Exosomes in the brain – from diagnostics to therapeutic potential

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Efficient delivery of drugs to the brain poses many challenges, mostly due to the special characteristics of the blood-brain barrier which prevents most therapeutics from reaching their target. Additionally, accurate diagnosis of many neurodegenerative and psychiatric disorders is often difficult and clear biomarkers are missing. Nanoparticles which have recently gained attention for their potential use in these areas are exosomes – small biomimetic nanovesicles which resemble the protein content of their parent cell, and play an important role in intercellular communication. In this review their special characteristics, both for diagnostics and therapeutics, will be discussed. These include immunocompatibility, natural organotropism and the ability to cross the BBB. Being a natural RNA carrier, exosomes were shown to successfully deliver gene therapy in animal models of neurodegenerative disorders and brain tumours. They also show intrinsic therapeutic properties due to their role in communication and neuronal protection. First clinical trials with exosomes as the drugs for neurodegenerative and psychiatric disorders are currently taking place. Moreover, their ubiquitous presence in many bodily fluids, combined with their role in spreading misfolded protein aggregates and other pathologies, makes them a good potential diagnostic tool. Remaining challenges concerning exosomes discussed in this review include the need for improved isolation methods, increased production yield and purity.

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INTRODUCTION

Drug Delivery across the Blood-Brain Barrier

The blood-brain barrier (BBB) provides a unique protection against any potentially harmful agents, maintaining the brain's homeostasis by carefully regulating the transport of molecules to and from the bloodstream. Alongside endothelial cells, the BBB is composed of astrocytic end-feet lining up the barrier, and pericytes which surround the capillary walls and secrete proteins contributing to the basement membrane [1]. The main reason behind its special characteristics is the strong interconnection of neighbouring endothelial cells with tight junctions (TJs) which are located between adjacent cells and prevent most of the paracellular transport. Other parts of the membrane, such as astrocytes also play a crucial role in ensuring the level of permeability stays low, by e.g. regulating the differentiation of pericytes and expression of TJs proteins [2].

While the neurovascular unit is great at keeping potential pathogens away from the brain, these excellent barrier properties prove to be one of the main hurdles in reaching the blood with therapeutics [3]. It is estimated that around 98% of potentially effective therapeutics cannot reach the brain because of the barrier posed by its special endothelial layer [4]. The transport methods which the brain does use for e.g. obtaining nutrients and energy, are mostly heavily restricted in terms of what molecules can be trafficked (see Fig. 1).

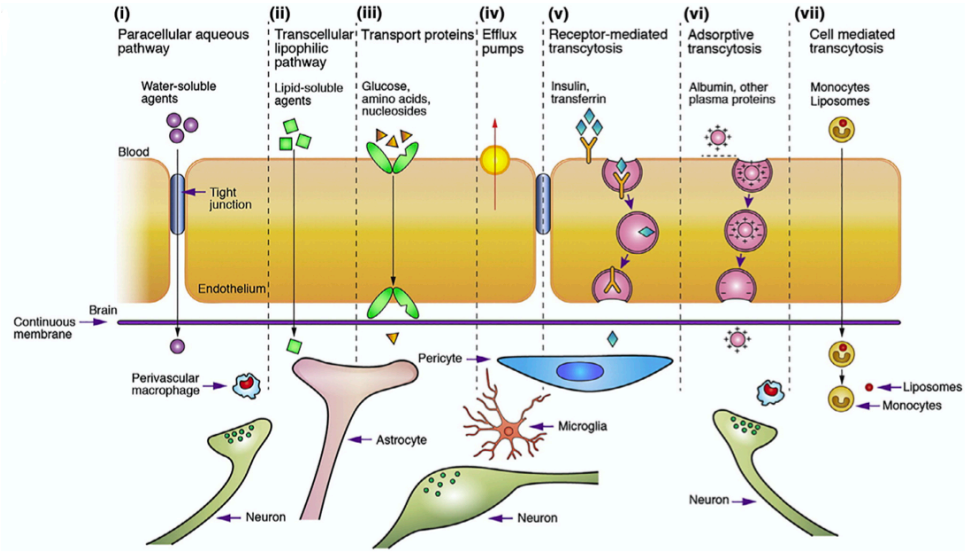


Fig. 1 Various transport mechanisms across the BBB (taken from: [5])

Due to the TJs, only small and hydrophilic molecules can pass paracellularly. The transcellular route also poses many challenges, with e.g. transporter-mediated transcytosis being highly selective for specific substances, such as glucose, making it hard to create therapeutics able to "fool" the transporters [1]. A route which has been used the most is the receptor-mediated transcytosis (RMT; "Trojan horse"). In this mechanism a given ligand, together with the receptor, form an intracellular vesicle created by means of membrane invagination [6]. After the vesicle is trafficked to the opposite side of the membrane its contents are released through exocytosis [7]. A common example of RMT is using the rabies virus glycoprotein (RVG), which binds to the nicotinic acetylcholine receptors on BBB, as a brain targeting peptide [8].

Designing a good therapeutic carrier to the brain poses many challenges. One possibility therefore is to circumvent the BBB altogether by administering drugs directly to the brain, however this is a highly invasive and risky procedure which additionally can only reach limited tissue depth [9]. Yet another method is to temporarily weaken the BBB by e.g. chemical compounds such as mannitol [10] or focused ultrasound [11]. The clear disadvantage is the high risk of damaging the BBB permanently. Approaching the issue from the other side involves modulating the characteristics of the drug molecules themselves, e.g. in an effort to make them more lipophilic. However, adjusting the components of the therapeutics can decrease their efficacy and targeting ability. Additionally, many drug molecules are being forced back to the bloodstream by multidrug resistance proteins or quickly cleared from the system by macrophages [12]. In this light, nanoparticles have emerged as a novel and promising drug carrier strategy.

Nanoparticles as Drug Carriers

Nanoparticles (NPs) have many useful characteristics in the context of carrying drugs into the brain, such as their small size and high drug-loading capabilities. They can also be engineered to release their content in a controlled and sustained manner and are easy to track and imagine [13]. They have to be tailored in many aspects, including size, shape, lipophilicity and surface chemistry, which also means their characteristics can be precisely designed for the purposes of brain-targeting [11]. Nanocarriers can increase the bioavailability and stability of the drug, as well as decrease its peripheral toxicity [13]. In recent years, there has been growing interest in NPs as a drug carrier to the brain (see Fig. 2).

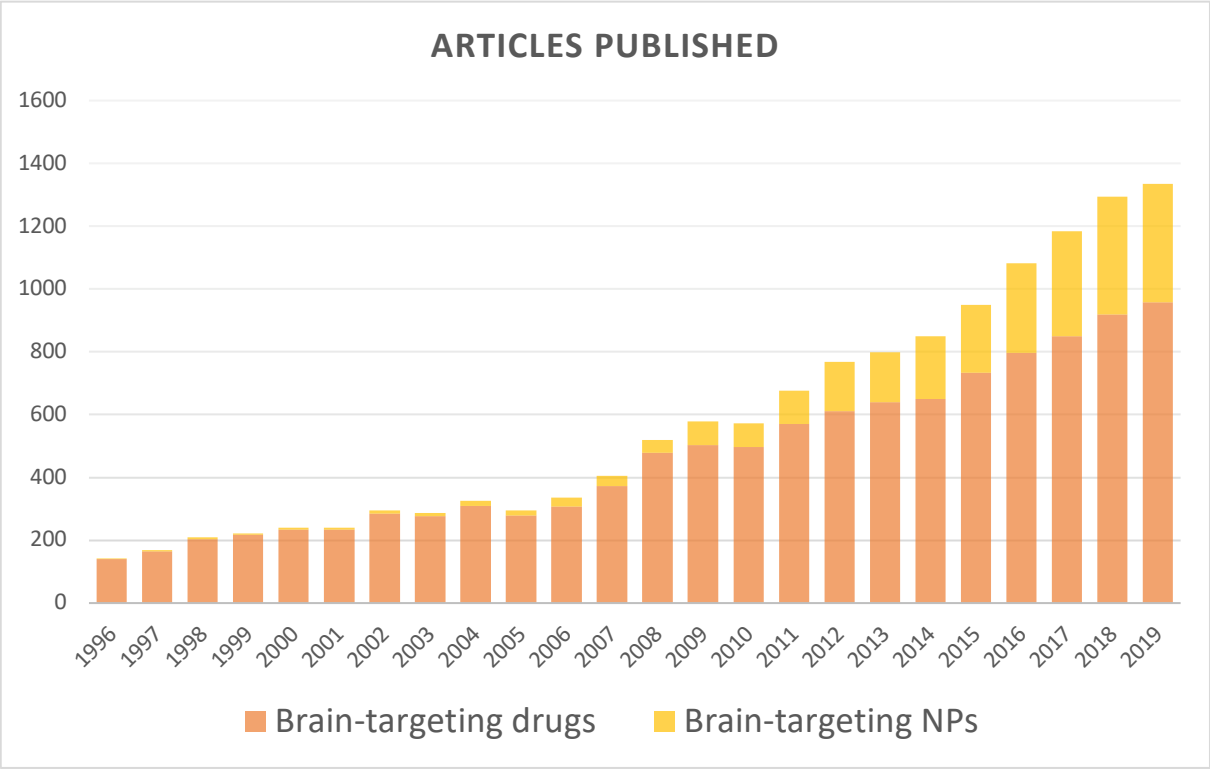


Fig. 2 Number of published papers per year on brain targeting drugs and the proportion of those discussing NPs-based therapeutics (from: webofscience; searched terms: "BBB"; "Drugs"; "Nanoparticles"; date accessed: 15.10.2020)

The three main classes of nanoparticles are: polymer-based, biomimetic-based and inorganic-based (see Fig. 3). In principle, any type of NPs can be modified with ligands for BBB-crossing, which allows it to use the RMT route and transport the drug encapsulated inside [6]. Currently the carriers which are used most often are liposomes and polymeric nanoparticles. While liposomes have low toxicity and are lipophilic, which makes it easier to cross the endothelial cell layer, among their limitations are low stability and the potential to cause immune reactions [14]. Using polymeric nanoparticles does provide more stability, however they are also less biocompatible which means they can accumulate in the body and cause adverse reactions in the long run [15]. Nevertheless, one of the polymeric nanoparticles, PLGA, has been recently approved by the FDA as a drug-delivery vehicle, and has also been shown to have beneficial effects as a carrier for curcumin in Alzheimer's Disease [16].

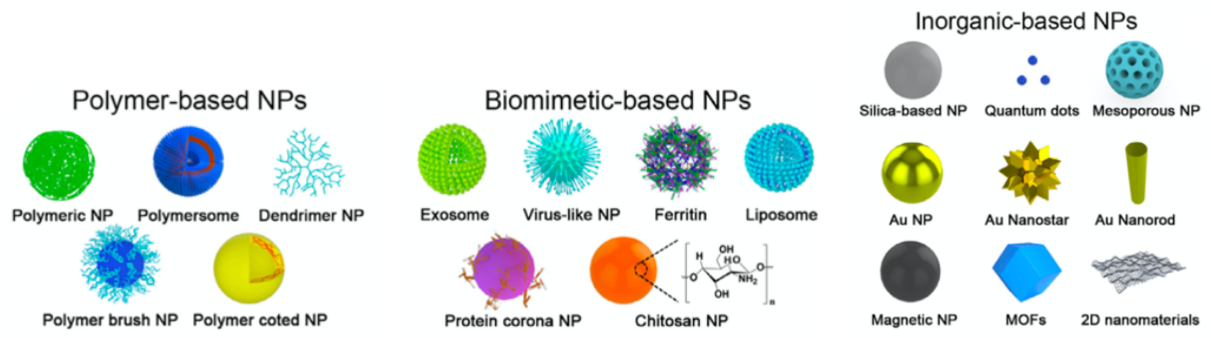


Fig. 3 Different types of NPs with examples (taken from: [6])

As mentioned before, both liposomes and polymeric NPs have to be coated with specific antibodies or peptides which define their target organ – in the case of the brain this can be e.g. transferrin or insulin [7]. In order to increase the time of blood circulation, as well as improve the efficiency, their surface can also be modified with PEG (polyethylene glycol) [14], however some reports show that this might not be as effective when they are administered for the second time [17]. One general problem with NPs is the "protein corona" they form by collecting on their surface various proteins encountered in the body. This can negatively impact their functioning as therapeutic carriers. To counteract this, PEG coating can be used to decrease non-specific interactions, however it can also decrease the uptake level at the target site [18]. The "protein corona" also undergoes drastic change once it crosses the BBB and encounters new molecules which makes it additionally hard to design compounds maintaining their characteristics once inside the brain parenchyma [19].

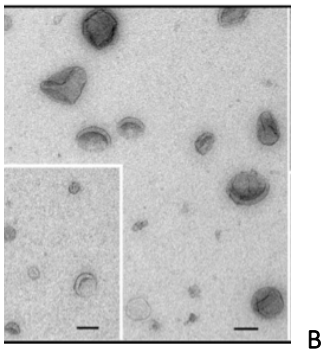
Overall, nanoparticles have so far not found great success in targeting the brain, with one of the main reasons being poor drug distribution, issues with accurately targeting the brain, and with differentiating between healthy and pathological tissue once there [11]. Peripheral safety is another concern, since many NPs (especially the inorganic ones which are harder to metabolise) can accumulate in other organs, e.g. kidneys and liver [20]. Here, the biomimetic-based NPs have the advantage of not being easily recognizable by the immune system and consequentially having a higher chance of staying in the circulation long enough to reach the brain. Additionally, they can also bind to the ligands relatively easily, and are not toxic. Therefore, a type of biomimetic NP, exosome, has recently gained attention as a potential brain-targeting drug carrier. In this review I will analyse its characteristics in the context of

delivering therapeutics to the brain, as well as its intrinsic therapeutic properties and diagnostic potential.

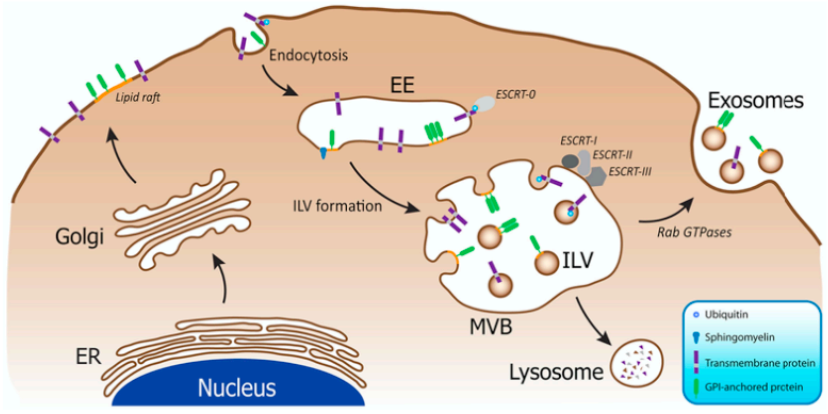
EXOSOMES: BASIC CHARACTERISTICS

Exosomes are small nanovesicles, ranging in size between 30-150 nm (see Fig. 4A), first reported in 1987 [13, 21]. Since then, exosomes have gained more and more attention, with the number of citations increasing from 28 in 1996 to 24,765 in 2016 [22]. Their precise characterisation is challenging due to the difficulties and disparities in isolation techniques. Originating in endosomes, they can be considered an endogenous nanocarrier with a lipid bilayer membrane, the composition of which heavily depends on the parent cell [15]. The formation of exosomes usually happens in three steps (see Fig. 4B):

- The endocytic vesicle is differentiated from the plasma membrane and forms an early endosome (EE)
- As a result of inward budding the vesicle forms a multivesicular body (MVB) which contains many intraluminal vesicles (ILVs)
- When MVB is fused with the membrane, these ILVs are released into the extracellular space and are further referred to as exosomes (alternatively the MVBs are fused with lysosomes and their cargo – ILVs, is degraded)



A



B

Fig. 4 A Negatively stained exosomes secreted from rat cortical primary cultures. Scale bars: 100 nm (adapted from: [23]) **B** Formation of exosomes. Early endosomes (EE) mature and form multivesicular bodies (MVB). Intraluminal vesicles (ILVs) are formed through inward budding of the limiting membrane of the MVB. When MVBs fuse with the membrane, ILVs are secreted into the extracellular space, and are now called exosomes (taken from: [24])

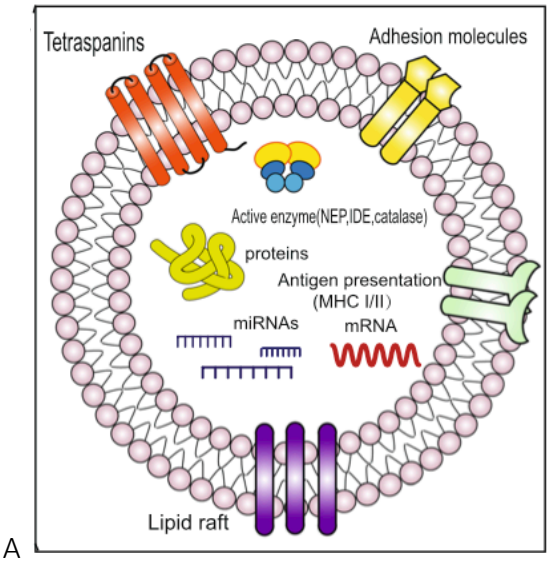
Since ILVs contain proteins of the cells in which they originated, so do the exosomes, reflecting their origin cell in terms of surface composition and its specialised functions. For example, exosomes derived from immune cells have molecules which are involved in antigen presentation, such as MHC II (major histocompatibility complex II), and exosomes secreted by B cells can induce T cell responses [25]. Moreover, through the mechanism of organotropism, they can selectively target cells similar to their parent one [26]. Even though they were first thought to be responsible for clearance of redundant proteins, it is now known that they in fact play an important role in distant intercellular communication [15]. They are secreted by many different cell types, including neurons, astrocytes and other cerebral cells, and can be found in almost all types of bodily fluids, such as blood or urine [13, 27]. While their precise function depends on the cell of origin, their ubiquitous presence in the body certainly points to their importance.

Potential as a therapeutic carrier

Since they are essentially a natural product of the body, exosomes do not induce a strong immune response [28]. Moreover, their size makes them ideal for drug delivery to the brain, since they are small enough to cross BBB – which has been shown both *in vitro* and *in vivo* [29], while at the same time they are able to carry enough of the therapeutic cargo [30]. The blood circulation time is generally assumed to be sufficient for drug delivery purposes since they are natural carriers [20]. Another substantial advantage of using exosomes is the fact that they can be derived directly from patient's cells, further increasing their biocompatibility.

Exosomes can transport various genetic and biochemical information, making them ideal potential drug carriers (see Fig. 5A). They are a natural RNA carrier, mostly for miRNA (micro) and siRNA (small interfering) which can regulate gene expression post-transcriptionally by influencing mRNA [31]. They are therefore perfectly fitted for gene therapy, since RNAs by themselves cannot cross membranes and are very unstable, needing a good carrier [32]. Once inside an exosome, the siRNAs and miRNAs are protected even from externally applied RNAase [33]. In the recipient cell, exosomes can start translation, protein secretion and consequentially induction of new functions [34]. Other than transporting nucleic acids, they are also appropriate both for small and larger therapeutic molecules, e.g. proteins [15]. In one of the first studies showing that exosomes can in fact encapsulate therapeutic molecules, anti-

inflammatory curcumin was delivered to the lungs [35]. Exosomes increased the specificity and stability of the drug, enhancing its overall therapeutic effects.



A

Fig. 5 Example model of an exosome derived from mesenchymal stem cell, with the lipid bilayer and various cargo materials inside such as RNAs, proteins and enzymes (taken from: [36])

Importantly, exosomes often do not need to be coated because of their natural organotropism in agreement with the parent cells. This is a great advantage, since often finding the right combination of receptors which agree with the carrier and do not change the therapeutic efficacy is complicated [37]. For example, exosomes from neuronal stem cells have natural targeting abilities to the brain [20]. Naïve macrophage-exosomes can also cross BBB and deliver their cargo [38]. How exactly this occurs is still not fully understood, and the question is discussed in more detail elsewhere [39]. Nevertheless, some exosomes (e.g. from dendritic cells) must also be additionally modified to specifically target the brain and enhance the tissue-specific homing, e.g. through transgene expression. For example, the aforementioned RVG has been used for brain targeting in dendritic-cell derived exosomes [40], however such modification might also increase the immune reactivity of the compound [38].

Naturally, the use of exosomes has also potential risks – their complexity means that it is often difficult to predict exactly what kind of effects they would have. Their isolation and loading techniques are also time- and cost-consuming, often do not yielding high purity samples [13, 20]. An important question is also how to track the exosomes once they are in the brain, with

many *in-vivo* (e.g. PET), and *ex-vivo* (e.g. fluorescence microscopy) techniques suggested, not discussed in further detail here [9].

Role in pathologies and disorders

In essence, exosomes are vesicles which can be used to transport all kinds of biological information – both the good and the bad. Therefore, apart from their role in usual functioning of various organs (including the brain), they have also been found to play an important role in diseases, such as cancer [41], where they are used by tumour cells to promote their growth by transporting RNAs and angiogenic proteins (see Fig. 6A). Exosomes are also crucial in the process of metastasis, guiding cancer cells to specific sites depending on their molecular composition – proteomic analysis revealed distinct integrin expression patterns for exosomes derived from tumours with propensities to spread to specific sites, such as lung and liver (see Fig. 6B).

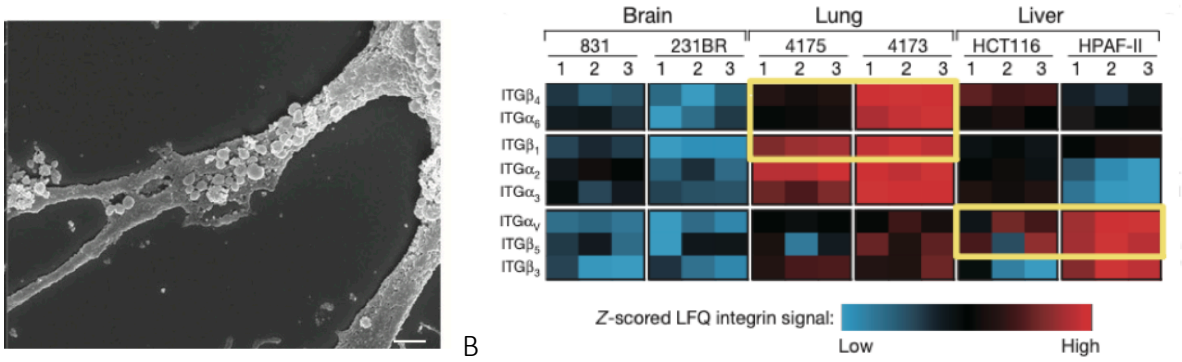


Fig. 6 A Exosomes produced by glioblastoma cells. The surface of the cells is visualized with scanning electron microscopy (taken from: [42]) **B** Heat map of integrin signals in metastatic tumour exosomes showing brain-, lung- and liver-tropism. Data from a mass spectrometry analysis: Z-scored label-free quantification (LFQ) values (taken from: [26])

In cases of neurodegeneration, exosomes are thought to contribute to spreading of the disease by carrying misfolded proteins, such as in prion disease [25] or Alzheimer's [43]. Remembering how the exosomes are formed, it is easy to see how they can contribute to spreading of the protein aggregates. When a multivesicular body is formed inside a neuron which already has the amyloid beta peptide inside, it is also encoded within the released exosome which is then transported to other neurons and release the peptide, propagating the aggregation process (see Fig. 7). Similarly, exosomes can transfer hyperphosphorylated tau from microglia to

neurons. Some have even hypothesized that the move towards more exosome-focused neuronal communication which occurs with age is one of the reasons for the occurrence of neurodegenerative disorders later in life [44]. When exosomes are more ubiquitous, they could inadvertently cause the expansion of neurodegeneration by "shipping" the toxic agents to a greater area in the brain (acting as "Trojan horses").

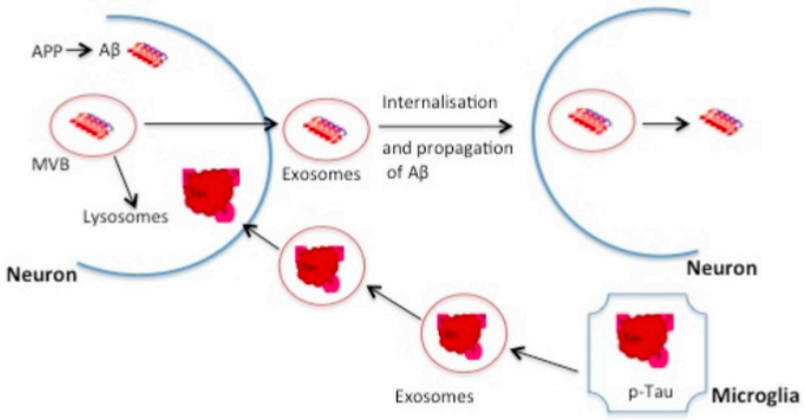


Fig.7 The role of exosomes in propagating AD pathology. Upon MVB-formation in an amyloid-beta infected neuron, the subsequently released exosomes can transfer the peptide further. Additionally, hyper-phosphorylated tau can also be transported in this way from microglia to neurons (taken from: [45])

The presence of exosomes in various pathologies means they can serve as diagnostic tools [13]. Since they are found in almost all bodily fluids, they can be collected in a relatively non-invasive manner, compared to e.g. biopsies [46]. Additionally, understanding exosomes will help us to understand the mechanisms behind the diseases, such as organ-specific metastasis in cancer. Moreover, we need to understand them fully to be able to use them as therapeutics, so as to avoid increasing the pathological transmissions and other side effects.

MANUFACTURING OF EXOSOMAL DRUG CARRIERS

Isolation

The isolation can be based on different traits, such as size, shape, density or presence of specific antigens [22]. The gold standard and the most common method in the field is size-based centrifugation. Disadvantages include not being able to differentiate between non-exosomal extracellular vesicles of the same size, and lack of precision meaning exosomes can still be mixed with parts of the culture medium or other proteins [47]. It is also a time-consuming

process and requires specialised equipment [15]. Another method is immunoaffinity, based on interactions between antibodies and exosomal surface proteins. This can yield higher purity than centrifugation, however antibodies are expensive, and since they will also bind to other molecules with the same antigens, the purity is not ideal. Precipitation is a method based on altering the solubility, however it has also relatively large contamination [20].

Overall, there is no single ideal solution and the production yield is usually quite low. Some ways to improve it could be e.g. through longer incubation times of the parent cells, which could increase secretion of exosomes or through adjusting the culture medium composition [20]. Instead of longer incubation times, heat stress was also shown to increase exosome production, it can however also change their composition [48]. While progress has been made in recent years, many challenges still remain in terms of obtaining high numbers of pure vesicles, mostly due to potential contamination from other extracellular vesicles with similar biochemical characteristics and sizes [20]. Therefore, a completely novel approach could be useful, such as integrating microfluidics and acoustics [49]. The proposed platform can isolate exosomes directly from blood in a quick and label-free way (see Fig. 9), with isolation yield of around 98,4%. Quality control of the obtained samples usually involves looking at size, morphology, surface charge, quantity and protein composition [29].

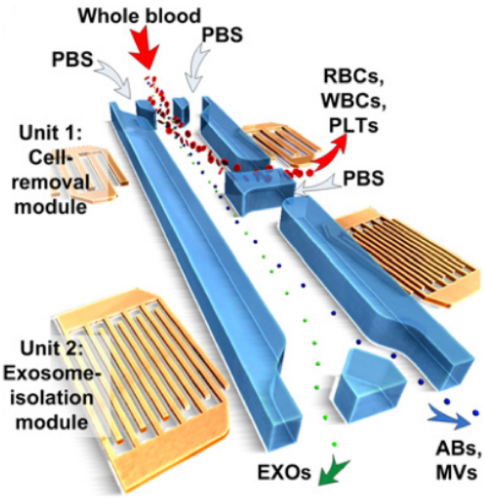


Fig. 9 Schematic illustration of the acoustofluidic model for exosome isolation. ABs – apoptotic bodies; EXOs – exosomes; MVs – microvesicles. (taken from: [49])

Loading

Optimal drug loading strategy is crucial for the therapeutic efficacy, both in terms of the amount of drug loaded and the speed of its release. Various methods can be used for loading the drugs inside the exosomes, which can be broadly divided into pre-loading and post-loading, sometimes also referred to as *in vivo* and *in vitro* loading [20, 50]:

Pre-loading (*in vivo*) → when the drug is incorporated into the parental cell (requires in-depth knowledge of biogenesis of exosomes; loading efficiency cannot be controlled)

- Engineering (transfection): introducing purified nucleic acids into cells – once the RNA is overexpressed in parental cell, it will also be passively loaded on to the exosomes
- Treatment with drugs

Post-loading (*in vitro*) → when the drug is incorporated into exosomes after isolation (most common way)

- Drug incubation: the simplest method of co-incubation at room temperature (RT); does not require any extra equipment, but it has low loading efficiency
- Assisted drug incubation: by creating membrane imperfections exosomes are destabilised and loading efficiency is increased (disadvantages include risk of permanently disrupting the integrity of exosomes); many different ways of achieving that exist, including:
    - Saponin-assisted loading: disadvantages include potential haemolytic activity *in vivo* [28]
    - Freeze/thaw cycles: disadvantages include low loading efficiency and risk of aggregates formulation
    - Electroporation: it is easy to overestimate how much of the compound is actually inside due to aggregate formation [51]
        - Using an optimised buffer to compensate and maintain the structural integrity can prevent aggregates and increase overall efficacy [52]
    - Sonication: has relatively large loading efficiency [53]
    - Extrusion: relatively large loading efficiency, but high risk of permanent membrane disruptions

Several of the above-mentioned methods were compared in a recent study by Haney and colleagues (2015) who loaded catalase onto macrophage-derived exosomes. The catalase activity was best preserved in the case of sonication, freeze/thaw cycle and incubation at RT (see Fig. 10A), which were also the most effective in terms of uptake (see Fig. 10C). For neuroprotection sonication, extrusion and incubation with saponin were most efficient *in vitro* (see Fig. 10B). Overall, sonication was deemed as the most efficient method in terms of preserving the activity, uptake, and increasing survival [28]. It was also more efficiently incorporated into the neurons than other NPs such as PLGAs or liposomes (see Fig. 10D), however the exact neuroprotection was not compared between these three conditions (since higher uptake does not necessarily mean higher effectivity).

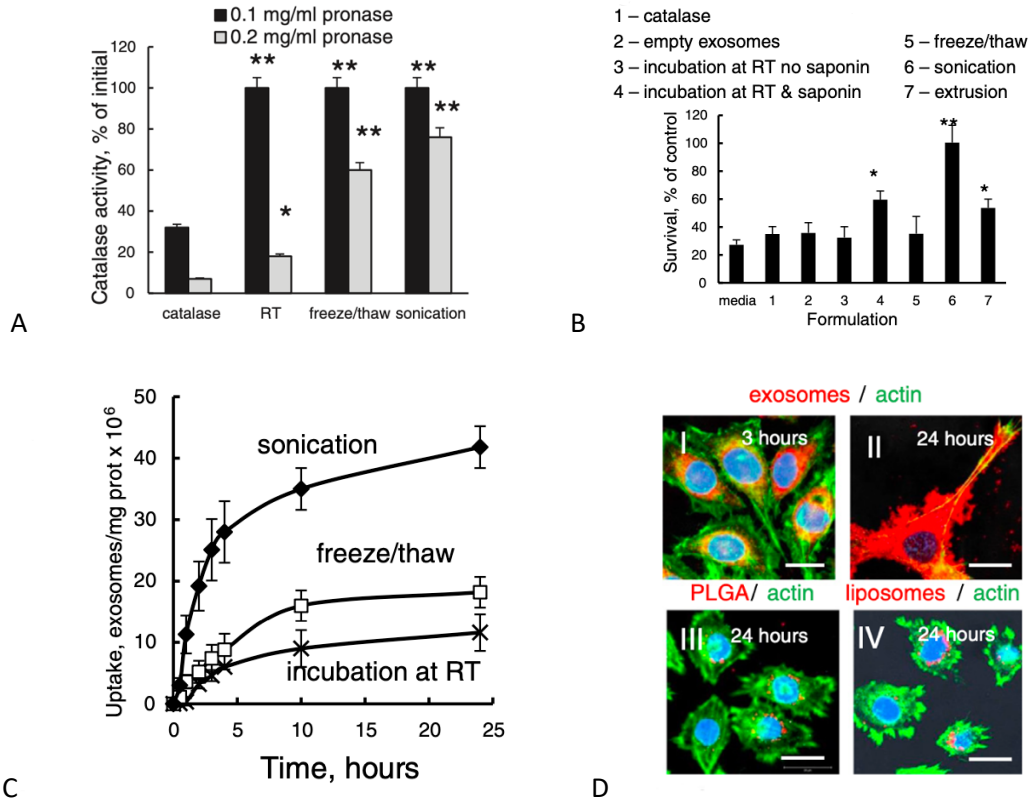


Fig. 10 Examining the characteristics of macrophage-derived exosomes loaded with catalase **A** How well was the catalyse activity protected depending on the loading method **B** Neuroprotection induced by exosomes with the cargo **C** Uptake by different methods examined with spectrofluorimetry **D** Accumulation of sonicated exosomes with catalyse by confocal microscopy (II). Comparably lower uptake of polymer-based PLGA (III) and liposomes (IV) at 24h (taken from: [28])

Parent Cell Type

As explained before, the type of cell from which exosomes originate has important consequences on their characteristics. Exosomes derived from neuronal stem cells should be able to cross BBB, due to the "inherited" organotrophic properties [29]. Other cells, such as dendritic ones, might require extra coating, e.g. with RVG [40]. Stem cells and their exosomes seem to have a special inherent tumour-tropic property, being able to infiltrate and target its individual cells [37]. Using exosomes instead of cells themselves has many advantages, since living cells are fragile and especially stem cells can differentiate uncontrollably causing a risk of tumour formation [9]. Exosomes are more stable, and there is no problem with maintaining the cell viability once inside the organism [22]. Moreover, a large proportion of intravenously injected cells does not even reach the brain due to due to capillary trapping and clearing [9, 54].

Mesenchymal stem cells (MSCs) can be a good source of exosomes, due to their immunomodulatory role which means they inherently migrate towards sites of lesions and pathologies [27, 55]. MSCs can also "push" their exosomes, packed with small RNAs, through the TJs. Mesenchymal exosomes have been shown to be the primary vesicles for miRNA therapeutic delivery to glioblastoma cells [56], and to mediate neurogenesis and functional recovery after traumatic brain injury [57]. Moreover, MSCs have large *ex vivo* expansions capabilities and can be immortalised without affecting the quality of produced exosomes which makes production reproducible and easier [50, 54]. Interestingly, all of the currently running clinical trials use mesenchymal-derived exosomes.

Neuronal stem cells (NSCs) have the inherent property to cross BBB and once inside, migrate to the injury site even when injected systemically [37, 58, 59]. In a brain cancer rodent model they were also found to "chase" the infiltrating calls originating from neuroblastoma to other anatomic locations, such as the liver [37]. Despite promising initial results, placebo-controlled studies of NSCs transplantation did not show significant improvement [60]. However, exosomes have been shown to decrease neuroinflammation and even be able to restore some neural functions [61], as well as to have beneficial effects on amyloid-beta clearance in AD model [62]. Recently, exosomes derived from neural stem cells have been shown to successfully transport a protein cargo across an *in vitro* BBB model [29], without damaging or otherwise negatively influencing the integrity of the barrier. Another brain-derived cells, namely the endothelium,

could also provide a good source. In a zebra fish model of brain tumour, when exosomes (from mouse endothelial cells) were loaded with siRNA and injected, they successfully crossed BBB and delivered their cargo [63].

Dendritic cells (DCs) are the antigen-presenting cell of the immune system which can release exosomes with various different characteristics. For example, when stimulated with interferon gamma, DCs produced exosomes with miRNA which could reduce oxidative stress and improve remyelination in an *in vivo* model of multiple sclerosis [64]. They were also used in seminal work on siRNA transport with exosomes [40]. Another immune cell type which can be used are macrophages, e.g. as in previously described Parkinson's Disease models [28], or in cases of brain inflammation [38].

Another interesting avenue is deriving exosomes from food, i.e. fruit and vegetables. Such edible plant-derived exosome-like nanoparticles (EPDELNs) could potentially be a safer source, and many of those have intrinsic anti-inflammatory properties [65]. They would have to be coated appropriately to be able to target the brain, but they were also found to encapsulate miRNAs which could regulate human mRNA *in vitro*. One study, currently in Phase 1, will trial plant exosomes as a vehicle for curcumin in cases of colon cancer [66], so it is feasible that in the future they might also be a carrier to the brain.

DIAGNOSTIC POTENTIAL

Most of the recent exosome research has focused on their role as diagnostic tools for various diseases, including those of the brain – from cancer to neurodegenerative and psychiatric disorders [13, 67]. In fact, the majority of miRNA detectable in bodily fluids (e.g. blood, saliva) is packaged into exosomes, which makes them ideal biomarkers, as they can also be collected in a relatively non-invasive manner [46, 68].

Glioblastoma

Most of the currently running clinical trials use exosomes as biomarkers for cancer. Since they are used for communication between tumour cells, an increase in their numbers and special characteristics has been found in various cancers [69, 70], including glioblastoma where the intercellular signalling was shown to be mediated by exosome-like extracellular vesicles [42].

The exosomes released by glioblastoma cells are taken up by healthy host cells, which then translate the information encoded within, and can e.g. promote angiogenesis, as well as stimulate self-proliferation. Specifically, cancer patients had a higher number of exosomes containing proteins and miRNAs correlated with hypoxia (one of the key markers of cancer microenvironment), as well associated with bad prognosis for the disease [41].

Neurodegenerative Diseases

In Alzheimer's Disease (AD), β-amyloid plaques activate microglia and astrocytes which contribute to constant inflammation and increased level of peripheral cytokines, exacerbating symptoms and contributing to neuronal degeneration [62]. The amyloid-beta peptides have been found to be released to the extracellular space together with exosomes [43], in a manner described earlier. Specifically, the endocytic system has been implicated in the abnormal cleavage process which leads to creation of these pathogenic peptides from the amyloid precursor protein [43]. Exosomal proteins were also found in the plaques of AD patients. This means that the brain-originating exosomes which can be found in blood can be the perfect "liquid biopsy" providing a definitive AD diagnosis, with high sensitivity and specificity, but low invasiveness [36].

This would be especially useful since current diagnostic process is based on subjective neuropsychological testing and brain imaging, with definitive diagnosis really only possible after autopsy [71]. Additionally, neurodegenerative changes are thought to start much earlier than clinical symptoms, so a way for early detection is needed. Specific miRNA have been identified from urine, CSF and blood of AD patients through deep sequencing [71]. However, "good" exosomes were also identified in the cerebrospinal fluid (CSF) of monkeys and mice models of AD [62]. The number of these exosomes decreased with age and infusion of the neuronal ones was found to decrease amyloid deposition in hippocampus (see Fig. 11). Therefore, it was actually suggested that their downregulation with age is what leads to protein accumulation in AD. Clearly, a set of exosomes is involved in the disease process, and even for diagnostic purposes it is necessary to correctly identify the up- and downregulated ones.

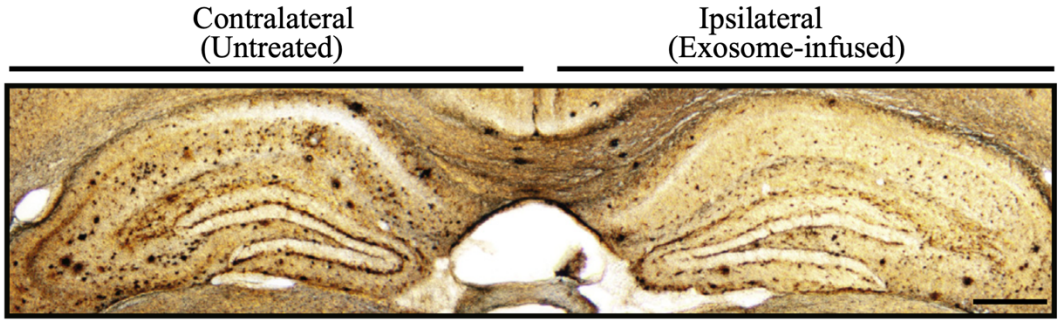


Fig. 11 Hippocampal section from an APP mouse infused with neural exosomes into one of its hemispheres, stained for the amyloid beta peptide. Bar, 200 µm (taken from: [62])

In Parkinson's Disease (PD) hyperactivity of a special molecule, namely leucine-rich repeat kinase 2 (LRRK2) which is linked to the disorder, could be identified by exosome from urine and CSF [72]. Similarly to AD, exosomes could also be markers for alpha-synuclein, and the two were associated *in vitro* [73]. Exosomal alpha-synuclein was also present in CNS *in vivo* (CSF from patients). The exosomes from CSF were also able to induce pathological process of alpha synuclein in healthy cells [73]. Importantly, like in AD, some PD exosomes can actually be neuroprotective [74].

Other

Exosomes can also be useful in diagnosing psychiatric disorders. In schizophrenia and bipolar disorder patients, different levels of specific miRNAs were found in prefrontal cortex-derived exosomes [75]. When cells are injured or undergo stress, e.g. as a result of traumatic brain injury (TBI), the nature of exosomes they release changes [76]. Therefore, since exosomes can mirror these physiological events in their structure, they can also enable real-time monitoring of remodelling and recovery after TBI.

THERAPEUTIC APPLICATIONS: CARRIER

Pathological Protein Aggregation

Seminal work on delivering siRNAs within exosomes was done almost ten years ago, with exosomes derived from dendritic cells in a mouse model of AD [40]. Cells were engineered to express RVG (a previously mentioned brain-targeting peptide) in Lamp2b plasmid. This allowed the resulting exosomes to cross BBB (see Fig. 12). The siRNA cargo was packaged with

electroporation, and targeted β-secretase. The compound was then injected intravenously and reached neurons, oligodendrocytes and microglia, resulting in decreased protein expression by 62%, as well as 60% reduction in its mRNA levels. Non-specific uptake was not observed in other tissues, and the study came to become a proof-of-concept that surface-modulated exosomes can target the brain after intravenous administration and successfully deliver RNA cargo.

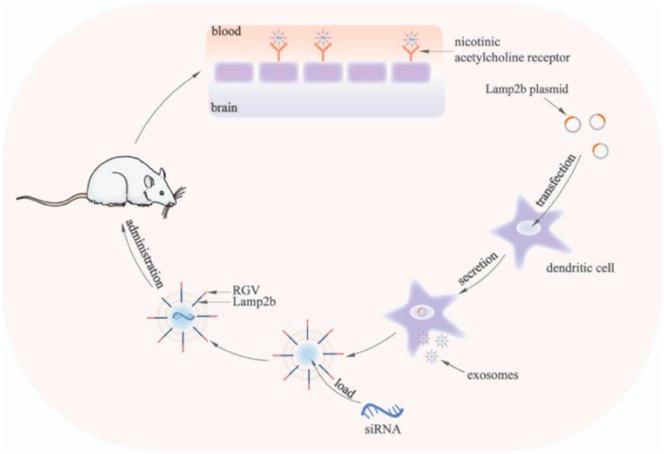


Fig. 12 Flow of the experiment of Alvarez-Erviti et al. (2011). Dendritic-cell derived exosomes were engineered to express RVG in Lamp2b protein, which allowed them to cross the BBB. Once inside the brain, their therapeutic cargo (siRNA targeting BACE-1) was released and resulted in decreased expression and mRNA levels of β-secretase (taken from: [13])

A few years later, the same group used this method (siRNA in RVG-exosomes derived from dendritic cells and administered systemically), but this time targeting α-synuclein which is the main component of Lewy bodies in PD [77]. It resulted in reduced mRNA of α-synuclein as well as reduction in protein aggregates in subcortical regions in one of the most important areas in PD, i.e. substantia nigra.

Exosomes with abnormally downregulated miRNAs were also able to restore normal gene regulation in Huntington's Disease, however without behavioural improvements [78]. In another study, unilateral infusion into the striatum of exosomes derived from glioblastoma cells and packaged with hydrophobically modified siRNAs targeting Huntingtin mRNA, led to the dose-dependent silencing of the mRNA up to 35% in both hemispheres [79]. Importantly, exosome packaging was crucial for this effect.

Neuroinflammation

Exosomes can also be used to counteract the effects of neuroinflammation. For instance, glioblastoma-derived exosomes with encapsulated (anti-inflammatory) curcumin were delivered intranasally in three different neuroinflammation mice models and were selectively taken up by microglia [80]. This worked to protect the mice in all three cases, namely through decreased inflammation from LPS (lipopolysaccharide), in a model of MS (experimental autoimmune encephalomyelitis), as well as through limiting brain tumour growth.

In a recent study, described before in the context of different loading methods, exosomes derived from macrophages were loaded with catalase (a potent antioxidant), which is hypothesized to have therapeutic effect on neuroinflammation through deactivating free radicals [28]. Different administration methods were compared: intranasal and intravenous, and while both resulted in delivery of the catalase-loaded exosomes to the mouse brain, the former was more efficient. As discussed before, the exosomes had a protective effect *in vitro* (see Fig. 10B), but also decreased the reactiveness of microglia *in vivo* (see Fig. 13A).

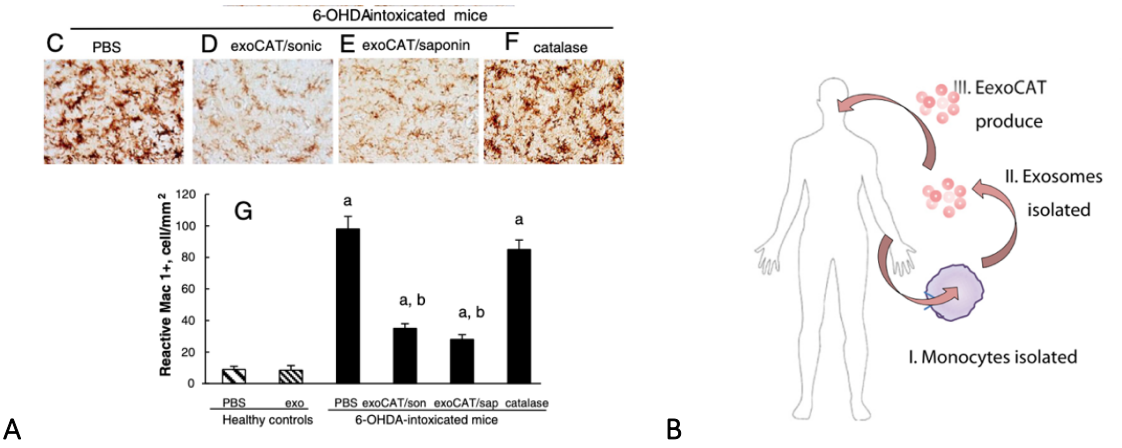


Fig. 13 A Effects of intranasal exosomes loaded with catalase on neuroinflammation. Decreased microglial activation when treated with exosomes (D, E) than when injected with PBS (C) or catalyse not encapsulated within the exosomes (F). Bottom panel shows the quantified effects with the number of activated microglial cells. **B** Proposed formulation flowchart of exosome-based therapeutics for clinical use (taken from: [28])

Haney and colleagues (2015) also proposed a way of using such an exosome-based drug delivery system in the clinic, which can first be harvested from peripheral blood monocytes, then loaded with a therapeutic and re-administered into the patient (see Fig. 13B).

Glioblastoma

Glioblastoma is challenging to treat, and most current methods are highly invasive and risky (e.g. surgery or diffusion-based implant system). siRNAs can silence the carcinogenic genes at the level of mRNA, effectively disrupting the homeostasis, self-renewal and multipotency of cancer cells [81]. Some glioblastoma-relevant siRNAs have already been identified [82], and seeing as exosomes are ideal carriers for nucleic acid, there is a growing interest in their application here. miRNAs are also crucial for chemo- and radioresistance of the tumours. Since mesenchymal stem cells migrate naturally to sites of cancer, modified exosomes derived from the cells were shown to counteract chemoresistance through miRNA delivery to the glioblastoma cells [56].

In a recent study using exosomes to target brain tumours, four types of parent cells were compared (three originating from tumours and one from brain endothelial cells), with the latter deemed the most effective at crossing into the brain due to its molecular signature (specifically CD63 protein). Exosomes derived from that cell type were then loaded with two known anti-cancer drugs (doxorubicin and paclitaxel), together with a fluorescent label (rhodamine 123) [83]. After confirming their ability to cross BBB and induce cytotoxicity of brain cancer cells *in vitro*, they were tested in xenotransplanted zebrafish model (embryos injected with human glioblastoma cells). When delivered by itself, doxorubicin did not have much effect on VEGF levels (vascular endothelial growth factor; marker for tumour growth), but once encapsulated within the exosomes, the RNA levels were significantly decreased (see Fig. 14).

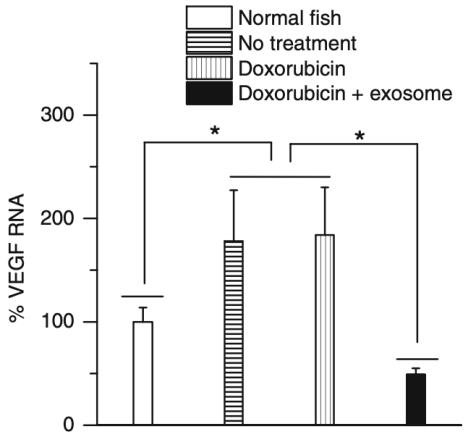


Fig. 14 Decreased level of VEGF when doxorubicin was encapsulated within an exosome in a xenotransplanted zebrafish model of glioblastoma; *: $p < 0.05$ (taken from [83])

A later study from the same group showed that the exosomes were also working when they encapsulated siRNAs for VEGF-knockdown purposes instead [63]. They inhibited VEGF protein and RNA in tumour cells *in vitro*, and in xenograft zebrafish model *in vivo*.

Addiction

Exosomes can have a wide range of application, including even in cases of addiction. A study by Liu et al. (2015) showed that RVG-modified exosomes effectively delivered opioid receptor mu (MOR) siRNA into the brain [84]. MOR is one of the main targets of opioid analgesics involved in primary reinforcing effects, so its blockage is thought to help in treating morphine addiction. Both *in vitro* and *in vivo*, the siRNA was efficiently delivered and the mRNA and protein levels were reduced. Importantly, on a functional level morphine relapse in mice was prevented through this downregulation [84]. These interesting results could pave the way for more investigation into exosomes in the context of drug addictions and relapses treatments.

THERAPEUTIC APPLICATONS: INTRINSIC

As described before, exosomes have been repeatedly shown to play a role in neuronal protection and development. This suggests that they might also be a useful therapeutic in their own right, not only as a carrier, for example through transferring functional molecules to damaged cells [30]. Outside of the brain, plasma-derived exosomes are currently investigated for wound healing [85]. Nevertheless, it is also important to remember about their role in propagating many diseases, and any intervention based on exosomes needs to be carefully evaluated at first. Exosomes derived from mesenchymal stem cells can restore neuronal functions by inhibiting oxidative stress and increasing density of nerve connections. For cognitive impairment in diabetes, they were shown to repair damaged neurons and astrocytes, as well as ameliorate cognitive impairments [86].

Alzheimer's Disease

Exosomes derived from mesenchymal stem cells are also investigated for Alzheimer's Disease [36]. After intracerebral injection in transgenic mice, the cognitive symptoms and hippocampal synaptic plasticity were measured. Clear beneficial effects were noted, both in terms of improved behaviour and rescued CA1 synaptic transmission impairments [87]. Similar

protective effects were observed when exosomes from the MSCs from the umbilical cord were used [88]. These effects were also corroborated by neurosphere models from neural stem cells from AD model mice [89].

Exosomes from MSCs can also be useful in early stages, before clinical manifestation, as shown by the protective effects of intracerebral injection in transgenic mice, at an age when cognitive symptoms have not yet started appearing [90]. As described before, some neuronal exosomes were shown to actually increase clearance of amyloid beta in AD models [62], so it might be worth further investigating those as therapeutic molecules. The same group also found that neuroblastoma-derived exosomes seem to decrease amyloid beta levels, and the common characteristic between the two were the abundant glycosphingolipids which captured amyloid beta [91].

Finally, there is currently a Phase 1&2 open-label clinical trial running aiming to investigate the safety and efficacy of therapeutic use of exosomes derived from mesenchymal stem cells in patients diagnosed with AD (with mild to moderate dementia) [92]. These will be administered as a nasal drip (low vs mild vs high dosage) twice a week for 12 weeks. The outcome measures include potential side effects (e.g. kidney functions), changes in cognitive functioning and neuroimaging.

Psychiatric Disorders

One of the three currently registered clinical trials using exosomes as therapeutics in the brain uses them for psychiatric disorders (treatment-resistant depression and anxiety), and dementia. It is an open-label study aimed at evaluating the safety and feasibility of concurrent treatment of transcranial focused ultrasound therapy applied just before administering the exosomes [93]. This is thought to facilitate the deployment to the subgenual cingulate in depression, amygdala in anxiety and hippocampus in dementia. Exosomes will be obtained from amniotic fluid stem cells, and primary outcome measures are mainly symptomatic improvements (depression/anxiety inventory; dementia scale).

Multiple Sclerosis

Exosomes can have different characteristics dependent on the kind of stimuli their parent cell was exposed to [64]. When dendritic cell-derived exosomes were stimulated with interferon gamma, the released exosomes containing miRNA which were preferentially internalised by oligodendrocytes and consequentially increased tolerance for oxidative stress in target cells *in vitro.* They also improved myelination both *in vivo* when administered intranasally and *in vitro* in slice culture (see Fig. 15) [64].

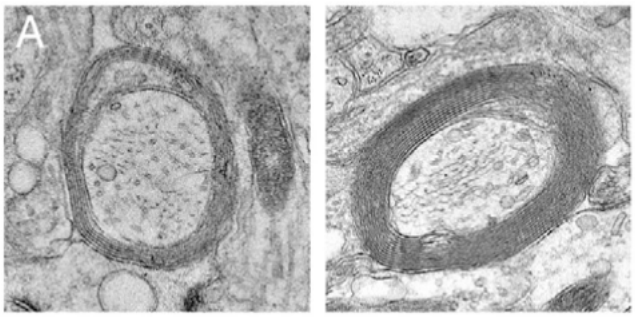


Fig. 15 Width of the myelin sheath in slice cultures as imagined by electron microscopy. Left image: control. Right image: treated with IFNγ-DC-Exo (taken from: [64])

Stroke and Traumatic Brain Injury

In animal models of stroke and TBI, the therapeutic potential of stem cells can also be mediated by the use of exosomes [94]. When MSCs-derived exosomes were injected into rats after TBI, functional recovery and reduced infarct volume were observed [57]. This was mostly due to reduced levels of oxidative stress and inflammation, as well as enhanced neurogenesis. Similarly, intravenous administration of exosomes from MSCs led to cognitive and sensorimotor functional recovery after induced stroke in rats [95]. They also increased the number of neurovascular remodelling (new endothelial cells and neurons) and decreased neuroinflammation. Additionally, a Phase 2 randomised, single-blind, placebo-controlled clinical study is currently recruiting, which will use MSCs-derived exosomes for acute ischemic stroke [96].

CONCLUSIONS

Overall, exosomes offer a new and exciting way of both diagnosing and treating brain disorders with several clinical studies already investigating their potential. They can cross the BBB without

causing adverse immune reactions and retain many useful cellular characteristics while not posing the same dangers and difficulties as cells. Since they are a natural RNA carrier, they are ideal for gene therapy, and they have also been shown to successfully carry anti-inflammatory compounds, and can even have therapeutic potential on their own – not only in neurodegenerative diseases, but also brain tumours and even psychiatric disorders. While many aspects of exosomes development remain to be improved, such as their isolation methods, the fast-development of the field to date suggests that these should also be overcome with new technologies, as seen with e.g. the aforementioned acoustofluidic isolating module.

Interestingly, most of the studies discussed in this review injected the exosomes directly into the brain of the animals, or investigated their effects *in vitro*. With some exceptions, such as successful intranasal administration in a mouse PD model, they did not answer one of the crucial questions about exosomal brain-targeting abilities. While exosomes derived from neural stem cells should be able to do so, they are actually not used that often, with most originating from other cell types and being conjugated, e.g. with RVG. That effectively means that one of their main potential advantages – organotropism, is actually not being used. For clinical applications, more studies investigating systemic administration of neuronally derived exosomes are needed.

Once we can identify and characterise exosomes properly, it will also help us in designing better and more effective synthetic nanoparticles. Since most likely only a few components of the complex mixture of exosomal surface proteins are necessary to obtain its desired characteristics, if we can identify them and engineer liposomal membranes accordingly, we should be able to functionalise their surface with targeting ligands to promote interactions with the brain tissue. Big advantages of such an approach include using known components and lower complexity of the resulting compounds, which would also be easily traceable. While identifying what components exactly would be necessary is still a big challenge, combing the advantages of liposomes and exosomes might allow us to get “the best of both worlds”.

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