Extracellular vesicle-based therapy application and efficacy in ophthalmic diseases: from origin to target

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List of abbreviations

BBB	Blood Brain Barrier
BMECs	Brain Microvascular Endothelial Cells
BMSC	Bone Marrow Stem Cell
BRB	Blood Retinal Barrier
ChorSC	Chorion Stem Cell
CKD	Chronic Kidney Disease
CNS	Central Nervous System
CNV	Choroidal Neovascularization
ESCRT	Endosomal Sorting Complex Responsible for Transport
EV	Extracellular Vesicles
FD	Fibroblast
FFFF	Flow Field-Flow Fractionation
HSPG	Heparan Sulfate Proteoglycans
IVT	Intravitreal
MenSC	Menstrual Stem Cell
MH	Macular Hole
MI	Myocardial Infarction
miRNA	micro-RNA
mRNA	messenger RNA
MS	Mass Spectrometry
MSC	Mesenchymal Stem Cell
MV	Microvesicle
ONC	Optic Nerve Crush
RAC	Retinal Astrocyte Cell
RGC	Retinal Ganglion Cell
RPE	Retinal Pigment Epithelium
RVE	Retinal Vascular Epithelium
SEC	Size Exclusion Chromatography
UCMSC	Umbilical Cord Mesenchymal Stem Cells

Abstract

The dysfunction and degeneration of retinal layers are the leading causes of vision impairment. Ophthalmic diseases, among which glaucoma and retinopathies, if diagnosed at late stages are often associated to irreversible consequences, such as blindness. The management of posterior ocular disease remains critical due to the complexity of ocular morphology and physiology, therefore new diagnostic and neuroprotective strategies are required. Advances in extracellular vesicle (EV) research has raised the potential use of these natural bi-layered vesicles as delivery systems in multiple tissues. EVs are heterogenous nanosized particles composed by a variety of biomolecules, including proteins, lipids and different RNA biotypes. It is hypothesized that EVs mediate intercellular communication by delivering their functional cargos to recipient cells, leading to phenotypic changes. The application of EVs in the retina has been demonstrated to elicit significant therapeutic effects. Recent evidence has indicated that EVs administration in retinal cultures and various animal models, including optic nerve crush (ONC) and diabetic neuropathy, promote retinal neuroprotection and regeneration. In this review results from currently available studies focusing on EV-based therapy application in ophthalmic diseases will be summarized, highlighting strategies to improve EVs uptake and quantifying EVs efficacy. Furthermore, possible solutions and future research efforts, concerning the clinical translation of EVs application in the ocular field, will be indicated.

Keywords: extracellular vesicles, exosomes, retina, mesenchymal stem cells, cargo, miRNAs

Introduction to ophthalmic diseases

Ophthalmic diseases have a direct impact on individuals' vision and quality of life, posing at the same time a significant financial burden on the global economy. In 2020 the prevalence of vision impairment was equal to 4.3%, representing a worldwide public concern. Estimations indicated that 43.4 million people are blind while 295 million are affected by moderate and severe vision problems (1). Of these the most indisposed age group are adults over 50, who are often affected by less treatable diseases such as glaucoma, diabetic retinopathy and age-related macular degeneration (2). These disorders are associated with photoreceptors damage, retinal ganglion cells (RGC) death, choroidal neovascularization (CNV) and vessels leakage, often leading to loss of vision (3). Currently there are interventions that seem to be effective in correcting and restoring mild impairments such as cataract and various uncorrected refractive errors (4). However, due to the complexity of ocular morphology and physiology, posterior ocular disease management remains challenging. For instance, it is known that the blood-retinal barrier (BRB), which is the biological barrier protecting the eye, has often hindered treatment specificity and reduced drugs distribution, thus causing a poor effect (5, 6). As a consequence, certain retinal conditions, such as the aforementioned ones, if diagnosed at late stages, are degenerative and irreversible (3). Retinal cells constitute the optic nerves that are involved in the visual phototransduction cascade, able to send visual information to the brain. These pathways can be permanently compromised in case of retinal dysfunction, similarly to neurodegenerative processes occurring in the central nervous system (CNS) (7). Therefore, further consideration in the ophthalmologic research should be placed in improving ocular disease diagnostic tools and in finding new neuroprotective strategies (8).

Alongside advances in stem cell biology and nanotechnology, a novel free cell-based therapy has been explored and employed in a variety of diseases. This alternative application consists in using a fraction of the secretome produced by cells, namely extracellular vesicles (EVs), as active cargo delivery systems able to target specific tissues. EVs are lipid bound vesicles detected in most body fluids, containing functional cargo that reflect the genetic material of the source cell (9, 10). Although, these vesicles were initially considered cellular waste, their potential biological activity and paracrine role in cell-cell communication is now being closely investigated. Besides their functions, EVs are emerging to be successful nanocarriers due to their natural origins, which renders these vehicles well tolerated by the human body, thus becoming safe and feasible for clinical implementation (11). Among the therapeutical applications of EVs, it is of interest its efficacy in ocular diseases, which was shown to induce *in vitro* and *in vivo* models neuroprotective, anti-

inflammatory and anti-angiogenic effects in the injured retina (10, 12). Although these outcomes exhibit promising use of EVs in preserving retinal function and survival, the underlying mechanisms in targeting the retinal tissue remain unclear.

In this review the effect of EV-based therapy application in ophthalmic diseases will be quantified by exploring in detail the paracrine role and transfer of EVs within the eye structures of interest including the vitreous chamber, the retina, and the choroid. To achieve this goal, a summary of existing *in vivo* studies, focusing on the posterior ocular disease management via intravitreal EVs administration, will be provided. By supplying an overview on the successes and failures of recent animal studies, this review will provide indications on how to approach toward human treatment.

What are EVs: origin, types, function

EVs are nanosized, bi-layered vesicles released from most types of cells into the extracellular space. There is a vast heterogeneity of EVs, which are generally characterized by negative surface charge and by the presence of transmembrane proteins such as tetraspanins, among which CD9, CD63 and CD81 (13, 14). Three EVs subclasses are categorized based on their particle size and biogenesis as exosomes (30-200nm), microvesicles (100nm-1μm) and apoptotic bodies (>1μm) (15), see Figure 1. Exosomes are originated from the endosomal route, consisting of the invagination of early endosome membrane that first matures in the multivesicular bodies, which are then released as small spheres via exocytosis. Microvesicles (MV) are derived directly from the outward budding of cell's plasma membrane, being secreted in irregular shapes. While apoptotic bodies, which may contain intact cell's organelles, are released by blebbing of cells undergoing death (16). Exosomes and MVs, often referred as small EVs, carry a large variety of components derived from their source cell, such as adhesion molecules, proteins, enzymes, lipids, nucleic acids and other small-molecule metabolites (16). Although it remains difficult to distinguish exosomes from MVs, due to compositional and particle size overlapping, it is possible to distinguish the firstly mentioned by the presence of specific biomarkers. For instance, proteins related to the Endosomal Sorting Complex Responsible for Transport (ESCRT), such as TSG101, Hsp70, Hsp90, are exclusively involved in the endosomal formation of EVs, thus of exosomes (17). EVs isolation and characterization will further be discussed in the following paragraphs.

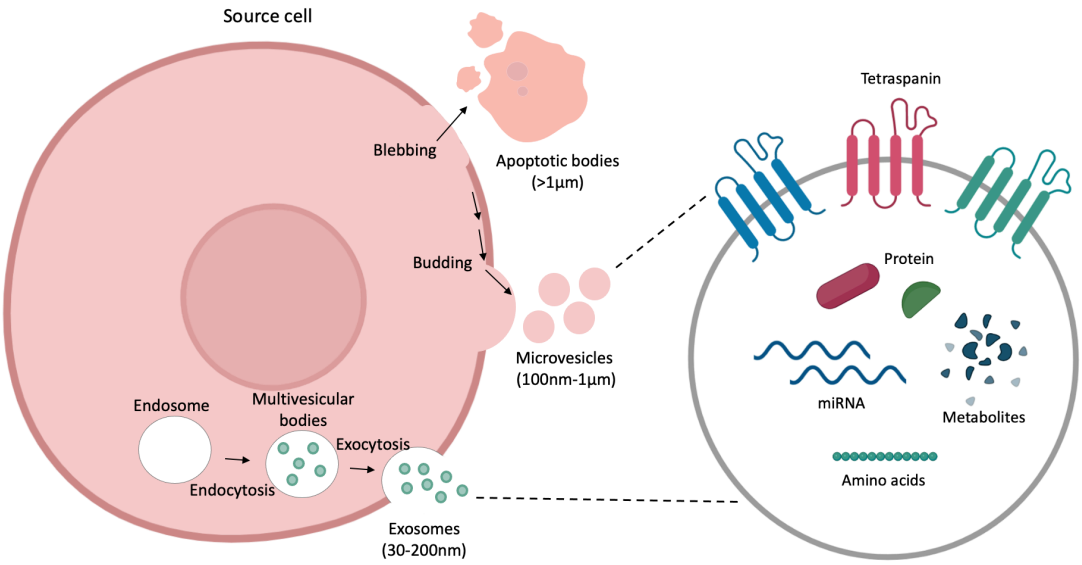

Figure 1. EVs biogenesis and classification. Source cells originate different types of EVs, among which the best distinguished are exosomes, derived from exocytosis of the multivesicular bodies; microvesicles derived from budding of the cell membrane and apoptotic bodies discarded via blebbing. Adapted from Gurung *et al.*, Cell Communication and Signalling (2021) (15).

The biology of small EVs is not well understood yet, however, their secretion has been associated to different cellular pathways, such as intercellular signalling, cell maintenance, response and development (16, 18). Significant consideration has been given to the role of RNA content in EVs due to the hypothesis that by transferring such biological information within cells, EVs acquire their function as mediators in intercellular communication. As anticipated, small EVs contain many RNA subtypes, among which the most characterized are messenger RNA (mRNA) and micro-RNAs (miRNA). Previous studies have reported different genetic material compositions and concentrations of EVs depending on the different cell source or different physiological conditions, including healthy, pregnancy or diseased state (18, 19). For instance, several studies have detected alteration in EVs secretion rates and EVs miRNA expression in correspondence to ocular disease, such as retinal detachment, glaucoma and uveitis (20-22). These findings indicate an involvement of EVs in both physiological and pathological state of the cells from which they are originated. Therefore, considering that EVs are found in many biological fluids including blood plasma, urine, breast milk, tears, bronchial, seminal and amniotic fluids (19), they could function as carriers of diseases biomarkers. EVs could be strong diagnostic tool candidates as they can be sampled in a non-invasive way and employed in monitoring patients state and response to selected interventions (16). Another clinical potential of EVs, that is being critically evaluated within the nanomedicine research, is their application as therapeutic vehicle. This role has been strongly supported by various evidences highlighting the ability of EVs to target specific tissues and to actively deliver their cargos

into recipient cells, leading to functional changes and gene expression alterations, such as protein knockdown (23, 24). Furthermore, studies of *in vivo* EVs biodistribution have further confirmed the involvement of these vesicles in cell-cell communication, including intracellular exchange with either distant or neighbouring targets (25). Regarding this exchange of information, three main mechanisms of material transfer between EVs and recipient cells have been disclosed: receptor-ligand mediated interaction, endocytosis and fusion, see Figure 2. Interactions mediated by receptor ligands consist in a direct communication mediated between EVs transmembrane protein, such as tetraspanins, and compatible recipient surface signalling receptors (26). Other EVs internalization methods are direct fusion and consequent delivery in the cytosol of the target cell, phagocytosis and endocytosis. This lastly mentioned is defined as EVs incorporation into the target followed by different fates, among which paracrine transfer to a neighbouring cell or degradation, through lysosome maturation (26, 27).

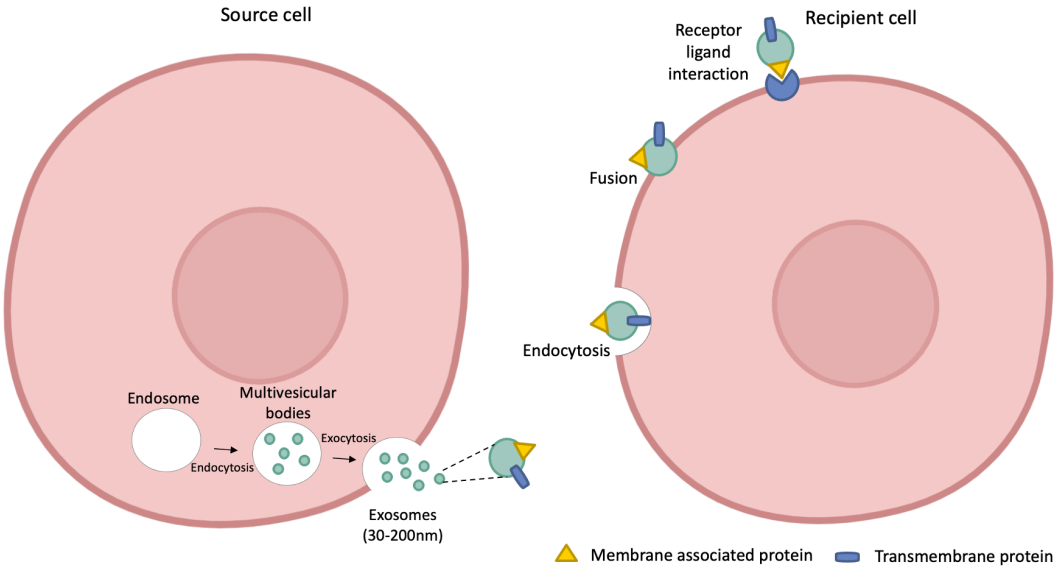

Figure 2. EVs interaction mechanisms. EVs communicate with recipient cells through different pathways, including receptor ligand mediated interaction, fusion and endocytosis. Adapted from Wang *et al.*, International Journal of Endocrinology (2020).

Within the drug delivery field, EVs are considered more powerful nanomedicine tools in comparison to other carriers for a series of reasons. Firstly, EVs are naturally occurring vesicles characterized by high stability and most importantly low toxicity and immunogenicity in the human body (11). Whilst synthetic nanoparticles, such as liposomes, have widely been implemented in clinical settings, they could still trigger immunological responses. According to their natural origin, EVs were shown to have intrinsic homing abilities, thus they are facilitated in penetrating biological barriers and in being internalized into target tissue (11, 28). Crossing such membranes remains one of the biggest

challenges of synthetic carriers, whereas EVs seem to overcome this limitation in a large variety of cases including Blood-Brain Barrier (BBB), BRB and Placental Barrier (5, 24).

Stem cell derived EVs

Although EVs can be derived from many cells, significant consideration is given to stem cell-derived EVs for resembling abilities and characteristics of source cell, with the advantage of representing a free-cell therapy. EVs in comparison to their cellular counterpart were shown to yield equal therapeutic effects in terms of tissue injury prevention. Besides its efficacy, this alternative application shows a higher safety profile, overcoming immunologic rejection or induction of tumour progression, and an enhanced delivery process, including cargo protection from degradation and uptake (29, 30). A fair comparison of the risks taken in the two applications was shown in the outcome of two clinical trials whose aim was to heal refractory macular hole (MH). Mesenchymal stem cell (MSC)-derived EVs stimulated closure of MH in all patients, however only one person participating to the pilot study experienced an inflammatory reaction which was attenuated by reducing the treatment dose, thus not causing any severe side effect (31). On the contrary, patients with analogous ocular problems, who were treated with MSC-based therapy suffered of irreversible vision loss due to unwanted differentiation of the transplanted cells (32), hence confirming the safety issue characterizing different stem cell employment.

Application of stem cell-derived EVs has been shown to yield positive effects in myocardial infarction (MI) and kidney disease animal model, indicating a potential underlying miRNA dependent-mechanism. C57BL/6J MI mice, when treated with MSC-derived EVs showed an increased angiogenic effect and cardiac function, with an additional reduction in the fibrotic areas, caused by the cardiac disease. These effects appeared to be related to the enriched expression of miR210 in MSC-EVs, which potentially targets and downregulates the Efna3 gene, responsible for angiogenesis inhibition. This dependency was further confirmed by administering MSC-EVs with silenced miR210, resulting in the loss of the previously mentioned effects (33). Furthermore, research focusing on brain tumours demonstrated that angiogenic effects were also retrieved in glioblastoma derived MVs in association to their mRNA and protein cargo, including angiogenin, IL-6 and IL-8 (34). Kog *et al.* observed, through *in vitro* assays, that mRNAs and proteins associated with cell proliferation and angiogenesis, carried by these MVs, were functionally delivered in brain endothelial cells leading to doubled tubule length within 16 hours from administration.

Nevertheless, miRNA dependency was also proven in BALB/c mice model affected by bilateral renal ischemia/reperfusion injury. Receiving $5x10^{10}$ bone marrow stem cells (BMSC)-derived EVs, via tail vein injection, led to attenuation of the condition and promotion of tissue regeneration and repair. EVs presented overexpression of miR-199a-3p (roughly 250-fold higher than HK-2 tubular kidney cells), which seemed to block the apoptotic process of cells induced by the injury model. In addition, BMSC-EVs also caused an improvement in kidney function by reducing certain negative biochemical indicators. Following an improvement in kidney architecture and anatomy, including amelioration of tubular dilation and brush border loss, was shown (35). In line with this study, the research performed by Nassar *et al*. on patients affected by chronic kidney disease (CKD) showed that MSC-EVs are safe and effective in slowing and attenuating the renal damage. The administration of EVs could significantly improve the immune inflammatory response and the renal function, by ameliorating the estimated glomerular filtration rate and the urinary creatinine ratio. Besides modulating the inflammatory response, EVs were also proven to have a clinical effect on kidney cells in terms of proliferation and differentiation (36). Additionally, Nassar *et al.* highlighted the importance of the administration route and the frequency of the injections of MSC-EVs. To obtain a better distribution and improved bioavailability of the EVs therapy, multiple doses and injection routes were chosen. Eventually a first dose was injected intravenously, modulating the inflammatory response of the circulating lymphocytes, while a second one was administered via intra-arterial injection, to maximize target specificity in the local kidney environment and diminish EVs clearance effect exerted by organs such as the liver and the spleen (25, 36).

EV-based therapy: from origin to ocular target

EVs isolation and characterization

EV-based therapies applied to ocular disorders are mostly derived from stem cells, such as umbilical cord mesenchymal stem cells (UCMSC), MSC, BMSC, with the exclusion of cases where they are originated from specific tissue, like fibroblasts (FD) and retinal astrocyte cells (RAC). EVs can be manufactured exogenously by first culturing the source cells of interest, under suitable conditions, and then by thoroughly isolating and characterizing the different debris and secretome fractions present in the culture medium, see Figure 3. A standard practice to isolate EVs fractions is to subject the medium to several ultracentrifugation or precipitation steps. To obtain further purification, in terms of size exclusion, the re-suspended supernatant is subjected to other filtration steps. This method has been commonly used in studies focusing exclusively on exosome-based therapies,

where they are separated from MVs through a 0.22μm filter passage (37-39). Following, various characterization techniques are employed to identify the isolated fractions in terms of particle size distribution, morphology and biomarkers detection. Currently available methods that are classified as more standardized techniques, are imaging, Nanoparticle Tracking, Flow Cytometry, Western Blotting and ELISA analysis; whereas more advanced technologies include Size Exclusion Chromatography (SEC), Flow Field-Flow Fractionation (FFFF) and Mass Spectrometry (MS)-Based Proteomic analysis (16). Next to all the advantages associated to EVs, also stand some drawbacks, among which there are their low productivity and limiting isolation and characterization processes. There is no gold standard for EVs isolation and characterization, since only partial purification can be achieved by the previously mentioned methods due to EVs heterogeneity and overlapping in physical characteristics with other biological nanoparticles (9). Therefore, the presence of impurities and unwanted EVs could be challenging and risky in clinical translation.

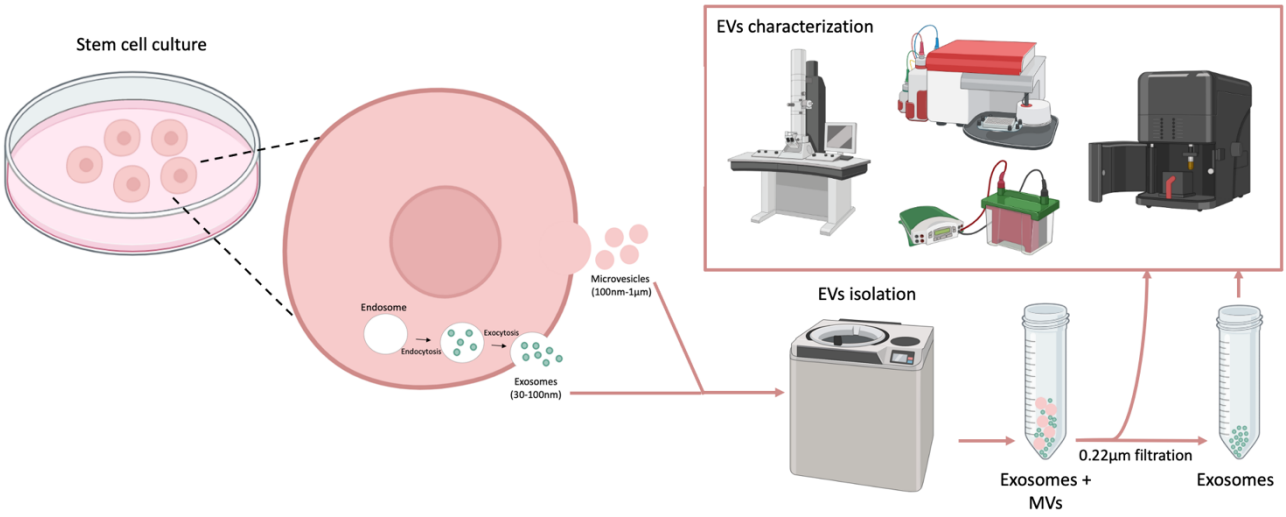

Figure 3. EVs isolation and characterization. EVs after they are exogenously produced from cell cultures of interest are isolated by ultracentrifugation and filtration steps. Physical characterization is obtained using standard techniques such as imaging, Western Blotting, Nanoparticle Tracking and Flow Cytometry.

Some studies have identified contrasting effects triggered by distinctive EVs fraction. A research group focusing on the effects of EVs derived from menstrual stem cells (MenSC) on cortical neuron cultures, showed EVs to be efficacious in promoting neurite growth. Interestingly, it emerged that treating cultures with different fractions of the MenSC secretome led to divergent outcomes. For instance, isolated MVs negatively impacted the cortical neurite length showing a 45% decrease in the length of longest neurite compared to the control condition, thus implementing an inhibitory effect. Whilst both isolated exosomes and combined exosomes with MVs fractions showed a

positive effect, yielding almost 40% increase in neurite growth compared to control condition. This evidence indicates the ability of exosomes to counteract and overcome the inhibitory effect of MVs (40). An analogue situation was also shown in the study conducted by Mead & Tomarev, where a dose of 3 x 10^9 EVs derived from BMSC promoted survival of RGCs and neuritogenesis *in vitro* retinal culture. In this case 37%/82% of the neuroprotective effect and 82%/92% neurites were lost once the dose was increased to respectively 1.5 x 10^{10} and 7.5 x 10^{10} EVs. However, if the EVs were filtered, thus containing only exosomes, the positive effect at the same high dosages was acquired back (37). In addition to the previous study, it resulted also that MVs tend to suppress even exosomes efficacy at high dosages. The different therapeutical outcome caused by the two EVs subtypes might be related either to the differences in binding and uptake capabilities, which could yield different effects depending on target cells, or to the cargo packaged in each system. However, many studies do not apply EVs size exclusion, due to the previously illustrated limitations, avoiding to specify whether they are administering exosomes or MVs, referring to the treatments as small EVs (31, 38).

Besides the differences among EVs subtypes, Lopez-Verrilli *et al.* in the same research designed a comparative study to understand the potential effect of purified exosomes derived from different stem cell sources, such as chorion (ChorSC), UCMSC, BMSC and MenSC. It was observed that all groups exerted a positive effect on the cortical neuron culture, however only BMSC and MenSC appeared to significantly promote neurite growth by inducing an increase of respectively 42% and 32% (40). This diversity might as well explain the affinity that certain EVs have in communicating and trafficking within specific tissues as their activity results to be dependent on their producer cells. In conclusion it appears that efficacy of EVs is dependent on both its origin and biogenesis and thus physical characteristics. So far MVs seem to have negative effects when administered exclusively or in combination with exosomes at higher dosages in retinal layers, while exosomes application seems to be consistently beneficial. To further outweigh the inhibitory character of MVs, additional *in vitro* testing and comparative studies involving different source cell lines should be performed.

EVs cargo enrichment and engineering

A study conducted by Li *et al.* on isolation and RNA analysis of exosomes concluded that in theory exosomes should be able to contain up to 25.000 small RNAs, of 100 nucleotides each, or protein molecules, ranging approximately 50kDa. However experiments have shown a significantly lower RNA concentration in individual serum derived exosomes, being equal to one or less RNA molecules

(41). This observation does not cancel the biological involvement in cell communication because, as previously mentioned, EVs and exosomes, are very heterogenous populations containing different cargos and having diverse functions. In agreement with these outcomes the stochiometric analysis of RNA content in exosomes, conducted by Chevillet *et al.*, indicated that the majority of singular exosomes retrieved by standard preparations do not carry sufficient RNA to lead to a functional delivery, suggesting that the effect is induced by a specific fraction of vesicles. To combine the biological role of exosomes and the stoichiometry results, four plausible scenarios were illustrated, see Figure 4. Both a high occupancy/high RNA and a high occupancy/low RNA concentration models reflect a theoretical and ideal exosome content, assuming that all exosomes are equally functional. Low occupancy models would be more representative of the heterogeneity and diverse characteristics of EVs. In a pool of EVs, low occupancy/low RNA concentration would stand for a small fraction of exosomes carrying a low genetic material concentration. This model would still induce certain effects, being more functional in case of rapid cellular uptake allowing accumulation of the vector. While the low occupancy/high RNA concentration model supposes the enrichment of certain genetic sequences in one or few vesicles, being successful in a highly selective targeting and uptake of the vehicle (42).

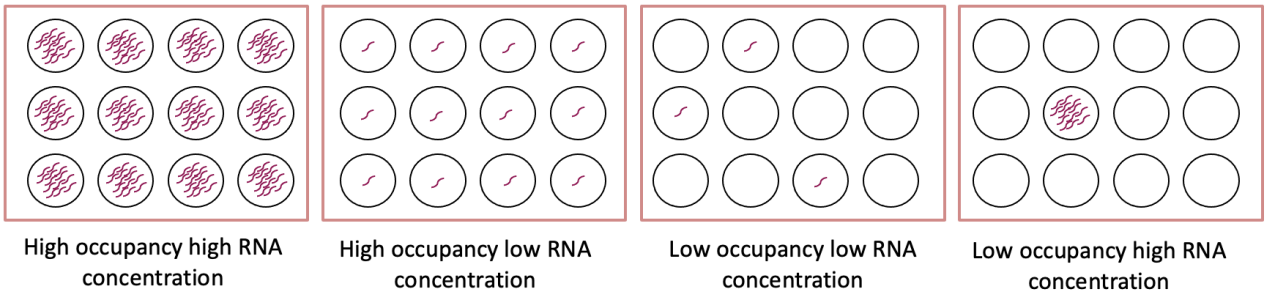

Figure 4. EVs cargo distribution model. Four different models of the RNA cargo are proposed. A uniform distribution of the cargo is shown in both the high occupancy models, indicating that all EVs are equally functional. Whereas the low occupancy models show that only scattered EVs are functional within the bulk. Adapted from Chevillet *et al.*, Proceedings of the National Academy of Sciences (2014).

Scientific publications on EVs application in the ocular research field, have shown that this topic is lagging behind compared to others (10), leading to a narrower range of studies. Most research that was performed on EVs employment in ocular disease showed a recurring strategy, in which EVs are purified and directly injected into animals or humans, as shown in the in macular hole healing clinical trial conducted by Zhang *et al.* (31). However, to boost and optimize EVs functional delivery as vectors into target tissues, several pre-clinical and few clinical studies have implemented EVs post-

purification modulation. These alternative approaches include exogenous cargo loading engineering and manipulation of EVs membrane to improve uptake rates (43). To increase the therapeutical success, these strategies should be performed in a way that EVs integrity, as well as safety, are maintained by avoiding use of antigenic or toxic components. Based on what is emerging concerning ocular targets and what is already known on EVs communication skills, it could be possible to extrapolate the use of these EV's alterations from other diseases research to achieve a better understanding of EVs efficacy on retinal targets.

Two main components could be modulated in order to boost the functional delivery of EVs: the cargo and the surface of the vesicle. To confer high specificity and achieve a better targeting, selected protein and RNA cargo, presenting affinity for sequences that will be identified by the RNA-binding motifs and receptors present in the recipient cells, should be loaded exogenously in the *in vitro* manufactured EVs. Currently, a range of proteins and miRNA cargos have been hypothesised to play a role in retinal disease, including endostatin, CXCL-1, MMPs, miR-126, miR-222, miR-21, miR-202-5p, miR543 and miR-27b (9, 44). Less is known or specified concerning mRNA delivery in ocular targets, however application of EVs in cancer treatment has demonstrated functional delivery of mRNA contributing to new proteins translation and target gene modulation (45). Post-isolation EVs cargo loading has been performed with a variety of techniques depending on the type of content included in the cargo. Among these methods, the most implemented in loading proteins, different RNA biotypes and other small molecules are electroporation, sonication, co-incubation and lipofection (46), see Figure 5A. Electroporation is a widely used technique that allows the introduction of genetic molecules into the cell membrane by creating pores with the use of electric pulses. Such practice was implemented by Alvarez-Erviti *et al.* to load exogenous siRNA in isolated exosomes for the silencing of BACE1, being a relevant target for Alzheimer's disease. Loading success was assessed indirectly, by the fact that following the systemic delivery of the electroporated exosomes in mice models, analysis of cortical tissues showed respectively 62% and 61% BACE1 protein and mRNA levels knockdown (24). This method was further optimized in terms of voltage, capacitance, exosome concentration and purification in other studies, which similarly were focusing on exogenous exosome loading (47, 48). Among this the research led by Momen-Heravi *et al.,* focusing on macrophage inhibition mediated by B cell-exosomes carrying miRNA-155, showed that 55% of the load was successfully recovered after optimizing the electroporation conditions resulting in functional delivery. To further improve loading capacity of EVs, it would be

of interest to conduct exploratory studies aiming to better understand to what extent EVs can be subjected to such stress without undergoing major cargo losses or physical damage.

Another comparable physical method is sonication which consists of deforming the vesicles membrane allowing the transfer of desired small molecule drugs or RNAs. High-loading efficiency of this approach were shown in animal models of breast cancer and pancreatic cancer (49-51). In addition, Haney *et al.* showed that a mild sonication yields a better cargo incorporation when compared to co-incubation method, which simply consists of incubating the vesicle and the exogenous cargo to be loaded under favourable conditions. This strategy is highly dependent on the lipophilic properties of the molecules which, also based on their gradient concentration should be able to first interact with the vesicle's membrane and then diffuse into it (52). Unlike the previously described physical treatments, transfection is a technology that allows the transfer of exogenous nucleic acid or proteins into cells, or EVs, by using chemical reagents (53). For instance, lipofection, known as lipid transfection, was implemented in the previously mentioned study of Alvarez-Erviti *et al.,* who loaded siRNA on exosomes targeting mouse model's brain. However, it is known that isolated EVs already contain a cargo derived from their cell source, thus a limited amount of additional material can be loaded in there (43), unless new strategies on partially replacing the original content with the exogenous one are explored.

Before delivering their cargo, EVs should interact with recipient cells. To increase the recognition and translocation of EVs through the acceptor cell, further EV packaging engineering should occur by displaying specific surface peptide or proteins, see Figure 5B. For instance, a wide range of fusion proteins that are abundantly found on EVs membrane are also present within the retinal tissues (43). Among these receptors cell-surface adhesion molecules, such as CD44 (6), membrane-based proteins (like caveolins and clathrins) (54-56), the already mentioned tetraspanins and Heparan Sulfate Proteoglycans (HSPG) could play an important role in the transfer of EVs into the retinal target (13, 14, 17), hinting toward the promotion of a selective uptake in the target and an eased crossing of the biological barriers surrounding it.

In line with the concept of modulating EVs to yield better results, another limitation that could be overcome is the low productivity of *in vitro* EVs, due to the already described drawbacks concerning the purification issues (9). Strategies to enhance the production and stability of EVs should further be explored to allow the growth and understanding of EVs therapeutical purposes. A couple of ideas have been indicated, such as increasing EVs control by focusing on EVs production boost, achievable by overexpressing certain proteins and modulators able to promote EVs biogenesis (43).

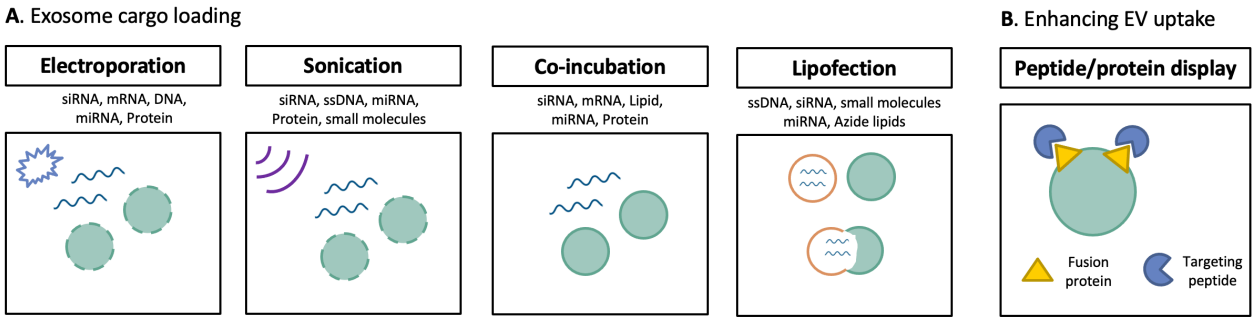

Figure 5. EVs engineering. Two strategies to enrich and modulate EVs uptake are shown: (A) different techniques, such as electroporation, sonication, co-incubation and lipofection are implemented to exogenously modulate the cargo of EVs; (B) displaying fusion proteins corresponding to target receptors or protein present on recipient cell could improve uptake of the vesicle. Adapted from Joshi *et al.*, Materials Today Nano (2021).

Despite loading and uptake enhancement, EVs content delivery to acceptor cells has not been defined yet, remaining neglected. Assuming the presence of scattered functional cargos in a batch of EVs, it is essential to quantify the amount of delivered material to estimate the physiologically relevant dose of EVs and cargo. The evaluation of cargo delivery in most EVs research, as it will be shown below in ophthalmic application, is assessed based on the phenotypic changes in the recipient cells. This approach, depending on the experiment design, which may include or not adequate controls and silencing elements, is not always reliable due to the presence of several factors influencing the outcome (57). To exclude cofounding, further research needs to be performed on functional EVs cargo transfer, implementing stoichiometric analysis and absolute quantification of proteins and RNA molecules of interest in recipient cell. This quantitative analysis requires the development of appropriate bioassays and the employment of various technologies applied on single EVs, ranging from quantitative or digital PCR to microfluidic platform (58, 59).

EVs delivery: intravitreal administration

The functional targeting of the posterior ocular tissues, such as the retina, is a challenging achievement. Besides the complexity of the eye's architecture, the drug delivery is highly impacted by the bi-layered blood BRB composed by the inner retinal vascular epithelium (RVE) and the relatively outer retinal pigment epithelium (RPE) (5, 60). Biological barriers, like BRB, apart from conferring protection to tissues and organs can also prevent treatments from being functionally delivered to the target by decreasing or even preventing their distribution in the protected areas (5). Different administration routes are available to treat ocular disease, including systemic, topical, periocular, sub-retinal and suprachoroidal delivery. Nonetheless most studies focusing on EVs

application in retinal disease appear to prefer intravitreal (IVT) injection (31, 37-39), which compared to the direct sub-retinal administration, employed in gene therapy and cell replacement, results less invasive and risky (61). IVT administration is a widely implemented technique to target the retina by directly injecting the drug into the vitreous humor of the eye. This lastly mentioned structure is a chamber consisting of water, heterogeneously distributed hyaluronic acid and collagen fibers (61). Compared to the BRB, also the vitreous chamber could act as a barrier for drug delivery, however due to its loose mesh size of an average of 500nm it represents a less restrictive environment (62). The vitreous does not appear to impede the mobility of nanoparticle systems whose size ranges below 500nm, although it is known that diffusion is reduced in case of increased particle size or presence of positively charged particles (61, 62). Therefore, based on these features, delivering EVs in the vitreous seem to be a valid choice due to the direct access to the target retinal sites, such as ganglion cells, photoreceptors, RPE and choroid. Even if IVT delivery seems to grant a better bioavailability of the cargos, it should be considered that the injected nanoparticle systems will be subjected to intravitreal clearance. This elimination process, in line with the retinal anatomy, has been shown to occur for larger particles only through the anterior route, via the aqueous humor flow rather than the posterior, which implements the permeation through endothelium and vessels of the iris (63). Although EVs could exhibit a longer circulating half-life compared to other non-natural drug delivery systems or smaller particles, Mathew *et al* confirmed that EVs half-life in the vitreous was equal to 2.5 days. In agreement with the conclusion of the author, these findings implicate that to increase the systems accumulation in the vitreous, yielding to a more powerful therapeutical effect, multiple injections or higher dosages could be required (55), whilst the risk of unwanted side effects could be encountered.

EVs targeting and effects: from vitreous to retina

The transfer of vehicles, such as EVs, from the vitreous chamber into the retina is followed by the gradual diffusion across the different retinal layers, being facilitated by their nanometre size. The retina, located between the vitreous chamber and the choroid, besides the BRB is composed, starting from the outermost portion, by ganglion cells, inner/outer plexiform, nuclear and photoreceptor layers (60). Animal studies focusing on EVs treatments in the ophthalmic research, have shown a variety of therapeutical effects regarding different sites of the retina, see Table 1. Different effects were promoted depending on the injured area of interest. However, the targeting mechanisms were not elucidated, seeming that recipient tissues and layers were targeted

indistinctively. EVs knowledge in combination with the outcomes of these pre-clinical studies, were utilized to roughly reconstitute the transfer of EVs through the retina, see Figure 6A.

Table 1. Summary of *in vivo* studies involving EVs application in ocular diseases

Effect	**Disease**	**Source cell**	**Target**	**Cargo**	**Reference**
Neuroprotection and regeneration	Optic therapeutic nerve crush Glaucoma	BMSC FD MSC	RGCs	miRNA	Mead & Tomarev, (2017, 2018a, 2018b) (37-39) Tassew *et al.* (2017) (64)
Vascular leakage suppression and CNV inhibition	Retinal Laser Injury Retinal Ischemia	RAC MSC	RPE and Choroid	Anti-angiogenic molecules	Hajrasouliha *et al.*, (2013) (44) Moissev *et al.*, (2017) (65)
Inflammation suppression	Diabetic Retinopathy	MSC	Photoreceptors	miR-126	Zhang *et al.,* (2019) (66)
Retinal architecture restoration	Diabetic Retinopathy	MSC	Various retinal layers	miR-222	Safwat *et al.*, (2019) (67)

The study conducted by Pan and collaborators, where rats with induced optic nerve crush (ONC) were treated with multiple intravitreal dosages (equal to 1 x 10^9) of exosomes derived from UMSC showed significant RGC survival, 12.5% higher compared to placebo group. Despite this, the neuroprotective effect appeared to be limited compared to what EVs deriving from other sources have shown in other studies. This study case needs to be considered as exosomes were labelled and tracked after being injected. In agreement with the exerted therapeutical effects the target site resulted to be the inner retina, however no specificity in staining certain structures such as neurons or astrocytes, within the same layer, was obtained (68). Mathew *et al.*, who studied the uptake and distribution of BMSC-EVs in the retina of glaucoma rat models, were able to define the vesicles destination and uptake time course. The majority of EVs were tracked in the inner plexiform and nuclear layers of the retina, while scattered were identified in the corresponding outer levels (55). This indicates a possible limitation in proceeding deeper in the retina, depending on the size-diffusion hindrance or the poor bioavailability reached at that level of the retina. Stained EVs were poorly localized in astrocytes and Muller cell's foot plates; rapid uptake, within a day, was observed in microglia, whose staining intensity peaked 7 days after administration, while the longest

residence time of the vesicles was attributed to the retinal ganglion cells peaking at day 14 (55). All the aforementioned retinal structures showed to have a dose-dependent saturable EV uptake kinetic and few or no EVs were visualized in any of them 28 days after receiving the IVT injection (55). These findings highlight, as in the residency time of EVs in the vitreous described previously, that to obtain significant neuroprotective effects in retina it should be required to find a strategy in elongating the half-life of these vesicles in the sites of interest. For instance, it could be interesting to understand which factors are involved in the different distribution and uptake kinetics among the RGCs, astrocytes and microglia and whether there is a way to modulate certain conditions or aspects to achieve a longer permanence, or on the contrary a reduced clearance.

Most of the *in vivo* studies that have been performed, have commonly targeted the inner retinal layer, more specifically RGCs. The frequent targeting of these cells, as witnessed by Pan and Mathew, could be a justified because of the neighbouring location among RGCs and the vitreous. The most feasible explanation on EVs translocation into the ganglion cells could be explained by a receptor-mediated internalization mechanism facilitated by the enrichment of the previously listed key proteins and cell-surface receptors that are often found on EVs membrane and retinal targets (see paragraph 3.2), see Figure 6B. Various studies conducted sequentially by Mead and Tomarev demonstrated the therapeutical effects of EVs derived from stem cells in mice affected by ONC and glaucoma, simulating retinal disease. The research published in 2017, besides highlighting the previously discussed efficacious differences between MV and exosomes, reported that ONC mice treated intravitreally with 3×10^9 exosomes showed RGC survival promotion. 21 days after injection BMSC-EVs caused only 30% reduction in the RGC count and function compared to healthy mice, being significantly beneficious against the 80-90% loss derived from untreated groups (37). Furthermore, this study was also able to understand the underlying mechanisms of the RGC survival. In fact, by transfecting the BMSC-EVs with siRNA against Argonaute-2, a successful knockdown of the miRNA effector protein Ago2 was achieved, leading to the loss of the previously obtained effects (37). Therefore, the driving force of the neuroprotective and axogenic effects in EV-based treatment was attributed to the miRNA cargo. Following research led by the same authors, demonstrated that the MSC-exosomes tend to preserve RGC count and function also when administered in mice and rats with differently induced glaucoma models. 56 days after since the beginning of the intervention, weekly administration of the exosomes seemed to be the most impactful. In both glaucoma models induced by microbeads and laser, the weekly therapy caused only 10% and 4% RGC loss respectively compared to control intact group, yielding almost 3-fold better neuroprotection compared to the

monthly administration (39). Different protection was reported between small EVs originated from different sources, such as BMSC, fibroblast. When comparing such treatments in a DBA/2J mice chronic hypertension ocular model, it emerged that BMSC-EVs were able to reduce RGC axonal injury and to significantly preserve RGC function only 6 months from the administration; however similar to FD-EVs or untreated group less effect was shown in preventing late RGC decline (38). Partly in contrast with these findings, Tassew *et al.* reported that FD-exosomes also significantly promote axonal regeneration in ONC animal models, showing a 2-fold increase in RGC survival compared to untreated group. Nonetheless, dose-dependency of exosome based treatment was confirmed by observing a significant regeneration trend in correspondence to higher doses in both *in vivo* and *in vitro* studies (64).

Reaching the other retinal layers, found deeper than the RGCs, should be more complicated due to the intermediate transfer from the vitreous through the inner layers and most importantly the crossing of the tightly regulated junctions characterizing the BRB. Both retinal capillaries and RPE are highly dependent on molecular size exclusion, in fact it was revealed that molecules whose diameter is larger than 2nm are not able to cross the BRB tight junctions via the paracellular pathways (61). However multiple studies focusing on the morbidity of retinal injury have reported that isolated exosomes were capable to target the choroid triggering retinal vascular leakage suppression and neovascularization inhibition (44, 65). Hajrasouliha *et al.* were able to track labelled exosomes derived from retinal astroglial cells (RAC) within the choroid of the retina, reporting that out of 24 CNV mice models none of the animal treated daily with RAC-exosomes suffered vascular leakage. Similarly, Moisseiev *et al.* proved that MSC-exosomes treatment contributes to the quantitative and qualitative attenuation of retinopathy, by reporting that exosomes improve by 10% retinal thickness compared to placebo group and reduce by roughly 40% the number of neovascular nuclei.

The fact that an effect invested the RPE and the choroid indicates that the exosomes successfully permeated into the barrier. Furthermore, considering the clearance rates and the different transfer steps affecting the injected vesicles prior to the targeting, it can be estimated that few exosomes are able to reach those deep retinal layers. Linking the exosome efficacy in the choroid to the cargo models, discussed in paragraph 3.2, the most feasible scenario would be that the vesicles originally would have had a low concentration and highly distributed cargo, thus increasing the chances of reaching the target. This model appears more realistic rather than few rare vesicles being highly loaded and highly specific in targeting such hindered layers. Concerning the transfer of EVs into the

choroid, two plausible hypothesis could be raised, see Figure 6 C-D. Firstly, the integrity of natural barrier was shown to be degraded by overexpression of miR-105, carried in EVs secreted by metastatic breast cancer cells (69). More specifically, Zhou et *al*. showed the ability of miR-105 to downregulate the tight junction-associated protein ZO-1 enhancing metastatic progression. Similarly it could be hypothesized that retinal disease, characterized by ocular ischemia and perfusion instability, compromise and downregulate the control of the gap junctions, therefore leading to a leaky BRB that allows the diffusion of larger molecules, as illustrated in the human glaucomatous eye model (70). This assumption indicates that under steady-state condition EVs would not be able to diffuse through the outer BRB due to size-exclusion issues, thus unless damaging or stressor stimuli are induced an alternative communication pathway implemented by EVs could be the transcellular transfer. Due to the analogies between BRB and BBB, exosomes uptake by the choroid could be associated to the crossing of RPE via lipid-raft mediated endocytosis. This model was suggested by a BBB *in vitro* study that confirmed the exosomes internalization in brain microvascular endothelial cells (BMECs) to be dependent on caveole and clathrin mechanisms (54). In addition, stem cells derived EVs appeared to be beneficious also in treating diabetic retinopathy. Zhang *et al.,* who further confirmed the miRNA-dependency on EVs therapeutical effect, demonstrated that miR126 expression enrichment in diabetic rats promoted inflammation suppression. This effect is hypothesized to involve the regulation of the HMGB1 protein found in the rod and con receptors layers of the retina (66). The same mechanism was observed also in a study performed on rabbits, where exosomes enriched in miRNA-222 were able to qualitatively restore the architecture of the retina conferring a regenerative response (67).

The ability of EVs to bypass biological barriers remains an open question in EVs biology. However, based on the currently available evidence it is possible to speculate that EVs are facilitated in crossing biological barriers under pathological conditions, responsible for the alteration of the paracellular pathway and thus causing the increase in permeability. To confirm or discard the crossing models that have been hypothesized, further research needs be conducted on the distribution of exogenously injected EVs in steady-state condition.

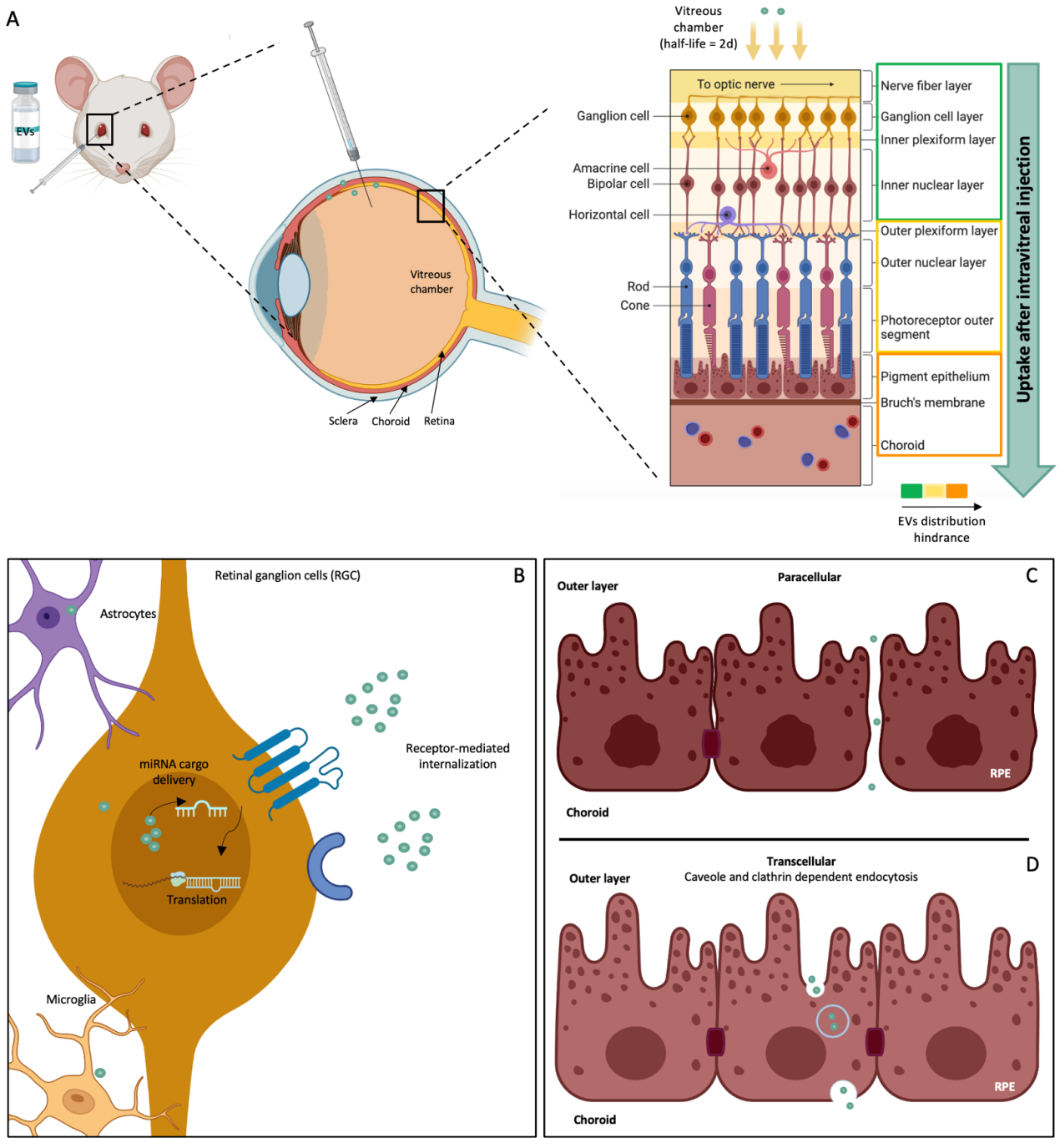

Figure 6. EVs targeting and internalization into the retinal tissues. (A) EVs after intravitreal injection are uptake gradually within the retinal layers. The distribution of the vesicles appears to be hindered the deeper the target is located (e.g., more complex to reach choroid due to clearance and other physical barriers); (B) Internalization in the most outer retinal layer composed by RGCs occurs via receptor mediated internalization. (C-D) Whereas choroid is hypothesized to be reached by both paracellular and transcellular (endocytosis-mediated) ways through the crossing of the RPE.

Conclusion

EVs are powerful candidates for treatment of retinal disease. Overall, their potential as cell-free therapy and natural delivery systems has been raised as they can circumnavigate the risks associated with toxicity and immunogenicity. In addition, compared to other nanocarriers, they appear to have higher penetration capabilities into tightly regulated biological barriers. Research concerning EVs application in the ophthalmic field is still underdeveloped, however based on the existing *in vitro* and *in vivo* studies performed on retinal disease models it emerged that different sites of the retina, such as RGCs and the choroid layers, are being functionally targeted. Multiple research groups demonstrated that EVs or exosomes have yielded neuroprotective and anti-angiogenic effects, leading to the overall attenuation of retinopathies and thus to the preservation of the retinal architecture and function. Although the underlying mechanisms are not clear yet, many findings indicate that EVs therapeutical effect is dependent on delivery of functional biomolecules able to modulate specific pathways. Currently, EVs reaching their targets in the posterior ocular segment have been tracked in a qualitative manner, thus it remains difficult to quantify the success rate of their delivery. However, based on the outcomes and on other system models, it is possible to estimate that due to the physical barriers encountered in the eye, only few vesicles can reach their target. To yield such significant effects, it must be that the therapeutical efficiency of the bulk of EVs is different from the one of single vesicle, not only in terms of power but in terms of heterogeneity and cargo occupancy. So far, due to the specificity and the screening limitations, it could be hypothesized that not all EVs are carrying functional cargos, however therapeutical effects could derive from a model of high occupancy and low RNA concentration in a pool of EVs. This model combined to the limited half-life of EVs in the eye may require higher or more frequent dosages to achieve the desired outcome, which may also trigger unwanted side effects. Before moving into the clinical translation of EVs application in retinal disease, several aspects need to be elucidated. For instance, better EV sub-population differentiation methods should be developed to define the therapeutical difference between exosomes and MV. Following, further research should be implemented on the stoichiometry and distribution of EVs into the retina targets. In conclusion to be able to quantify the delivery of EVs, a real time optical tracking tool could be developed to trace and quantify labelled EVs in their transfer from the vitreous to the retina, allowing an accurate estimation on the clearance rates and thus on the expected amount of EVs able to cause an effect in the human eye.

Plain language summary

Many eye diseases, for example the ones caused by increased eye pressure or damaged eye nerves, can lead to blindness. In some cases, it is not possible to cure this illness due to the rapid decline of vision. For this reason, it is necessary to prevent such decay by protecting the nerves that connect the eye to the brain. At this moment, the extracellular vesicle-based therapy has attracted many oculists and researchers for its capacity to heal various diseases. Extracellular vesicles are the waste materials produced by many types of cells in the human body. These vesicles composition mirrors the content of the cell that expelled them, within these components some, such as proteins and RNAs, are thought to restore the healthy condition of the eyes. It is believed that by inserting these vesicles in the eye, their content will be able to reach and act on the diseased portion of the eye, therefore recovering the vison. Many studies have shown that the application of extracellular vesicles works well on eye's cells grown in the laboratory or in animal's diseased eyes. In this review we will summarize all the results that these studies have collected on extracellular vesicles application on eye disease, and we will also highlight what can still be improved in this approach before moving toward treatment of human's eyes.

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