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REVIEW ARTICLE

Extracellular vesicles and their synthetic analogues in aging and age-associated brain diseases

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Abstract Multicellular organisms rely upon diverse and complex intercellular communications networks for a myriad of physiological processes. Disruption of these processes is implicated in the onset and propagation of disease and disorder, including the mechanisms of senescence at both cellular and organismal levels. In recent years, secreted extracellular vesicles (EVs) have been identified as a particularly novel vector by which cell-to-cell communications are enacted. EVs actively and specifically traffic bioactive proteins, nucleic acids, and metabolites between cells at local and systemic levels, modulating cellular responses in a bidirectional manner under both homeostatic and pathological conditions. EVs are being implicated not only in the generic aging process, but also as vehicles of pathology in a number of age-related diseases, including cancer and neurodegenerative and disease. Thus, circulating EVs—or specific EV *cargoes*—are being utilised as putative biomarkers of disease. On the other hand, EVs, as targeted intercellular shuttles of multipotent bioactive payloads, have demonstrated promising therapeutic properties, which can potentially be modulated and enhanced through cellular engineering. Furthermore, there is considerable interest in employing nanomedicinal approaches to mimic the putative therapeutic properties of EVs by employing synthetic analogues for targeted drug delivery. Herein we describe what is known about the origin and nature of EVs and subsequently review their putative roles in biology and medicine (including the use of synthetic EV analogues), with a particular focus on their role in aging and age-related brain diseases.

Keywords Extracellular vesicles · Exosomes · Neurodegeneration · Aging · Drug delivery · Nanomedicine

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Introduction

Safe, efficacious and specific drug delivery is integral to modern therapeutic medicine. The ability to optimise the bioavailability, stability, and targeted uptake of a therapeutic agent while simultaneously mitigating toxicity, immunogenicity and off-target/side effects is of utmost priority to in development of more effective drugs, and in the treatment of otherwise *incurable* diseases. Extensive efforts are being made in the

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modification or derivation of existing drugs, or the development of new drug-delivery platforms, to achieve these goals, often inspired by physiological mechanisms.

One such phenomenon is that of extracellular vesicles (EVs), a type of naturally occurring nanovesicles that envelop, protect and shuttle their bioactive cargo between cells in different systems (Thery 2011). These extracellular organelles are no longer considered to be mere cellular debris (Cocucci et al. 2009), nor are they just being proposed as circulating diagnostic markers that mirror their parental cell's physiologic statuses, rather they appear to be central players in a diverse, complex, and specific intercellular communication network (Simons and Raposo 2009). As EVs are implicated in a plethora of physiological and pathological processes, a thorough understanding their origin and function is of great importance to medical science. Furthermore, their role as natural molecular cargo carriers provides inspiration for the design of new and improved therapeutic platforms, be they emulating EVs or repurposing them for medicinal applications.

Herein we review the current state of knowledge of EVs, describing their various classes, and providing examples of their function in disease, health, and during the processes of brain aging. A broad overview of the therapeutic potential of EVs is also provided, as is a rundown of current synthetic nanotherapeutic drug-delivery platforms that mimic the properties of EVs. While the field of EV study is still largely in its infancy, the therapeutic potential of EVs (and their analogues) in aging and age-related disease, particularly neurodegeneration, is plain to see.

Extracellular vesicles (EVs)

Characterisation of EVs

EV is a broad term used to describe membrane structures secreted by cells into the extracellular space to be later taken up by an target/acceptor cell (Raposo and Stoorvogel 2013). Despite the lack of definitive evidence for their physiological function in vivo, EVs appear to constitute a newly recognized means of communication found to be shared by almost every cell type (Thery 2011).

While the description of EVs has historically been burdened by a Byzantine nomenclature (Gould and Raposo 2013), a systematic classification based on the mechanisms of biogenesis and release of EVs (Akers et al. 2013) allows for the categorization of EVs into four broad groups:

(i) *Exosomes* homogenous saucer-shaped EVs 30–100 nm in diameter, from multivesicular bodies (MVBs) of the endosomal pathway;
(ii) *Shedding vesicles* (or *microvesicles*) heterogeneous EVs 50–2,000 nm in diameter, from direct blebbing of the cellular plasma membrane;
(iii) *Retrovirus-like particles* (*RLPs*) sized 90–100 nm, with a typical subset of retroviral proteins but non-infectious, due to the lack of genes required for full viral propagation; and
(iv) *Apoptotic bodies* 50–5,000 nm in diameter, vesicles arising during the apoptotic fragmentation of cells.

Other classes of EVs that fall outside these classifications have recently been identified. For instance, *gesicles*, approximately 100 nm in diameter and slightly less dense than exosomes, are highly fusogenic due to their origins in cells induced to overexpress the spike glycoprotein of the vesicular stomatitis virus (VSV-G) (Mangeot et al. 2011). Moreover, *exosome-like vesicles* (20–50 nm) expressing the full-length 55-kDa tumour necrosis factor (TNF) receptor 1 have been identified and may originate from multivesicular internal compartments (not necessarily being part of the endosomal system), but their nature is not well defined (Hawari et al. 2004).

Considering that a single cell type can secrete multiple EV classes (Heijnen et al. 1999; Deregibus et al. 2007; Muralidharan-Chari et al. 2009), one of the key challenges in the field is to establish methods allowing for their discrimination and—in perspective—their characterization and fractionation. Differences in properties such as size, morphology and density are not fully sufficient for a clear distinction (Bobrie et al. 2011). Further characterization requires biochemistry, qualitative and quantitative protein, RNA and lipid characterization, and imaging such as electron microscopy. Complementary to that, nanoparticle-tracking analysis allows for the determination of EV size distribution based on the Brownian motion of vesicles in suspension (Soo et al. 2012). Furthermore, a novel high-resolution flow cytometry–based approach has been developed for quantitative high

throughput analysis of immunolabeled vesicles (Nolte-'t Hoen et al. 2012; van der Vlist et al. 2012).

Nevertheless, while there is promiscuity in the expression of protein markers between EV classes, distinct combinations of markers are used to distinguish between different types of EVs. *Exosomes* are characteristically enriched into endosome-associated proteins [e.g., Rab GTPase, Soluble NSF Attachment Protein (SNAP) receptors (SNAREs), annexins, and flotillin], some of which are involved in MVB biogenesis (e.g., Alix and Tsg101 van Niel et al. 2006). CD63 and CD9, members of the tetraspanin family (Hemler 2003), are also potential markers of *exosomes* (Escola et al. 1998; Bard et al. 2004). Moreover, compared with plasma membrane-derived vesicles, *exosomes* are highly enriched in cholesterol, sphingomyelin, and hexosylceramides, at the expense of phosphatidylcholine and phosphatidylethanolamine (Wubbolts et al. 2003; Laulagnier et al. 2004; Subra et al. 2007; Brouwers et al. 2013). Furthermore, the constituent fatty acids of *exosomes* are primarily saturated or monounsaturated.

General markers of *microvesicles* are less well-defined, perhaps due to the diversity inherent in this class, but recently ADP-ribosylation factor 6 (ARF6) and vesicle-associated membrane protein 3 (VAMP3) have been proposed as potential candidates (Muralidharan-Chari et al. 2009). *Shedding vesicles,* ostensibly a sub-type of microvesicle, typically exhibit high levels of phosphatidylserine and are enriched in lipid raft-associated proteins such as tissue factor and flotillin-1, as well as various selectins and integrins, CD40 ligand, complement receptor-1, and the matrix metalloproteinases (MMP)-2 and -9 (Lee et al. 2011; Théry et al. 2009).

Retrovirus-like particles are less well studied, though Gag protein (together with other endogenous viral proteins) may be a general marker (Bronson et al. 1979; Boller et al. 1993; Mueller-Lantzsch et al. 1993; Dewannieux et al. 2005).

Finally, thrombospondin (TSP), complement subunit C3b and annexin V (all bound by phagocytes for the final clearance), together with histones and fragments of genomic DNA, are generally accepted markers of *apoptotic bodies* (Théry et al. 2009).

Biogenesis of EVs

Exosomes are formed in MVBs (El-Andaloussi et al. 2013), whereas *microvesicles* originate by direct budding from the plasma membrane (Raposo and Stoorvogel 2013). Thus, the overall molecular machineries involved in their formation and release are likely to be different (Fig. 1). Nevertheless, it should be noted that some aspects of their biogenesis might overlap. For instance, it has been suggested that *microvesicle* generation may necessitate factors also involved in *exosome* generation (Nabhan et al. 2012). Specifically, it was observed that a class of microparticles known as arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs) form and bud from the plasma membrane following an interaction between the tumour susceptibility gene 101 (TSG101), an endosome-associated protein implicated in exosome formation, and ARRDC1, localised to the plasma membrane. Moreover, actin-myosin interactions seem to play a critical role in the formation of all four types of EVs described above (Gladnikoff et al. 2009; Piper and Katzmann 2007; Sebbagh et al. 2001; Muralidharan-Chari et al. 2009). For instance, increased phosphorylation of the myosin light chain (MLC) has been shown to promote the actin-myosin contraction force leading to membrane blebbing; inhibitors of the MLC kinase were found to decrease blebbing (Mills et al. 1998).

Exosomes

First identified by Rose Johnstone as a part of the reticulocyte maturation (Johnstone et al. 1987), these EVs were described as being secreted to remove membranes and proteins in a process of *reverse endocytosis*, and for this reason called *exosomes*. The biogenesis and trafficking of *exosomes* is not fully understood. They originate with the invagination of clathrin-coated domains on the plasma membrane, and then enter the cell to be developed by the endosomal network, a membranous compartment that sorts vesicles towards their appropriate sub-cellular destination. The endosomal sorting complex required for transport (ESCRT) machinery (Simons and Raposo 2009; Baietti et al. 2012) is required for transport into early endosomes. Subsequent budding of intraluminal vesicles into the endosomes themselves results in the maturation of the complex into large MVBs. These MVBs are ultimately trafficked to lysosomes for degradation (*degradative* MVBs) or they fuse with the plasma membrane of the cell (*exocytic* MVBs), releasing their intraluminal vesicles, at this stage termed *exosomes*, into the extracellular space.

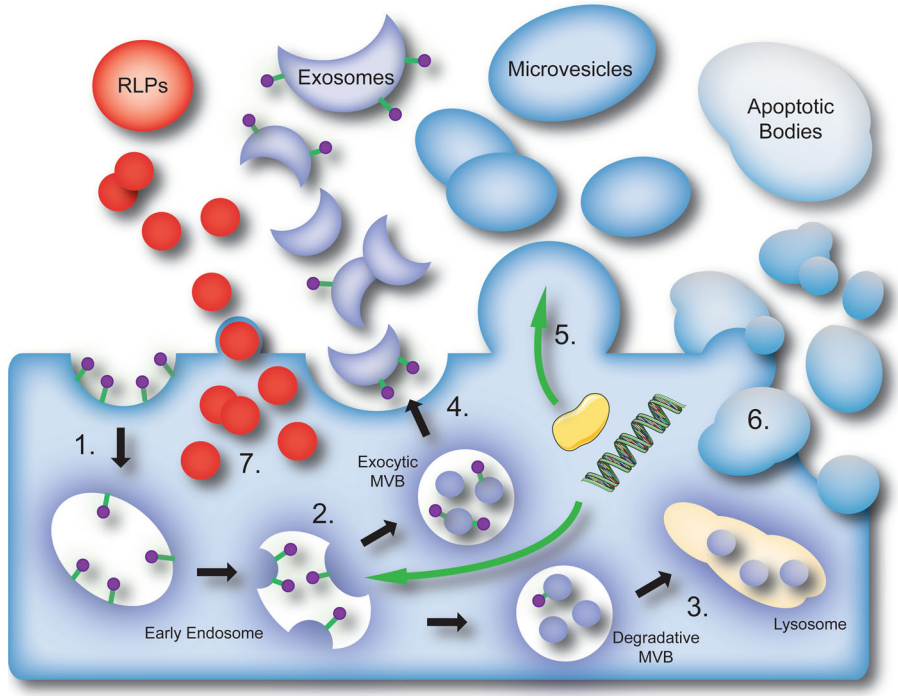

Fig. 1 The four general pathways of membrane vesicle biogenesis. *1* Exosomes arise from an endocytic pathway that begins with the invagination of receptor-coated plasma membrane to form an endosome (endocytic receptors are depicted in *purple*). *2* Intraluminal vesicles bud off into the endosome, passively or actively incorporating bioactive molecules as they do so. *3* The endosome matures into a MVB, which is subsequently destined for either degradation within a lysosome, or *4* exocytosis whereby exosomal EVs are released into the extracellular milieu. *5* Microvesicles (shedding vesicles) arise from direct budding and fission of portions of the plasma membrane, encapsulating a cargo of cytoplasmic proteins (depicted in *yellow*) and nucleic acids from the cytosol as they do so. Variables such as the nature or pathological state of the parent cell will influence the type and contents of EVs. *6* The shrinkage and fragmentation of apoptotic cells gives rise to so-called apoptotic bodies or blebs, *7* while an unknown mechanism believed to involve transcription of endogenous retroviruses leads to the formation of RLPs. (Color figure online)

These latter passages seem to be ESCRT-independent and are instead governed by the distribution of the sphingolipid ceramide and a tetraspannin tertiary structure within raft-based microdomains on the MVB (Trajkovic et al. 2008). This process accounts for the enrichment of ceramide (among other specific lipids and proteins derived from the MVB membrane) observed in *exosomes*, and also for the abundance of endosome-associated proteins such as Alix and TSG101 (Théry et al. 2002b). However, the relative importance of the ESCRT-dependent or -independent mechanisms is not yet fully elucidated. While the fusion of MVBs with the plasma membrane responsible for the release of *exosomes* into the extracellular space is reportedly controlled by Rab GTPases (Hsu et al. 2010; Ostrowski et al. 2010), recently an alternative mechanism for the secretion of Wingless-related integration site (Wnt)-bound *exosomes* was proposed involving the R-SNARE protein YKT6 (Gross et al. 2012).

Microvesicles

The mechanism behind the generation of *microvesicles* is largely unknown. They represent a heterogeneous population of vesicles that are formed by the outward budding and fission of the cell membrane. Secretion of *shedding vesicles* may be controlled by cholesterol-rich lipid rafts in the plasma membrane (Del Conde et al. 2005). Moreover, the asymmetric distribution of proteins and phospholipids is tightly regulated by aminophospholipid translocases (Zwaal and Schroit 1997; Bevers et al. 1999; Leventis and Grinstein 2010). Microvesicle formation is induced by

translocation of phosphatidylserine to the outer-membrane leaflet (Zwaal and Schroit 1997; Hugel et al. 2005), and the budding process is completed through contraction of cytoskeletal structures by actin–myosin interactions (McConnell et al. 2009; Muralidharan-Chari et al. 2009), regulated in turn by the small GTPase ADP-ribosylation factor 6 (ARF6) (Muralidharan-Chari et al. 2009). Acid sphingomyelinases have also been implicated in microvesicle secretion, notably in glia following adenosine triphosphate (ATP) stimulation: upon ATP activation of the P2X7 receptor, acid sphingomyelinases relocate to the outer leaflet of the plasma membrane, immediately preceding microparticle and interleukin-1β (IL-1β) secretion (Bianco et al. 2005). Inhibition or knockout of acid sphingomyelinase was found to reduce or block, respectively, ATP-induced secretion. Interestingly, a recent study provided evidence for the recruitment of TSG101, an ESCRT subunit, to the plasma membrane and into *microvesicles* (Nabhan et al. 2012).

Thus, the molecular machineries for *exosome* and *microvesicle* biogenesis may share mechanistic elements.

Retrovirus-like particles (RLPs)

The origin of *RLPs* is still uncertain. They may arise from transcription of human endogenous retrovirus sequences, which represent approximately 8 % of the human genome but are normally silent. Derepression of such sequences can occur following cellular stress (e.g. cytokine stimulation or cancer) (Depil et al. 2002; Reiche et al. 2010; Golan et al. 2008; Wang-Johanning et al. 2003; Taruscio and Mantovani 2004). *RLPs* arise by directly budding from the plasma membrane with a mechanism involving the interaction of retroviral proteins (i.e. Gag) with components of the plasma membrane (Bieda et al. 2001; Pincetic and Leis 2009) and the cytoskeleton (Gladnikoff et al. 2009). However, their biogenesis is thought to be distinct, even if the size overlaps with *exosomes* and makes difficult the differential purification.

Apoptotic bodies

Whereas other EVs are secreted during physiological cellular processes, apoptotic bodies arise only during programmed cell death. Like shedding vesicles, a flip-flopping process during vesicle *blebbing* results in high levels of phosphatidylserine on their outer surface. These translocated phosphatidylserines bind to Annexin V, which is subsequently recognized by macrophages for phagocytic clearance (Martinez and Freyssinet 2001).

Thus, elucidation of the mechanisms that give rise to the various types of EV (possibly hindered by an unwieldy and inconsistent designation system) is still far from complete. Only with a full knowledge of the molecular machineries required for the EV biogenesis will researchers be able to thoroughly illuminate the specific origins of each class of EV, and to resolve their respective functions.

The functions of EVs

The content of EVs

EVs contain a broad range of molecules, primarily RNAs, proteins and lipids; according to Vesiclepedia (Kalra et al. 2012), a manually curated database of EV contents, 43,731 different proteins, 20,196 different mRNAs, 2,400 different microRNAs (miRNAs) and 342 different lipids have been described at least once within EVs (database accessed 30th Jan 2013). Some of these are found in most EVs, or are specific markers for a particular EV class, while other vary according to the organism, organ, cell-type and condition of the cell of origin (Théry et al. 2009).

Taking the example of *exosomes,* trafficked proteins include the numerous components of the endosomal compartment, such as proteins involved in membrane transport, tetraspannins (e.g. CD9, CD63, CD81), MVB proteins (Alix, Tsg101) and Heat Shock Proteins (e.g. Hsp90).

In addition to proteins, evidence is available that several classes of RNAs can be profiled within exosomes. These include mRNAs, miRNAs, viral RNAs and other non-coding RNAs (ncRNAs) (Belting and Wittrup 2008; Janowska-Wieczorek et al. 2005; Nguyen et al. 2003; Skog et al. 2008; Valadi et al. 2007; Zomer et al. 2010), and in some cases exosomal RNAs have been shown to be intact and functional by means of in vitro translation (Valadi et al. 2007). In 2006, Ratajczak et al. demonstrated that EVs derived from embryonic stem cells are

enriched in mRNA for several early pluripotent transcription factors capable of reprogramming recipient hematopoietic progenitor cells (Ratajczak et al. 2006). Similarly, EMVs derived from human endothelial progenitor cells were shown to be enriched in a specific subset of cellular mRNAs associated with angiogenic pathways, such as the PI3K/AKT and eNOS signalling pathways, thus potentiating them towards triggering an angiogenic program in target endothelial cells (Deregibus et al. 2007). The functional transfer of miRNAs has been demonstrated by Montecalvo et al., who showed that exosomal miR-148a, abundant in exosomes from bone marrow-derived dendritic cells (DCs), could downregulate an artificially-induced miR-148a target sequence in a miR-148-deficient DC2.4 dendritic cell (DC) line (Montecalvo et al. 2012).

In some instances, the repertoire of proteins and RNAs contained within EVs matches closely that of the cell of origin. However, it has also been found that extracellular signalling is able to modulate the RNA and protein content of EVs. For example, it was shown that stress conditions such as hypoxia alter the protein and RNA composition of *exosomes* derived from endothelial cells (de Jong and Verhaar 2012). Levels of the mRNAs N-myc downstream regulated 1 (NDRG1) and BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), stress and apoptosis-related respectively, were significantly upregulated in exosomes from hypoxic cells, whereas cold inducible RNA binding protein (CIRP) mRNA was downregulated. The array of proteins overexpressed in these same exosomes includes lysyl oxidase homolog 2, fibronectin and collagen, suggesting a role in cytoskeletal and extracellular matrix rearrangement. Furthermore, stress conditions, such as heat stress, oxidative stress, or hypoxia, induce the exosomal secretion of heat-shock proteins (HSPs) in several cell types (Clayton et al. 2005; Eldh et al. 2010; Gastpar et al. 2005; Gupta and Knowlton 2007; Lancaster and Febbraio 2005; Taylor et al. 2007; Zhan et al. 2009). Similarly, it has been shown that the content of *exosomes* changes under a diversity of conditions: reticulocyte activation induces changes in proteolipidic composition (Carayon et al. 2011); viral infection results in the trafficking of viral miRNAs, such as the secretion of immunosuppressive miRNAs by Epstein–Barr virus infected B cells (Pegtel et al. 2010); and in response to signalling pathway activation, with proteins such as maspin, cyclophilin A, and phosphoglycerate kinase 1 upregulated in exosomes in a p53-dependent manner (Yu et al. 2006). All these evidences point in the direction that there is a cellular machinery able to sort specific proteins and/or RNAs towards exosomes. As such, a recent work by Villarroya-Beltri et al. has showed that the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) specifically binds to a 4-nucleotide motif present in a subset of miRNAs and mediates their loading into exosomes (Villarroya-Beltri and Gutiérrez-Vázquez 2013), reinforcing the idea the *exosomal cargo* is the result of an active and regulated process. While exosomal miRNA-loading was found to be modulated by changes in hnRNPA2B1 expression, how extrinsic factors might influence this process is still unknown. Moreover, hnRNPA2B1 is also implicated in intracellular trafficking and localisation of specific mRNAs in neurons (Munro et al. 1999) and HIV genomic RNA (Levesque et al. 2006), however the role, if any, of hnRNPA2B1 in loading mRNAs into EVs remains to be elucidated.

Mechanism of EV-mediated cell-to-cell communication

According to the type of EV and to the biological context, different mechanisms of interaction between EVs and target cells have been described, including ligand–receptor interactions, internalisation and direct membrane fusion.

DC-derived exosomes containing MHC-peptide complexes are efficiently recruited by T cell and mediate T cell inhibition without being internalised or fusing with the plasma membrane (Nolte-'t Hoen et al. 2009), providing an example of ligand-receptor interactions. Alternatively, other works have shown that EVs, and in particular exosomes, can also be internalised by target cells via endocytosis and macropinocytosis. For example, circulating exosomes are taken up by DCs, phagocytes of the spleen and Kupffer cells in the liver via clathrin-dependent endocytosis (Morelli et al. 2004), while exosomes secreted by oligodendrocytes can be internalised by microglia via macropinocytosis (Fitzner et al. 2011). Also within the brain, Frühbeis et al. showed that glutamate triggers the release of exosomes from oligodendrocytes, the secretion of which is modulated by Ca^{2+} uptake by *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-

5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Fruhbeis et al. 2013b). Exosomes also play an important role in the signalling between oligodendrocytes and neurons, potentially contributing to the myelination and long-term axonal survival of the latter. Similarly, dedifferentiated Schwann cells are found to secrete exosomes, which are taken up selectively by dorsal root ganglia axons, enhancing regeneration in injury models (Lopez-Verrilli et al. 2013). An additional mechanism of EV uptake that has been described is the direct fusion of the vesicle with the plasma membrane (Del Conde et al. 2005), while a recent work by Christianson et al. showed that heparan sulfate proteoglycans (HSPGs) act as receptors for cancer cell-derived exosomes and are required for their internalisation and function in the target cells (Christianson et al. 2013).

EVs as intercellular mediators of physiology and pathology

Recent works have also started to shed light on the function of EVs in physiology and pathology. One of the earliest insights into EV function dates back to the 1980s, when Johnstone and colleagues described EV secretion by sheep reticulocytes, suggesting that it could be a mechanism of protein clearance during reticulocyte maturation (Johnstone et al. 1987). More recently, most of the research efforts on EV function shifted towards immunology and immunotherapy. In a pioneering work Raposo et al. have shown that B cell-derived exosomes are capable of modulating the immune response by spreading MHC-antigen complexes (Raposo et al. 1996). Subsequently, it was shown that the injection of antigen-bearing exosomes derived from DCs induces the activation of antigen-specific $CD4^+$ T cells in vivo, causing an amplification of the primary immune response (Théry et al. 2002a). Additionally, the EV-mediated transfer of MHC-peptide complexes between DCs and from DCs to T cells enhances T cell activation in vitro (Nolte-'t Hoen et al. 2009; Arnold and Mannie 1999; Bedford et al. 1999; Patel et al. 1999). On the other hand, there are also several works reporting that EVs—and in particular tumour derived exosomes—have an immunosuppressive effect in vitro on T cells and NK cells, and promote the induction of T regulatory cells (Clayton et al. 2007; Andreola et al. 2002; Huber et al. 2005; Szajnik et al. 2010; Zeelenberg et al. 2008; Valenti et al. 2006; Liu et al. 2006). It has been shown that bioactive Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) are expressed in tumour-derived exosomes and induce apoptosis in activated tumour-specific T cells (Iero et al. 2007), while NK cells lose their cytolytic potential through an exosome-mediated inhibition of perforin release (Liu et al. 2006). Furthermore, tumour-derived exosomes are known to impair the capacity of $CD14^+$ monocytes to differentiate into functional DCs, leading to an abundance of $CD14^+$ cells with low levels of expressed human leukocyte antigen-DR (HLA-DR) that serve as myeloid suppressor cells (Valenti et al. 2006). These data support the idea that tumour-derived exosomes might induce immune tolerance and contribute to tumour growth.

Similarly, others have described that several other cell-types also secrete exosomes carrying immune-suppressive agents. For example, exosomes derived from the placenta carry immunosuppressive FasL and UL-16 binding proteins that modulate the activity of maternal cytotoxic T and NK cell, respectively, inducing tolerance toward the foetus (Hedlund et al. 2009; Taylor et al. 2006). Given the amount of evidence supporting both the immune-stimulatory and immune-suppressive role of EVs, their effect is probably very much dependent on the cell of origin (and therefore the content of the EV), on the state of the target cell, and on the biological context in which the interaction between EVs and target cells takes place.

In addition to their immune-modulatory effect, EVs were also shown to be involved in cytokine activity. For example, exosome-like vesicles mediate the release of full-length TNF receptor 1 (Hawari et al. 2004) and are considered a major mechanism through which murine bone marrow derived macrophages (BMDM) secrete Interleukin-1β (IL-1β) (Qu et al. 2007). A similar mechanism has been observed in microglia following stimulation by astrocyte-derived ATP (Bianco et al. 2005). Additionally, EVs mediate the transfer of the chemokine receptor CCR5 from peripheral blood mononuclear cells to cells that do not express it. The efficient infection of cells by the human immunodeficiency virus-1 (HIV-1) requires the presence of CD4 and a specific chemokine co-receptor, a role served by CCR5 in macrophage-tropic (M-tropic) HIV-1 strains. M-tropic HIV-1 is known to infect multiple cell types, however the expression of CCR5

Table 1 A selection of miRNAs identified in EVs as relevant to pathophysiological conditions or putative therapeutic applications

Disease	EV source	miRNA content[a]	Putative effect/target	Reference
Pathological roles of EV miRNAs				
HIV-associated neurodegeneration	Astrocytes treated with pathogenic HIV Tat protein and morphine	miR-29b	Decreased PDGF-B expression and viability in recipient neurons	Hu et al. (2012)
Prion disease	Prion-infected neuronal cells	let-7b, let-7i, miR-128a, miR-21, miR-222, miR-29b, miR-342-3p, miR-424	Increased expression of the cellular prion protein gene	Bellingham et al. (2012a, b)
		(miR-146a)		
Asthma	Bronchoalveolar lavage fluid	let-7 and miR-200 families	Biomarkers of mild nonsymptomatic asthma	Levanen et al. (2013)
Cardiovascular disease	Injured/dying cardiomyocytes	miR-133a	Biomarker; regulation of cardiac hypertrophy	Kuwabara et al. (2011)
	Urine	miR-4516, miR-3183, (miR-3940-5p), (miR-4649-5p)	Biomarkers of salt sensitivity or inverse salt sensitivity index	Gildea et al. (2013)
	Plasma of atherosclerosis patients	miR-150	Reduced c-Myb expression and increased cell migration in recipient microvascular endothelial cells	Zhang et al. (2010)
Liver disease	Serum/plasma	miR-122, miR-155	Biomarkers of alcoholic liver disease and inflammatory liver injury	Bala et al. (2012)
Kidney disease	Urine	(miR-29c)	Biomarker correlating with renal function and degree of histological fibrosis	Lv et al. (2013)
Sjögren's syndrome	Glandular saliva	miR-23a*	Diagnostic/biomarker, salivary gland pathologies	Michael et al. (2010)
Metabolic diseases	Large adipocytes	miR-16, miR-27a, miR-146b, miR-222	Stimulation of lipid synthesis, lipid droplet biogenesis and cell growth in recipient *small* adipocytes	Mueller et al. (2011)
	Blood	miR-130a, miR-195	Biomarkers of hypertension	Karolina et al. (2012)
		miR-197, miR-23a, miR-509-5p	Biomarkers of hypercholesterolemia	
		miR-27a, miR-320a	Biomarkers of type 2 diabetes	
Schizophrenia	Post-mortem prefrontal cortices	miR-497	Putative biomarker; pathogenesis of neoplasms, neurodegenerative diseases and heart disease; promotion of ischemic neuronal death by negatively regulating anti-apoptotic proteins, bcl-2 and bcl-w	Banigan et al. (2013)
Bipolar disorder	Post-mortem prefrontal cortices	miR-29c	Putative biomarker; regulation of cell-adhesion machinery components	Banigan et al. (2013)

Table 1 continued

Disease	EV source	miRNA content[a]	Putative effect/target	Reference
Colorectal cancer	Serum of colorectal cancer patients	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	Biomarkers	Ogata-Kawata et al. (2014)
	Plasma of mice with colorectal cancer xenografts	miR-92a	Enhanced proliferation and motility in recipient endothelial cells, down-regulation of target Dickkopf-3 tumor-suppressive gene	Yamada et al. (2013)
Melanoma	A375 melanoma cells	let-7a, miR-182, miR-221, miR-222, miR-31, miR-19b-2, miR-20b and miR-92a-2, miR-21, miR-15b, miR-210, miR-30b, miR-30d, miR-532-5p, miR-185	Melanoma progression and metastasis	Xiao et al. (2012)
Glioma	Primary human glioblastoma cells	miR-21, let-7a, miR-16	Tumorigenesis, promotes angiogenesis in target human brain microvascular endothelial cells	Skog et al. (2008)
Liver cancer	Hepatocellular carcinoma cells	miR-584, miR-517c, miR-378, miR-520f, miR-142-5p, miR-451, miR-518d, miR-215, miR-376a*, miR-133b, miR-367	Downregulation of transforming growth factor b activated kinase-1 (TAK1) in recipient cells, promotion of hepatocarcinogenesis	Kogure et al. (2011)
Kidney cancer	Renal cell carcinoma	miR-29a, miR-650, miR-151, miR-19b, miR-29c, miR-200c, miR-92, miR-141	Induction of an activated angiogenic phenotype in recipient endothelial cells, formation of a pre-metastatic niche	Grange et al. (2011)
Ovarian cancer	Ovarian tumor cells	miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214	Biomarkers, tumorigenesis	Taylor and Gercel-Taylor (2008)
	Ovarian cancer cell lines	let-7 family	Levels indicative of parent cell invasiveness	Kobayashi et al. (2014)
Prostate cancer	Plasma and serum from prostate cancer patients	miR-375, miR-141	Metastasis	Bryant et al. (2012)
		miR-107, miR-574-3p	Diagnostic biomarkers	
Lung cancer	Plasma from NSCLC patients	(let-7f), (miR-20b), (miR-30e-3)	NSCLC diagnosis and prognosis	Silva et al. (2011)
	Plasma from lung SCC patients	miR-205, miR-19a, miR-19b, miR -30b, miR-20a	SCC biomarkers; oncomiRs	Aushev et al. (2013)
Tumorigenicity of Epstein–Barr Virus (EBV)	Nasopharyngeal carcinoma cells	EBV miRNAs	Angiogenesis, cell proliferation, tumor-cell invasion, and immune evasion in recipient human umbilical vein endothelial cells	Meckes et al. (2010)
Gastric cancer	AZ-P7a metastatic gastric cancer cells	let-7 family	Depletion of tumor-suppressive let-7 miRNA in parent gastric cancer cells, maintaining oncogenesis	Ohshima et al. (2010)
	Gastric cancer tissue-derived mesenchymal stem cells	miR-221	Promotes proliferation and migration in recipient human gastric cancer cells	Wang et al. (2014a, b)

Table 1 continued

Disease	EV source	miRNA content[a]	Putative effect/target	Reference
Breast cancer	Activated tumour-associated macrophages	miR-223	Increases invasiveness of co-cultured breast cancer cells	Yang et al. (2011)
	Breast cancer cell lines	miR-210	Enhanced angiogenesis and induction of a metastatic niche in recipient endothelial cells	Kosaka et al. (2013)
	MDA-MB 231 breast cancer cell line	miR-130a	Tumorigenesis through regulation of TGF-β/Smad signalling	Kruger et al. (2014)
		miR-328	Targets CD44, reducing cell adhesion, enhancing cell migration, and regulating the formation of capillary structure	
	MCF-7 breast cancer cell line	miR-301a	Considered a negative prognostic indicator in lymph node negative invasive ductal breast cancer	
		miR-34a	p53 regulation	
		miR-106b	Downregulation of BRMS1 and RB, promoting breast cancer invasion and metastasis; mediation of TGF-β-induced epithelial-mesenchymal transfer, an early process in tumor metastasis	
	Malignant mammalian epithelial cells	miR-451	Tumour suppression (sequestration thereof); proliferation; cell polarity; dysregulation of oncogenic pathways	Palma et al. (2012)
		miR-1246	Induction of p53 dependent apoptosis	
Cancer (angiogenesis/ metastasis)	Hypoxic K562 leukaemia cell lines	miR-210	Induction of angiogenesis in recipient endothelial cells	Tadokoro et al. (2013)
	Brain metastatic cancer cell lines	miR-210, (miR-19a), (miR-29c)	Biomarkers; potential prognostic agent for brain metastatic breast cancer and melanoma	Camacho et al. (2013)
	Metastatic rat adenocarcinoma	miR-494, miR-542-3p	Downregulation of cadhedrin-17 and concomitant up-regulation of matrix metalloproteinase transcription, preparation of a pre-metastatic niche	Rana et al. (2013)
	K562 leukaemia cells	miR-92a	Reduced expression of integrin α5 in recipient HUVECs, enhancing cell migration and tube formation	Umezu et al. (2013)
Therapeutic potential of EV miRNAs				
Cancer (angiogenesis)	MSCs	miR-16	VEGF down-regulation in recipient tumour cells, anti-angiogenic effect	Lee et al. (2013)
	Highly metastatic A549 adenocarcinoma subpopulation enriched in miR-192	miR-192	Repression of pro-angiogenic IL-8, ICAM and CXCL1 in HUVEC co-cultures in vitro, impairs tumour-induced angiogenesis in bone metastasis in vivo	Valencia et al. (2014)

Table 1 continued

Disease	EV source	miRNA content[a]	Putative effect/target	Reference
Glioma	Marrow stromal cells engineered to overexpress miR-146b	miR-146b	Reduced glioma xenograft growth	Katakowski et al. (2013)
Breast cancer	Human embryonic kidney cell line engineered to express EGFR-binding peptide, lipofected with let-7a	let-7a	Inhibited tumour development in vivo (xenografted breast cancer cells)	Ohno et al. (2013)
Prostate cancer	COS-7 fibroblast-like kidney cells transduced with miR-146a	miR-146a	Knockdown of ROCK1 in recipient PC-3 M metastatic prostate cancer cells, attenuating proliferation	Kosaka et al. 2010
	Epithelial prostate PNT-2 cells	miR-143	Knockdown of KRAS and ERK5 in recipient PC-3 M metastatic prostate cancer cells, attenuating proliferation	Kosaka et al. (2012)
Stroke	MSCs	miR-133b	Enhanced neurite remodelling; Increased functional recovery and neurovascular plasticity in rat stroke models	Xin et al., 2012 Xin et al. (2013a), Xin et al. (2013b)
Multiple sclerosis	Sera of young rats or rats exposed to environmental enrichment	miR-219	Myelin production, oligodendrocyte differentiation	Pusic and Kraig 2014
	IFN-γ stimulated DCs	miR-219	Myelin production, oligodendrocyte differentiation	Pusic et al. (2014)
		miR-106a, miR-124, miR-181a, miR-451, miR-532-5p, miR-665	Anti-inflammatory response	
Atherosclerosis	KLF2-transduced or shear-stressed HUVECs	miR-143/145 cluster	Regulation of smooth muscle cell function, reduced atherosclerotic lesion formation	Hergenreider et al. 2012
Kidney disease	Endothelial progenitor cells	miR-126, miR-296	Protective effects in models of ischemia–reperfusion injury	Cantaluppi et al. 2012

BRMS1 breast cancer metastasis suppressor 1; *DC* dendritic cell; *EBV* Epstein–Barr Virus; *EGFR* epidermal growth factor receptor; *HIV* human immunodeficiency virus; *ERK5* extracellular-signal-regulated kinase 5; *IFN* interferon; *HUVEC* human umbilical vein endothelial cell; *KLF2* Krüppel-like factor 2; *KRAS* V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *MSC* mesenchymal stem cell; *NSCLC* non-small cell lung cancer; *PDGF-B* platelet-derived growth factor beta; *RB* retinoblastoma; *ROCK1* Rho-associated, coiled-coil containing protein kinase 1; *SCC* squamous cell carcinoma; *TGF* transforming growth factor; *VEGF* vascular endothelial growth factor

[a] miRNAs enriched into EVs relative to non-pathological conditions; miRNAs in parentheses are depleted

by target endothelial cells, astrocytes and renal cells is still debated, raising the question as to how such cells become infected. Mack et al. report a potential explanatory mechanism whereby functional CCR5 is transferred via EVs to endothelial cells that do not normally express CCR5 (Mack et al. 2000).

In the last few years it was also reported that EVs can mediate the spread of infections. Wiley and Gummuluru have shown that HIV-1 particles can be endocytosed by DCs and released within exosomes, which in turn can spread the infection to T cells with an efficiency 10-fold higher than cell-free viral particles (Wiley and Gummuluru 2006). Similarly, other works have shown that prion-infected cells release the prion protein (PrP^{C}) and its abnormally folded version scrapie (PrP^{Sc}) inside exosomes, which in turn are able to spread the infection to other cells (Fevrier et al. 2004). In addition to the direct spreading of infections there's also evidence that some pathogens can exploit EVs to modulate their host. For example, Epstein–Barr Virus (EBV)-infected B cells release exosomes that contain viral microRNAs, which in turn are transferred to non-infected cells of the immune system (Pegtel et al. 2010), making this a mechanism by which viruses could potentially modulate the immune response of the infected organism.

As well as being exploited for the spreading of viral infections, EVs were also shown to mediate the intercellular transfer of anti-viral activity. In fact, Li et al. recently showed that IFN-α stimulation of macrophages and liver sinusoidal endothelial cells induces the secretion of exosomes that block Hepatitis B Virus (HBV) replication in infected cells (Li et al. 2013a). This observation suggests the existence of a mechanism that allows uninfected cells to overcome the HBV-mediated blockage of IFN activity in infected cells.

In addition to their role in infections and immunity, EVs can also mediate the acquisition of new functional properties by recipient cells, such as migratory, adhesive or metastatic abilities. For example, in gliomas EVs mediate the transfer of an oncogenic, truncated form of the epidermal growth factor receptor (EGFR) to cells that do not express it, and thus they promote the activation of transforming signalling pathways inducing morphological transformation and promoting growth (Al-Nedawi et al. 2008). Following the discovery that tumour-derived EVs contain oncogenes, other groups have investigated the possibility of using EVs as biomarkers. For example, Skog et al. have found that EVs purified from the serum of glioblastoma patients contain the mRNA for the oncogenic form of EGFR (EGFRvIII) highlighting their potential as diagnostic markers (Skog et al. 2008). In addition to glioblastoma, the diagnostic potential of exosomes is under investigation also for prostate cancer, with various studies having identified altered levels of specific miRNAs in exosomes derived from the serum of prostate cancer patients (Hessvik et al. 2013; Brase et al. 2011; Lodes et al. 2009; Mitchell et al. 2008; Moltzahn et al. 2011). Study into the pathological role of EV-mediated miRNA transfer, and their potential application as disease biomarkers or even therapeutic agents, is a burgeoning field of interest and many potential targets have been identified (see Table 1).

In parallel, numerous works have identified distinct proteins, mRNAs and lipids in exosomes purified from blood or urine of prostate cancer patients, offering additional possibilities for the use of exosomes as disease biomarkers or indicators of treatment efficacy (Soekmadji et al. 2013).

EVs in the brain

The physiological processes of the brain require a highly complex array of intercellular communications between a diversity of cell types over variable distances and time-scales. Mechanisms implicated in neural communications networks include the development of gap junctions, cell adhesion processes, and the secretion of bioactive signalling molecules, neurotransmitters and growth factors. In recent years, mounting evidence has implicated EVs as an additional route of communication within the brain (and, by extension, the broader CNS) (Sharma et al. 2013; Lai and Breakefield 2012; Von Bartheld and Altick 2011). EV secretion has been observed in nearly all cell types that constitute the brain: neurons (Faure et al. 2006; Putz et al. 2008; Schiera et al. 2007), astrocytes (Guescini et al. 2010; Taylor et al. 2007), Schwann cells (Lopez-Verrilli et al. 2013; Lopez-Verrilli and Court 2012), neural stem/progenitor cells (Huttner et al. 2008; Marzesco et al. 2005), microglia (Bianco et al. 2005; Bianco et al. 2009; Potolicchio et al. 2005; Tamboli et al. 2010), oligodendrocytes (Fitzner et al. 2011; Hsu et al. 2010; Trajkovic et al. 2008) and endothelial cells (Simak et al. 2006; Jung

et al. 2009) can communicate with each other within the brain and, by extension, the broader CNS.

EVs released from neurons have been confirmed as being involved in synaptic function (Faure et al. 2006), with their release being stimulated by enhanced glutamatergic activity and resulting in increased spontaneous neuronal activity with the presence of glutamate receptor 2 subunits in EVs (Lachenal et al. 2011). Furthermore, EVs have been shown to regulate the synaptic transfer of Wnt morphogens at the neuromuscular junction (Korkut et al. 2009), hinting at a potential role in broader Wnt-mediated developmental processes. Additionally, EVs are implicated in various mechanisms within the specialised immune system of the brain (Cossetti et al. 2012). Microglia, prime components of the intrinsic brain immune response, secrete EVs exhibiting MHC class II molecules, the expression of which is upregulated upon stimulation with interferon (IFN)-γ (Potolicchio et al. 2005). Thus, they may reflect the non-professional antigen-presenting activity of their progenitor cells. Moreover, EVs of microglial origin are found to propagate inflammatory signals in vitro and in vivo, with cerebrospinal fluid (CSF) levels of myeloid EVs exhibiting a positive correlation with neurodegenerative disease activity. Mice in which EV secretion had been inhibited showed protection against experimental encephalomyelitis, an animal model of multiple sclerosis (MS) (Verderio et al. 2012), and significantly elevated levels of neurotoxic myeloid EVs have been detected in the CSF of Alzheimer's disease (AD) patients (Joshi et al. 2014). These observations highlight the role of microglial EVs as not only markers of neuroinflammation, but also putative therapeutic targets for the treatment of neurodegenerative disease. Like microglial EVs, endothelial cell-derived EV levels are particularly responsive to the immune state of the CNS, with increased secretion under inflammatory conditions making them putative biomarkers of cerebrovascular disorders and neuroinflammatory diseases such as MS (Minagar et al. 2001). Levels of circulating endothelial EVs are being correlated with the severity and prognostic outlook of disease (Simak et al. 2006; Jung et al. 2009), and the EVs themselves are being attributed possible roles in the propagation of inflammation (Chironi et al. 2009; Morel et al. 2011) by stimulating the trans-endothelial migration of monocytes (Jy et al. 2004). Such migration is believed to be facilitated by the binding to and activation of monocytes via a specific phenotypic subset of $CD54^+$ EVs.

Nevertheless, EVs are also believed to serve a protective role with respect to brain injury and regeneration. Trophic support for neurons by oligodendrocytes has been ascribed to exosome-mediated transfer of genuine myelin proteins and stress-protective proteins (Kramer-Albers et al. 2007). Conversely, the neuron-modulated release of auto inhibitory oligodendrocyte-derived exosomes have also been implicated in the inhibition of myelin membrane sheath formation (Bakhti et al. 2011), The cargoes carried by oligodendroglial exosomes, including metabolites, protective proteins, glycolytic enzymes, mRNA and miRNA may serve to maintain axonal integrity (Fruhbeis et al. 2013a). Neuron-derived exosomes are also attributed a role in the sequestration of unwanted Nedd4-family metal cation-transporting proteins during times of stress (Putz et al. 2008), while endothelial cell and astrocyte-derived microvesicles have been found to be enriched in nucleoside triphosphate diphosphohydrolases, imbuing them with the capacity to suppress toxic levels of ATP after an ischemia-related breach of the blood brain barrier (Ceruti et al. 2011). EVs are also found to be a means of degradation of toxic β-amyloid (Aβ) protein, the accumulation of which is implicated as a causative factor in AD, when taken up by microglia; however, pathologic accumulation of Aβ neurons restarts when that clearance pathway is overwhelmed (Yuyama et al. 2012) and EVs are considered to be putative vehicles by which toxic protein aggregates are spread in several neurodegenerative diseases (see below).

EVs in biogerontology

Senescence

With EVs having been attributed a role in intercellular communication, it stands to reason that they too play a significant part in the propagation of senescence/aging-related processes. Indeed, while the study of the role of EVs in aging (at the cellular or organismal level) is still in its infancy, there is evidence that senescent cells do undergo specific changes in EV trafficking, particularly with regards to exosome trafficking.

Cellular senescence, induced by triggers such as shortening of the telomeres, commonly operates via

tumour-suppression pathways, notably the p53 pathway. Upon activation, p53 up-regulates secreted factors such as insulin-like growth factor-binding protein 3 (IGFBP-3, a growth factor regulator), maspin (Yu et al. 2006) and plasminogen activator inhibitor 1 (PAI-1, inhibitors of protease activity in the extracellular matrix), and TSP (an antiangiogenic), all modulators of the microenvironment. Moreover, p53 is known to regulate the transcription of several genes involved in the biogenesis and secretion of exosomes, enhancing the extracellular release of exosomes upon senescence-correlated activation (Yu et al. 2006; Yu et al. 2009). p53 is reported to up-regulate the expression of caveolin-1 and charged MVP protein 4C (CHMP4C), two genes involved in the regulation of the endosomal compartment (Yu et al. 2009; Feng 2010). Caveolin-1, the primary component of caveolae plasma membranes, facilitates endocytosis and internalisation of surface receptors such as EGFR (Yu et al. 2009), while CHMP4C plays a role in MVB formation as a part of the ESCRT-III complex (Saksena et al. 2007). Similarly, p53 stimulates the expression of genes ascribed roles in vesicle secretion, notably tumour suppressor-activated pathway 6 (TSAP6) which has been shown to be integral to competent exosomes release (Lespagnol et al. 2008). Thus, senescence-associated chronic activation of the p53 tumour suppression pathway and the associated up-regulation of an array of auto-, para- and endocrine-acting secreted factors, including exosomes, is implicated in the propagation of the aging phenotype from the cellular to organismal levels. Unfortunately, the contents of senescence-evolved exosomes have yet to be characterised to any significant extent, and therefore the specifics of their function in influencing recipient cells during aging in vivo remains ambiguous. Nevertheless, exosomes have been associated with a number of age-related pathologies, both as diagnostic biomarkers and putative propagators of disease.

Chief amongst these aging-related disorders is cancer. An accumulation of mutations (and diminished genetic repair efficacy) and senescence-induced pro-oncogenic tissue changes during aging results in an exponential increase in the occurrence of cancer in older organisms (Krtolica and Campisi 2002). EVs and their contents are established as biomarkers of a number of cancer types (Vlassov et al. 2012; Principe et al. 2013; Gabriel et al. 2013; Lau et al. 2013; Wittmann and Jäck 2010), as well as being putative agents of tumour cell proliferation and metastasis (Azmi et al. 2013; Simona et al. 2013; Shin-ichiro et al. 2013). Tumour-derived EVs are known to traffic a variety of proteins, mRNAs, miRNAs and metabolites that can promote an oncogenic niche, obstructing immune responses, promoting angiogenesis, and yielding an environment more conducive to tumour cell mobilisation. This age-related increase in cancer risk is manifested largely as an increased incidence of epithelial carcinomas (DePinho 2000), with lung, colon, breast and prostate cancers being responsible for the highest cancer mortalities in the elderly (Cancer Research UK: http://www.cancerresearchuk.org/cancer-info/cancerstats/mortality/age/). However, given the brain-related focus of this review we will focus upon glioma, the most common adult-onset brain tumour (Stoll et al. 2013).

Brain cancer

Glioma increases in incidence with age and exosomes have been implicated in its malignancy. Microvesicles secreted by glioma cells are characteristically enriched in tumour-characteristic miRNAs, and proteins and mRNAs capable of inducing pro-angiogenic phentotypic modulation in target brain endothelial cells and stimulating proliferation in an autocrine manner (Skog et al. 2008). Glioma-derived exosomes were found to contain angiogenin, fibroblast growth factor (FGF)-α, IL-6, IL-8, issue inhibitors of metalloproteinases (TIMP)-1, TIMP-2 and vascular endothelial growth factor (VEGF), angiogenic proteins that are envisioned to exert their biological function on recipient endothelial cells, In addition to angiogenesis, ontology analyses reveal high levels of expression within these glioma EVs of mRNAs involved in cell migration, cell proliferation, immune response and histone modification, all potential avenues through which tumours might modulate their stroma and facilitate growth. As described earlier, these trafficked mRNAs also include the oncogenic variant of EGFR, EGFRvIII, a characteristic biomarker of some clinically distinct glioblastoma subtypes (Pelloski et al. 2007). Glioma-derived EVs are found to promote oncogenic transformation of neighbouring cells via trafficked EGFRvIII which in turn enhanced angiogenesis through induced VEGF expression and a resultant autocrine stimulation of

VEGF receptor 2 (Al-Nedawi et al. 2008), These EVs were are also found shuttle the protein cross-linking enzyme, tissue transglutaminase (tTG), which imbued non-transformed fibroblasts and epithelial cells with cancer-like properties including anchorage-dependent growth and enhanced survival capability (Antonyak et al. 2011). Exosome secretion and tumour aggressiveness are once again seen to increase under hypoxic conditions (Svensson et al. 2011), with an enrichment in exosomal MMP, IL-8, platelet-derived growth factors, caveolin-1 and lysyl oxidase (the increased expression of which have been associated with cancer progression and poor prognosis) relative to normoxic secretions (Kucharzewska et al. 2013). Thus, hypoxic conditions appear to drive cancer cells to modulate their microenvironments via microvesicle secretions, yielding a state more conducive to tumour growth and metastasis (Park et al. 2010).

Neurodegenerative disease

Exosomes are implicated in many facets of neuron-to-neuron, neuron-to-glia, glia-to-glia, and glia-to-neuron communication within the CNS (Fruhbeis et al. 2013b), but from a pathophysiological standpoint they are best characterised as conveyors of inflammatory signals and vehicles by which toxic protein aggregates (or their precursors) are propagated (Schneider and Simons 2013; Vella et al. 2008; Kalani et al. 2013). Cellular and molecular changes that occur during the aging process, such as the accumulation of oxidative damage and diminished adaptive immunity, make the elderly more susceptible to neurodegenerative disease, while impaired neurogenesis limits self-repair (Hung et al. 2010).

AD is the most common form of dementia (Querfurth and LaFerla 2010), and is associated with the accumulation of Aβ peptides into potentially neurotoxic extracellular plaques. The earliest signs that exosomes might be involved in this process came from observations in the 1970s that MVBs were more abundant and larger in cortical dendrites obtained from AD patients (Paula-Barbosa et al. 1978). The Aβ peptide undergoes extensive processing and subcellular trafficking, with the amyloidogenic Aβ42 fragment ultimately accumulating in MVBs (Takahashi et al. 2002) and subsequently being secreted into extracellular space via exosomes (Rajendran et al. 2006). Aβ, its parent protein amyloid precursor protein (APP), and β- and γ-secretases (proteases responsible for cleavage of APP into the Aβ peptide) have all been found enriched in exosomes obtained from AD patients, hinting at the possibility that processing of APP into pathogenic forms might be occurring in the exosomal pathway (Vella et al. 2008). Furthermore, exosomal proteins such as Alix and flotillin-1 have been found in association with plaques in the brains of AD patients, implying a potential role of exosomes in the formation of these deposits (Rajendran et al. 2006). Several reports describe the potential role of EV-associated lipids in shifting the equilibrium between monomeric Aβ units, soluble Aβ oligomers, and insoluble Aβ aggregates. Microglia-derived EVs, levels of which are elevated in AD patients, were found to promote the formation of the neurotoxic, soluble oligomeric form of Aβ from insoluble aggregates (Joshi et al. 2014), further implicating EVs in neurodegeneration. On the other hand, neuron- and astrocyte-derived exosomes have been demonstrated to promote aggregation of monomeric Aβ into insoluble plaques, suggesting that the parent cell-dependent lipid composition may influence an EV's effect on Aβ aggregation (Dinkins et al. 2014; Yuyama et al. 2008). Indeed, exosomes may present a clearance mechanism by which potentially pathogenic deposits are shuttled to microglia for degradation (Yuyama et al. 2012) or degraded by EV-shuttled proteases such as the insulin degrading enzyme (Tamboli et al. 2010). It is also noteworthy that microvesicles isolated from the cerebrospinal fluid of AD patients exhibit some 60 miRNAs that are differentially expressed relative to healthy controls, however the significance of these specific markers has yet to be established (Cogswell et al. 2008). Hyperphosphorylation of the tau microtubule-associated protein results in the disruption of tau's normal axonal transport function as well as the formation of neurofibrillary tangles and toxic species of soluble tau, and these effects have been associated with a number of neurodegenerative diseases, including AD. Indeed, secretion and interneuronal transfer of toxic tau species is believed to play a role in the spread of AD lesions, and selectively phosphorylated tau has been shown to be actively secreted via exosomes into the CSF during early stages of the disease, not just as refuse from dying neurons (Saman et al. 2012).

Parkinson's disease (PD), the second most common neurodegenerative disease after AD, is also

characterised by the accumulation of protein aggregates (Lees et al. 2009; Russo et al. 2012). The pathological progression of the disease, selective degeneration of dopaminergic neurons in the substantia nigra pars compact, is accompanied by the formation of Lewy bodies, deposits primarily consisting of fibrillar α-synuclein (α-Syn), in surviving neurons (Danzer et al. 2012). While the exact mechanism of PD pathogenesis is yet to be elucidated, toxic α-Syn aggregates are implicated and intercellular transport of α-Syn from overexpressing neurons to recipient neuronal cells has been observed (Russo et al. 2012; Danzer et al. 2012). Excess α-Syn secreted by neurons can be phagocytised by astrocytes and microglia in a putative waste clearance mechanism (Lee et al. 2010; Lee et al. 2008), however excessive accumulation in these recipient cells can lead to the formation of inclusions and trigger an inflammatory response (Vekrellis et al. 2011; Lee et al. 2010; Halliday and Stevens 2011). α-Syn can be transferred between neurons, result in aggregation within the recipient neurons, inducing cell death (Desplats et al. 2009; Hansen et al. 2011; Emmanouilidou et al. 2010; Schneider and Simons 2013). Exosomes are known to play a role in this intracellular transfer and propagation of α-Syn (Alvarez-Erviti et al. 2011a; Emmanouilidou et al. 2010; Schneider and Simons 2013) in an active, energy-dependent manner (Bellingham et al. 2012b; Aguzzi and Rajendran 2009). Moreover, it is reported that α-Syn oligomers that are associated with exosomes are more likely to be taken up by recipient cells and are more toxic than free α-Syn (Danzer et al. 2012). PD has been linked to mutations in a number of genes involved in the endosomal-lysosomal pathway, thus it might be speculated that resultant alterations to vesicle secretion and trafficking mechanisms might play a role in disease progression (Russo et al. 2012; Schneider and Simons 2013). One such example is leucine-rich receptor kinase 2 (LRRK2), which is involved in exosome secretion (Alegre-Abarrategui et al. 2009; Dihanich and Manzoni 2011; Shin et al. 2008; Piccoli et al. 2011); mutations to this protein yield abnormally large MVBs which may potentially release large numbers of exosomes bearing α-Syn (Alegre-Abarrategui et al. 2009; Russo et al. 2012).

Like AD and PD, prion disease is associated with misfolded proteins and is more prevalent in the elderly. Indeed, the neuropathology implicated in the various types of prion disease may coexist with the protein aggregates described above (Kovacs and Budka 2002). Prion disease is a fatal, transmissible neurodegenerative disease that involves the conversion of the prion protein PrP^{C} into an abnormal, misfolded and protease-resistant, pathogenic isoform, PrP^{Sc} (Brown and Mastrianni 2010). The infectious form of the disease begins in the periphery before spreading to the brain via a yet unknown mechanism (Fevrier et al. 2004). Once acquired, the toxic form of the protein catalyses conversion of the non-toxic form to the pathogenic state, however the means of dissemination of the disease was, until recently, a mystery since no plausible vector by which PrP^{Sc} could spread to uninfected tissue could be identified (Vella et al. 2007). Experiments demonstrating that the culture medium of infected cells was itself infectious hinted at an extracellular route of transmission, with subsequent characterisations revealing exosomes to be the likely carriers of the toxic prion (Vella et al. 2007; Alais et al. 2008). PrP^{Sc}-laden exosomes from infected cells were found to be of greater density than those from healthy cells, containing only the PrP^{C} form, due to the formation of toxic protein aggregates (Vella et al. 2007). Moreover, exosomes from infected neuronal cells have been described as being more spherical in shape, but diverse in size and internal structure (Coleman et al. 2012), while infected platelets are described as releasing PrP^{Sc} via both microvesicles *and* exosomes (Robertson et al. 2006). In the case of the neuronal cell line, prion packaging into exosomes is believed to involve N-terminal modifications to a distinct subtype of PrP glycoforms (Vella et al. 2007).

Therapeutic applications of EVs

Although the significant and broad role played by EVs has only recently come to receive due attention, and is still far from being thoroughly elucidated, the therapeutic potential of these extracellular delivery vectors is already under intense investigation. Numerous studies have demonstrated the in vivo and in vitro loading of EVs with a diversity of drugs, enzymes, genes and RNAi agents and, furthermore, seen their subsequent application as putative therapeutic vectors in a variety of disease models. Therapeutic applications of EVs are perhaps most promising within the CNS, where conventional drugs have traditionally

exhibited low efficacy due to a number of biological barriers to their delivery. The tissue/cell specificity and low immunogenicity of biogenic EVs, coupled with an appropriate cargo of bioactive molecules (be they naturally derived or artificially loaded), makes for a potent therapeutic vector in the treatment of neurodegenerative disease and brain cancers for which age is a prominent risk factor.

Therapeutic potential of EVs in brian repair

Delivery of therapeutic agents into the brain is a challenging task due to the major obstacle of the blood–brain barrier (BBB). Numerous studies have shown the advantages of biological EVs for brain repair (Lakhal and Wood 2011; Zhuang et al. 2011; Alvarez-Erviti et al. 2011b), since they possess the ability to cross BBB, as well as ability to deliver therapeutic cargoes, inherent targeting ability to certain cell types, and immune tolerance. EVs often manifest selective cell homing that, like many key EV features, is often specifically derived from the parent cell. Furthermore, they possess effective protective ability for bioactive cargoes including mRNA, siRNA, miRNA, proteins and drugs, thus making them potential natural vehicles in drug delivery system. Furthermore, advances in genetic engineering allow for the functionalization of otherwise naturally occurring EVs to enhance e.g. their targeting capability or to bolster their therapeutic cargoes. These properties lend themselves to the development of EV-based cell free therapies for brain diseases.

Naturally occurring EVs have innate therapeutic potential due to their diversified bioactive cargoes, thus making them novel candidates as cell-free therapy. Yu et al. describe the isolation of a sub-class of DC-derived exosomes expressing TGF-β1 in their membranes which purportedly exert a potent immunosuppressive effect capable of inhibiting the development and progression of experimental autoimmune encephalomyelitis (EAE) in recipient mice when delivered systemically (Yu et al. 2013). The importance of the host cell type can be seen in the experiments described by Hajrasouliha et al. wherein exosomes obtained from retinal astroglial cells (RACs) were able to suppress retinal vessel leakage and inhibit choroidal neovascularisation, whereas exosomes from retinal pigmental epithelium were not (Hajrasouliha et al. 2013). The anti-angiogenic properties of these RAC-derived EVs were attributed to the exclusive presence of endogenous angiogenesis inhibitors in those exosomes.

There is considerable interest in EV-based RNA-interference (RNAi)-based therapy, with EVs representing an ideal platform for RNA delivery, opening a new route for gene modulation. Recent reports have demonstrated that systemic administration of exosomes derived from mesenchymal stem cells (MSCs) promoted neurovascular remodelling and functional recovery after stroke in rats (Xin et al. 2013a). The authors' initial hypothesis, that the MSC-derived exosomes were exerting this neurological recovery via transfer of miR-133b, known to enhance neurite remodelling and be at high levels in MSC-derived exosomes (Xin et al. 2012), was supported by follow-up experiments (Xin et al. 2013b). Exosomes were found to transfer miR-133b to neurons and astrocytes, and cause a knockdown in the expression of connective tissue growth factor and ras homolog family member A at the ischemic boundary zone in rat stroke models, enhancing functional recovery. The application of EVs as drug delivery vehicles is another focus of developing EVs as therapeutics. For instance, Sun et al. were the first to devote their efforts to load curcumin, a polyphenol anti-inflammatory compound, into EVs derived from EL-4 lymphoma lines, with this *exosomal curcumin* affording protection against LPS-induced inflammation in mice through delivery to activated myeloid cells (Sun et al. 2010). Remarkably, the same group reported intranasal administration of *exosomal curcumin* or exosomal JSI-124, a signal transducer and activator of transcription 3 (Stat3) inhibitor, could across BBB and resulted in suppression of a range of inflammation-driven disease models, including LPS-induced inflammation, myelin oligodendrocyte glycoprotein-induced EAE and GL26 glioma (Zhuang et al. 2011).

Despite the promising properties of naturally occurring EVs, there is considerable interest in improving their therapeutic utility through genetic engineering, with the goals of improving specificity or enrichment in the bioactive cargo(es) of interest. Genetic engineering of EV producer cells or direct modification of the EVs themselves, with insertion of therapeutic agents into the lipid layer or loading into their aqueous core, are proposed as means to modulate the specificity and activity of EVs as targeted delivery vehicles (Lai et al. 2013).

The inspiration for the targeted delivery of EVs was perhaps first envisioned in EV-mediated immunotherapy (Trumpfheller et al. 2012), particularly for cancer vaccines, EVs have been investigated to pulse DCs with antigens to activate an immune response against tumor cells (Tan et al. 2010). Work by Viaud et al. showed that DC-derived highly immunogenic, clinical grade EVs expressing CD40, CD80, CD86, and ICAM-1 on their membranes could prime $CD8^+$ T cells in a peptide-dependent manner (Viaud et al. 2011). By utilizing the ligand-receptor interactions, studies have demonstrated EVs that express ICAM-1 can bind DCs and T cells, while EVs from B cells that carried selected galectins can target T cells (Théry et al. 2009). These encouraging findings laid the foundation for further steps into targeting EVs to brain tumours (Lai and Breakefield 2012) and CNS inflammatory disease. The combinatorial EV-based therapy (El-Andaloussi et al. 2013) that couples DC-derived EVs presenting tumour antigens to T cells with tumour-targeted EVs loaded with RNAi effectors is also expected to be a potent therapeutic approach to CNS diseases.

The first proof-of-concept for applying modified EVs in targeted drug delivery for the brain was from the work done by Alvarez et al., in which the host DCs were engineered to express Lamp2b fused to the neuron-specific peptide rabies virus glycoprotein (RVG), imbuing the daughter EVs with BBB-traversal capabilities and facilitating their subsequent uptake into neurons, microglia and oligodendrocytes. These engineered EVs were able to deliver siRNA into the mouse brain where they achieved strong knockdown of beta-site APP cleaving enzyme 1 (BACE1) mRNA and protein, a therapeutic target for AD (Alvarez-Erviti et al. 2011b). This proposed method, along with identification of targeting peptides selectively binding to the cell type or tissues of interest in the brain, is a significant step towards realising the therapeutic potential of EVs, particularly as RNAi-delivery platforms (El-Andaloussi et al. 2012). In addition, strategies utilised in the modification of artificial nanoparticles, such as utilizing monoclonal antibodies complementary to receptors that are naturally expressed on the BBB (Roberts et al. 1993) or inflamed tissues, could be adopted for EV modification.

Despite the modification of EVs for targeted drug delivery, the target loading of cargoes into EVs is also addressed as an important issue requiring further development. For the loading of nucleic acids, several strategies including transfection-based approaches and electroporation have been utilized. The basic idea of transfection method is to construct suitable expression vectors that can be transfected into donor cells and finally induce overexpression of desired short RNAs enriched into EVs (Kooijmans et al. 2013). Several studies have evidenced successful loading of siRNAs and miRNAs into EVs with constructed vectors as well as utilizing transfection reagents (Olson et al. 2012; Zhang et al. 2010b; Kosaka et al. 2010; Ohno et al. 2013). For instance, in a rat model of primary brain tumour, exosomes derived from MSCs engineered to over-express anti-tumour miR-146b significantly reduced glioma xenograft growth upon intra-tumour injection (Katakowski et al. 2013a). Interestingly, synthetic spherical nucleic (SNAs) acids endocytosed into PC-3 prostate cancer cells were naturally sorted into exosomes to a small degree (<1 %), while transmission electron microscopy results indicated SNAs were internalized into exosomes as well as bound to the membrane surface (Alhasan et al. 2014). Nevertheless, questions remaining in the transfection-based approach revolve around not only the level of desired small RNAs enriched in EVs independent of sequences (Batagov et al. 2011), but also the changing encapsulation process and behaviour of EVs (Kooijmans et al. 2013). As for the electroporation method, loading efficiency may vary among sequences of small RNAs (Kooijmans et al. 2013). Wahlgren et al. showed up to 85.2 % of EVs loaded with exogenous siRNA successfully induced gene knockdown in monocytes or lymphocytes (Wahlgren et al. 2012). Alvarez-Erviti et al. demonstrated that RVG-exosomes loaded with approximately 25 % of the electroporated siRNA induced up to 60 % mRNA and protein knockdown, predominantly in the midbrain, cortex and striatum (Alvarez-Erviti et al. 2011b). However, there is a debate about the efficiency of electroporation. Kooijmans et al. argued that electroporation is far less efficient than previously described since electroporation of EVs with siRNA is accompanied by extensive siRNA aggregate formation, which may cause overestimation of the amount of siRNA actually loaded into EVs (Kooijmans et al. 2013). Therefore, there is an urgent need to develop efficient approaches to load exogenous cargoes into EVs. Such efforts could be devoted to synthesizing EV-targeted vectors, as well

as screening RNA targeting to EVs, since multiple motifs were found specifically enriched in secreted RNAs (Batagov et al. 2011) and a "zip code-like" sequence may direct mRNAs targeting into EVs (Bolukbasi et al. 2012).

Nanotherapeutic synthetic analogues of EVs

The exploitation of natural EVs and their biogenic cargoes as therapeutic agents is a very promising avenue of cellular medicine research. As potent vehicles by which to deliver potentially therapeutic miRNAs and proteins to dysfunctional cells, microvesicles may prove invaluable in treating a wide variety of diseases and disorders, including those that are age-associated. Advances in synthetic biology and genetic engineering will further progress our ability to evolve and develop these therapeutic delivery platforms with tailored contents and targeting, but current applications of natural exosomes are limited (Koppers-Lalic et al. 2013; Kosaka et al. 2013; Lai et al. 2013; Munoz et al. 2013; Tan et al. 2013; van Dommelen et al. 2012; Kalani et al. 2013). A number of factors need to be carefully considered in the application of naturally occurring EVs. The composition and contents of the vesicles can be complex and difficult to characterise which may confound predictions of in vivo activity; the abundant and diverse bioactive contents of vesicles may exert a plethora of effects, intended and unintended. Furthermore, obtaining pure microvesicle preparations can be tedious, and scalable production of vesicle from mammalian cells is problematic due to low yields (van Dommelen et al. 2012; Lakhal and Wood 2011). Thus, while advances continue in modulating the expression, composition and contents of natural microvesicles towards more efficacious therapeutic activity, parallel efforts are being made in the development of biomimetic or synthetic drug-delivery platforms (Kooijmans et al. 2012).

There have been a number of novel approaches to generating artificial vesicles that are nonetheless cell-derived, including stem cell *nanoghosts* and nanovesicles (Jo et al. 2014; Toledano Furman et al. 2013; Jang et al. 2013). However, the recent emergence of nanomedicine, the utilisation of cell- and molecule-specific interactions for medicinal applications, has led to the adoption of a plethora of diverse technologies and synthetic constructs as putative platforms for the cellular delivery of therapeutic and diagnostic agents (Tennyson and Clemens 2012; Duncan and Gaspar 2011; Devadasu et al. 2013; Ganta et al. 2008; Collet et al. 2013; Gao et al. 2013). The makeup of these *nanovehicles* spans a broad range of physical and chemical compositions (see Fig. 2), including liposomal and polymersomal EMV analogues (Akbarzadeh et al. 2013; Christian et al. 2009; Lee and Feijen 2012; Theresa and Pieter 2013), micelles (Deng et al. 2012; Xu et al. 2013), polymer, protein and lipid complexes (Zia ur et al. 2013; Wasungu and Hoekstra 2006; Ge et al. 2012; Zhang et al. 2012), dendrimers (Deng et al. 2012; Zhu and Shi 2013), and nucleic acid-based nanoparticles and nanostructures (Shu et al. 2014; Roh et al. 2011). Beyond these biomimetic and bioinspired delivery platforms, there has been also considerable interest in the use of surface-functionalised inorganic nanoparticles, nanocrystals, nanotubes and quantum dots for nanomedicinal applications. Such agents are thoroughly reviewed elsewhere (Sekhon and Kamboj 2010a; Malmsten 2013; Rajendra and Hae-Won 2013; Son et al. 2007a, b; Sekhon and Kamboj 2010b) but are beyond the scope of this review. Below we describe some of the synthetic nanoscale drug-delivery systems most reminiscent of biological EMVs, and provide examples of their application in the treatment of age-related disease.

The *nanovehicles* most resembling natural microvesicles are liposomes and polymersomes. Both are synthetic vesicles of adjustable size (typically tens to hundreds of nanometres in diameter), usually enclosing and protecting an aqueous compartment, however the membrane of the former consists of a lipid bilayer (typically comprised of phospholipids) while the latter is self-assembled from amphiphilic block copolymers (Chandrawati and Caruso 2012; LoPresti et al. 2009; Allen and Cullis 2013). Hydrophilic cargoes are enclosed within the aqueous compartment, whereas hydrophobic species can be sequestered within the membrane; the vesicles, like all drug delivery vehicles, serve as vectors by which to enhance drug pharmacokinetics, uptake, stability or solubility, or as a means to mask the (off-target) toxicity of the cargo. Both classes of vesicle are tailorable in composition, however liposomes generally benefit from a high biocompatibility and a soft and fluid bilayer, which can facilitate direct interaction with cell membranes, whereas polymersomes typically possess a greater mechanical and chemical stability making them

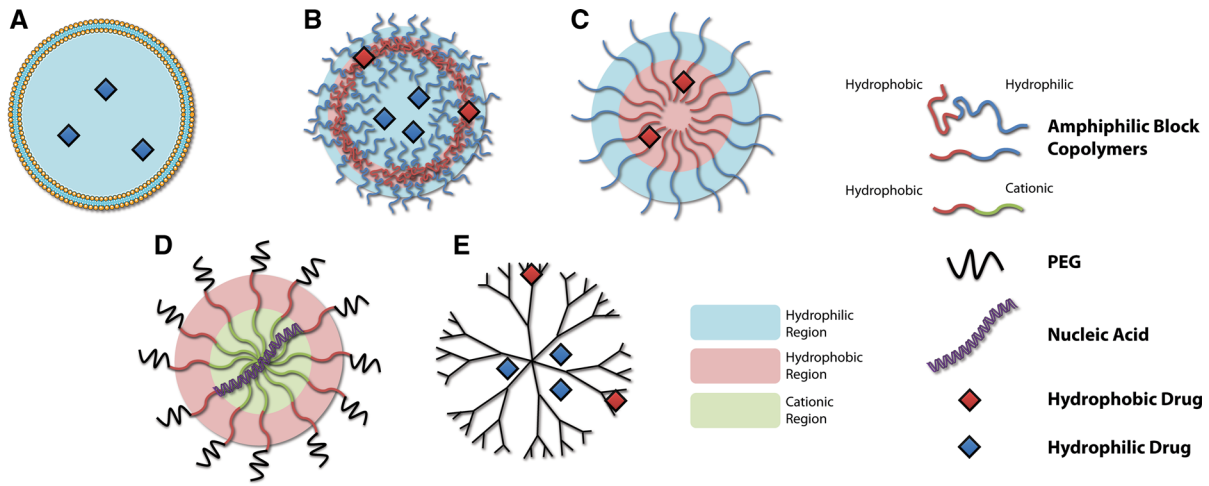

Fig. 2 Types of synthetic EV analogue nanovehicles. Liposomes (**a**) are membrane bilayers enclosing an aqueous/hydrophilic interior. Polymersomes (**b**) are comprised of amphiphilic block copolymers that self-assemble into a sphere with a hydrophobic layer sandwiched between a hydrophilic core and surface. Micelles (**c**) also consist of amphiphilic block copolymers, but assembled into a sphere with a hydrophobic core and hydrophilic exterior. Polyplexes (**d**), like their lipid or protein-based analogues, complex polyanionic nucleic acids via electrostatic interactions with cationic polymers. Dendrimers (**e**) are unimolecular, branched spherical assemblies with dense, hydrophobic surfaces but relatively empty pockets nearer the core in which to encapsulate drugs. (Color figure online)

potentially more robust in regards to functionalization. While some liposomes can enter cells via direct membrane fusion (dependent on liposome membrane composition) (Lee et al. 2005), many liposomes and most polymersomes are proposed to enter cells via an endocytic pathway; passively targeted vesicles are believed to be taken up through pinocytosis (or phagocytosis) whereas those actively targeted towards a specific cellular surface marker benefit from receptor-mediated endocytosis (Allen and Cullis 2013; Templeton 2002; Christian et al. 2009).

Early liposomes applications involved the use of neutrally-charged vesicles for the delivery of proteins and drugs (Gregoriadis and Ryman 1971), and later genes (Tai-Kin et al. 1980). More recently, cationic PEGylated (that is, coated in bio-inert poly(ethyleneglycol)) liposomes have been employed as the du jour standard for greater transfection ability and biocompatibility (Collet et al. 2013; Boado 2007), and liposomal cargoes have expanded to include RNAi agents (Spagnou et al. 2004; Kanasty et al. 2013; Buyens et al. 2012). Complexes of cationic lipids and polyanionic nucleic acids are sometimes referred to as *lipoplexes* (Wasungu and Hoekstra 2006; Zhang et al. 2012). Over the years, there has been considerable research into further optimising the pharmacokinetics of liposomes and improving the encapsulation extent, release rate, and *intra*cellular delivery of liposome-delivered therapeutics (Allen and Cullis 2013). Through appropriate modification and functionalization of the lipidic membrane, liposomes can be engineered to release their contents under appropriate trigger conditions, such as a specific pH range, elevated temperatures, irradiation, sonication, or enzymatic degradation (Allen and Cullis 2013). Furthermore, there have been extensive efforts to develop actively targeted liposomes through the attachment of cell-specific ligands, or their incorporation into the lipid formulation, with the intent of enhancing drug delivery to the tissue of interest. This is typically approached by using monoclonal antibodies (mAbs) to direct so-called *immunoliposomes* against surface receptors on the cells of interest (Noble et al. 2014), however enzymes (Blume et al. 1993), small molecules (Lee and Low 1994), and nucleic acid aptamers (Cao et al. 2009) have also been employed. Nevertheless, the efficacy of targeted liposomes has to date generally not been considered sufficiently improved over passively-targeted liposomes to warrant the extra preparative work and cost (Allen and Cullis 2013).

Liposome-drug formulations have been approved for clinical applications, especially the delivery of anti-cancer chemotherapeutics via a variety of administrative routes, or are presently in clinical trials (Allen

and Cullis 2013). Indeed, PEGylated, doxorubicin-loaded liposomes—Doxil—became the first nanomedicine to be approved by the FDA in 1995 (Gabizon et al. 1994; Barenholz 2012). Of specific relevance to age-related diseases, liposome-delivered doxorubicin has also been approved for use in treating early and metastatic breast cancers (Lao et al. 2013), with HER2-targeted (Hendriks et al. 2013) and hyperthermia-triggered (Staruch et al. 2011) liposomes undergoing clinical testing as delivery vectors. Anti-tumour applications are not restricted to the delivery of doxorubicin: for instance, liposomes have been used to deliver paclitaxel to breast cancers (Fasol et al. 2012), cisplatin and anti-MUC vaccines in non-small cell lung cancers (Fantini et al. 2011; Bradbury and Shepherd 2008), irinotecan (CPT-11) to colon and breast cancers (Drummond et al. 2006), and combinatorial treatments (irinotecan and floxuridine) in colorectal cancers (Batist et al. 2008). Overexpression of miR-7 in tumour models through the liposomal delivery of a miR-7 plasmid was found to lead to the suppression of EGFR tyrosine kinase inhibitor-resistance in lung cancer cells (Rai et al. 2011), while *immunoliposomes* targeted towards human insulin receptor and mouse transferrin receptor (TfR) delivered an anti-EGFR shRNA plasmid, knocking down EGFR expression and increasing survival in murine glioma models (Zhang et al. 2004). Liposome-based treatments for cardiovascular disease are also undergoing clinical trials, with the vesicles being employed as vehicles to deliver RNAi-based therapeutics, such as anti-proprotein convertase subtilisin/kexin type 9 (PCSK9) siRNA for tackling hypercholesterolemia (Jayaraman et al. 2012).

To date, liposomes have received relatively little attention as therapeutic delivery agents for the treatment of neurodegenerative disease. Some success has been achieved with directing liposomes across the BBB using receptor-mediated transcytosis and appropriate mAbs (e.g. the insulin and TfR antibodies mentioned above) (Boado 2007). Therapeutic outcomes were achieved in rats with experimental PD by delivering TfR-targeted liposomes across the BBB and into neurons, delivering a glial-derived neurotrophic factor (GDNF) plasmid. Expression of the GDNF was restricted to catecholaminergic neurons by means of a region-specific tyrosine hydroxylase promoter, with dosed GDNF expression having trophic effects in dopaminergic neurons (Xia et al. 2008). Most applications of liposomes in the treatment of PD are symptomatic, with efforts devoted to developing optimal pharmacokinetics of L-DOPA and analogues (Spuch and Navarro 2011; Di Stefano et al. 2006). A number of liposomes have been developed as putative AD therapeutics, with a common approach being the use of the vesicle to target (via specific membrane lipids, antibodies or curcumin) and sequester potentially toxic extracellular Aβ (Bereczki et al. 2011; Canovi et al. 2011; Gobbi et al. 2010; Taylor et al. 2011; Mourtas et al. 2011). Intranasal delivery of liposomal formulations of rivastigmine, an acetyl cholinesterase inhibitor used in AD treatment, have been found to exhibit a longer in vivo half-life and effect higher drug concentrations in the brain than the free drug (Mutlu et al. 2011; Arumugam et al. 2008). Furthermore, liposomes have been employed as vaccination vectors against protein misfolding diseases such as AD, by delivering short peptides mimicking pathological epitopes of Aβ or Tau with the intent of eliciting a robust and specific antibody response against the toxic form of the peptide and a subsequent clinical improvement in disease models (Hickman et al. 2011; Muhs et al. 2007; Nicolau et al. 2002; Theunis et al. 2013).

Polymersomes differ from liposomes in the nature of their membrane composition, with the coblock polymers of polymersomes yielding a thicker, more robust membrane (Christian et al. 2009). Accordingly, the membranes of polymersomes are generally considered to be more amenable to modification and functionalization, with many more examples of vesicles with triggered-release mechanisms. This commonly takes the form of a membrane, which degrades in the acidic environment of the endosome, ensuring that large quantities of the polymersome's therapeutic cargo are delivered into the cytosol (LoPresti et al. 2009). Other vesicles are engineered to release their payload under external stimuli such as UV irradiation, elevated temperature, or appropriate redox conditions (Rijcken et al. 2007; Lee and Feijen 2012). Polymersomes are commonly PEGylated to enhance pharmacokinetics and circulation half-lives, as per their liposomal counterparts, and active targeting is accomplished through the incorporation of antibodies, peptides and small molecule ligands into the external membrane (Christian et al. 2009; Lee and Feijen 2012). One novel example of active targeting involves the incorporation of polyguanylic acid, thus targeting the polymersomes

to the macrophage scavenger receptor A1, upregulated in activated tissue macrophages (Broz et al. 2005). Also like liposomes, polymersomes have been most extensively investigated as a means to deliver anti-cancer chemotherapeutics; doxorubicin is once again the archetypical cargo (Waterhouse et al. 2001), however the thick hydrophobic membranes of polymersomes facilitates co-delivery of a more lipophilic drug such as paclitaxel as well (Ahmed et al. 2006). Other putative payloads include genes, RNAi agents, and proteins/enzymes that, with the optimal polymersome composition, remarkably maintain their structure and activity when incorporated into the vesicle membrane or when encapsulated within (Christian et al. 2009). The relatively recent development of polymersomes means that few thoroughly proven examples of their therapeutic utility are available. Nevertheless, they show promise in preliminary applications, including systems of relevance to age-related disease. Polymersomes functionalised with the PR_b peptide, a fibronectin mimetic targeting $\alpha_5\beta_1$ integrin, were developed to deliver siRNA down regulating the Orai3 calcium channel protein in breast cancer cells, inducing cell death (Pangburn et al. 2012). Likewise, incorporation of hyaluronic acid into the external membrane of polymersomes has been found to enhance their delivery to CD44-overexpressing breast cancer cells (Upadhyay et al. 2010), with potential applications in other CD44-overexpressing cancers such as glioma (Knupfer et al. 1999). Transferrin and lactoferrin have also been employed in targeting doxorubicin-laden polymersomes towards glioma, in vitro and in vivo (Pang et al. 2011; Pang et al. 2010). Lactoferrin-bearing polymersomes were also found to cross the BBB to deliver the neuroprotective peptide S14G-humanin to the neurons of Aβ-treated rats, with a protective effect (Yu et al. 2012). Similarly, polymersomes modified with the TfR antibody OX26 and loaded with NC-1900, a vasopressin fragment analogue known to improve spatial memory impairment, crossed the BBB and accumulated in the brain of scopolamine-lesioned rats, which subsequently performed better in the Morris water maze test (Pang et al. 2008). Studies with neurotensin-modified polyplexes have demonstrated that neurotrophic genes such as GDNF can be delivered with high specificity to neurotensin receptor 1-expressing dopaminergic neurons, a potential therapeutic avenue in PD (Martinez-Fong et al. 2012). More generally, polymersomes functionalised with imaging moieties are also being investigated in the role of diagnostic probes, through the delivery of fluorophores or magnetic resonance imaging (MRI) contrast agents (LoPresti et al. 2009; Levine et al. 2008; Pourtau et al. 2013; Chiang et al. 2013).

Micelles, monolayered spherical arrangements of lipids or (more typically in therapeutic applications) amphiphilic block copolymers, typically with a hydrophobic core, have seen therapeutic applications in the delivery of poorly water-soluble drugs (Xu et al. 2013). For instance, PEGylated polylactide-based micelles loaded with the hydrophobic drug paclitaxel (Kim et al. 2004) are approved for clinical use in the treatment of breast, lung, and ovarian cancers in South Korea (and undergoing Phase III trials elsewhere) as Genexol-PM. Many other micelle formulations, incorporating a diversity of different chemotherapeutics, are undergoing clinical trials for treating a variety of cancers (Deng et al. 2012). Combinatorial approaches are also being investigated; for instance, micelles loaded with a combined payload of doxorubicin and lapatinib yielded an enhanced doxorubicin uptake in drug-resistant breast cancer cells in vitro, and reduced tumor growth relative to doxorubicin monotherapy in vivo (Wang et al. 2014a). Micelles (polyplexes) have also been employed as nucleic acid delivery agents, typically through incorporation of cationic polymers by which to complex the polyanionic biopolymers (Liu et al. 2013) (Jeong et al. 2011). MRI-active micelles have been used to deliver plasmid DNA into the brain via a compromised BBB after intranasal delivery in mice with traumatic brain injury (Das et al. 2014), while micelles functionalised with the Tat cell-penetrating peptide were able to deliver siRNA to the (intact) brain via the same administration route (Kanazawa et al. 2013). As with the vesicular drug delivery platforms can be engineered with stimulus-responsive drug-release mechanisms (Ganta et al. 2008; Liu et al. 2013), and can be actively targeted by appending and appropriate targeting moiety (e.g. an antibody) to the external polymers (Sawant et al. 2012). BBB-crossing micelles have been developed by targeting nicotine acetylcholine receptors on the capillary endothelium of the brain using the peptide CDX (derived from snake neurotoxin candoxin). These vehicles efficiently delivered paclitaxel into the brain and inhibited intracranial glioblastoma growth in mouse models. Co-delivery of the tumour necrosis-factor related apoptosis-inducing

ligand gene enhances the anti-glioblastoma effect even further (Zhan et al. 2012). Haney et al. report on a novel alternative to targeting moieties wherein they macrophages to deliver *nanozymes* (micelles incorporating the redox enzyme catalase) across an artificial BBB and into microvesicle endothelial cells, neurons, and astrocytes. The antioxidant properties of the *nanozymes* resulted in efficient reactive oxygen species decomposition, implying potentially useful therapeutic applications in diseases with a neuroinflammatory component, such as AD or PD (Haney et al. 2011).

Various dendritic and polymeric nanoparticle preparations have also been used to deliver chemotherapeutics, nucleic acids, and proteins. Dendrimers are unimolecular, highly branched spherical macromolecules that mimic micelles in their external topology, but also with a liposome-like interior voids (Ganta et al. 2008; Esfand and Tomalia 2001; El Kazzouli et al. 2012). The hydrophilic exterior can be extensively functionalised for enhanced bioavailability and targeting (Kesharwani et al. 2014; Zhu and Shi 2013), a property extensively exploited for tumour-specific delivery of chemotherapeutics (Agarwal et al. 2008). They are also potent siRNA and gene-delivery vectors (Wu et al. 2013). Sialic acid-functionalised dendrimers have been found to mimic cell surface sialic acid clusters, mitigating Aβ-induced neurotoxicity (Patel et al. 2007). Tang et al. describe a dendrimer comprised of multiple hydrolysable L-DOPA units, enhanced in stability and solubility relative to free L-DOPA while facilitating slow release (Tang et al. 2006). This represents a potentially useful *prodrug* for the treatment of dopamine deficits seen in PD. Dendrimers have also been investigated as potential agents by which to directly destabilise the neurotoxic protein aggregates associated with neurodegenerative disease (Heegaard et al. 2007).

Biodegradable polymeric nanoparticles, commonly based on poly(lactic-*co*-glycolic acid) (PLGA), have been used to deliver anti-cancer drugs, proteins, genes and RNAi (Danhier et al. 2012). They have also shown promise in treating neurodegenerative disorders (Gao et al. 2013). For instance, PLGA nanoparticles functionalised with the BBB-penetrating peptide TGN delivered the neuroprotective peptide NAP into the brains of mice with model AD, eliciting improved spatial learning and acetylcholinesterase/cholinacetyltransferase activity (Li et al. 2013b). Wheat germ agglutinin-functionalised PLGA nanoparticles enhanced the brain delivery of encapsulated vasoactive intestinal peptide relative to unfunctionalised nanoparticles, and this was again reflected in improved spatial memory and acetylcholinesterase activity in dementia mice (Gao et al. 2007). Lactoferrin-modified polymeric nanoparticles have been used to enhance the delivery of the cytoprotectant urocortin across the BBB of PD-model rats when administered intravenously (Hu et al. 2009; Hu et al. 2011). Odorranalectin has been employed in intranasal administrations for a similar increase in brain-delivery efficiency of urocortin-loaded nanoparticles, alleviating the loss of dopaminergic cells in PD-model rats (Wen et al. 2011).

In recent years, nanostructures that are constructed from nucleic acids have become an increasingly popular prospective therapeutic platform. Such constructs benefit from the fact that both the therapeutic and targeting moieties – siRNA and aptamers, for instance – can be constructed from the same material as the platform without requiring additional synthetic and conjugation steps. RNA nanotechnology(Guo 2010) has demonstrated considerable potential, with one recent approach involving packaging RNA (pRNA) nanostructures, discrete and stable stem-loop structures derived from the DNA-packaging motor of the phi29 bacteriophage (Shu et al. 2014). The pRNA molecule is readily modified to include therapeutic, diagnostic, and targeting moieties, and can be engineered at the supramolecular level to generate multimeric species via loop–loop interactions (Shu et al. 2013a) or multi-armed junctions (Haque et al. 2012; Shu et al. 2011). Each of the helical arms of these structures can be designed to function as siRNAs, miRNAs, aptamers, or ribozymes, or can be functionalised to append small molecule drugs or ligands, fluorophores, peptides, or additional nanostructures or nanoparticles (Shu et al. 2013b). The crux of the pRNA nanostructure demonstrates remarkable chemical and thermodynamic stability, low immunogenicity and toxicity, and excellent in vivo halflife and biodistribution properties. pRNA nanotechnology has shown considerable promise in laboratory studies, largely in tumour-specific delivery of siRNA. For instance, systemically-delivered folate-functionalised pRNA three-way junctions have been shown to accumulate in folate receptor-overexpressing tumour xenografts in mice (Shu et al. 2011). Similarly, DNA-based self-assembled architectures have also proven highly functionalizable and effective platforms for the

delivery of diagnostics and therapeutics (drugs and siRNA/antisense oligonucleotides) in vitro and in vivo (Bhatia et al. 2011; Keum et al. 2011; Walsh et al. 2011; Roh et al. 2011; Lee et al. 2012a; Zhu et al. 2013).

On a larger scale, RNAi microsponges are polymers of repeating hairpin RNAs that self-assemble into pleated sheets, which themselves arrange into a spherical formation. The approximately half-million RNA hairpins are cleavable into siRNA moieties by the RNAi machinery after cellular uptake (with the aid of a polycationic escort), making for potentially potent gene silencing (Lee et al. 2012b). Spherical nucleic acids (SNAs) are densely packed, highly oriented oligonucleotides covalently attached to an inorganic (usually gold) nanoparticle core. They are highly stable and nuclease resistant, capable of autonomous transfection, and capable of strong and persistent gene silencing with minimal immune response or off-target effects (Choi et al. 2013; Cutler et al. 2012). SNAs bearing siRNA targeting the oncoprotein Bcl2Like12, overexpressed in glioblastoma multiforme (GBM), effectively knocked down Bcl2L12 mRNA and protein in GBM cells and, when systemically administered to GBM xenografted mice, increased intratumoral apoptosis and decreased tumor burden (Jensen et al. 2013). As described earlier, SNAs have been delivered via natural exosomes. Exosomal inclusion of SNAs appears to greatly enhance their functional effect: SNAs comprised of anti-miR-21 oligonucleotides were endocytosed into PC-3 prostate cancer cells and subsequently naturally sorted into exosomes. The secreted exosomes, when re-introduced into the same cell type, knocked down the miR-21 oncomiR with an efficacy vastly greater than free SNAs (Alhasan et al. 2014).

While RNA-based therapeutics, particularly those acting via the RNAi pathway, possess great potential, specific and efficient cell delivery to target cells represents a major hurdle to widespread clinical applications. These obstacles are particularly large in the CNS, where the blood–brain and blood–spinal cord barriers obstruct the efficacious uptake of many conventional therapeutics. Encouraging results are being obtained using cell-specific and/or BBB-crossing functionalities, be they nucleic acid aptamers, cell-penetrating peptides, or small molecule receptor-binding ligands, however EVs may be a particularly convenient means to deliver RNA-based therapeutics to their site of action. As described previously, EVs often possess an inherent recipient cell specificity when trafficked in vivo which can be exploited or enhanced for the delivery of therapeutics. Moreover, as delivery vehicles they possess very low immunogenicity, particularly if derived from autologous cells. These aspects are well-illustrated by the DC-derived EV-mediated delivery of siRNA across the BBB and into neural cells, detailed earlier in this review. When administered systemically, these EVs, further functionalised with a brain-penetrating RVG peptide, demonstrated selective and effective delivery of siRNA into the brain (Alvarez-Erviti et al. 2011c). Presently, the in vitro and in vivo delivery of RNAi agents (or other nucleic acids) commonly relies upon generic liposome preparations, the transfection efficacy and toxicity of which varies largely between recipient cell types. It is envisioned that EVs might serve as a means to greatly enhance the efficacy and safety of such transfections, particularly with advances in the ease of EV isolation, purification and characterisation. Thus, EVs represent a potential high-specificity, low-immunogenicity option for the targeted delivery of multifunctional RNA therapeutics.

Conclusions

Our emergent understanding of the diverse and significant roles played by EVs hints at a powerful new therapeutic avenue to exploit in the treatment of disease, including those typically associated with the aging process. This is perhaps most evident in age-related neurodegenerative disorders as EVs tantalise researchers with properties conducive to surmounting the barriers plaguing the generally *undruggable* ailments of the brain. While advances in bioengineering lead us towards the ability to modulate and adapt naturally occurring EVs, directing their packaging with therapeutic molecules and directing them towards specific recipient cells, concurrent advances in nanoengineering furthers our ability to emulate these properties in synthetic drug-delivery vectors.

Nevertheless, an efficacious exploitation of the EV system will require greater understanding of the intricate in vivo intercellular communication network that extends well beyond the unidirectional processes studied in most in vitro systems. This is of particular significance in multi-factorial, complex conditions such as cellular senescence and organismal aging, especially in immune-specialised environments such

as the CNS. Moreover, elucidation of the natural processes of biogenesis, packaging and trafficking will serve to strengthen our efforts at effecting targeted therapy via synthetic routes. Thus, while studies into the nature of EVs are still in their infancy, the significance of these agents of extracellular communication in matters physiological and pathological is abundantly clear.

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