complement activation supports the model of a crosstalk between the EV and complement systems. It was reported that C1q and C3 (essential proteins of the immune system) bind to EVs, followed by activation of complement with binding of complement fragments and, further downstream, formation of the membrane attack complex on the EVs' surface (Stein & Luzio, 1991). The deposition of complement fragments on EVs can be mediated by three possible mechanisms. In one mechanism, native surface moieties (e.g., membrane lipids and surface proteins) mediate the binding of complement fragments to EVs. For instance, it has been described that C1q electrostatically binds to anionic liposomes through the highly cationic $C1qA_{14\text{-}26}$ region, thus anionic membrane lipids might also drive C1q adsorption on EVs (Figure 4a) (Bradley et al., 1999). Although speculative, C1q could also bind to the EVs' surface proteins displaying negative charges (Németh et al., 2017). In a second mechanism, complement fragments bind to the EVs' surface after it undergoes modifications through the adsorption of soluble proteins. For instance, small-sized EVs released by APCs through a pathway involving antigen-processing endolysosomal compartments are likely to contain antigens (Figure 4b) (Clayton et al., 2003). The presence of surface antigens might trigger the binding of immunoglobulins and, in turn, the binding of C1q in the extracellular milieu, initiating the complement classical pathway (Dunkelberger & Song, 2010). The activation of the complement system on EVs can also be driven by a more complex process involving the conformational change of EV surface proteins (native or soluble), as it has been recently described for C-reactive protein (CRP) on monocyte-derived medium-sized EVs (Braig et al., 2017).

Pentameric CRP (pCRP) is synthesised by the liver in response to tissue injury and inflammation (Volanakis, 2001). Upon binding to lipid membranes, pCRP can undergo conformational changes, which lead to the generation of neoepitope-expressing (pentameric) CRP (pCRP*). pCRP* can either bind with C1q (as well as other interacting proteins) and activate the complement cascade or dissociate into its neoepitope-expressing monomeric sub-units (mCRP) and trigger clearance by cell uptake (Agrawal

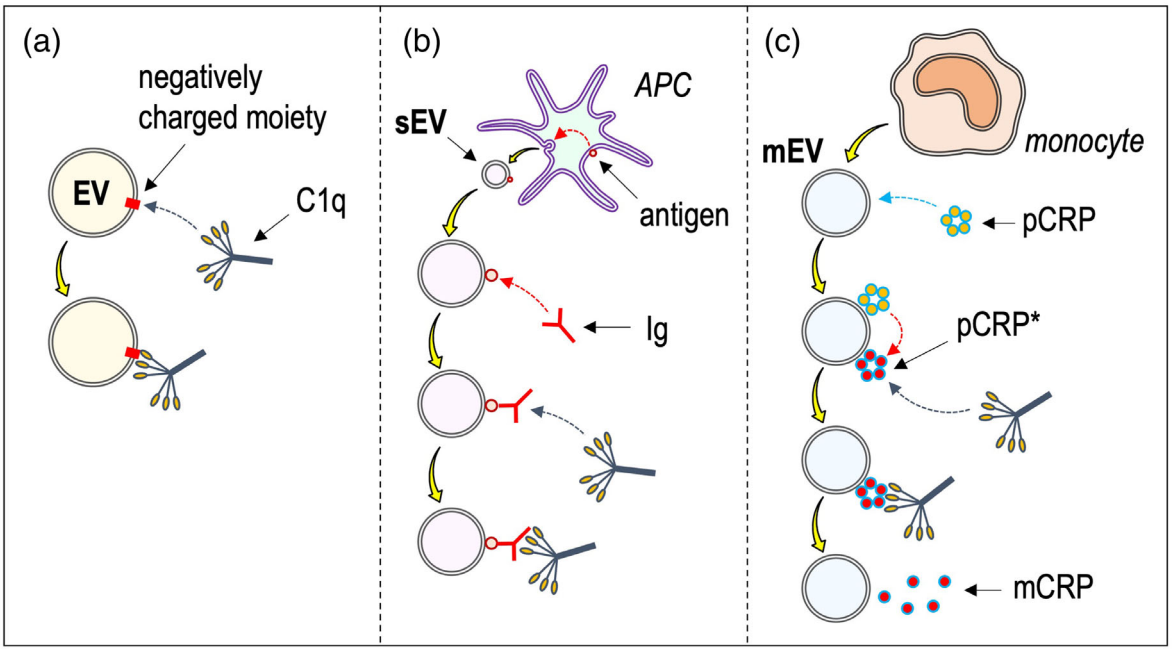

FIGURE 4 Binding of C1q to extracellular vesicles. Three possible mechanisms of C1q binding to extracellular vesicles are depicted. In the first mechanism, C1q binds to vesicle's native surface biomolecules with exposed negative charges (Bradley et al., 1999; Németh et al., 2017) (a). In the second mechanism, C1q binds to (acquired) immunoglobulins bound to antigens on the surface of small-sized EVs released by antigen presenting cells through a pathway involving antigen-processing endolysosomal compartments (Clayton et al., 2003) (b). In the third mechanisms, acquired pentameric C-reactive protein on monocyte-derived medium-sized EVs converts to neoepitope-expressing pentameric C-reactive protein, which enables the medium-sized EV cloaking by C1q and the triggering of a pro-inflammatory cascade, eventually terminated by the dissociation of neoepitope-expressing C-reactive protein into monomers (Braig et al., 2017)

Abbreviations: APC, antigen presenting cell; CRP, C-reactive protein; sEV, small-sized extracellular vesicle; EV, extracellular vesicle; Ig, immunoglobulin; mCRP, monomeric CRP; mEV, medium-sized extracellular vesicle; pCRP, pentameric CRP; pCRP*, neoepitope-expressing pCRP.

et al., 2001). Braig et al. found that human monocyte and monocytic THP-1 cells incubated with lipopolysaccharide and pCRP released pCRP-expressing medium-sized EVs (Braig et al., 2017). The authors also showed that pCRP could bind to medium-sized EVs purified from activated THP-1 and Jurkat cell lines as well as human monocytes, polymorphonuclear leukocytes and platelets. The medium-sized EV-pCRP interaction was Ca^{2+}-dependent via the phosphocholine head groups of phospholipids (Thompson et al., 1999). While the THP-1 cells' membrane expressed only native CRP (pCRP), medium-sized EVs derived from THP-1 cells expressed both native as well as neoepitope-expressing CRPs (pCRP*/mCRP) (Braig et al., 2017). Neoepitope-expressing CRPs were also secreted on medium-sized EVs derived from leukocytes, platelets and activated platelets from patients after myocardial infarction (Braig et al., 2017). Medium-sized EVs derived from THP-1 cells were able to bind C1q and activate the complement cascade (Braig et al., 2017). Molecular modelling showed that C1q could be docked onto pCRP*, but not onto pCRP (Braig et al., 2017). Braig et al. also described the effects of the presence of the neoepitope-expressing CRP isoforms on the adsorption of complement components on medium-sized EVs incubated with normal human sera: pCRP* led to the deposition of C3, C3b and iC3b whereas mCRP led to minor binding (Braig et al., 2017). Finally, the authors showed that monocyte-derived pCRP*-expressing medium-sized EVs were pro-inflammatory by their ability to activate the complement system, upregulate the expression of adhesion molecules in endothelial cells in vitro and induce leukocyte transmigration in the microcirculation of rat muscle in vivo. This suggested that monocyte-derived medium-sized EVs express pCRP, which was either native or acquired from the extracellular milieu, and its conversion to pCRP* triggered a pro-inflammatory cascade by activating the complement, which was eventually terminated by the dissociation of pCRP* into mCRP (Figure 4c).

From this point, two considerations shall be discussed. Firstly, Braig et al. confirmed the concept that the medium-sized EVs' small membrane curvature is essential for the conversion of pCRP to pCRP* (Braig et al., 2017; Wang et al., 2012). This would explain why, although they contain pCRP, large-sized EVs do not elicit a pro-inflammatory effect (Gershov et al., 2000). Large-sized EVs have a slight membrane curvature that might be responsible of the poor conversion of pCRP to pCRP*. Secondly, the presence of C3, C3b and iC3b on medium-sized EVs suggests that pCRP* might mediate a complex interaction mechanism with the alternative pathway. It may be possible that pCRP*-expressing medium-sized EVs may recruit factor B and factor D, resulting in C3b formation, as well as regulatory proteins [serine protease factor I and the co-factors complement receptor 1 (CR1/CD35) and factor H] to process bound or circulating C3b to iC3b (Dunkelberger & Song, 2010). Factor H adsorbs on complement-C3 marked EVs from retinal pigmented epithelium cells (Wang et al., 2009). Braig et al. found that, even in the absence of pCRP,

C3 bound to monocyte-derived medium-sized EVs and was processed to iC3b (Braig et al., 2017). Although the mechanism driving the formation of iC3b on pCRP-devoid medium-sized EVs remains elusive, it is probable that C3(H_2O) was generated on the medium-sized EVs' surface, leading to the activation of the complement alternative pathway and, in the presence of complement regulatory proteins, the formation of iC3b. Alternatively, C3b and iC3b generated in the extracellular milieu might bind to medium-sized EVs. Further investigations are warranted to validate the adsorption of pCRP as well as C1q in biological fluids, and unravel the mechanisms driving the presence of C3, C3b and iC3b on medium-sized EVs in the presence and in the absence of pCRP.

3.4 | Coagulation

During the development of thrombotic events, EVs are released from platelets as well as other types of cells (e.g., monocytes and endothelial cells) and act as procoagulant surfaces enhancing the rate of coagulation (Klaihmon et al., 2017; Mallat et al., 1999; Owens & Mackman, 2011; Palta et al., 2014; Ramacciotti et al., 2009; Siwaponanan et al., 2019). Tumour cell-derived medium-sized EVs isolated from the plasma of patients with pancreatic carcinoma have been also reported to be central to the pathogenesis and progression of venous thromboembolic events (Zwicker et al., 2009). The key component driving the procoagulant function of EVs is phosphatidylserine. Redistribution of phosphatidylserine to the cell surface can be observed during apoptosis and platelet activation, or in pathological conditions, such as the release of medium-sized EVs or small-sized EVs from tumour tissues (Park & Kang, 2019). Platelets are the major source of phosphatidylserine-rich medium-sized EVs in the blood (Hron et al., 2007). Phosphatidylserine mediates the deposition of clotting factors containing an N-terminal γ-carboxyglutamic acid (Gla)-rich domain on the EVs' surface. Gla-containing clotting factors include factor VII, factor IX, factor X and prothrombin (Owens, 2011). Another key component driving the procoagulant function of EVs is factor III (or tissue factor) (Owens, 2011). Monocytes and endothelial cells are likely to be the major source of tissue factor-rich medium-sized EVs in both healthy and diseased patients (Drake et al., 1989; Satta et al., 1994; Shet et al., 2003). Tissue factor can be found in a low- and high-activity conformation and, in the high-activity state, it is a receptor for factor VII/factor VIIa (Bach, 2006). The formation of the tissue factor:factor VIIa complex initiates the coagulation cascade leading to the propagation of thrombin (factor IIa) from prothrombin catalysed by the prothrombinase complex (factor Xa and its protein factor Va) formed on the phosphatidylserine-rich membranes of platelets and platelet-derived medium-sized EVs (Bach, 2006; Owens, 2011). Majumder et al. proposed that phosphatidylserine acts not only as a scaffold for the formation of the prothrombinase complex but also as a regulator of the complex's activity (Majumder et al., 2008). Eventually, the presence of phosphatidylserine in the proximity of tissue factor may favour the factor's high-activity state and the formation of thrombin (Bach, 2006). The formation of thrombin leads to the activation of factor XIII (fibrin stabilizing factor), which catalyse the formation of fibrin clots (Palta et al., 2014).

An additional function of thrombin is the activation of the complement system by its C5 convertase activity (Huber-Lang et al., 2006). Zecher et al. showed that erythrocyte-derived medium-sized EVs failed to bind immunoglobulins, C1q, and mannose-binding lectin in plasma; however, the vesicles became cloaked by C3 fragments, which was due to the activation of the prothrombinase complex on medium-sized EVs and the formation of thrombin (Zecher et al., 2014). This suggests that the deposition of coagulation factors on medium-sized EVs and formation of thrombin can trigger the binding of complement fragments to the vesicles. Alternatively, the activation of the complement system can trigger the deposition of coagulation factors on medium-sized EVs. The treatment of endothelial cells with C5b-9 proteins led to the release of medium-sized EVs that expressed C5b-9 proteins and factor Va binding sites and provided the principal catalytic surface for the prothrombinase complex (Hamilton et al., 1990). Activation of prothrombinase can lead to the formation of thrombin, which, drives the development of clots as well as complement activation, triggering a positive feedback loop. Thus, the medium-sized EVs' surface can work as a platform between the coagulation and complement systems (Amara et al., 2010).

3.4.1 | The functional role of fibronectin binding to EVs

Fibronectin is a high-molecular weight protein involved in tissue repair (Singh et al., 2010). Upon tissue injury, fibronectin is incorporated into fibrin clots and exerts effects on platelet function and hemostasis (To & Midwood, 2011). Fibronectin also exerts key functions in cell adhesion and motility (To & Midwood, 2011). Fibronectin is secreted from Golgi-derived vesicles in a soluble non-adhesion promoting form and it becomes converted to a fibrillar adhesion-promoting form by the interaction with integrins on the cells' plasma membrane (Amara et al., 2010). Fibronectin mediates EV functions by binding biomolecules on the EVs' membrane and on the plasma membrane of cells (Purushothaman et al., 2016). Purushothaman et al. found that fibronectin mediates the in vitro targeting of myeloma cell-derived small-sized EVs to RPMI 8226 myeloma cells and HS-5 bone marrow stromal cells by bridging heparan sulphate proteoglycans on both the vesicles' and target cells' surface through its C-terminal heparin/heparan sulphate binding site (Hep-II) (Figure 5a) (Purushothaman et al., 2016). However, the authors did not assess if fibronectin was pre-assembled on EVs before their release from the parent cells or it bound to the vesicles' surface in the extracellular milieu.

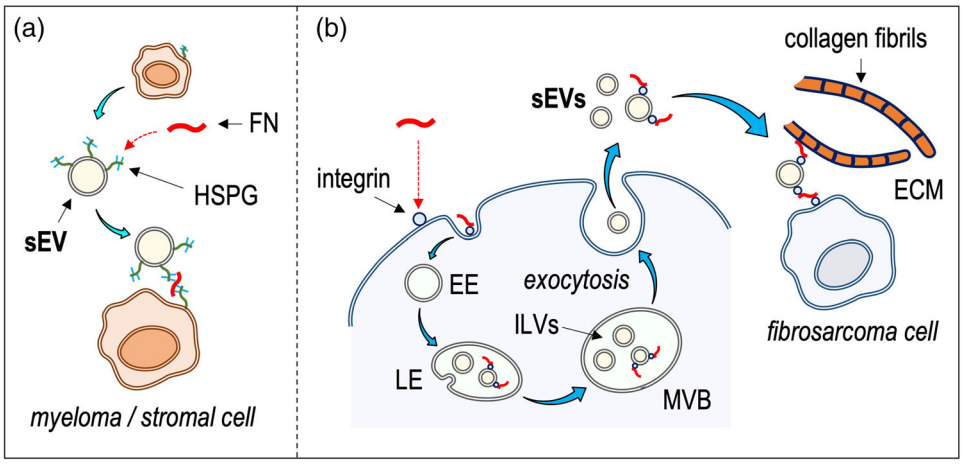

FIGURE 5 The functional role of fibronectin binding to extracellular vesicles. Fibronectin mediates the binding of extracellular vesicles to cells and components of the extracellular matrix. Fibronectin acts as a bridge between myeloma cell-derived small-sized EVs and myeloma/bone marrow stromal cells by binding heparan sulphate proteoglycans on both the vesicles' and target cells' surface (Purushothaman et al., 2016) (a). Alternatively, fibronectin binds to integrins on the plasma membrane of fibrosarcoma cells and the fibronectin-integrin complexes are recycled on the outside of small-sized EVs in the correct topology to mediate cell adhesion to the extracellular matrix by bridging integrins on the cell membrane and collagen fibrils (b) (Sung et al., 2015) Abbreviations: EE, early endosome; ECM, extracellular matrix; FN, fibronectin; HSPG, heparan sulphate proteoglycan; ILV, intraluminal body; LE, late endosome; MVB, multivesicular body; sEV, small-sized extracellular vesicle.

Fibronectin might also mediate EV functions by binding to the integrins on the EVs' surface (Laragione et al., 2003; Rieu et al., 2000; Sung et al., 2015). Rieu et al. found that during maturation of reticulocytes into erythrocytes, downregulation of integrin $\alpha_4\beta_1$, also known as very late antigen 4 (VLA-4), takes place through the release of VLA-4$^+$ small-sized EVs (Rieu et al., 2000). The authors also found that VLA-4$^+$ small-sized EVs bind to fibronectin immobilised on microtiter plates in a divalent cation dependent manner ($Mn^{2+} > Mg^{2+} > Ca^{2+}$) (Rieu et al., 2000). However, if free fibronectin can bind to VLA-4$^+$ small-sized EVs in the extracellular milieu was not established by the authors. Sung et al. described that fibronectin-integrin complexes are endocytosed by HT1080 cells and recycled on the outside of small-sized EVs in the correct topology to interact with moieties on both the cell membrane (e.g., integrins) and the extracellular matrix (e.g., collagen fibrils), thus mediating the adhesion and motility of HT1080 fibrosarcoma cells (Figure 5b) (Sung et al., 2015). A study by Laragione et al. suggested that soluble fibronectin can bind to small-sized EVs' integrins after they undergo a conformational change (Laragione et al., 2003). The authors described that treatment of Jurkat cells with N-acetyl-L-cysteine reduced integrin α_4 on cells' surface and augmented integrin α_4-dependent cell adhesion to fibronectin-coated plates and aggregation (Laragione et al., 2003). If treatment with N-acetyl-L-cysteine (as well as other thiol antioxidants) also increases the density of surface SH on EVs and promotes the vesicle cloaking by soluble fibronectin in the extracellular milieu has yet to be assessed. Finally, the interaction between fibronectin and EVs can be mediated by DNA. Németh et al. described that treatment of Jurkat cells with the antibiotic quinolone induced the release of small-sized EVs displaying both mitochondrial and chromosomal DNA on their surface and these vesicles were able to bind fibronectin immobilised on the sensor's surface (Németh et al., 2017). However, they did not establish if fibronectin bound to small-sized EVs by the exofacial DNA or by associated DNA-binding proteins and if free fibronectin can bind to the vesicles' surface in the extracellular milieu.

3.5 | Viral infection

During the viral infection, infected cells can release EVs which contain viral and host components to promote viral spread (Barberis et al., 2021; Borowiec et al., 2021; Giannessi et al., 2020; Hassanpour et al., 2020). In this section, in a speculative manner, we will interpret several pieces of experimental evidence that suggest the both small- and medium-sized EVs in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection may acquire specific properties after they adsorb soluble extracellular proteins.

It was suggested that small-sized EVs from SARS-CoV-2 virus-infected cells may induce immune cell response and use endocytosis to spread the infection (Barberis et al., 2021; Hassanpour et al., 2020). The increase of CRP (122-fold increase) in small-sized EVs of COVID-19 patients which is correlated, although at less extend, with the increase in CRP in the serum of COVID-19 patients (40-fold increase) in comparison to those of healthy patients, indicated that CRP level could be associated with the disease severity (Barberis et al., 2021). We may suggest that the small-sized EVs enriched with CRP could increase the viral

infection as compared to that induced by the small-sized EVs lacking CRP. In this respect, it was suggested that the cytokine storm in COVID-19 patients might affect the surface of circulating EVs (Balbi et al., 2021). Tissue factor is detected in EVs from plasma of patients with COVID-19 by measuring its activity or by western blotting (Balbi et al., 2021; Rosell et al., 2021). Tissue factor activity in plasma EVs from patients with COVD-19 is higher than plasma EVs of healthy patients (Rosell et al., 2021). Tissue factor-enriched EVs may act as clotting initiation agent and contribute to disease severity consistent with the occurrence of tissue factor-enriched EVs associated with SARS-CoV-2 infection severity and mortality (Balbi et al., 2021; Rosell et al., 2021). It was speculated that tissue factor-enriched EVs originate from activated monocytes, endothethlial and perivascular cells (Rosell et al., 2021). However, it is unclear if extracellular tissue factor can be adsorbed on EVs after their release from the cells or if it is present on the EVs when they are formed. Although EVs can facilitate the viral infection, they can also induce an antiviral response (Giannessi et al., 2020). EVs in plasma of COVID-19 patients contain an enriched amount of angiostensin-converting enzyme 2 (ACE2) in comparison to seronegative control (El-Shennawy et al., 2022). ACE2-enriched EVs from plasma of COVID-19 patients have better efficacity to block SARS-CoV-2 infection on Vero-6-cells, than EVs lacking ACE2 from plasma of seronegative patients (El-Shennawy et al., 2022). Recombinant ACE2 can inhibit infectivity and prevent COVID-19 (Batlle et al., 2020; Davidson et al., 2020; Hassler et al., 2021; Monteil et al., 2020; Wysocki et al., 2021), while ACE2-enriched EVs can bind to SARS-CoV-2 spike protein (Cocozza et al., 2020; Zhang et al., 2021). This suggest that ACE2-enriched EVs can inhibit SARS-CoV-2 by preventing the binding of SARS-CoV-2 to the ACE2 of host cells in a competitive manner (El-Shennawy et al., 2022). Since there are soluble and cleaved ACE2s, their eventual occurrence has been ascertained in EVs (El-Shennawy et al., 2022). Only full-length ACE2, which contains a transmembrane domain has been detected in EVs, while the soluble ACE-2 was not detectable in EVs (El-Shennawy et al., 2022). It is therefore unlikely that soluble ACE2 in the plasma could adsorb on EVs and modify their property.

Another antiviral response is the one induced by EVs carrying the non-coding RNA miR-146a-5p, which can inhibit the toll-like receptor 4 and may limit excessive inflammatory response to virus (Meidert et al., 2021). Higher amount of EVs associated miR-146a-5p were found in patients with COVID-19 pneumonia and with COVID-19 severe acute respiratory corona virus-2 syndrome in comparison to those of healthy patients and to non-COVID sepsis (Meidert et al., 2021). However, the circulating EV precipitates may contain cell-free proteins, including argonate 2 or lipoprotein particles which can carry circulating miRNAs (Meidert et al., 2021). Whether such proteins with their miRNAs are adsorbed on EVs under native conditions and can modulate the immune response remains to be investigated.

Medium-sized EVs are central in the pathophysiological mechanism of pulmonary coagulation in COVID-19 patients with cancer by driving a positive feedback loop between coagulation and inflammation (Jing et al., 2022). In the advanced stage of COVID-19, the burst of lung vascular endothelial cells by viral infection activates immune cells (macrophages, neutrophils, dendritic cells and natural killer cells) to release chemokines and cytokines (Lowery et al., 2021). The local increase in chemokines and cytokines leads to the recruitment of more immune cells, further sustaining the release of cytokines (cytokine storm) (Jing et al., 2022). In COVID-19 patients with cancer, tumour cells aggravate the cytokine storm by stimulating the release of pro-inflammatory cytokines by immune cells (Jing et al., 2022). In turn, the cytokine storm induces the release of medium-sized EVs from tumour cells (medium-sized EV storm), promoting microvascular coagulation and microthrombi formation due to phosphatidylserine exposure on the EVs' surface (Jing et al., 2022). The formation of microthrombi induces blood cell apoptosis (Jing et al., 2022). The release of large-sized EVs from apoptotic cells further sustains the release of medium-sized EVs from tumour cells. Ultimately, the positive feedback loop of events driven by the sustained storms of cytokines and tumour cell-derived medium-sized EVs leads to pulmonary coagulation, pathology progression and a poor prognosis (Iba et al., 2021). The exposure of phosphatidylserine, coordinated by flippases and scramblases in the parent cells' plasma membrane, on the EVs' surface is essential for blood coagulation initiation, amplification and propagation (Yeung et al., 2008). It is worth noting that all fibrinogen components in plasma EVs were less present in COVID-19 patients, suggesting an altered coagulation activity that could reflect a compensatory response to potential thrombotic manifestations (Jing et al., 2022). Not only the presence, but the absence of secreted soluble proteins on the surface of EVs may modulate EV properties. However, the complete biochemical machinery driving the procoagulant function of tumour cell-derived medium-sized EVs is currently unknown. More studies are warranted to assess the biologic role of soluble proteins acquired by EVs from the local milieu.

3.6 | Biomineralisation

3.6.1 | Mineralizing EVs in physiologic processes

Matrix vesicles are medium-sized EVs (100–300 nm diametre) released by mineralisation-competent cells, including hypertrophic chondrocytes, fully differentiated osteoblasts and odontoblasts, during physiologic biomineralisation (Bottini et al., 2018). Matrix vesicles are different from other types of EVs for their release mechanisms, unique ability to bind to collagen fibrils, and high activity of tissue non-specific alkaline phosphatase (TNAP), a key enzyme in biomineralisation (Figure 6 and Table 1) (Bottini et al., 2018). Due to their strong binding to collagen fibrils, matrix vesicles are extracted after a collagenase digestion of

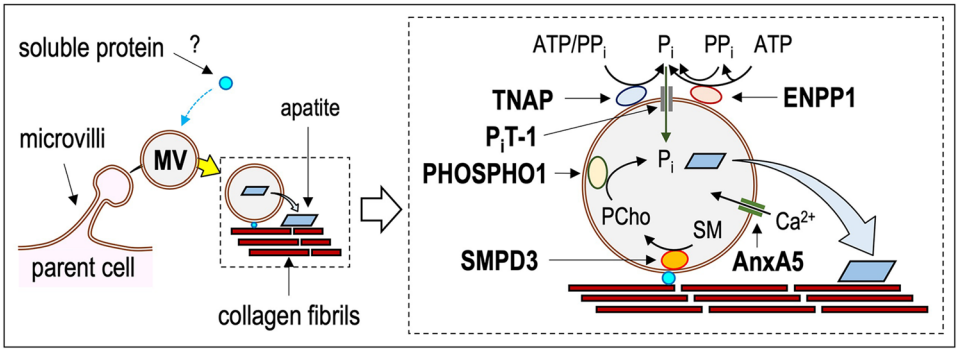

FIGURE 6 The biochemical machinery of matrix vesicles. Matrix vesicles are released by outward budding from the apical microvilli of mineralization-competent cells and are equipped with the biochemical machinery necessary to bind to collagen fibrils and allow the intraluminal accumulation of inorganic phosphate and Ca^{2+} to form apatite minerals that will be propagated onto collagen fibrils (Bottini et al., 2018). MVs bind to collagen through a mechanism that is still not well understood. Soluble proteins might adsorb (acquired soluble proteins) on the EVs' surface and mediate the vesicle binding to the collagen fibrils either directly by acting as bridge between the EVs and collagen, or indirectly by modulating the activity of native proteins. This hypothesis is indicated by the question mark in the figure

Abbreviations: AnxA5, annexin A5; ATP, adenosine triphosphate; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; MV, matrix vesicle; PCho, phosphocholine; PHOSPHO1, orphan phosphatase 1; P_i, inorganic phosphate; P_iT-1, phosphate transporter 1; PP_i, inorganic pyrophosphate; SM, sphingomyelin; SMPD3, sphingomyelin phosphodiesterase 3; TNAP, tissue-nonspecific alkaline phosphatase.

TABLE 1 Properties of mineralizing EVs released by different types of cells during physiologic and pathologic calcification

	Matrix vesicles		**Media vesicles**
Origin	Chondrocytes Osteoblasts	VSMCs	VSMCs M1 macrophages
Isolation with collagenase	Yes		No
Class	Medium-sized	Small-sized	Small-sized
Membrane topology	Phosphatidylserine inside	(?)	Phosphatidylserine outside
TNAP activity	Very high	Medium-low	Low
Presence of minerals	Intraluminal		Intraluminal and extraluminal
Apatite formation	Yes	(?)	(?)
Bind to collagen	Yes		(?)
Enzyme-driven Ca/P ions accumulation	Yes	(?)	(?)
References	Bottini et al. (2018)	Chen et al. (2008, 2010, 2018, 2021); Chaturvedi et al. (2015)	Cui et al. (2017); Kapustin et al. (2011, 2015, 2017); New et al. (2013); Reynolds et al. (2004); Shroff et al. (2010)

Abbreviation: TNAP, tissue-nonspecific alkaline phosphatase ; VSMCs, vascular smooth muscle cells; (?), to be confirmed.

the extracellular matrix to release them (Bottini et al., 2018). Their properties are distinct from small-sized and large-sized EVs extracted without collagenase digestion which lack of TNAP activity and exhibit poorer mineral properties (Bottini et al., 2018). The membrane leaflets of MVs are positioned in the same orientation than plasma membranes with the GPI-anchor protein TNAP pointing outside and phosphatidylserine pointing inside (Table 1). Matrix vesicles are thought to drive biomineralisation through a multi-step process: after their release by outward budding from the apical microvilli of mineralisation-competent cells (Figure 1), matrix vesicles bind to collagen fibrils and establish the appropriate ratio of inorganic pyrophosphate (PP_i), a potent mineralisation inhibitor, to inorganic phosphate (P_i), a mineralisation promoter, to allow the intraluminal formation of apatite minerals (Bottini et al., 2018). The latter will propagate onto collagen fibrils after the release in the extracellular milieu (Bottini et al., 2018). Although the exact mechanisms driving the ability of matrix vesicles to bind to collagen fibrils and generate a PP_i/P_i ratio conducive to biomineralisation remains elusive, these processes are explained as being driven by the vesicles' biochemical machinery (Figure 6).

Several key proteins on matrix vesicles may regulate the PP_i/P_i ratio (Figure 6). Among them, TNAP hydrolyses extracellular ATP and PP_i which are both inhibitor of apatite formation (Bottini et al., 2018). Extracellular PP_i originates partly from the hydrolysis of extracellular ATP by an ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) (Bottini et al., 2018).

Phosphate transporter 1 (P_iT-1) incorporates perivesicular P_i into the matrix vesicles (Bottini et al., 2018). P_i inside the matrix vesicles is also sustained by the activity of an orphan phosphatase 1 (PHOSPHO1) which generates P_i from the hydrolysis of phosphocholine. Phosphocholine is produced by sphingomyelin phosphodiesterase 3 (SMPD3) from sphingomyelin (SM) (Figure 6). Annexin A5 or an unidentified calcium channel contribute to the entrance of Ca^{2+} inside matrix vesicles (Bottini et al., 2018). The further accumulation of both P_i and Ca^{2+} favours the formation of apatite inside matrix vesicles (Bottini et al., 2018). The phosphatidylserine-mediated nucleation of amorphous calcium phosphate species could be a predominant mechanism to produce and stabilise the very first mineral nuclei during matrix vesicle-driven bone/cartilage biomineralisation (Simão et al., 2019). Once apatite is formed inside matrix vesicles, mechanical forces induced by apatite formation and/or the action of phospholipase contribute to the rupture of the matrix vesicles' membrane and release of apatite in the extracellular medium, inducing the second step of mineralisation process within collagen fibrils (Bottini et al., 2018) (Figure 6).

The matrix vesicles' proteins have been traditionally envisaged as 'native', that is, they are prearranged on the leaflets or within the lipid bilayer of the parent cells' microvilli before the matrix vesicles are released. However, by comparing the proteome of matrix vesicles and apical microvilli isolated from osteoblast-like cells, Thouverey et al. found that several proteins of matrix vesicles, including chondroitin sulfate proteoglycan 2, cysteine-rich angiogenic inducer 61, immunoglobulin J chain, macroglobulin α2, syndecan 2, syntaxin 4A and vitronectin, are not of microvilli origin (Thouverey et al., 2011). This suggests that soluble proteins are acquired by the matrix vesicles' surface in the extracellular milieu (Figure 6). However, if the acquired soluble proteins have a biological function, or they are contaminants from the extracellular medium, is currently unknown.

Matrix vesicles have been traditionally considered a special class of EVs acting solely as mineralisation nanoreactors, however several reports revealed that information cargoes, including bone morphogenetic proteins (BMPs) and micro RNAs (miRNA), are also present in the matrix vesicles' lumen (Bottini et al., 2018; Lin et al., 2016; Yoshiko & Minamizaki, 2020). The role of these cargoes was not assessed. Recently, Minamizaki et al. described that osteoblast-released matrix vesicles can regulate osteoclastogenesis during skeletal development by harbouring miR-125b (Minamizaki et al., 2020). This suggests that matrix vesicles might also function as signalosomes as reviewed (Shapiro & Landis, 2015). Matrix vesicles are currently described as vesicles steadily anchored to collagen fibrils and producing apatite minerals within one-to-three hours of their release in a microenvironment (the growth plate) populated by dying cells (Bottini et al., 2018). This model implies that if matrix vesicle-mediated cell-cell communication exists, it would be of limited occurrence and of a short range. Thus, other mechanisms should be taken in consideration to explain the ability of matrix vesicles to function as signalosomes. One mechanism is that mineralisation-competent cells release not only matrix vesicles but also other types of EVs, including small-sized EVs and medium-sized EVs, – more suitable vectors for the cell-to-cell communication than matrix vesicles – loaded with information cargoes. Alternatively, although speculative, binding of soluble proteins to matrix vesicles' surface may result in altered ability of the vesicles to bind to collagen. TNAP, an ecto-phosphatase anchored to the matrix vesicles' outer surface, is crucial for biomineralisation by controlling the PPi/Pi ratio (Millán, 2013). Recently, by using matrix vesicle-biomimetic lipid monolayers harbouring TNAP, we have shown that the lipid bilayer's composition and fluidity modulate the TNAP activity and mineralisation (Derradi et al., 2019; Favarin et al., 2020). We have also shown that matrix vesicle-biomimetic proteoliposomes harbouring TNAP and annexin A5 (a medium-sized EV protein) bind to collagen fibrils *via* annexin A5 only in the absence of TNAP (Bolean et al., 2020). These findings suggest that binding of matrix vesicles to collagen is a dynamic process driven by interactions among the matrix vesicles' components, both native and acquired. This model would imply that soluble (acquired) proteins are involved in matrix vesicle homing, either indirectly by modulating the affinity of native proteins for collagen, or directly by forming a molecular bridge between the vesicles and the collagen fibrils, or both. More experiments are warranted to assess the role of soluble proteins in the matrix vesicle binding to collagen. These studies will help to shed light on the biochemical mechanisms driving the ability of matrix vesicles to function as either mineralisation nanoreactors or signalosomes.

3.6.2 | Mineralizing EVs in cardiovascular diseases

Matrix vesicles are not the only class of EVs acting as biomineralisation nanoreactors (Aikawa et al., 2021; Bakhshian Nik et al., 2017; Blaser & Aikawa, 2018; Bottini et al., 2018; Chen & Moe, 2015; Demer & Tintut, 2014; Kapustin & Shanahan, 2016; Li et al., 2022; Schurgers et al., 2018; Zazzeroni et al., 2018). A seminal study from New et al. described the release of mineralizing EVs from macrophages in human atherosclerotic plaques (New et al., 2013). The authors found that macrophages co-localised with areas of the plaque rich in mineralizing $CD68^+$ EVs in a greater extent than vascular smooth muscle cells or $CD43^+$ cells. TEM images using gold nanoparticle-labelled antibodies showed that macrophages released $CD68^+$ mineralizing EVs in plaque regions of early and mid-stage calcification (New et al., 2013). In vitro experiments showed that macrophagic cells stimulated with Ca^{2+} and P_i acquired a pro-inflammatory M1 polarisation and released EVs with a distribution of diametres in the 30–200 nm range with a main peak at around 50 nm (New et al., 2013) (Table 1). It is worth noting that these EVs were isolated without collagenase digestion, thus they did not bind to the extracellular matrix and shall be classified as "media vesicles", rather than "matrix vesicles".

The media vesicles showed the nucleation of minerals, both on the membrane and in the vesicle's lumen (Table 1). However, it is unclear if the electron dense particles, as observed by TEM and near infrared fluorescence imaging could correspond to apatite (New et al., 2013). Stimulation of macrophages induced the formation of complexes of annexin A5 and S100 calcium-binding protein A9 (S100A9) docked on phosphatidylserine but whether the nucleation of CaP minerals occurred inside or outside the media vesicles remain undetermined (New et al., 2013). If the nucleation of CaP minerals is driven by a biochemical machinery made of native and/or acquired enzymes would be of crucial importance to shed light on the molecular mechanisms regulating the formation of microcalcifications in the intima of atherosclerotic plaques.

Mineralizing EVs are also released by vascular smooth muscle cells at the sites of medial vascular calcification (Aikawa et al., 2021; Bakhshian Nik et al., 2017; Blaser & Aikawa, 2018; Bottini et al., 2018; Chen & Moe, 2015; Kapustin & Shanahan, 2016; Li et al., 2022; Schurgers et al., 2018; Zazzeroni et al., 2018). Medial vascular calcification is a condition associated with the deposition of apatite minerals in the arterial wall's medial layers, which can cause vascular stiffening and heart failure (Durham et al., 2018). It was first recognised around 40 years ago that EVs are secreted by vascular smooth cells within the extracellular matrix (Tanimura et al., 1983). This result was later supported by a couple of seminal papers (Chen et al., 2008; Reynolds et al., 2004). Since matrix vesicles from chondrocytes and osseous cells are extracted after a collagenase digestion due to their strong affinity to collagen fibrils, a clear distinction shall be made between the EVs from vascular smooth muscle cells isolated with (matrix vesicles) (Chaturvedi et al., 2015; Chen et al., 2008; Chen et al., 2010; Chen et al., 2018; Chen et al., 2021) and without (media vesicles) collagenase digestion (Chen et al., 2018; Cui et al., 2017) (Table 1; Kapustin et al., 2015; Kapustin et al., 2017; Reynolds et al., 2004; Shroff et al., 2010). Vascular smooth muscle cell-derived matrix vesicles have TNAP activity (Chen et al., 2008), while media vesicles have low TNAP activity (Kapustin et al., 2011) (Table 1). Matrix vesicles express CD63 (Chen et al., 2018), whereas media vesicles express CD81 and CD9 (Chen et al., 2018), but CD63 is detected (Kapustin et al., 2015). Matrix vesicles induced calcification of the recipient vascular smooth cells, while media vesicles did not (Chen et al., 2018). Matrix vesicles have a diametre of approximately 100 nm (Chen et al., 2018), while media vesicles have been described to have a diametre of approximately 120 nm (Reynolds et al., 2004), 150 nm (Kapustin et al., 2015) and 150 to 250 nm (Cui et al., 2017), thus they can be mostly classified as small-sized EVs (Table 1). Both matrix and media vesicles released by vascular smooth muscle cells can accumulate calcium intraluminally (Chen et al., 2018; Kapustin et al., 2011; Kapustin et al., 2015; Reynolds et al., 2004). TEM of matrix vesicles showed the presence of electron dense particles in the vesicles' lumen (Chen et al., 2018), which may indicate the initiation of mineral complexes, while TEM studies of media vesicles revealed electron dense particles localised both in the lumen and on the surface of the vesicles (Kapustin et al., 2015) (Table 1). Matrix vesicles can calcify collagen fibrils (Chen et al., 2018). It was proposed that phosphatidylserine exposure on the surface of media vesicles together with annexin A6 could drive the mineralisation process (Kapustin et al., 2015). However, experimental evidence of apatite formation induced by EVs (both matrix and media vesicles) released by vascular smooth muscle cells is lacking, except for one report indicating apatite in circulating calciprotein particles from the serum of patients with chronic kidney disease, while there was no mention of its occurrence in EVs from this serum (Viegas et al., 2018). Fetuin-A, an inhibitor of mineralisation, can be accumulated in EVs from vascular smooth muscle cells and modulate their mineralisation property (Chen et al., 2008; Kapustin et al., 2015; Reynolds et al., 2004; Viegas et al., 2018). However, fetuin-A is not expressed in vascular smooth muscle cells, suggesting that either it is endocytosed and recycled in the EVs by the cells, or it adsorbs on the EVs' surface in the extracellular milieu (Kapustin et al., 2015). Indeed, it has been proposed that under physiological conditions, vascular smooth muscle cells release EVs loaded with calcification inhibitors such as matrix Gla protein (MGP) and fetuin-A circulating proteins, while under pathological conditions, depletion of MGP and fetuin-A, along with the presence of phosphatidylserine, Ca^{2+} and annexin A6 on the EVs' surface, could drive the mineralisation process (Kapustin & Shanahan, 2016). This is consistent with the fact that pro-mineralizing matrix vesicles contain less fetuin-A than the less-mineralizing media vesicles (Chen et al., 2008). Prothrombin, a vitamin K-dependent coagulation factor, harbours a Gla-rich domain and can interact with the exposed phosphatidylserine of the media vesicles from vascular smooth muscle cells where its distribution overlapped with that of fetuin-A and it can disrupt the EV-induced calcification (Kapustin et al., 2017). Whether media vesicles from vascular smooth muscle cells may protect against calcification, while matrix vesicles may induce calcification remains to be investigated. Although earlier studies have suggested that medial vascular calcification is driven by the differentiation of vascular smooth muscle cells to bone-forming osteoblasts, calcifying vascular smooth muscle cells differ from mature osteoblasts (Patel et al., 2019). Such differences are emphasised by the distinct properties of mineralizing EVs secreted by vascular smooth muscle cells and osseous cells (Table 1). Current knowledge suggests that mineralizing EVs released from M1 macrophages or vascular smooth muscle cells have distinct characteristics: [small-sized EVs, membrane topology with phosphatidylserine in the outer membrane leaflet (especially media vesicles from smooth muscle cells), high to low TNAP activity, formation of minerals outside and, possibly, in the lumen) than those of matrix vesicles produced by chondrocytes or osseous cells (medium-sized EVs, membrane topology with phosphatidylserine in the inner membrane leaflet, TNAP specific activity greater than that of the parent plasma membrane, formation of apatite in the lumen, bind strongly to collagen fibrils and harbour a complex biochemical machinery that enable to accumulate Ca^{2+} and P_i ions in the lumen) (Table 1). To this end, assessing the functional role of soluble proteins adsorbed onto the surface of mineralizing EVs may help develop diagnostic and/or therapeutic strategies for medial vascular calcification.

TABLE 2 Functional role of soluble proteins acquired by extracellular vesicles in biologic processes

Biologic process	Type of EV	Soluble protein	Functional role	Refs.
Innate and adaptive immune response	sEV	ICAM-1/CD54	T cell stimulation	Segura et al. (2005)
	mEV	MFGE8	Binding to APCs (?)	Hanayama et al. (2002); Morelli et al. (2004); Oshima et al. (2002)
		MFGE8/Gas6	Indirect binding to immune cells (?)	Graham et al. (2014)
Rheumatoid arthritis	mEV	Fibrinogen, vimentin, immunoglobulins	Formation of pro-inflammatory mEV-containing immune complexes	Cloutier et al. (2013), Fortin et al. (2016)
		Prdx1	Remove oxidized molecules from plasma membrane	Szabó-Taylor et al. (2017)
Complement activation	mEV	pCRP	Activation of inflammation	Braig et al. (2017)
			Trigger clearance by cell uptake	Agrawal et al. (2001)
Coagulation	mEV	iC3b	Activation of the complement system	Zecher et al. (2014)
SARS-CoV-2	EV	CRP	Activation of infection	Barberis et al. (2021)
		TF	Clotting initiation	Balbi et al. (2021), Rosell et al. (2021)
Vascular calification	sEV	Fetuin-A	Inhibit calcification	Kapustin et al. (2015), Reynolds et al. (2004), Viegas et al. (2018)
		Prothrombin	Disrupt the EV-induced calcification	Kapustin et al. (2017)

Abbreviations: CRP, C-reactive protein; EV, extracellular vesicle; Gas6, growth arrest-specific protein 6; ICAM-1, intercellular adhesion molecule 1; mEV, medium-sized extracellular vesicles; MFGE8, milk fat globule epidermal growth factor factor 8; pCRP, pentameric C-reactive protein; Prdx 1, peroxiredoxin 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sEV, small-sized extracellular vesicles; TF, tissue factor; (?), to be confirmed.

4 | CONCLUSIONS

Only a few in vitro studies have shown experimental evidence that soluble proteins are acquired by EVs from the extracellular milieu and mediate vesicle functions in biologic processes (Table 2). These findings shed light on the model that the EVs' biological functions depend on a complex relationship between the local and temporal changes in the protein composition of the extracellular milieu and the pattern of the native biochemical (e.g., membrane lipids and surface proteins) and physical (e.g., membrane curvature) properties of the EVs' surface. The validation of this model would need additional investigations to assess whether the EVs' surface proteins are pre-assembled by the parent cells, or they were acquired from the extracellular milieu in vivo. Although in vitro studies have shown that soluble proteins can adsorb on EVs in the extracellular milieu, a microscopy-based approach on cultured cells has unraveled that an endocytosis-resecretion process is possibly used by cells to dock fibronectin on the surface of secreted EVs (Sung et al., 2015). Thus, further work to validate that surface proteins are acquired from the extracellular milieu by EVs after they have been shed in vivo is warranted. It is worth noting that, while current spectroscopic and microscopic methods [described in Section 2 of this review and in Hartjes et al. (2019)] are adequate to probe proteins adsorbed on the surface of EVs, they do not indicate if the adsorbed proteins originate from the extracellular medium or if they were present on the surface of EVs during their secretion. Analysis of the components in the extracellular medium and EVs, as well as their relative proportions, shall indicate if EVs are enriched in extracellular proteins during their exposure to the extracellular medium. The comparisons of the compositions of the surface proteins of freshly secreted EVs and those of EVs exposed to the conditioned media from selected cells shall complement the strategy to determine the origin of adsorbed proteins. Culture media could be a possible source of contaminated soluble proteins, which could adsorb on the surface of EVs and affect the EV properties. Thus, studies on the biologic functions of the EVs' acquired surface proteins should rule out the presence of protein contaminants in cell culture media.

Finally, consideration should be given to the biologic functions of the EVs' acquired surface proteins. It cannot be excluded that soluble proteins adsorb on the EVs' surface to carry out a function different from the modulation of the EV interaction with molecules in the extracellular milieu, the extracellular matrix, and the plasma membrane. For instance, the adsorption of fetuin-A on the surface of EVs could affect the mineralisation property of EVs released from vascular smooth muscle cells, which could be a determinant factor to prevent vascular calcification (Kapustin & Shanahan, 2016). The deposition of soluble proteins has been described to affect the mobility of EVs by tuning the vesicles' size (Skliar et al., 2018). Thus, although speculative, we can hypothesise that some of the soluble proteins acquired by EVs in body fluids have the role to

finely modulate the vesicles' mobility in the extracellular microenvironment. For instance, Kowal et al. described that tetraspanin negative EVs isolated from the conditioned medium of human dendritic cells were enriched in several soluble extracellular proteins, including fibronectin, factor II, C3, and serum albumin (Kowal et al., 2016). It could be hypothesised that, while fibronectin, factor II and C3 mediated the role of dendritic cell-derived EVs in coagulation (and complement) activation, albumin regulated the vesicles' mobility. However, more investigations are warranted to validate the hypothesis that acquired soluble proteins can also regulate the EVs' motility and to assess the molecular mechanisms driving their docking on the EVs' surface.

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CONFLICT OF INTEREST

The authors report no conflict of interest

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