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Reviews

Neuronal Enriched Extracellular Vesicle Proteins as Biomarkers for Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is a major cause of injury-related death throughout the world and lacks effective treatment. Surviving TBI patients often develop neuropsychiatric symptoms, and the molecular mechanisms underlying the neuronal damage and recovery following TBI are not well understood. Extracellular vesicles (EVs) are membranous nanoparticles that are divided into exosomes (originating in the endosomal/multi-vesicular body [MVB] system) and microvesicles (larger EVs produced through budding of the plasma membrane). Both types of EVs are generated by all cells and are secreted into the extracellular environment, and participate in cell-to-cell communication and protein and RNA delivery. EVs enriched for neuronal origin can be harvested from peripheral blood samples and their contents quantitatively examined as a window to follow potential changes occurring in brain. Recent studies suggest that the levels of exosomal proteins and microRNAs (miRNAs) may represent novel biomarkers to support the clinical diagnosis and potential response to treatment for neurological disorders. In this review, we focus on the biogenesis of EVs, their molecular composition, and recent advances in research of their contents as potential diagnostic tools for TBI.

Keywords: extracellular vesicles (exosomes) and biomarkers; traumatic brain injury (TBI)

Introduction

Traumatic brain injury (TBI) is the leading cause of death and long-term disability in the developed world. Annually, in excess of 10 million people suffer a TBI event worldwide.[1,2] Projections reveal that TBI will comprise the third largest portion of the total global disease burden by the year 2020.[1] Within the United States, an estimated 1.7 million people per year incur a TBI, and approximately 5.3 million people live with a TBI-induced disability.[3,4] By far the majority of sustained TBIs are mild to moderate in nature and account for some 80–95% of incidents, with severe TBI comprising the balance.[5] This stratification of injury is based on the widely used Glasgow Coma Scale (GCS) for determining the severity of neurological injury in TBI patients by evaluating three functions: motor responsiveness, verbal performance, and eye opening. A score ranging from 1 to 6 points is assigned to each function, and their sum indicates the TBI grade: mild TBI (GCS 13–15), moderate TBI (GCS 9–12) and severe (GCS ≤8).

With improvements in survival rate following initial injury, TBI can give rise to substantial and lifelong cognitive, physical, and behavioral impairments that necessitate long-term access to health care and disability services.[5,6] Particularly vulnerable are the elderly, in which the same insult leads to greater disability and can result in a dramatic rise in the risk for neurodegenerative[7,8] and neuropsychiatric disorders.[9] TBI symptoms can sporadically resolve within the first year following injury, but 70–90% of patients continue to exhibit protracted and often unending neurocognitive dysfunction. It is now established that TBI represents a time-dependent process, rather than a single event. Emerging evidence reveals that this process may in some individuals lead to early dementia onset,[7,8] but predicting which individuals and the sequence of TBI-induced cellular cascades that underpin this has been difficult to determine in the absence of easy to sample, well-validated biomarkers. Finding and evaluating the utility of such biomarkers has become critical as, clinically, TBI is one of the most powerful environmental risk factors for development of Alzheimer's disease (AD). Recent gene expression studies have identified the upregulation of pathways leading to AD and Parkinson's disease that are triggered by mild TBI (mTBI), let alone moderate or severe forms of TBI, in animal models.[10–12] In light of the lack of any available therapeutic options,[13] it is imperative to understand the mechanisms that underlie head injury and the ensuing neuronal dysfunction and loss, as well as triggered degenerative pathways to support the development of effective therapeutic strategies. These mechanisms are time-dependent, and biomarker technologies to accurately and quantitatively follow them to select subjects that

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might best respond to a particular treatment or to optimize a treatment are sorely needed.

TBI-associated brain damage can be classified into two key phases. First, an initial primary damage phase occurs at the moment of insult, and includes contusion and laceration, diffuse axonal injury, and intracranial hemorrhage that can result in instantaneous (necrotic) cell death.[10,14] This is followed by a protracted second phase that encompassess cascades of biological processes, initiated at the time of injury, which may persist over much longer times consequent to ischemia, neuroinflammation, glutamate toxicity, astrocyte reactivity, and apoptosis.[15–18] As this secondary brain injury may be reversible, it is crucial to understand the biological cascades that drive the delayed secondary phase that occurs following TBI.[10,13]

Routinely used neuroimaging techniques, such as single photon emission computed tomography (CT) scanning and magnetic resonance imaging (MRI), are available to assess TBI, but, in broad terms, have not proved effective in the diagnosis of brain injury in relation to the true intensity, cell death, blood–brain barrier breakdown, neuroinflammation, and excitotoxicity that ensues. MRI scans are relatively costly and cannot be performed in a repeated manner over short intervals owing to their potential adverse effects in humans. Single-photon emission CT scanning has proved useful in the diagnosis of regional blood flow abnormalities, but although relatively cost-effective to apply has not been of utility in detecting TBI-associated structural damage.

Protein biomarkers potentially available in biological fluids, such as blood, plasma, and serum as well as cerebrospinal fluid (CSF; if available), can be sampled frequently and inexpensively, and offer the promise of providing information about TBI severity. How such damage time-dependently progresses, and whether predictive signatures can be found in relation to the development and progression of TBI-induced disorders, or, more positively, to gain treatment-induced improvements and drug target information would be hugely useful. Clearly, biomarkers that are linked to the extent of injury, as well as to molecular pathways associated with either degenerative or regenerative processes would be of great benefit to patients and clinicians to aid appropriate decision making regarding treatment options—particularly, if these were early biomarkers that showed substantial and predictable changes.

The present review focuses on identifying and monitoring potential protein biomarkers in biological fluids, such as blood, plasma, and serum, with the promise to aid in understanding and better treating TBI.

Biomarkers of mTBI Diagnosis

Diagnostic markers are indicative of disease status, progression, severity, and potential therapeutic interventions.[19] Although several biomarkers exist for TBI diagnosis, there is no single clinical diagnostic marker to estimate injury severity.[20] Whereas the GCS rating system is often used to assess the severity of TBI,[21] it is unable to provide insight into the origin of TBI (i.e., by alcohol, drug use, or polytrauma).[22]

Bodily fluids including blood, CSF, and urine as reservoirs for diagnostic markers may offer the potential to evaluate neurological deficits in a specific, time-dependent, and sensitive manner. Markers in bodily fluids hold the advantage of being an objective and quantitative measure of biological changes.[23] Currently, multiple available markers are under investigation to better understand the biological mechanisms via which mTBI occurs, as well as to assess the severity of the disease.

In this regard, numerous potential markers have been evaluated for their ability to diagnose mTBI, including some with moderate success (Table 1). S100B, a low affinity calcium binding marker expressed in Schwann and glial cells within 6 h of mTBI, is one such candidate.[24–27] S100B expression is found to be elevated in the serum of TBI patients; however, the fact that other pathologies that often accompany TBI also drive the expression of S100B, calls into question the protein's specificity as a marker.[28–32] Recent studies indicate S100B and glial fibrillary acidic protein (GFAP) levels are increased in TBI patients, and could potentially differentiate mTBI patients from those with severe TBI.[33,34] Indeed, GFAP as a potentially promising marker holds the advantage of specific expression in nervous tissue, and can be correlated with CT or MRI images.[35] The disadvantages of utilizing GFAP expression include the inability to predict its levels 6 months post-TBI, as well as that GFAP cannot be used to assess the pathophysiology or mechanism underpinning a TBI due to its inability to identify axonal and glial damage.

Currently available markers include neuron-specific enolase (NSE), myelin basic protein (MBP), and hyperphosphorylated neurofilament heavy chain (NFH). MBP has been found to be a more specific TBI marker than NSE[36] primarily because NSE can also be elevated in CSF due to lysis of erythrocytes. NFH is found to be expressed in TBI patients at 2 to 4 days post-injury, but its expression levels drastically alter.[37]

Tau's phosphorylated form is a well-established CSF biomarker for AD patients[38]; however, phosphorylated tau (P-tau) is also reported in CSF of patients suffering from chronic neurodegenerative disorders, such as Pick disease and progressive supranuclear palsy.[39–41] Tau is a central nervous system (CNS)-specific protein, and a reliable marker found to be elevated 24 h following hypoxic brain injury, with delayed elevations observed up to 48 h. Ultra-sensitive immunoassays are currently available, and have the potential to measure less than 10 pg of P-tau protein, potentially allowing correlations with outcome measures.[42] Additional research on P-tau protein levels, and in particular specific types of the protein (e.g., the *cis* vs. *trans* form[43,44]), are warranted to evaluate its potential as a reliable marker for TBI.

Spectrin is a non-erythrocytic protein found in neurons, axons, and presynaptic terminals.[45] Calpain and caspase-3 are expressed in TBI and during neuronal apoptosis, and promote the breakdown of αII spectrin.[45–47] Spectrin's breakdown products are increased in severe TBI, and can potentially be correlated with patient outcome.[48–50]

Ubiquitin carboxy-terminal hydrolase isoenzyme L1 (UCH-L1), a deubiquinatase that is highly expressed in neurons and a tissue distribution that is almost exclusively restricted to brain, is another potential biomarker for TBI.[51] Research indicates UCH-L1, in tandem with spectrin levels could provide prognostic information in TBI.[49,50] S100B and GFAP levels increase in a manner similar to UCH-L1 and spectrin breakdown, and similarly could be used to assess the severity of TBI using peripheral blood.[48,50] UCH-L1 was detected in serum within 1 h of injury, and was found to be correlated with GCS score and CT-assessed lesions in a 96-patient TBI study.[52]

In synopsis, recent investigations have identified sets of interesting potential biomarkers that can be linked with and provide insight into pathobiological processes instigated by TBI (e.g., caspase-3 and calpain-mediated spectrin breakdown,[48] and/or brain structural elements (as epitomized by UCH-L1 and GFAP), that are symbolic of trauma-induced brain injury. The evaluation of and combined use of such markers, when related to a comprehensive

Table 1. TBI Biomarkers

TBI biomarker	*Function*	*References*
GFAP	Serum glial fibrillary acidic protein breakdown products in mild and moderate TBI are associated with intracranial lesions and neurosurgical intervention.	141, 142
S100β	S100β is a 21 kDa calcium-binding protein (Ca2+), found mainly in the cytosol of astroglial cells and Schwann cells. S100B may be a reliable marker of brain damage in TBI without multiple trauma at 24 h.	143–145
Myelin-basic protein (MBP)	TBI-induced axonal injury could cause damage to the myelin structure, resulting in secondary myelin sheath instability and demyelination, which may increase the vulnerability of the axons.	146
NSE	Enolase is a glycolytic enzyme and NSE is one of its five isoenzymes. NSE levels are particularly elevated in severe TBI.	36, 142
NFH (neurofilament heavy chain)	NFH is an axonal injury marker. Serum NFH levels are significantly elevated in diffuse axonal injury (DAI).	37, 147
Tau	Microtubule-associated structural protein in axons. Shearing of axons leads to the disruption of Tau binding to tubulin, and subsequent Tau hyperphosphorylation can lead to the formation of Tau oligomers. These can cause self-propagating Tau pathology under specific conditions.	148
Tumor necrosis factor-alpha (TNF-α)	Pro-inflammatory cytokine that plays an essential role in the immune response observed in patients post-TBI. When TNFα levels are unregulated and chronically elevated, they can drive neurodegenerative cascades.	149
Interleukin-6	Pro-inflammatory cytokine that has been implicated in TBI patients and, when elevated levels are not regulated, then can drive neurodegenerative processes.	150
Ubiquitin C-terminal hydrolase-L1 (UCH-L1)	The addition or removal of ubiquitin from proteins that are destined for metabolism; this is a key part in the removal of excessive, oxidized or misfolded proteins during both normal and pathological conditions in neurons. It is abundant in neurons and has been thought of as a possible biomarker for TBI. It has previously been associated with severe TBI, an increased mortality rate at 6 weeks, and a poor 6-month GOS score.	151–153
Alpha II-spectrin (SBDPs)	Principally found in neurons and is in plentiful supply in axons and pre-synaptic terminals. The protein is processed into breakdown products (SBDPs) - specifically, SBDP150 (150 kDa size) and SBDP145 (145 kDa) by calpain, and into SBDP120 (120 kDa) by caspase-3. SBDPs are found across cellular and animal models of TBI, and levels are elevated in the CSF of humans particularly after severe TBI. SBDP150, −145, and −120 show a different temporal pattern after TBI, particularly in differentiating survivors from non-survivors.	50, 154
Estradiol and testosterone	Female TBI patients, in general, recover better than male TBI patients – postulating that differential levels of progesterone and other sex hormones may act as neuroprotective agents. However, increased oestradiol and testosterane levels over time have been associated with increased mortality and worse global outcome for both men and women.	155, 156

CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; GOS, Glasgow Outcome Scale; NSE, neuron-specific enolase; TBI, traumatic brain injury.

physical examination may be useful in determining the full extent of injury and possible outcome scenarios.

Extracellular Vesicles

Extracellular vesicles (EVs) are membranous nanoparticles that are found in all biological fluids investigated to date including amniotic fluid, blood, urine, saliva, breast milk, CSF, and ascetic fluid. EVs are divided into exosomes (smaller EVs in the range of 30–150 nm originating in the endosomal/multi-vesicular body [MVB] system) and microvesicles (larger EVs in the range of 100–300 nm that are produced through budding of the plasma membrane). Both types of EVs are secreted by a variety of cell types including lymphocytes, mast cells, platelets, endothelial cells, neurons, and dendritic cells via direct release from the plasma membrane.[53] Crucial discoveries regarding exosome structure were made in 1992. The finding that exosomes contain the transferrin receptor, allowing them to be segregated from other membrane proteins and externalized, shed light on the role that exosomes play in intercellular signaling.

EVs are lined by a lipid bilayer, and contain numerous types of proteins and lipids. (For specifics, the website www.microvesicles.org provides a detailed catalog of proteins, RNAs, and lipids associated with EVs.) The additional website Vesiclepedia (formerly ExoCarta; http://microvesicles.org/index.html) lists 92,897 proteins, 27,642 messenger RNAs (mRNAs), 4934 microRNAs (miRNAs), and 584 lipids associated with EVs. The protein and lipid profiles of EV reveal a great deal about their functional role. Notable proteins in EVs include tetraspanins (CD9, CD63, CD81, and CD82), membrane transport and fusion proteins (GTPases, annexins, and flotillin), and heat shock proteins (Hsc70 and Hsp90).[54] Proteins of the Rab family, annexins, and heat shock proteins play a key role in intracellular assembly and trafficking of EVs. The tetraspanins that are frequently found in exosomes mediate cell migration, fusion, cell–cell adhesion, and signaling. Integrins, also found abundantly in exosomes, regulate

the adherence of vesicles to their target cells.[53] Likewise, many further proteins are associated with EV formation and aid in cargo for cell–cell communication, and when widely expressed in EVs have the potential to be used as markers.

Lipids such as phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, phosphatidylinositol, and monosialotetrahexosylganglioside (GM3), are abundantly present in the EV lipid bilayer.[55,56] Phosphatidylserine, in particular, is involved in the signaling and fusion of EVs to the plasma membrane, and docking the proteins expressed on the EV membrane by acting through different phospholipid transportation enzymes.[57] GM3 and sphingomyelin are reported to be involved in the rigidity of EVs,[58] and ceramide, cholesterol, and phosphoglycerides, along with saturated fatty-acid chains appear also to be present in EVs.

Biological Functions of Extracellular Vesicles

EVs are secreted by cells and via biological fluids that include the blood, CSF, synovial fluid, urine, and amniotic fluid, act as key role players in implementing intercellular communication and initiating physiological responses. They express major histocompatibility complex (MHC) class I and II molecules on their cell surface, which are secreted from antigen-presenting cells and aid in triggering specific immune responses by activating CD8+ and CD4+ T cells.[59–62] EVs also carry nucleic acids, mRNA, and miRNA that can potentially be transferred to recipient cells. As an example, in the case of EVs deriving from glioblastoma cells, their mRNA and miRNA have the potential to trigger angiogenesis.[63]

EVs have been described as impacting a broad array of biological processes that include cellular waste removal, the maturation of erythrocytes,[64] inflammation,[65] coagulation,[66] angiogenesis,[63,67] and immune responses.[68] They play a key role in cell to cell communication in a target-specific manner, and are important in the promotion of thrombosis, tumor proliferation, and angiogenesis through transfer of mRNA and KRAS protein.[69]

In addition to potential beneficial and physiological roles, EVs are likely involved in the spread of disease within organ systems. This is exemplified by the role EVs play in Parkinson's disease pathogenesis through the transport of misfolded proteins.[70] Likewise, potential roles of EVs in other disorders such as cardiovascular disease and infectious diseases such as human immunodeficiency virus (HIV) have been reported.

Biogenesis of Extracellular Vesicles

EVs display a round or cup-shaped morphology, and are released from a diverse group of both non-neuronal cells, exemplified by monocytes and lymphocytes,[71,72] and neuronal cells including microglia,[73] neurons,[74] and astrocytes.[75] EVs are generated primarily through one of two routes: via endocytic vesicles from the plasma membrane that form early endosomes and subsequently MVBs by inward budding of endosomal vesicles, which contain intraluminal vesicles that are released as exosomes by the fusion of MVBs with plasma membranes; and by budding of the plasma membrane (Fig. 1). Studies have indicated the involvement of SNARE proteins and Rab GTPases such as Rab27, 35, 11 in the regulation of EV secretion. These proteins aid in the process of tethering, docking, and the fusion of EVs at the plasma membrane.[76,77] Although mechanisms such as endocytosis, receptor ligand binding, and fusion of EVs with plasma membranes have been proposed as mechanisms by which target cells receive EVs, the definitive mechanisms of interaction and secretion have yet to

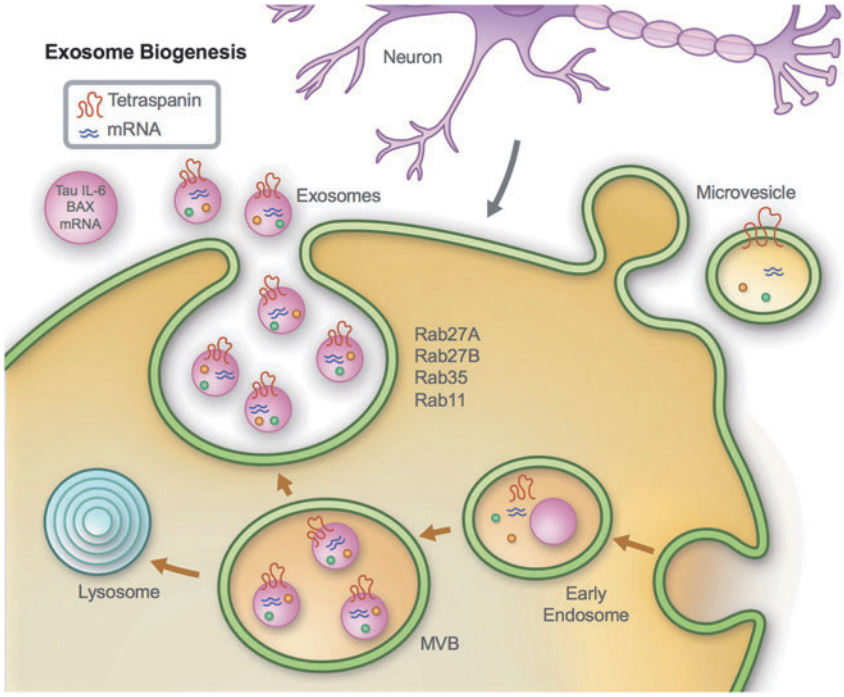

FIG. 1. EV (exosome) biogenesis. EVs are generated in late endosomes or multi-vesicular bodies (MVB). The components of the endosomal sorting complex required for transport (ESCRT) are involved in MVB and EV biogenesis, and aid in cargo loading and vesicle release.[157] RabGTPases, such as Rab27, 35, 11 and the SNARE proteins are involved in tethering and fusion to the plasma membrane.[76,77,158,159] The cargo of EVs contains proteins and RNAs reflective of ongoing intracellular processes, and thereby provide a window to cellular events.

be fully elucidated.[53,78,79] Recent research evidence does, however, support the concept that the internal cargo of EVs contributes to the health and pathology of cells within the CNS.[80]

Extracellular Vesicle Isolation

Multiple methods have been developed to support the isolation of EVs from biological fluids by exploiting their physical properties in relation to other consituents within the same sample. Such methods include centrifugation, chromatography-filtration, polymer-based precipitation, and immunological separation techniques to permit relatively consistent EV isolation (Fig. 2). These methods have assorted advantages and disadvantages of relevance to pre-clinical and clinical research. Notably, they can be combined in various manners to enrich EV populations deriving from select cell types.

Centrifugation-based isolation methods

Differential centrifugation is a common method used to isolate EVs from biological fluids and media. The basis of differential centrifugation is the application of successive centrifugation steps of increasing centrifugal force and duration to sequentially isolate smaller from larger objects. The larger particles sediment faster and, by leaving the majority of smaller ones in the supernatant, can be progressively removed in the initial centrifugation steps (Fig. 2A). Subsequent successive rounds of centrifugation ultimately provide the particulate of interest. For most common EV isolation protocols, there are four consecutive centrifugation steps: low speed (10 min at 300*g*) is initially used to sediment cells and debris, (10 min at 2000*g*) to remove further debris and apoptotic bodies, (30 min at 10,000 to 20,000*g*) to isolate microvesicles (generally with diameters in excess of 100–150 nm), followed by pelleting of smaller EVs with predominance of exosomes (100,000g for 70 min). Thereafter, washing and a repeat 100,000*g* centrifugation step of the re-suspended pellet is commonly applied to further purify the EV sample from any free proteins.[81]

Essentially, the method provides a reasonable purity of EVs but at a moderately low yield. The primary challenge for this purification technique is the separation of EVs from smaller microvesicles consequent to their close size similarity. Additionally, the

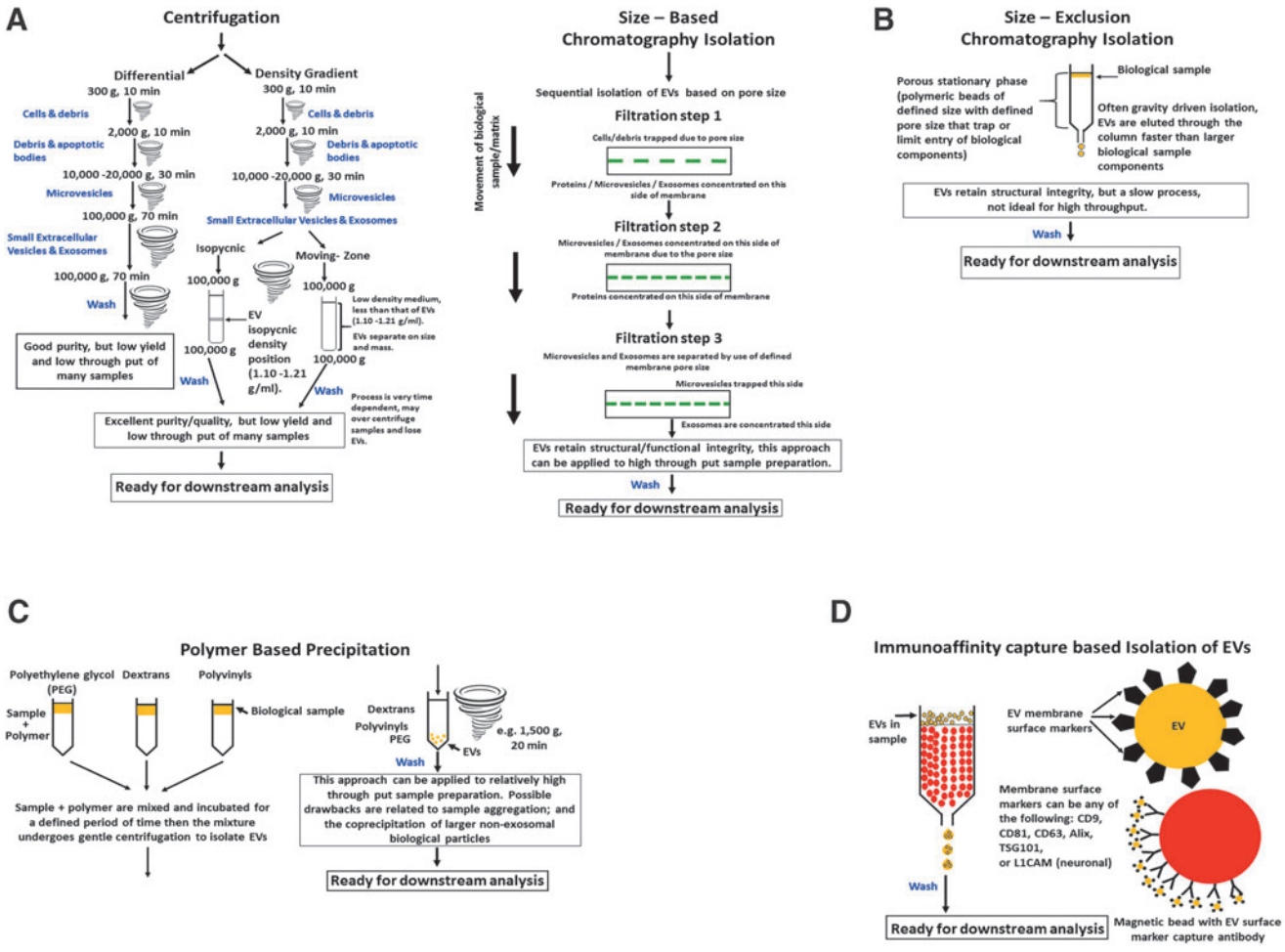

FIG. 2. Commonly used methods to isolate exosomes (EVs) from biological samples. **(A)** The stepwise process of centrifugation-based EV isolation is shown. Centrifugation can utilize differential or density gradient-based methods. **(B)** Size-based chromatography-filtration isolates EVs based on the size differences of the different biomolecules present in the samples. **(C)** Polymer-based precipitation of EVs is accomplished by exploiting protein–protein interactions of the EVs and the polymers. **(D)** Immunoaffinity capture-based isolation of EVs takes advantage of the presence of EV surface marker proteins that are selective for global EVs or even cell type-specifc surface markers, such as L1CAM (a neuronal cell-type marker).

method is not particularly efficient for handling large numbers of biological fluids, such as would be expected with time-dependent serum or plasma samples deriving from a clinical trial for EV analysis of potential biomarkers.

Density gradient centrifugation, a variation of ultracentrifugation, described above, is the use of a density gradient during the centrifugation process.[82] There are essentially two types, isopycnic and moving-zone (Fig. 2A). The use of the former, density gradient ultracentrifugation, is being increasingly applied to the isolation of EVs where separation is achieved based on their size, mass, and density within a selected density gradient medium of increasingly higher density from top to bottom. The biological sample is added as a narrow band onto the top of the density gradient, and subjected to ultracentrifugation to allow centrifugal force to move the assorted components within the sample, including EVs, as different discrete zones depending on their specific sedimentation rate. These zones can then be collected and cleaned by re-suspending them in physiological buffered saline (PBS) and ultracentrifuged (100,000*g*) to provide EVs for further analysis. In the isopycnic process, a density gradient medium is selected to cover the entire range of densities of sample constituents, and thus zone separation depends on density differences between EVs versus other components, with each sedimenting along the density gradient that matches its own, the isopycnic position—which, in general, lies between 1.10 and 1.21 g/mL for EVs.[83]

In moving-zone ultracentrifugation, the biological sample is added as a thin layer to the top of a prepared gradient density medium of lower density than any of the sample's components. EVs hence gradually separate from other elements based on their different size and mass, rather than their density difference (as in isopycnic ultracentrifugation), and thus the moving-zone process permits the separation of EVs with similar densities but diverse sizes. When centrifugal force is applied, the separation process is dynamic for the moving-zone procedure as, over sufficient time, all components will eventally pellet together at the bottom of the centrifuge tube, and hence time and the application of centrifugal force need to be optimized to maximize EV separation. This contrasts with the isopycnic procedure, in which static zones are ultimately achieved at the isopycnic position of the alike density components.

In synopsis, when applied optimally, density gradient ultracentrifugation procedures can be advantageouly applied to differential ultracentrifugation to improve the quantity and purity of the EVs harvested, particularly when there is an expected heterogeneity in the EV population and an overlap in size in relation to other elements within the biological sample.[84] A disadvantage is that such techniques are relatively labor-intense and cannot readily be applied to large numbers of samples.

Size-based chromatography and size-exclusion chromatography isolation

Size-based EV isolation techniques are widely used for blood and urine samples,[85,86] particularly the application of ultrafiltration. In this, EVs can be separated by sequentially passing them through a series of membrane filters of specific molecular weight or size exclusion limits,[84,87] (Fig. 2B). In several procedures, a low force is applied to speed up the EV isolation process, which has to be selected to avoid breaking or deforming larger vesicles as this may detrimentally impact later analyses.[88] Several commercial EV isolation kits have been developed for plasma, serum, urine, and CSF samples.[84] Such consecutive filtration permits EV isolation with relatively high purity and functional integrity. The combination of this technique with ultracentrifugation procedures has been employed to isolate therapeutic EVs for clinical studies, and appears to be scalable to handle large sample numbers.[89,90]

An additional size-based separation technique utilized to harvest EVs is size-exclusion chromatography (SEC). In SEC, a porous stationary phase (often polymeric beads) within a column is applied to isolate components according to their size (Fig. 2B). Components with a smaller hydrodynamic radius are capable of passing into and through the bead pores, and thus take a longer time to elute. In contrast, those with a greater hydrodynamic radius (i.e., EVs) are unable to transfuse through as many pores, and hence elute from the column more quickly, permitting their isolation. As this is often achieved using gravity flow, EV structure and integrity are retained, albeit long run times ensue that impact the scalability of SEC techniques.[88,91] For both forms of isolation the eluted EV samples are washed and prepared for downstream analysis or biological assays.

Polymer-based precipitation

The basis of polymer-based precipitation is the use of polymers, such as polyethylene glycol (PEG), dextrans, or polyvinyls that, when added to a biological sample, attract water molecules away from the solvation layer around proteins. This increases protein–protein interactions and enhances precipitation[92] (Fig. 2C). Taking advantage of this phenomena, several commercial isolations kits are available that use polymers to permit EVs to be pelleted under a low centrifugal force, producing a relatively high yield of useable EVs both quickly and in a scalable manner to support evaluation of large sample numbers. Available commercial kits include ExoQuick, ExoSpin, and the Invitrogen Total Exosome Purification Kit, or can be potentially replaced by use of the ExtraPEG method (8% PEG + wash).[93,94] In synopsis, a small volume of precipitant is added to a biological sample, which is then incubated (for between 30 min and 12 h, 4°C, depending on the manufacturer). Thereafter, the EV fraction is then pelleted by low-speed centrifugation ($1500 \times g$, 20 min, 4°C). This pellet is then washed and resuspended in distilled water with a protease inhibitor cocktail and phosphatase inhibitor cocktail. Potential disadvantages of such techniques, are that polymer-based precipitation solutions can result in aggregates, the co-precipitation of larger non-exosomal elements, and precipitants deriving from the kits.[95] Recent studies have demonstrated the utility of this EV isolation technique, particularly in relation to clinical sample analyses.[96–99]

Immunoaffinity capture-based isolation

The presence of numerous proteins and receptors within the membrane of EVs provides the opportunity to isolate EVs based on immunoaffinity capture-based techniques focused on interactions between these exposed surface proteins (antigens) and their specific antibodies (Fig. 2D). Clearly, such antigens should ideally be expressed in a highly enriched and abundant manner on EVs, versus other components within the same biological samples, be membrane-bound and lack soluble counterparts.[84] Magnetic bead technology, together with other techniques, have been applied to further increase EV purity. Notably, such techniques can be advantageously combined with other isolation procedures to enrich for EVs deriving from select tissues, such as neurons, astrocytes, and/or capillary endothelial cells.[82,98,100] After elution of EVs the samples are washed and prepared for downstream analysis of biological assays.

Extracellular Vesicles Characterization

EVs are classified as exosomes, microvesicles, and apoptotic bodies based on their size or origin.[101,102] Exosomes are small in size and derived from the endosomal system[103–106]; they bear surface markers such as tetraspanins (CD9, CD81, and CD63), Alix, or TSG101[107] (Fig. 1). Microvesicles (also previously named as ectosomes) are generated by evagination of plasma membranes into the extracellular space and carry cytoplasmic contents (Fig. 1). Microvesicles do not appear to arise from the endosomal pathway. The size and number of EVs are normally determined by nanoparticle tracking analysis (NTA) or tunable resistive pulse sensing (TRPS).[108] Exosomes and microvesicles can be distinguished to some extent based on size, although significant overlap occurs, but cannot be easily distinguished based on protein markers on the vesicle membrane. Tetraspanins, which are commonly used to define EVs, are enriched in both exosomes and microvesicles.[109,110] Apoptotic bodies are diverse in size ranging from 50 nm to 5000 nm and are released from dying cells undergoing programmed cell death. More accurate clues are required to distinguish all these EVs; even these are classified based on size and origin.

Extracellular Vesicles Involved in Neurological Diseases

EVs are secreted by cells throughout the CNS, including astrocytes, neurons, microglia, and oligodendrocytes, and their involvement in regeneration, the modulation of synaptic function, and neuronal development has been described.[111–113] Similar to other organ systems, CNS EVs appear to be key players in intercellular communication and the initiation of physiological responses regulating the immune response, in eliminating cellular waste, and in communication between neural cells.[65,114,115] Notable among recent studies, is that EVs appear to be involved in promoting communication between glia and neurons.[116] For example, EVs from human CSF or derived from N2a cells can mitigate the synaptic plasticity disruption caused by both synthetic and AD brain-derived Aβ.[117] *In vitro* and *in vivo* studies have reported that microglia EVs can promote the spread of tau protein, and inhibiting EV synthesis appears to reduce such tau propagation.[113]

Recent studies suggest that EVs can be used as biomarkers for diagnosing neurodegenerative disorders such as Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis, and AD. These disorders are associated with the occurrence of misfolded proteins that can be found and quantified in EVs deriving from the CNS.[118] An increasing number of studies are hence evaluating the utility of EVs as diagnostic tools to characterize disease progression.[119] Their ability to cross the blood–brain barrier allows their time-dependent collection and enrichment from the systemic circulation, saliva, and urine, making their contents an attractive target to quantify for diagnostic applications (Fig. 3).

Extracellular Vesicles as a Tool for Diagnosis and Therapy

As noted, the relative ease of collecting EVs from biological fluids, and enriching them for cellular origin, such as neuronal or astrocytic, make them a useful tool for non-invasive disease diagnosis.[79] As an example among several, a study of the classical AD markers Aβ_{42} and P-tau (phosphorylated at the S396 or T181 sites) found them significantly increased in plasma derived EVs enriched for neuronal origin (enrichment was obtained by use of the neuronal cell surface marker L1CAM) that allowed the differentiation of AD patients from age-matched controls.[120] Notably, these markers appeared to be significantly higher as early as 10 years prior to AD clinical diagnosis.[121] Recent analyses of other proteins within the cargo of plasma derived, L1CAM neuronally enriched EVs are providing insight into mechanisms involved in AD pathogenesis, with the occurrence of heightened protein profiles involved in brain insulin resistance[122] and declines in the levels of cellular survival factors[123] and synaptic proteins.[124] Notably, these protein profiles can be likewise interrogated in TBI, as there appear to be shared mechanisms that underpin AD and TBI. Indeed, elevations in P-tau, Tau, Aβ_{42}, and IL-10 within neural-enriched EVs have recently been reported in TBI subjects.[125,126] Such processes, together with measures of neuroinflammation by evaluating astrocyte-derived[127] and/or microglial-derived EVs[128] may provide insight into time-dependent mechanisms occurring in TBI, as well as the factors involved in TBI progression to later AD, and responses to interventions.

Growing experimental evidence suggests that EVs hold a potentially useful role in therapeutic interventions. It has become increasingly clear over the last decade that EVs provide an efficient mechanism for cell–cell communication by delivery of RNA and proteins both within the local microenvironment of their release as well as at a distance, by trafficking their contents through the systemic circulation. Interaction and binding to recipient cells can occur via multiple processes, including receptor–ligand interactions and antigen presentation. Additionally, EVs may attach and fuse to their target-cell membrane or become internalized by endocytosis. Whatever the mechanism, it has become clear that the transferred EV contents are functional at their new location. As an example, mRNA within mouse EVs has been demonstrated to be taken up and successfully translated within human cells.[129] siRNA containing EVs have been used to successfully deliver short interference (siRNA) to target tissues when injected into mice.[130] Plasmacytoma-derived EVs have been reported to successfully reduce the inflammatory and autoimmune responses in arthritis patients and prevent tumor development.[79,131] Finally, recent studies have demonstrated that EVs derived from mesenchymal stem cells can augment functional recovery, mitigate pattern separation and spatial learning impairments, enhance neurovascular remodeling through actions on neurogenesis and angiogenesis, and diminish neuroinflammation in animal models of TBI.[132–134] Such research hence opens many windows in relation to new diagnostic and therapeutic approaches. For the former, by allowing the time-dependent collection and evaluation of the contents of EVs generated in cells (neurons and/or astrocytes) impacted by physiological/environmental challenges and/or disease states, and for the latter by aiding the transport and delivery of specific proteins (as well as pharmaceutical and biotherapeutics) to recipient cells to support specific regenerative functions.

Conclusion

TBI symptoms can occasionally resolve within the first year after injury and in many cases largely do so, but 70–90% of patients continue to manifest prolonged and sometimes permanent neurocognitive dysfunction. Albeit a majority of subjects that experience a single mTBI recover completely with sufficient time, some 25% largely fail to do so.[134,135] Those experiencing repetitive concussive mTBIs or blast impact mTBIs are less likely to fully recover.[136,137] Perhaps more important still, how and in whom these single and repetitive forms of TBI increase the vulnerability of the recipient to later neurodegenerative disorders that manifest as AD or Parkinson's disease remain largely unknown. Under such circumstances, readily accessible biomarkers are clearly needed for

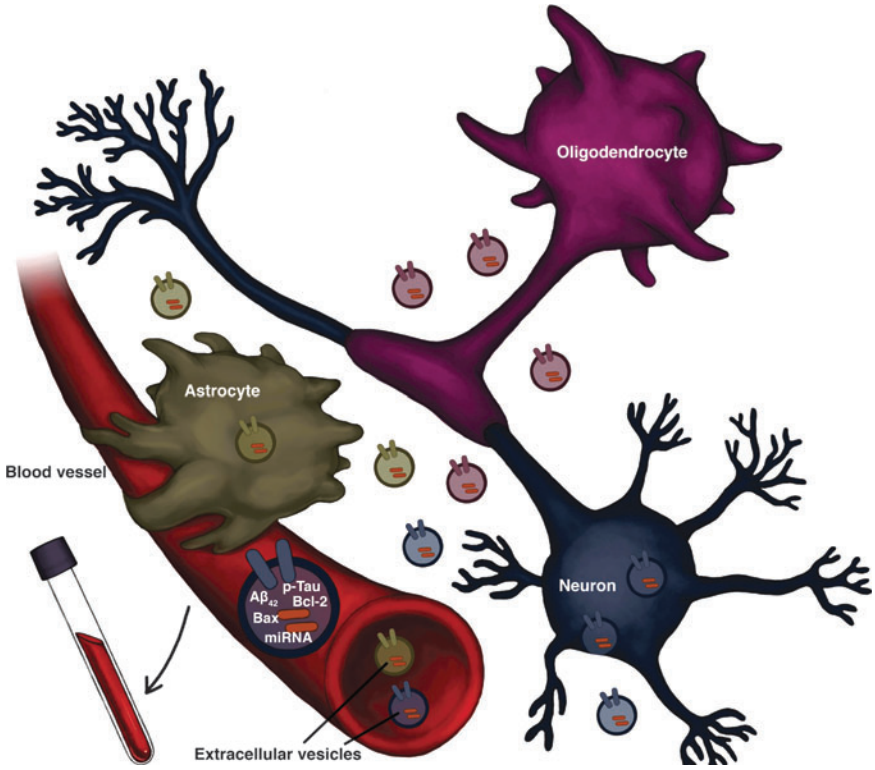

FIG. 3. Plasma extracellular vesicles (EVs) enriched for neuronal origin, astrocyte, or oligodendrocyte origin. Schematic showing the assembly and release of EVs from cells within the brain. EVs are generated by the inward budding of endosomal membranes, with the associated recruitment and internalization of cellular protein and RNA cargo. Following their cellular release EVs travel within the interstitial fluid to neighboring cells or, via the circulation, to distant targets to potentially provide modulatory actions. EVs are found in and can be isolated from plasma/serum samples from animal models as well as humans. Enrichment for their cellular origin (whether neuronal, astrocyte, or a different cell type—such as oligodendrocytces) can be achieved by immunoprecipitation with biotinylated antibodies against surface markers to isolate sub-populations from different central nervous system (CNS) cell types. The quantitative evaluation of their protein and RNA content provides a platform for their use as biomarkers to changes in neurological function, disease state, and treatment conditions.

identifying underlying pathobiological changes, and could additionally support early diagnosis and optimize treatment for TBI. Recent studies indicate EV protein profiles contain injury-specific biomarkers.[138]

Whereas advances have been made in the identification and characterization of a selection of blood and CSF biomarkers that appear well-replicated in acute and more severe TBI, there remains an insufficient understanding of the dynamic changes that transpire during the hours, days, weeks, and months that follow different forms of head injury. Elucidation of these dynamic changes would clearly improve our understanding of the pathobiologies that result from acute TBIs. They may define windows of opportunity for therapeutic interventions, define mechanisms of drug action that might best be suited as treatment options within these windows, and also define which subjects might best respond and, importantly, when its safe for them to return to work or play.[139] In this regard, neuronal- and astrocyte-enriched plasma EVs appear particularly promising for identifying blood-based biomarkers[140] that are more specific to CNS pathobiology as the contents of these stable, cell-derived, small phospholipid bilayer-enclosed vesicles largely mirror the attributes of their parent cells; they can cross the blood–brain barrier and are available for time-dependent sampling from biological fluids, such as blood, saliva, and urine in addition to CSF. Current methodologies allowing the relatively rapid isolation of plasma EVs enriched for CNS origin by the targeting of CNS-specific cellular markers available on their surface, such as the neural adhesion proteins NCAM and L1CAM (CD171) for neurons or glutamine aspartate transporter (GLAST) for astrocytes, permit their immunoprecipitation and collection to enable analysis of their protein and RNA contents. The current availability of technologies to reproducibly quantify within a single sample multiple proteins and/or RNAs derived from focused biochemical cascades permits the interrogation of mechanisms theoretically underpinning TBI, host homeostatic responses to TBI, and the potential of therapeutic options in a time-dependent manner. How these correlate to the more classical markers of TBI sampled from plasma and/or CSF, described in Table 1, remains to be elucidated. Such prior markers have often been evaluated at a single time-point that is often different between studies, and thereby offer only a snapshot of events arising following a head injury. These same markers can be time-dependently evaluated within EV samples alongside gateway proteins/RNAs regulating mechanistic pathways. Such studies in well-orchestrated cellular, animal, and exploratory clinical trials have the ability to provide the field truly useful biomarkers for TBI and other neurodegenerative disorders.

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