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1 **Interest of extracellular vesicles in regards to lipid nanoparticle based systems for**
2 **intracellular protein delivery**

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13 **Abstract**

14 Compared to chemicals that continue to dominate the overall pharmaceutical market, protein therapeutics offer the
15 advantages of higher specificity, greater activity, and reduced toxicity. While nearly all existing therapeutic proteins
16 were developed against soluble or extracellular targets, the ability for proteins to enter cells and target intracellular
17 compartments can significantly broaden their utility for a myriad of exiting targets. Given their physical, chemical,
18 biological instability that could induce adverse effects, and their limited ability to cross cell membranes, delivery
19 systems are required to fully reveal their biological potential. In this context, as natural protein nanocarriers,
20 extracellular vesicles (EVs) hold great promise. Nevertheless, if not present naturally, bringing an interest protein
21 into EV is not an easy task. In this review, we will explore methods used to load extrinsic protein into EVs and
22 compare these natural vectors to their close synthetic counterparts, liposomes/lipid nanoparticles, to induce
23 intracellular protein delivery.
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26 **Keywords: exosomes - microvesicles - therapeutic proteins - cytoplasmic delivery - liposomes - macromolecules**
27 **delivery - vectorisation**
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1. Introduction

Extracellular vesicles (EVs) are vesicles produced by most cells and present in biological fluids. These natural biological carriers have a structure consisting of a shell of phospholipid bilayer including surface and transmembrane proteins surrounding a hydrophilic core (lumen) containing lipids, carbohydrates, proteins and nucleic acids. In healthy cells, EVs are commonly classified in two subcategories according to their size and biogenesis (Figure 1): (i) microvesicles (100 nm - 1 μ m), which bud directly from the plasma membrane of healthy cells; (ii) exosomes, the smallest EVs (50-150 nm), which are released by the fusion of multivesicular endosomal bodies (MVBs) with the plasma membrane [1]. Some molecules were reported to be common to exosomes and MVs while others were described as more specific to one or another type of vesicles (in italics in Figure 1). Nevertheless, despite this classic categorisation, numerous studies have evidenced the complexity and heterogeneity of these vesicles, and revealed the existence of subtypes depending on several parameters (cell source, state, isolation protocols, etc.) [2-4]. We therefore chose in the following sections to use the term EVs in general and to use exosomes or microvesicles when specified in described work.

It is now well established that EVs participate to homeostasis and control of many cellular and biological functions [5]. It was therefore intuitive that EV intrinsic properties would arouse interest to be used as therapeutic agents, as natural vehicles of beneficial biological cargo, mainly for regenerative medicine in a panel of pathologies (i.e for cartilage repair [6], cardiac injury protection [7, 8]). In parallel to their use for their intrinsic properties, the natural ability of EVs to transport biological molecules has prompted several teams to explore their potential as delivery vehicles mainly for nucleic acids or chemicals [9, 10]. Indeed, EVs have major advantages compared to synthetic nanoparticles: they are naturally equipped to cross extra- and intra-cellular barriers, and are able to vectorise functional biomolecules (nucleic acids, protein, lipids) from one cell to another, even if these cells are far apart in the body. These advantages, along with promising *in vivo* results [11-13], have clearly support the potential of EV. Today, these advantages have led more and more teams to use EVs for the delivery of active therapeutics [8, 14, 15]. Alongside the interest of academic researchers, a growing number of companies have emerged to exploit EVs as therapeutic agents (Evax Therapeutics, Codiak BioSciences, Carmine Therapeutics, Capricor Therapeutics, EverZom, Ciloa...). Recently, several agreements have been concluded between these start-up companies and major actors in the pharmaceutical field (such as Lilly, Takeda or Bayer) [16, 17]. Nevertheless, as new systems, and coming from living source, EV have to face practical challenges, such as high scale manufacturing or storage issues be clinically relevant. The complexity of EV suspensions comprising different EV subpopulations, other secreted products (therapeutic proteins) and sometimes contaminants is another challenge in terms of quality, together with the issue of natural EV content. In terms of safety and standardization, these complexities place EV-based therapies somewhere between cell therapies and biologics (therapeutic proteins). Even if these issues are currently being addressed, the development of EVs in therapeutics will probably lead to expensive treatments. Another crucial point will therefore be to figure out if EVs could be more efficient than other innovative treatments and if the efforts to use them in therapeutics have a real added-value, particularly in regards to synthetic nanocarriers. It is in this

context that we decided, in this review, to compare interest in EVs in regards to their closest synthetic counterparts, liposomes/lipid nanoparticles (LNP), for intracellular protein delivery, as no reference system exists to date, especially *in vivo*.

Indeed, while commercially available therapeutic proteins were developed to interact with extracellular or soluble targets [11], intracellular targets could be a powerful alternative to treat many pathologies such as cancers, immune disorders, but also infectious diseases. In regards to their specificity, proteins are less prone to off-target effects compared to small-molecule drugs, and brought for greater activity and less toxicity [12]. Compared to other biologics, such as nucleic acids, which required a transcription/translation (plasmid DNA, double stranded DNA) or a translation (mRNA) step before being functional, proteins are fast acting / highly specific actors. In regards to stability, even if proteins are particularly complex molecules requiring formulation work, robust methods have been developed over several decades and, to our knowledge, the field is, for now, more mature than for nucleic acid therapeutics (as an example in regards to long term storage). From a safety perspective, protein therapy may be seen as safer than gene therapy (especially using DNA molecules), because no genetic modification occurs. Advances in molecular biology and recombinant protein production technology have also made it possible to remove immunogenicity issues encountered with first generation of protein therapeutics. By contrast, even if mRNA chemical modifications have allowed alleviating this issue, these molecules are still more prone to immunogenicity compared to protein therapeutics. Therefore, if mRNA therapeutics are becoming an exciting option, especially after local administration, these very promising molecules still have drawbacks to overcome, and in this context, the use of protein remains an interesting therapeutic option. Among different therapeutic strategies, cytoplasmic delivery of proteins could allow: (i) supplying an extrinsic protein originally missing or ineffective (ex: a functional tumor suppressor protein while the natural form is mutated in cancer), (ii) introducing a previously inexistent function (ex: providing gene editing tools) or (iii) interfering with protein-protein interactions to modify intracellular pathways (ex: intrabodies or other non-antibody protein scaffolds) [13, 14]. Altogether, these needs have called for the development of efficient protein delivery systems to the cytoplasm which would be particularly useful both for researchers but also for pharmaceutical development. In this context, synthetic delivery systems, such as lipid or polymer based nanocarriers (liposomes, polymer nanoparticles (NPs), polymersomes, micelles, nanogels), inorganic NPs (mesoporous silica NPs, gold NPs, carbon nanotubes) or protein mediated carriers (cell penetrating peptides) have been investigated [15-17]. While the first generation, mainly relying on cationic lipid and/or polymer, was often restricted to *in vitro* use, some recently developed synthetic systems have been used *in vivo* with local [18] or systemic administration achieving convincing protein accumulation in targeted organs [19, 20]. Nevertheless, to our knowledge, no universal system exists to date and previously presented synthetic systems often failed to efficiently cross intracellular barriers and especially to escape from the endosomes to reach the cytosol or other targeted organelles [21].

By contrast, the ability of EVs to carry and deliver proteins at the intracellular level is particularly interesting [22-24]. Yet, little research work has reported EV use as extrinsic protein delivery vectors. Indeed, using EV intrinsic proteins for a therapeutic effect is possible, but artificially delivering an extrinsic protein of interest with EVs remains a particularly challenging task. The main difficulties come from dealing with protein size, complexity and, above all,

stability. To permit protein loading, two main strategies have been carried out: i) transfecting the producing cells in order to obtain modified EVs, which can be called “pre-production loading” with biological manipulation or ii) modifying EVs after isolation or “post-production loading / EV engineering” relying on physico-chemical methods. These two strategies will be described and discussed in this review. We have chosen to explore the interest of EV use for protein intracellular delivery in opposition to membrane associated protein delivery used to modify EV biodistribution (for review on this topic see [25]). We have chosen to compare EV ability to deliver proteins to that of their closest synthetic counterparts, in regards to the similarity with EV composition: liposomes/LNP. Moreover, these systems are among the more evaluated nanocarriers so far and are therefore legitimate to be used as reference systems. Finally, even though this task is delicate in regards to differences in used methodologies (production, characterization, biological read-out, etc.), we tried to compare, in different therapeutic areas, the use of liposomes or EVs in terms of protein loading, endosomal escape, and therapeutic efficiencies. In the conclusion section, we aimed at identifying the pros and cons of using EVs or lipid-based nanoparticles for intracellular protein delivery and to discuss potential improvements that could be done in the field.

2. State of the art on EV-mediated intracellular delivery of proteins

Different proteins of interest were evaluated with various objectives. Thus, reporter proteins were used, such as mCherry, luciferase [26], or Cre recombinase coupled with a fluorescence system [27]. However, in the majority of studies, proteins were chosen for their therapeutic role: compensation for a pathological absence of said protein [28, 29], tumour progression impeding proteins [26, 30, 31], inhibition of apoptosis protein [32], enzyme used to activate a pro-drug [33-35], antioxidant activity [36]. Such proteins have been loaded into EVs according to different strategies, which will be described in the following sections. The proportions of studies using the various strategies are detailed in Figure 2 (17 studies analysed).

2.1. Biological, pre-production methods

Among those biological methods used to load protein cargo into EVs, several strategies have been tested which are summarised in Table 1. First studies dealing with biological loading mainly relied on ‘classic’ plasmid transfection to induce overexpression of the desired protein and subsequent loading into EVs without specific strategy to be loaded into EV (passive biological loading, section 2.1.1). By contrast, in recent studies, a broad spectrum of methods were used to increase protein loading into EV by co-transfection, or fusion strategy with specific protein inducing a tropism of cargo proteins toward plasmic / endosomal membranes to be loaded in microvesicles / endosomes respectively (active biological loading, section 2.1.2).

2.1.1. Passive biological loading

Transfected cells were most often HEK293T (n= 8/13 studies). Nevertheless, other cell types were also used such as melanoma cells, mesenchymal stem cells, lung cancer cells, or macrophages. Transfection was performed

1 mainly with synthetic vectors (Lipofectamine® 2000, Lipofectin®, GenePorter® 3000, polyethyleneimine (PEI), etc.)
2 (n=9) or adenoviral vectors (n=2). Strategies described thereafter have been applied for different therapeutic goals:
3 the field of oncology in preliminary *in vitro* or advanced *in vivo* studies for the treatment of glioblastoma, pancreatic
4 adenocarcinoma, but also in the field of cardiology and genetic diseases.

5 HEK293T cells were transfected using Lipofectamine 2000® to overexpress suicide therapeutic mRNA/protein
6 encoding cytosine deaminase (CD) fused with uracil phosphoribosyltransferase (UPRT) to create EVs loaded with this
7 suicide mRNA/protein cocktail. Indeed, CD could convert pro-drug 5-fluorocytosine (5-FC) into anti-cancer 5-
8 fluorouracil (5-FU) especially toxic toward UPRT expressing cells [34]. Loaded EVs combined with pro-drug 5-FC
9 induced significant cell death (~80%) *in vitro* in human Schwannoma NF2 cells; however, a decrease of this cell death
10 by half was observed when purifying EVs with a sucrose gradient. This suggests a large contribution of aggregated
11 protein overexpressed by EV-producing cells, which could have pelleted with the EVs during the ultracentrifugation
12 process. Endosomal scavenging has not been specifically evaluated in this study, but the action of CD tends to prove
13 that EVs carrying the protein, or the protein alone, were able to catalyse the insertion of 5FU into DNA, blocking
14 replication and inducing apoptosis. *In vivo*, intra-tumoural injection of loaded EVs and pro-drug 5-FC resulted in
15 complete tumour growth inhibition. CD-UPRT EVs and 5-FC were also evaluated in glioblastoma cells in which they
16 induced more moderate cell death *in vitro* (~30%) in U87-MG cells [35]. However, CD-UPRT EVs and 5-FC induced a
17 similar level of cell death in 3D spheroids from human glioblastoma cell lines (U87-MG, E98), and they clearly
18 reduced spheroid volume (by 33-41%). Finally, this treatment induced a 70% reduction in tumour growth in a
19 subcutaneous xenograft (U87-MG cells) model of glioblastoma in nude SCID mice after EV intratumoural injection. In
20 another study, still with the goal of providing anticancer activity, murine melanoma cells (YUSAC 2 cells) were
21 transfected using Lipofectin® reagent to produce EVs loaded with survivin-T34A (15 kDa), a dominant mutant of
22 survivin known as an inhibitor of apoptosis [30]. This modified survivin is assumed to block wild type survivin,
23 inducing caspase activation and apoptosis. *In vitro*, survivin loaded EV (150 µg/mL total proteins), used
24 concomitantly with gemcitabine (10 µM), were able to induce apoptosis in 30% of human pancreatic
25 adenocarcinoma cells (MIA PaCa-2).

26 The protein kinase B (Akt; 56 kDa) is known to play important roles in numerous signalling pathways, it is
27 involved in promoting cell proliferation and inhibiting apoptosis as well as stem cell-induced cardio protection [37,
28 38]. Human umbilical cord mesenchymal stem cells (HucMSCs) were therefore engineered to overexpress Akt using
29 adenoviral vectors. HucMSCs derived EVs were collected from cell culture supernatant (48h in 10% EV depleted FBS),
30 concentrated by ultrafiltration (100KDa) before purification with sucrose cushion. Thereby produced Akt-loaded EVs
31 were used to treat acute myocardial infarction in rats [32]. Unloaded and Akt-exosomes (Akt-Exo), both 100 nm in
32 diameter were intravenously injected after ligation of the left anterior decedent coronary artery (LAD) performed to
33 induce myocardial infarction. Akt-Exo seem to (i) improve heart function and reduce apoptosis *in vivo* in a model of
34 myocardial infarction, (ii) increase cell migration *in vitro*, (iii) increase blood vessel formation (Chick Chorioallantoic
35 Membrane assay). This action was shown to involve cytokine PDGF-D. However, some results obtained with Akt-Exo
36 were also (albeit to a sometimes lesser extent) found with GFP-Exo or exosomes from non-transduced MSCs. This
37 suggests the effects seen are partly due to EVs and not only to Akt protein, which is consistent with previous studies

1 reporting MSC-EV positive effects on myocardial repair [39-41]. Akt-overexpression can thus be seen as a means of
2 potentiating EV natural effects.

3 High molecular weight cystic fibrosis (CF) transmembrane conductance regulator (CFTR; 200 kDa)
4 glycoprotein fused to GFP was loaded into EVs to restore chloride channel function in CF15 mutant cells deficient in
5 this function [29]. Loaded EVs were generated from transduced human A549 lung cancer cells (adenoviral vectors).
6 Over 80% of EVs were GFP positive when cells were transfected with a GFP only construct; however, this number
7 dropped to 4% (exosomes) and 12% (microvesicles (MVs)) for EVs generated from CFTR-GFP cells. This difference can
8 be attributed to the membrane-bound nature of CFTR in opposition to GFP which is purely cytoplasmic, and may
9 thereby be more easily located in EV lumen. Moreover, no separation methods to eliminate free GFP or GFP-CFTR
10 protein was used in this study. EVs were delivered more efficiently to homologous A549 cells than to CF15
11 heterologous cells while in both cell type, MVs were consistently more internalised than exosomes. Interestingly, the
12 two types of vesicles accumulated in cells in different vesicular compartments, as only exosomes were shown to co-
13 localise with lysosome-associated protein Lamp1. CFTR-coding mRNA was also found in MV and exosomes and both
14 vesicles efficiently restored chloride channel function in CF15 cells. It was established EVs contained mRNA, protein
15 and even vector DNA, but it was not determined which of these entities was responsible for the gain of function.
16 Interestingly, the kinetics of EV-mediated delivery of GFP-CFTR were U-shaped whereas a steady increase was
17 observed for GFP-EVs, which was rationalized by the authors as mature glycoproteins being responsible for early GFP
18 signal and *de novo* synthesized protein from mRNA responsible for retarded GFP signal. Nevertheless, the
19 restoration of chloride channel function in CF cells transfected by CFTR-loaded EVs was evidenced in a dose
20 dependant manner, and this effect was found to persist at detectable levels for up to 5 days, highlighting the
21 delivery of functional CFTR.

22 Lysosomal enzyme tripeptidyl peptidase-1 (TPP1; 61 kDa) was recently loaded in EVs derived from
23 macrophages. These EVs were used to treat disorder due to TPP1 deficiency leading to accumulation of storage
24 material in neural tissues and progressive neurodegeneration in models of Batten disease [28]. EVs derived from
25 macrophages transfected (GenePorter® 3000, cationic lipid) with a pDNA encoding TPP1 (EVs-t) were compared to
26 EVs loaded with TPP1 using physico-chemical methods: saponin chemical treatment (EVs-sa) or sonication (EVs-so).
27 All EVs were roughly the same size (EVs-t: 133.8 ± 4.1 nm; EVs-so: 130 ± 8 nm; EVs-sa: 135 ± 12 nm), slightly larger
28 than naïve EVs (106.3 ± 9.3 nm). EVs-t were less efficient in delivering TPP1 to TPP1-deficient CLN2 cells (less TPP1
29 protein was found in the cells after incubation with EVs) than EVs-so and EVs-sa. This is consistent with the fact that
30 EVs-t cells contained 5 to 7 times less TPP1 protein (based on catalytic activity measurement) than EVs-so or EVs-sa.
31 Indeed, drug loading was estimated, after removal of free protein on Sepharose CL-4B, to be 987 molecules/EV for
32 EVs-t; 6,908 molecules/EV for EVs-so; and 4,934 molecules/EV for EVs-sa. These results support the interest of
33 exploring physico-chemical loading methods over the continued use of biological methods for this application.

34 To allow gene editing, *Streptococcus pyogenes* CRISPR (Clustered Regularly Interspaced Short Palindromic
35 Repeats) associated protein 9 (Cas9) nuclease (SpCas9) was overexpressed in HEK293 cells via transfection with
36 appropriate plasmids encoding both Cas9 and single guide RNA (sgRNA) to allow this Cas9 - ribonucleoprotein (RNP)
37 complex loading into EVs [42]. Culture supernatants were collected 24h after transfection (performed in DMEM 10%

EV depleted FBS) and concentrated using differential ultracentrifugation. It is important to note that no transfection medium withdrawal step was reported by the authors: PEI polyplexes were therefore, to our knowledge, still present in EV enrichment steps without a purification stage. Nevertheless, obtained suspensions, named GEDEX, were able to induce DNA double-stranded breaks (DSB) resulting in insertions/deletions (Indels) close to 10% both *in vitro* and *in vivo* after intravenous administration, as evidenced with various targeted genes. However, the more innovative aspect of this work relies on the combination of a catalytically inactive Cas9 (dCas9) -sgRNA system with a composite tripartite activator domain VP64-p65-Rta (VPR) to activate gene expression. GEDEX were therefore used to carry a combination of sgRNA:dCas9-VPR with the goal to upregulate gene transcription. This was confirmed *in vitro* with a reporter gene (luciferase): GEDEX carrying sgRNA_{luc}:dCas9-VPR induced a 470-fold increase in luciferase activity on treated cells compared to transfection of the same cells with a plasmid encoding the same construct. Interestingly, this increase was identified earlier with GEDEX (24h) compared to pDNA treated cells (48h), probably because of transcription/translation time required for Cas9-VPR activity when using plasmid. This system was then evaluated in a mice liver damage model. Hydrodynamic delivery of GEDEX carrying Cas9-VPR construct associated to sgRNA targeting Hepatocyte Growth Factor (HGF) gene resulted in a significant increase in HGF expression and a beneficial therapeutic effect on liver regeneration. Altogether, these results identified GEDEX as a versatile platform to downregulate or upregulate gene expression, efficient in different cell type *in vitro* and *in vivo*, which could be particularly relevant for multiple therapeutic applications.

2.1.2. Active biological loading

In parallel to “simple” transfection, thereafter described studies used motifs/proteins fused with protein of interest to favour its loading into EVs (microvesicles generated from membrane budding or exosome generated from inward budding of endosomes). Different strategies described in this section are illustrated in Fig3.

A specific vesicle type, Arrestin domain containing protein 1 [ARRDC1]-mediated microvesicles (ARMMs), was evaluated to transport tumour suppressor protein p53, as well as the CRISPR-Cas9/ sgRNA complex (Fig.3A) [43]. ARRDC1, localised on the cytosolic side of the plasma membrane, is responsible for TSG101 recruitment to the cell surface leading to outward membrane budding. As a simple overexpression of the ARRDC1 protein increases the production of ARMMs in cells, cargo proteins were fused to the C-terminus of this protein to allow loading into microvesicles. HEK293 cells were therefore transfected with p53-fused ARRDC1. EVs were then produced in DMEM – 10% EV depleted DMEM during 72h, collected by differential centrifugation – filtration steps, and purified on a sucrose gradient. Obtained vesicles were less than 100 nm and were loaded with an average of 540 p53 proteins per ARMM vesicle. Interestingly, GFP-standardized western blotting was used to estimate the amount of ARMM transferred into treated cells (H1299 cells). It appeared that each cell received an average of 3.1×10^6 cargo proteins from 5.8×10^3 ARMMs. p53 loaded ARMMs were detected in both cell cytoplasm and nucleus of p53 null H1299 cells and were able to induce p53-dependent gene expression *in vitro* (increased transcription of MDM2 and p21 gene). After intravenous injection of ARRDC1-p53 ARMMs in p53 KO mice, a significant induction of apoptosis induced by ionizing radiation was observed, whereas mice injected with ARMMs containing ARRDC1-GFP were almost completely resistant to irradiation-induced apoptosis. This study established ARMMs as a promising and versatile

platform for packaging and *in vivo* delivery of protein. In the same work, ARMMs were evaluated for delivery of Cas9 and its associated single-guide RNA (sgRNA) (RNP complex). Cas 9 molecular weight (160 kDa) prevents from using the previously described ARRDC1 fusion strategy. An approach relying on ARRDC1 ability to interact with WW-domain containing protein was used. A fusion construct of Cas9 linked to WW domains (Cas9-WW) was therefore used to allow loading into ARMM. ARMM-mediated Cas9/sgRNA delivery was evidenced into U2OS cells (stably expressing GFP protein) leading to a significant decrease of GFP negative cells suggesting inactivation of GFP gene by Cas9-sgRNA-GPE-ARMM.

Another gene editing protein, Cre recombinase (Cre), has been loaded into EV using light-induced dimerization (Fig.3B). In 2016, Yim *et al.* described a system named 'EXosomes for Protein Loading via Optically Reversible protein-protein interactions' (EXPLORs). HEK293T cells were transfected (Effectene® transfection reagent, lipid reagent) with a plasmid encoding photoreceptor cryptochrome 2 (CRY2)-conjugated cargo proteins and CRY-interacting basic-helix-loop helix 1 (CIB1) truncated version (CIBN) conjugated with EV-associated tetraspanin protein CD9 [26]. When cells grown under blue light illumination, cargo protein-CRY2 linked could bind to CIBN-CD9 protein: the cargo protein was linked to CD9 and thus loaded into EVs through CIBN-CRY2 photo-induced bonding. Once EVs were produced, blue light could be turned off and the CRY2-cargo protein could be released into the hydrophilic core of EVs. Transfected HEK 293 were grown in FBS free or 10% EV depleted FBS DMEM for 48h. EV were then collected either by PEG precipitation (ExoQuick TC, System Biosciences) or by centrifugation/ultracentrifugation. Unfortunately, the authors did not clearly specify when one method or another was used, and this could strongly hinder the interpretation of obtained results. By using luciferase as a reporter protein and measuring luciferase activity in thus isolated EVs, the authors reported that this method allowed loading of 1-2 molecules of Cre enzyme per EVs. The authors evidenced intracellular delivery of a set of functional protein (luciferase, mCherry, Bax, super-repressor IKB) as well as Cre enzyme into target cells *in vitro* and *in vivo* (into brain parenchymal cells). Although promising, this system allowed low protein loading. Indeed, an average 1.4 molecule of luciferase (61 kDa) was associated per EV, which is ~1000 times lower than other studies loading proteins of a similar size (TPP1: 61 kDa [28]; catalase: 60 kDa [36]): this could be explained by the linkage of CRY2 protein to luciferase, which increases the size of loaded protein by 57 kDa. While establishing a smart proof of concept, this promising system therefore need to be improved in order to become a real therapeutic option.

Still to load Cre recombinase, a vesicle-trafficking mechanism involving late-domain (L-domain) proteins was explored (Fig.3C) [27]. L-domain proteins recruit endosomal sorting complex required for transport (ESCRT) components to cell membrane which results in MVB formation, followed by exosome generation and loading. L-domain-containing protein Ndip1 was thus used to load a WW tagged exogenous proteins into these vesicles, as WW motifs were identified as capable of interacting with Ndip1 L-domains. Constructs coding for tagged proteins were transfected in LN18 or HEK293T cells using TurboFect® transfection reagent before collecting EVs in cell culture supernatant by differential centrifugation. WW-Cre was shown to be loaded inside EV lumen, as proteinase K treatment only degraded WW-Cre when used in combination with Triton X-100 (which degrades the exosomes). Unfortunately, no EV/free protein separation step were included to our knowledge and no loading efficiency value

1 was provided. Nevertheless, EV-delivered WW-Cre induced recombination activity in target mouse embryonic
2 fibroblast cells *in vitro* as well as *in vivo* in neuronal cells after nasal administration.

3 In the context of a collaborative work between AstraZeneca, GE healthcare and Cambridge university,
4 another strategy was used to load Cre recombinase in EVs (Fig.3D) [44]. Two chimeric proteins, CD81FKBP and
5 CreFRBT2098, were co-transfected and overexpressed in Expi293F™ cells (Thermofisher). The FKBP and FRB protein
6 domains are able to dimerize when a small molecule, named rapalog is present, bringing the CreFRB cargo into the
7 vicinity of membrane protein CD81 where EV biogenesis is supposed to take place. After transfection, cells were
8 incubated 48h into Expi293F expression media (+/- rapalog) before collecting EV by differential UC. This resulted in
9 active loading of Cre into EVs, even if rapalog active loading was not identified as crucial for Cre loading.
10 Unfortunately, EVs derived from CreFRB and CD81FKBP expressing cells could only deliver CreFRB in the presence of
11 endosomal escape enhancing compounds such as chloroquine, which prevents a rational use in therapy.

12 Based on the observation that cells overexpressing the viral fusion protein VSV-G were able to produce
13 increased amounts of EVs, and that these EVs expressed the VSV-G protein, Zhang's team chose to use this protein
14 to obtain EVs loaded with a chosen protein (Fig.3E) [45]. HEK293T cells were therefore transfected (PEI) to induce
15 overexpression of the viral fusion protein VSV-G fused to GFP. Thereafter, the same cells were incubated for 48h in
16 DMEM or Freestyle 293 expression media (ThermoFisher), after 24h/48h, this media was collected and a differential
17 ultracentrifugation protocol was performed. To select modified EVs, a SEC purification (qEV, IZON) was first
18 performed followed by incubation of EV containing fractions with magnetic beads exposing VSV-G targeting
19 antibodies to retain EV exposing VSV-G at their surface. Unfortunately, no information was provided concerning the
20 yield of VSV-G positive cells. Using this protocol, the GFP loading in EVs was increased by 1000-fold compared to EV
21 produced by cells transfected only with GFP (lacking VSV-G protein). To develop an active loading, a split GFP system
22 was used. Indeed, GFP could be split in two separate constructs of a 16 amino acid fragment (GFP11) and the rest of
23 the protein (GFP1-10); these constructs nevertheless have a peculiar affinity and could reassemble to allow
24 functional GFP fluorescence. VSV-G was therefore fused to GFP11 while a reporter β -lactamase-vpr (BlaM-vpr) was
25 fused to GFP1-10. Only a co-transfection of these 2 constructs in cells could therefore induce GFP fluorescence,
26 proving that VSV-G is associated to its intended cargo in cells. EV isolated from supernatant of HEK293 cells
27 transfected with this split GFP construct were then evaluated for fluorescence by NTA, evidencing that a proportion
28 of produced EVs was fluorescent ($\sim 3 \times 10^8$ EV/ml). These EVs were able to deliver functional BlaM into target cells
29 (HeLa). It is important to note that VSG deficient peptide could induce protein loading into EVs but produced EVs
30 were less efficiently internalised by target cells. Cre recombinase loaded Gectosomes (Cre-Gectosomes), with the
31 active split GFP loading, were able to enter target cell nucleus and to mediate Cre-lox recombination (26-fold more
32 than when no GFP split loading was used). These results are consistent with the well-known implication of VSV-G in
33 inducing endosome escape of viral particles [46, 47]. This versatile platform was then engineered to deliver Ago2 and
34 *Staphylococcus aureus* Cas9 (SaCas9). SaCas9 packed with a sgRNA targeting the mitochondrial kinase PINK1 gene
35 was loaded into Gectosomes. These vesicles were able to significantly decrease PKN1 expression (40%) in treated
36 cells (HeLa). *In vivo*, intravenous injection of Gectosome carrying SaCas9 and a sgRNA targeting proprotein
37 convertase subtilisin/kexin type 9 (PCSK9) were able to induced both deletion and mutation in mice liver tissues.

1 Although transduction of Cas9-RNP complex with VSV-G ectosomes has already been described [48-50], this study
2 differs by presenting a smart active loading method associated with more specific cargo encapsulation and reduction
3 of non-specific cellular components.

4 Still relying on fusion with viral protein, a mutant version of a Human Immunodeficiency Virus (HIV) protein,
5 Nef^{mut}, described as able to strongly accumulate in EV lumen, was used to allow loading of cargo protein into EVs
6 (Fig.3F). Interestingly, in mice, an intramuscular injection of a pDNA construct, encoding Nef^{mut} fused with HPV-E7
7 protein, allowed secretion of EV loaded with Nef-E7 proteins by transfected cells *in vivo* [51]. Nef^{mut}-E7 thus loaded
8 EVs were taken up by antigen presenting cells, subsequently presented to cytotoxic T lymphocytes and were able to
9 induce an antigen specific immune response against both E7 and Nef. However, especially as this system is evaluated
10 *in vivo* after pDNA intramuscular injection, it is difficult to identify the mechanism of this therapeutic effect. It is
11 indeed challenging to define "who does what" between fusion protein that can be secreted alone or protein actually
12 associated with EV. Following the same strategy, a single chain variable fragment (scFv) targeting the oncogenic
13 protein human papilloma virus (HPV)16-E7 protein was fused to Nef^{mut} to allow its loading into EVs [52]. For this, 24h
14 after HEK293 cell transfection, cells were cultured for 48-72h in 5% EV depleted FBS media before performing
15 differential centrifugation-ultracentrifugation steps [53]. Nef^{mut}/anti-HPV16-E7 scFv loaded EVs were able to inhibit
16 the proliferation of HPV16-E7-expressing cells. Despite being low (20%), this anti-proliferative effect demonstrated
17 the possibility to engineer EVs with scFvs, and that these vesicles have the potential to induce an effect in target cells
18 and could be used as a novel approach. Indeed, scFvs have already been associated with EVs to allow targeting [54-
19 56], but to our knowledge, this study is the only one describing intracellular delivery of scFv via EVs [52] and such a
20 therapeutic option presents exciting promise [57-59].

22 2.2. Physico-chemical, post-production methods

24 Biological methods have proven effective, but sometimes flawed: they require multiple steps that are liable
25 to biological variation, making it difficult to control the yield and reproducibility of protein loading. This has
26 prompted researchers to recently explore alternative, non-biological, physico-chemical, physical or chemical loading
27 methods inspired by the synthetic vector field. Such processes (illustrated in Fig. 4) will be described thereafter and
28 are summarised in Table 2. So far, to our knowledge, only four studies have used physico-chemical methods to load
29 proteins into EVs for intracellular delivery [28, 31, 60, 61]. We therefore also chose to discuss two additional studies
30 using physico-chemical loading processes [33, 62], even though their application is not dedicated to intracellular
31 delivery. Among these studies, strategies used to allow membrane permeabilisation/rearrangements, are mainly
32 based on EVs permeation, sonication, extrusion, freeze-thaw (F/T) cycles and electroporation. Used EVs were
33 produced from macrophages (IC21, Raw 264.7), HEK293 cells and HeLa cells.

35 2.2.1. Saponin permeation

36 First explored by Fuhrmann *et al.* for the loading of porphyrins, Haney *et al.* were the first to report the use
37 of saponin, an amphipathic natural glycoside, for EVs protein loading [36]. Raw264.7 derived EV were collected from

cell supernatant, followed by differential ultracentrifugation protocol [53]. Co-incubation of antioxidant catalase (60 kDa) (0.5 mg/mL) and murine Raw 264.7 macrophage derived EVs (0.1 mg/mL total protein) was performed in 0.2% saponin for 20 min at Room Temperature (RT) before SEC purification (Sephacrose CL-6B) to separate free catalase from loaded one. Loading efficiency was reported to be $18.5 \pm 1.3\%$, slightly lower than other evaluated methods: sonication ($26.1 \pm 1.2\%$), extrusion ($22.2 \pm 3.1\%$). A neuroprotective effect was reported upon nasal administration of loaded EVs in a Parkinson's disease mouse model; it should be noted that among the evaluated loading methods, this was the most effective *in vivo*. More recently, saponin was used to load the lysosomal enzyme tripeptidyl peptidase-1 TPP1 (61 kDa) in an aforementioned (see section 3.1) model of lysosomal storage disorder [28]. IC21 macrophages derived EVs (1×10^{11} particles/mL) were mixed with TPP1 (0.2 mg/mL) and saponin (0.4 mg/mL) and incubated for 30 min at RT. Saponin-loaded EVs (EVs-sa) were shown to contain 5 times more TPP1 protein (based on catalytic activity) than EVs derived from TPP1-overexpressing cells (EVs-t) (4 934 molecules/EV vs. 987 for EVs-t). Consistently with this last result, EVs-sa delivered TPP1 more efficiently to CLN2 cells than EVs-t. The authors hypothesized permeabilisation with saponin might result in satisfactory surface uniformity with intact membrane proteins [36], or create pores in the EV bilayer by removing membrane-bound cholesterol [28]. However, saponin permeabilisation induced a 52% drop in EV concentration, which is not convenient for a therapeutic development

Although not dedicated to protein intracellular delivery, saponin-permeation (0.1 mg/mL, incubation for 10 min) was also used to load β -glucuronidase (1.5 mg/mL) into EVs derived from human mesenchymal stem cells (hMSC) [33]. After elimination of free protein by SEC (Sephacrose CL-2B), EVs (loaded or not) were then embedded in implantable, biocompatible poly(vinyl alcohol) (PVA) hydrogels. EV-mediated enzyme protection allowed for an increased retention of enzymatic activity upon gel recycling. In an *in vitro* inflammation model (macrophages stimulated by bacterial lipopolysaccharide), enzyme loaded EV-hydrogels were more efficient than enzyme-hydrogels in converting the pro-drug curcumin glucuronide into curcumin resulting in macrophage death. However, they were as efficient as enzyme-loaded liposome-hydrogels used as a control. No information was provided regarding the effect of non-loaded EVs. Interestingly, the same protocol was used to load β -glucuronidase into EVs from different origins. Such as protocol ensured that EVs were not altered as assessed by size and concentration profiles measured by NTA [63]. β -glucuronide-EVs were used to study the effects of freeze-drying on EVs size, concentration and enzyme activity. Results evidenced that this process did not disturb enzymatic activity of loaded β -glucuronidase.

2.2.2. Sonication

When loading catalase, the use of a sonication probe yielded the highest amount of loaded protein, with 940 molecules/EV, compared to 109, 437 and 809 for incubation at RT, F/T cycles and extrusion respectively [36]. The protocol used (500 V, 2 kHz, 20% power, 6 cycles by 4 s pulse/2 s pause, 2 min cooling period) significantly increased EVs size (162.4 ± 6.1 nm vs. 99.5 ± 11.2 nm for native EVs). Sonicated EVs were internalised into neuronal PC12 cells to a higher extent than otherwise modified EVs as well as PC:PEG-PE (95:5 mol/mol%) liposomes or polymer-based poly(lactic-co-glycolic acid) (PLGA) nanoparticles. Interestingly, DiD labelled EVs were observed in the whole cytoplasm of PC12 cells by contrast to liposomes or PLGA NP treatment which resulted in a punctiform staining

suggesting endosomal accumulation. Sonicated exosomes resulted in great neuroprotective activity *in vitro* as well as *in vivo* after nasal administration in a model of Parkinson's disease, albeit to a lesser extent than saponin-permeabilised exosomes.

By contrast, probe sonication (2kHz, 5% power, 20 cycles with 2 s pulse/ 1 s pause) of HEK293 cell derived EVs with a solution of Cas9 RNP complexes led to a significant size increase (152 ± 22 nm vs. 105 ± 16.2 nm for native EVs) [61]. Cas9-RNP loading was identified to be 15% (mass) with this process. By contrast, another physico-chemical process, use of F/T cycles led to higher loading rates (30%, see section 2.2.5). EVs loaded with Cas9-RNP via sonication were therefore not evaluated for their ability to deliver their cargo to target cells.

When loading TPP1 [28], sonication in a water bath (30 min, RT) (EVs-so) led to a loading of 6,908 molecules/EV (1.4 times more than EVs-sa (saponin permeation) and 7 times more than EVs-t (transfection)). Sonicated EVs were slightly larger than naïve EVs (130 ± 8 nm vs. 106.3 ± 9.3 nm), and sonication induced a 41% decrease in EVs concentration. It is interesting to note that protein loading using sonication bath proved to be more efficient compared to sonication probe, whereas EVs were derived from a similar source (mouse macrophages) and proteins were of a similar size (61 kDa for TPP1 vs. 60 kDa for catalase). These differences could be due to distinctions in protein properties (such as hydrophobicity, isoelectric point, etc.) as well as differences in protein stability regarding sonication strength (more important with sonication probe). Nevertheless, EVs loaded by water bath sonication were intraperitoneally injected, migrated to the brain and increased the lifespan of Batten disease model mice in a significant manner by contrast to control with non-loaded EVs.

2.2.3. Extrusion

EV extrusion through 200 nm-pore polycarbonate membranes was studied by Haney *et al.* [36]. This method allowed loading of catalase, previously solubilised in the EV suspension, giving loading results equivalent to those obtained by sonication (809 molecules / EV compared to 940 for sonication, the highest of all tested methods for catalase). Extruded EVs were polydisperse, aggregated and larger than naïve EVs (149.4 ± 3.3 vs. 99.5 ± 11.2 nm) although still exposing a round shape (AFM imaging). Extruded EVs showed similar levels of neuroprotection *in vitro* compared to sonicated EVs. Surprisingly, catalase activity preservation, cellular accumulation and *in vivo* neuroprotection were not studied for extruded EVs despite promising results in terms of loading and neuroprotective effect.

Extrusion was also briefly studied in the previously mentioned EXPLOR work of Yim *et al.* [26]: EVs extruded through 200 nm pores at a concentration of 2×10^{10} EVs/mL were compared to EVs loaded with EXPLOR technology. Luciferase loading was shown to be 40 times lower by extrusion than with the EXPLOR system. However, extruding 200 nm EVs through 200 nm-pore membrane might not allow sufficient reshaping, as the pore size is not sufficiently smaller than EV size. Protein association may therefore not be possible using this method. Finally, even if the extrusion method is feasible for small molecules, it is probably difficult to envisage generalization of this procedure for protein loading, as evidenced by mitigated results exposed above.

2.2.4. Freeze-thaw cycles

Still with the goal to induce membrane remodelling, F/T cycles were explored to load catalase into EVs [36]: the protein was incubated for 30 min with EVs, rapidly frozen at -80°C, and thawed at RT (repeated three times). This resulted in a catalase loading lower to the one obtained with extrusion or sonication (evaluated at 437 molecules / EV), and a similar EV size (130 ± 11 nm measured by NTA). Accumulation in cells was also inferior compared to other processes, and freeze-thawed EVs shown to be prone to aggregation.

A slightly different protocol was used to load Cas9-RNP complexes into HEK293 derived EV [61]. Cas9-RNP were co-incubated with EVs for 30min at RT, rapidly frozen in liquid nitrogen and thawed at RT (repeated three times). Obtained vesicles (EVs-RNP) have a larger size compared to native EVs (129 ± 9 nm vs. 105 ± 16 nm). After ultracentrifugation step ($100\,000 \times g$, 70min) was performed to separate free RNP complexes from encapsulated ones, Cas9 -RNP complex loading efficiency was reported to be over 37%. However, it is important to not that free RNP complexes, specifically after undergoing freeze-thaw cycles, could also have formed aggregates able to pellet with EVs at $100\,000g$. As a control, it would have been interesting to see if the complexes alone gone through F/T cycles, were all present in the supernatant after this ultracentrifugation step. In parallel, EV-RNPs surface was functionalised with DNA aptamers targeting HepG2 cells anchored in EV membrane thanks to a tri-cholesterol anchor (TDN1; tetrahedral DNA nanostructures). TDN1-EVs-RNP associated with sgRNA targeting an exon of WNT10B (oncogene found in hepatocellular cancer) were used to treat HepG2 cancer cells. The protein expression levels of WNT10B were significantly reduced in cells treated with TDN1-EVs-RNP, whereas no (saline, lipo-RNP) or lower (EVs-RNP) differences in WNT10B expression were observed in other groups (saline, lipo-RNP). This diminished expression resulted in a decrease in cell viability ($\sim 40\%$) of TDN1-EVs-RNP treated cells. In BALB/c nude mice bearing HepG2 xenograft tumours, intravenous injection of TDN1-EVs-RNP also led to a significant decrease in tumour weight compared to saline and even lipo RNP treatment. This study is to our knowledge, the only one reporting Cas9-RNP complexes loading into EVs via physico-chemical tools.

2.2.5. Electroporation

Electroporation, frequently used to load nucleic acids, was explored to load ribosome-inactivating protein saporin into HeLa cell-derived EVs [31]. EVs (25 μg) were loaded with different amounts of saporin (0, 5, 50 μg) following thereafter described protocol: poring pulse: twice pulses (100 V, 5 ms); transfer pulse: five pulse (20 V, 50 msec). Unfortunately, the effect of electroporation on EVs size and morphology was not reported. PBS washing of EV-saporin suspension followed by ultrafiltration (100 kDa) was performed to discard non loaded protein, and encapsulation efficiency was identified to be 0.1%. Yet, few details are provided on the method (indirect vs. direct method) used to calculate this value. Non-functionalised EVs loaded with saporin did not show cytotoxicity towards CHO-K1 cells. The results obtained in cell death assay with non-functionalised EVs do however correlate with the low internalisation level seen in flow cytometry. In parallel, EVs with arginine rich peptides could enhance cell uptake. Consistently with their established superior cell uptake, ArgCPP-modified saporin-loaded EVs had a significantly higher cytotoxicity (83%). However, to make these results even more convincing, a control using electroporated saporin alone would have been interesting, as protein electroporation is a delicate process that can induce

1 aggregation and thus the formation of particles that are difficult to separate from EVs. These modified EVs will also
2 have to be evaluated *in vivo*, as the presence of cationic charges on EV surface could lead to a diffuse biodistribution
3 and a lack of *in vivo* specificity.
4

5 **2.3. Comparison of protein loading strategies into EVs**

6 Despite some promising results, biological methods, historically among the first evaluated for protein
7 loading, have proven effective but also exhibited limitations. They usually involve numerous steps that are liable to
8 biological variation, which leads to difficulties in controlling yield and reproducibility of protein loading. Altogether,
9 these points led to difficulties in imagining a rational translation to the clinic. The majority of presented studies used
10 EVs isolated from HEK293 cells due to their high EV production capacity, minimal intrinsic biological cargo and ease
11 of transfection. However, this is not translatable to all cell types, especially “hard-to-transfect” cells, such as
12 mesenchymal stem cells that are nonetheless an important source of EVs for therapeutic use.

13 In contrast to biological methods, in which the protein is supplied during vesicle biogenesis, physico-chemical
14 strategies rely on getting macromolecules through an already formed membrane, a particularly delicate task [64]. To
15 allow protein loading, a permeabilisation step is therefore a prerequisite. This EV permeabilisation step must be
16 transitory and not deleterious to EV stability and yield. It is therefore required to find optimal conditions to induce
17 transitory EV membrane permeation allowing protein loading while working in conditions (concentration,
18 temperature, agitation) compatible with protein stability and integrity. As described in the previous section, physico-
19 chemical tools induce sometimes harsh conditions (temperature cycles, shear stress induced by sonication, elevated
20 temperature, etc) that could induce protein aggregation and even EV physical or chemical denaturation and could
21 lead to low EV yield, even if loading ratio is sometimes more important than with biological loading methods. The
22 physico-chemical properties of the protein to be loaded is probably also more important when envisaging physico-
23 chemical loading vs. biological loading, as size, charge, hydrophily/lipophily affinity will be critical factors in allowing
24 membrane crossing.

25 A delicate task, this time common to both biological and physico-chemical loading methods, is to find a
26 reproducible method to separate loaded EVs from non-incorporated protein cargo. The contamination of free cargo
27 as well as protein forming nano-aggregates, may lead to misinterpretations of overall conclusions about the
28 efficiency of EV-based drug formulations. Unfortunately, in the majority of studies dealing with genetically-induced
29 overexpression of a protein by producing cells, no density gradient purification step were performed. Moreover, an
30 important point is to determine if proteins are encapsulated inside EV lumen and/or associated with EV membrane;
31 using controls such as proteinase K treatment could help answer this question, but unfortunately this is not often
32 done.

33 When dealing with protein loading, the choice of the strategy to be used should therefore depend on many
34 parameters including the cells used to produce EVs, as well as the protein to be loaded. Comparing biological and
35 physico-chemical methods was a delicate task as encapsulation efficiencies were rarely given and many parameters
36 differed, such as EVs isolation protocols, EVs nature, etc. (Tables 1 & 2). Only one study presented results to this
37 effect: as previously mentioned, Haney *et al.* compared transfection of a plasmid into producing cells to sonication

or saponin-permeation of EVs for TPP1 enzyme loading [28]. Their results suggested superiority of physico-chemical methods over biological overexpression in terms of: (i) loading efficiency (6,908-4,934 vs. 987 molecules/EV); (ii) *in vitro* delivery of cargo protein (amount of TPP1 protein found in the TPP1-deficient CLN2 cells after incubation with EVs). Until now, most teams initially focused on pre-production/biologic modifications (Fig. 2); these proved feasible but could be burdensome and time-consuming [65].

Additional studies on physico-chemical methods are needed to draw further conclusions, as, to our knowledge, only 4 articles have been published so far (vs. 13 studies for biological methods): the encouraging results obtained by Haney *et al.* [28, 36] and Zhuang *et al.* [61] nevertheless indicate the promise of such processes, which do not require genetic engineering and could be more relevant for an upscale therapeutic production.

3. Lipid nanoparticles and intracellular protein delivery

In a broader context, not necessarily for intracellular delivery, liposomes/LNPs have been used for protein delivery for decades, starting with reference articles published more than 40-50 years ago, followed by numerous studies until this day. The first enzyme encapsulated in liposomes was lysozyme, in 1970 [66], then many other examples followed. β -glucuronidase encapsulated in liposomes and injected intravenously in mice remained active for more than a week [67]. β -galactosidase containing liposomes degraded GM1-ganglioside in lysosomes of feline fibroblasts presenting pathological accumulation of this molecule [68]. Interestingly, liposomal encapsulation allowed delivery of enzymes through the blood-brain barrier: liposomal horseradish peroxidase [69], liposomal glucose oxidase [70] (5% of activity detected in the brain of rats after intravenous injection in the tail). Following these reference studies, other enzymes and proteins were encapsulated and investigated: catalase [71]; L-asparaginase, an anti-cancer enzyme [72]; haemoglobin [73]; blood clotting factor VIII, to treat haemophilia [74]; growth factors (fibroblast [75], nerve [76]); human gamma-globulin [77]; superoxide dismutase [78]. These studies represent a few examples among a large number of publications in this field. Proteins have been incorporated into liposomes using multiple processes such as reverse-phase evaporation, injection, freeze-thaw cycles, extrusion, dry lipid hydration, and leading to variable association efficiencies [79]. Nevertheless, to this day, no commercial liposomal protein delivery system has reached the market, especially for intracellular delivery. Research in this field is however very active, as underlined by the following studies describing different lipid nanoparticle (LNP) compositions, mainly based on cationic lipids [18, 80-90] but also on neutral lipid or lipopolymers [91-97].

3.1. Cationic lipid nanoparticles for intracellular delivery

Commercial synthetic vectors, initially developed for nucleic acid delivery, such as Lipofectamine® RNAiMAX or 2000, have been used to deliver Cas9-RNPs and single stranded DNA oligonucleotides (ssODN) donor template inducing homology directed repair (HDR) with 10 to 20% efficiencies *in vivo* after local administration in mouse ears. The authors hypothesized that an interaction of cationic lipid headgroup with endosomal membrane could trigger endosomal membrane destabilization and release of Cas9-RNPs complexes in cell cytosol [18]. Another

lipofectamine formulation, dedicated to CRISPR Cas9 delivery (Lipofectamine® CRISPRMAX) was used to co-deliver donor DNA and achieved up to 17% HDR *in vitro* in HEK293 cells [89]. Cationic lipid-based commercial vectors could therefore be relevant for *ex-vivo* modification (depending on cell type) or for local administration *in vivo*, IV injection being difficult in regards to the potential toxicity of these cationic lipids.

In parallel to commercial options, other cationic lipid formulations were investigated to deliver proteins to cell cytoplasm. Among the first developed, BioPorter (trifluoroacetylated lipopolyamine (TFA-DODAPL (2,6-diaminohexanoic acid [5-amino-5-[5-[2-amino-6-(2,6-diaminohexanoylamino)-hexanoylamino]-1-(3-diocadecylamino-propylcarbamoyl) pentylcarbamoyl] - pentyl] - amide)) cationic lipid associated with DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine)) exposed protein (FITC-Ab or β -gal) delivery efficiencies between 70 and 75% in NIH3T3 fibroblasts compared to less than 5% delivery for other evaluated formulation ((DOTAP:DOPE, DMRIE (1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide), DOPG (1,2-dioleoyl-sn-glycero-3-phosphoglycerol), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), Trans-IT®, FuGene® 6, Tranfast®, Lipofectamine® and Lipofectin®) [85]. In this study, fluorescent microscopy along with cell fractionation and protein function assays, evidenced that TFA-DODAPL:DOPE Bioporter system allow effective protein delivery in cell cytoplasm. The BioPorter® system was investigated to allow efficient cytoplasmic and nuclear delivery of a protein nucleoside kinase to allow gene suicide therapy [86]. This delivery strategy allowed an increase in cytotoxic nucleoside analogues sensitivity, such as Ganciclovir™, into human osteosarcoma and CHO cell lines.

A set of synthetic systems dedicated to nucleic acid delivery were also evaluated for protein delivery. The classic DOTAP/DOPE lipid couple was associated with apolipoprotein (APOA-I) to form small nanoparticles (20-30 nm) able to deliver cyt c in non-small cell lung tumor cells [98]. To allow its loading, cyt c were mixed with a membrane permeable sequence (MPS) before a PEGylation step to increase NP stability. After a separation step using SEC (Sephacrose CL 6B), the NP loading efficiency of MPS-cyt c was determined to be 64-75%. After 6h of incubation with cells, PEGylated NP loaded with Cyt c were co-localised with mitochondria, and such treatment induced apoptosis (Annexin V staining) in 24.4% of treated H460 cells. *In vivo*, this resulted in a significant (3 fold) decrease in tumor growth after intravenous injection of PEGylated-NP loaded Cyt c. Another nucleic acid transfection reagent, dioctadecylglycylspermine (DOGS) was used to intracellularly vectorise a panel of negatively charged proteins (FITC-IgG- α actin, FITC-IgG- α tubulin, R-Phycoerythrin, BSA) with various efficiencies depending on vectorised proteins. [87]. However, important charge ratio (CR) were used to obtain protein complexation and delivery (from CR 32 to 1100 for BSA) leading to highly charged complexes prone to aggregation. The authors evidenced that not only electrostatic interactions, but also Van der Waals attractive interactions were responsible for protein complexation, explaining differences in delivery efficiencies in regard to complexed protein. As an example, while delivering FITC-IgG to CHO cells, a diffuse intracytoplasmic fluorescence signal was evidenced when using DOGS complexation, while BSA-FITC/DOGS treatment led to a more punctiform staining (probably showing endo/lysosomal retention). Complexes composed of SAINT-2 cationic lipids (N-methyl-4(dioleoyl)methyl-pyridinium-chloride) and lipid helper DOPE proved to be more efficient than the reference BioPorter® for β -galactosidase (β -gal) and antibody cytoplasmic delivery in the presence of serum [88]. The same liposome composition also allowed nuclear delivery and functionality of DNA methyl transferase. Nevertheless, fluorescent microscopy observation of

cells treated with SAINT-DOPE/IgG1 antibody MOC31-Alexa488 evidenced a majority of endo/lysosomal retention of the protein. Chatin *et al.* evaluated different nucleic acid dedicated reagents (bis (guanidinium)-tren-cholesterol (BGTC), dioleoyl succinyl paromomycin (DOSP),imidazole-based helper lipid MM27, or DOPE) to vectorise proteins (β -gal and antibody targeting cytokeratin K (K8)) and highlight the necessity of adapting the lipid assortment to the vectorised protein [99]. Using a lipid:protein molar ratio of 400, DOSP-MM27 were able to fully complex FITC-antiK8 antibody. These complexes allowed FITC-antiK8 delivery in 67% of HeLa cells (vs. 14 and 8% with DOSP:DOPE and BGTC:MM27 respectively). Moreover, a diffuse, cytoplasmic staining was observed. Nevertheless, formed complexes were micron size particles which should restrict their use to *in vitro* application.

In the same way, a set of lipid-like molecules, lipidoids [100], exposing less distinct/binary structures compared to cationic lipids composed of a cationic head and aliphatic tails, were screened to find optimised candidate molecules able to complex and deliver proteins into cells. To allow their complexation, proteins were transiently modified with *cis*-aconitic anhydride (protein-Aco) [81]. This modification was particularly crucial for complexation of positively charged and hydrophilic RNase A protein. The modification was shown to be reversible when performed with RNase A in endolysosomal-like environment (pH 5.2), restoring active protein. Among evaluated lipids, EC16-1 was the most efficient to deliver RNase A-Aco, Saporin and Saporin-Aco and reduced cell viability to 30%. After 4h treatment with FITC-RNase A/EC16-1 complex, a significant amount of protein was visible in the cytoplasm, non-co-localised with endo/lysosomal compartment, evidencing that this system bypasses degradation by endo/lysosomal system. Saporin was formulated in lipid NPs composed of EC16-1, DOPE, cholesterol, ceramide-mPEG₂₀₀₀ to allow *in vivo* IV administration in BALB/c mice bearing 4T1 breast cancer tumours. 28 days post-injection, tumour volumes were significantly reduced compared to free Saporin (by 80 %) with no side effects (no body weight variation, no increase in TNF- α or IFN- γ levels). This efficiency was confirmed more recently *in vivo* using two models of colon (SW620/AD300) and lung (NCI-H460/MX20) carcinoma multidrug resistance tumours [80]. RNase A lysine residues were modified using 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzyl carbonate (NBC) to trigger reactive oxygen species (ROS) responsive release of RNase A and reduce protein charge to allow complexation with cationic entities [101]. Another member of EC16 lipids (EC16-80) complexed with RNase A-NBC and decreased cell viability to at least 40 % using a panel of cancer cell lines (HeLa cervical cancer cells, B16F10 melanoma cells, PC-3 prostate cancer cells, and MDA-MB-231 breast cancer cells). This effect was not observed in non-cancer cells suggesting that the ROS level, increased in cancer cells, could led to a ROS responsive protein cytotoxic activity. The same lipid (EC16-80) was used to complex tumour suppressor protein PTEN, and led *in vitro* to a decrease in cell viability to 20% into PC3 cells [82]. Delivered PTEN negatively regulated the AKT pathway and induced apoptosis (20% of annexin V positive PC3 cells after PTEN/EC16-80 treatment).

A library of 12 bio-reducible, GSH sensitive lipids was evaluated to complex genome editing proteins [83]. Negatively charged Cas9-RNP complexes exposed high efficiency to target and inhibit genomic EGFP reporter gene expression into HEK cell line (70% EGFP expression loss). Thanks to the presence of bio-reducible disulfide bonds in lipids, the intracellular dissociation of complexes was favoured, enabling nucleus delivery and avoiding endo-lysosomal degradation. Still to deliver Cas9-RNP, a library of chalcogen-containing lipids was synthesized by reacting lipophilic tails containing O, S or Se ethers (named O17O, O17S or O17Se) with various amine heads [84]. One lipid

(81-O17Se) was particularly interesting to allow Cas9-RNP delivery in reporter GFP-HEK cell line, resulting in GFP knockout levels comparable to commercial standards (50% vs. 63%) but with lower cytotoxicity (24% vs. 34%). These results, while encouraging, will have to be completed with findings exhibiting therapeutic potential.

Recently, a new lipid-containing oligoaminoamides library was synthesized to allow Cas9-RNP complexation by electrostatic interactions [97]. Interestingly, the presence of one hydroxyl group in the fatty acid was evidenced as crucial to form stable well-defined nanoparticles and increase cellular uptake, endosomal escape as well as nuclear delivery. A GFP reporter gene was used as a read-out to assess the knock out efficiency of Cas9-RNP targeting this gene. The optimised construct gave 40% GFP knock out in Neuro2A-GFP cells after a single Cas9/sgRNA treatment. After a proof-of-concept in artificial reporter cell lines, this system was also evaluated to knock out an endogenous gene, the folate receptor 1 gene. Into Hela cell line, FolR1 knock out of around 30% was detected. These promising results now call for formulation optimisation adapted to *in vivo* evaluation.

By contrast, *in vivo* delivery of Cas9/sgRNA RNPs was evidenced after complexation with smart ionizable LNPs [90]. A dendrimer-based ionizable lipid (5A2-SC8) with pKa around 6.4 was used to bind negatively charged protein at the pH of mixing (e.g., pH 4 when the amines are protonated), to lose charge at neutral pH before cellular uptake, and then acquire charge again in acidic endosomal compartment to fuse with endosomal membranes and enable protein release into cytoplasm. In parallel, DOTAP cationic lipid, positively charged at neutral pH, allowed for complexation of RNPs complex at a physiological pH. These lipids were associated with cholesterol and DMG-PEG to allow increase in LNP stability. By mixing an ethanol solution of lipids (5A2-SC8/DOPE/Chol/DMG-PEG/DOTAP = 15/15/30/3/7 (mol/mol)) with an aqueous solution of Cas9/sgRNA RNPs, formed LNP were inferior to 200nm in hydrodynamic diameter and exposed a neutral charge. Using GFP-fusedCas9, the authors shown that LNP-RNPs were endocytosed by an energy-lipid raft dependent mechanism. Subsequently, Cas9-GFP protein was first observed in cell cytoplasm before gradually migrating into nucleus within 6h, probably due to the presence of a nuclear localisation signal on Cas 9. Treatment of HeLa-GFP cells with LNP complexing Cas9/sgGFP induced small nucleotide insertion or deletion (Indel) into GFP DNA as a knock-out of nearly all GFP expression was observed (80% indels after 6 days). Moreover, these formulations were shown to be stable for 2 months at 4°C. By adjusting the molecular components and ratios, a tissue-specific gene editing was evidenced selectively in the livers and lungs of mice following systemic injection. A particularly high edition efficiency was shown in the lungs in a reporter model of Td-Tomato mouse when injecting LNPs encapsulating Cas9/sgTOM RNP complexes. Focusing on therapeutic application, interesting results were obtained after local administration (intramuscular) in a mouse model of Duchene Muscular Dystrophy. Indeed, the expression of dystrophin in injected muscles was restored after intramuscular injection of LNPs encapsulating Cas9/sgDMD RNPs (4.2% evaluated by western blot). After iv administration of LNP formulated with less amount of DOTAP (5A2-DOT-5) and encapsulating Cas9/sgPCSK9, the PCSK9 (a well-known target for treating hypercholesterolemia) level in serum and liver tissues were significantly decreased. T7E1 assays evidenced indel generation at PCSK9 loci with efficiencies from 5 to 7.8%. This formulation is therefore among the most promising to date. Perspective of using these LNP after a simple intravenous administration for precise gene correction of various genetic diseases is particularly desired.

3.2. Non cationic lipid nanoparticles for intracellular protein delivery

As described in the previous section, the majority of studies aiming at intracellular delivery of proteins rely on cationic lipid use. By contrast, few studies described the use of non-cationic lipids or lipidoids. As an example, non-cationic EC16 derived lipidoids containing nitrilotriacetic acid (NTA) groups were recently formulated with divalent nickel ions and helper lipids (cholesterol, phospholipid, and macromolecular lipids) to complex His-tagged proteins [91]. His-tagged-NLS-cas9-RNP/lipidoid complexes formed well dispersed 150-350 nm nanoparticles. Already evocated GFP-HEK cell line was used to evaluate the genome editing efficiency, leading to GFP knock-out (KO) in the range of 15 %, a lower amount compared to the one obtained with GSH sensitive cationic lipidoids systems previously developed [83].

Diphtheria toxin A chain (DTA) (70 kDa) and an endosome fusogenic peptide resembling the N-terminal domain of influenza virus hemagglutinin (diINF-7) were co-encapsulated into PC: Chol liposomes [94]. Interestingly, the cytoplasmic delivery of diINF-7/DTA-containing immunoliposomes (DTA-425-FIL) led to 8-fold higher cytotoxicity compared to free DTA, or liposomes loaded with only diINF-7 or DTA.

As an innovative source of lipid, a mix of neutral to negatively charged lipids isolated from spinach chloroplast thylacoids was used to form small unilamellar liposomes and complex mitochondrial membrane protein VDAC (voltage dependent anion channel) and pro-apoptotic Bak protein [92]. Recombinant Bak and/or VDAC proteins, obtained by the Rapid Translation System (Roche) were mixed with purified spinach lipids in organic solvent (methanol, dichloromethane). After formation of a lipid film by solvent evaporation, the dry lipids were rehydrated and sonicated to obtain protein-loaded liposomes. Non associated proteins were separated from liposomes by a sucrose gradient. Bak, VDAC or Bak-VDAC loaded liposomes, called proteoliposomes were used to treat HCT116 colon cancer cells. After 24h, 20% of treated cells were annexin V positive vs. 8.5% for unloaded liposomes and 48% for doxorubicin treated cells). A pro-apoptotic effect was also evidenced in GL26 glioblastoma cells treated with Bak loaded proteoliposomes using a different lipid source but the same loading strategy [93]. *In vivo*, this formulation induced tumour regression in 40% of mice carrying ectopic GBM model (GL26) after intra-tumoural injection. Systemic administration also led to an increased tumour cell apoptosis and animal life span, but only a slight delay in tumor growth, maybe due to a lack of efficiency to reach tumour cells, a well-known drawback encountered with liposomes.

4. Comparison of EVs and liposome-based protein delivery systems

In this section, we aim to compare liposomal formulations described in the previous section and EV formulations described in section 2 for their efficiency in delivering therapeutic proteins. We will discuss these studies according to their application/therapeutic strategy: (i) delivery of protein for anti-cancer therapy, (ii) restoration of ion channel function for cystic fibrosis (CF) therapy and (iii) delivery of gene engineering tools.

4.1. Delivery of protein for anti-cancer therapy

Studies focusing on delivering proteins were carried out on a multitude of cancer cell types (Table 3): for EVs, schwannoma [34], glioblastoma [35], pancreatic [30], cervical [26]; for liposomes, colorectal [102], cervical [85], leukaemia [85], prostate [85, 103], lung [98], ovarian [104]. Most studies expressed efficacy in terms of percentages of decrease in cell survival. Survivin-T34A loaded EVs resulted in 30% decrease in pancreatic cancer cell survival [30]. Cytosine deaminase loaded EVs (associated with pro-drug treatment) induced 80% of cell death in Schwannoma cells [34] and 30% in glioblastoma cells and spheroids [35] while Bax loaded EVs were reported to induce cytochrome c release [26]. Arg- functionalised saporin-loaded EVs induced 83% cell death in hamster cells: these results will need to be confirmed on human cancer cells, but are nevertheless very promising [31]. Regarding liposomes, liposomal Bak induced 20-40% apoptosis [102], liposomal granzyme B 40-60% depending on cell type [85], liposomal PTEN up to 90% [103], liposomal cytochrome c 24% [98]. *In vitro* results were thus similar for EVs and liposomal formulations; it is worth noting that when it comes to saporin delivery, functionalised EVs-delivered saporin seemed to induce a higher degree of cell death (83%) than EGFR-targeted liposomal saporin (25%). Regarding *in vivo* antitumoural effect, the cytosine deaminase loaded EVs [34] fared slightly better than their cytochrome c loaded liposome counterparts [98], resulting in 70% tumor growth inhibition (TGI) in a glioblastoma tumor model and total TGI in Schwannoma tumour model, when liposomal induced TGI was around 73%.

4.2. Cystic fibrosis (CF) therapy

Vituret *et al.* [29] and Chatin *et al.* [105] both explored a possible therapeutic strategy for cystic fibrosis through the restoration of ion channel function thanks to expression of CFTR at the plasma membrane in F508del-CFTR expressing cells (Table 4). Their approach was however different: for EVs, restoration was performed through the delivery of wild type CFTR whereas in the case of liposomes, an anti-K8 antibody was delivered using liposomes to disrupt F508del-CFTR-K8 interaction and its subsequent entrapment of CFTR. Target cells also differed: for EVs, an epithelial cell line CF15 was used, whereas for liposomes, cervical cancer HeLa cells were genetically modified to express the mutant protein. Outputs to control ion channel function restoration were distinct (EVs: iodide efflux; liposomes: MEQ fluorescent probe), making results difficult to confront: comparing deficient cells treated with the formulation to normally functioning cells, EVs were able to restore iodide efflux to 25%, and liposomes to 75%. However, cystic fibrosis affects primarily lungs so CF15 cells, which are nasal epithelial cells, seem like a more appropriate model to study a therapy for this disease than cervical cancer HeLa cells.

4.3. Gene engineering

Various EVs and lipid formulations were recently investigated to allow transport of gene engineering proteins such as Cre recombinase (Cre), Transcription Activator-Like Effector Nucleases (TALEN), and above all, the promising CRISPR associated protein 9 (Cas9).

To be functional, Cas 9 should be associated to a single guide RNA (sgRNA) to form RiboNucleoProtein (RNP) complexes. CRISP-Cas9 can be used to create knock-outs (KO) (by creating a double stranded break (DSB) at the DNA

target site followed by repair of this break by non-homologous end-joining (NHEJ) pathway. NHEJ will induce insertion of small nucleotide insertion or deletion (Indel) at the DSB site leading to mutation, inducing premature stop codon and loss of function of the target gene. CRISPR-Cas9 can also be used to introduced a modified gene sequence ("true" edition) after DSB by Homology directed repair (HDR) if a DNA repair template is provided (containing the desired sequence to be edited surrounded by homologous sequences named homology arms). Nevertheless, the percentage of effective HDR is generally low (inferior to 10%). The majority of DSB will therefore be repaired by NHEJ. In studies evocated in this section, CRISPR-Cas 9 efficiency was measured by regarding mainly indels percentages, or KO efficiencies, and, for only two studies [18, 89], HDR percentages with commercial transfection reagents.

Regarding Cre-recombinase, 4 studies were identified regarding delivery with EVs ([26, 44, 45, 106] and 3 regarding delivery with lipid nanoparticles [18, 83, 84]. In these last 3 studies, Cre association required a fusion with a negatively charged form of GFP, (-27)GFP or (-30)GFP, to promote electrostatic interactions with cationic lipids. This evidences one drawback encountered with synthetic lipid nanoparticles: complexation with proteins mainly relies on electrostatic interactions. Cationic lipids can therefore only interact with negatively charged proteins, except if biological or chemical modifications are performed to allow interactions. Regarding *in vitro* recombination, Cre loaded EVs led to recombination efficiencies ranging from 40 to 95%, values that are similar to the one obtained with lipid nanoparticle based systems (45-80%). Two EV-based Cre delivery systems were evaluated *in vivo* by nasal [106], or local brain [26] administration and exhibited up to 30% recombination after nasal administration (no quantification was given in the other study). Similarly, effective recombination was described after (-27)GFP-Cre delivery with cationic bio-reducible lipids but without any quantification. Interestingly, Gectosome ability for intracellular Cre delivery was compared to that of liposomes one [45]. Liposomes encapsulating Cre recombinase were synthesized following Yu *et al.* work [107, 108]. Interestingly, with these liposomes, 510 nM of Cre delivered by liposomes was required to induce nearly 20% of Cre recombination. In contrast, a similar recombination percentage was obtained with only 0.81 nM of Cre-Gectosome treatment: Gectosomes thus have a 630-fold higher capacity to induce recombination than liposomes. To explain this capacity gap, the authors the authors referred to the difficulties that liposomal formulations can have in terms of endosomal escape, evidencing here the advantage of EVs which are naturally equipped to do so [109].

Regarding Cas9 delivery, as the global charge of Cas9-sgRNA RNP complex is negative, no further modification was required to interact with cationic lipid formulations. Concerning Indels percentage: 8 (in MCF7 cells) to 85% (in HCT116 cells) of Indels were obtained with commercial reagent Lipofectamine® CRISPRMAX vs. 10% for EVs (GEDEX) in HEK293 treated cells. Lipid nanoparticles were the only systems evaluated which allowed HDR *in vitro* with 8-11% [18] and 17% [89] restoration of eGFP reporter system expression. No HDR was evaluated in the two reported studies used to transport Cas9-RNP [42, 45] with EVs. A set of cationic lipids were evaluated to induce KO via DSB induction followed by NHEJ repair, with KO efficiencies ranging, *in vitro*, from 30 to 70% [83, 84, 97], while non-cationic lipids based delivery led to 15% KO efficiency [91]. To summarise, regarding *in vitro* delivery, cationic lipids have been more evaluated (5 studies vs. 2 studies for EV, Table 5) and tended to be more efficient. In contrast, regarding *in vivo* evaluation, both EV systems have been evaluated after intravenous [42, 45] and

intraperitoneal [42] administration while cationic lipids have only been evaluated after local administration [18]. Cas9-RNP vectorised by EVs induced a 50% decrease in targeted gene expression (PCK9, Proprotein convertase subtilisin/kexin type 9) after intravenous injection [45] and 50% of KO in reporter GFP expression after intraperitoneal injection [42]. Cationic lipids were administered in mouse inner ears leading to 20% Cas9-mediated KO in hair cells. This evidenced another drawback of cationic lipids: their *in vitro* efficiency is not translated once in *in vivo* complex environment. In contrast, EVs administered *in vivo* led to promising delivery of functional CRISPR-Cas9, and hold great promise for the future.

5. Conclusion

In regards to the previous section, protein delivery using EVs proved feasible, and EV-based formulations were able to compete with cationic lipids acting as *in vitro* reference systems (Tables 3, 4, 5), showing even more effective in some *in vivo* applications (Table 5). As evidenced in the present review, the majority of lipid-based nanoparticles used to complex proteins are cationic systems. This implies, if the proteins to deliver are not naturally negatively charged, the use of biological or chemical modifications to allow electrostatic interactions. Such modifications are not applicable to all proteins and could hinder the biological functions of the proteins as well as increase system complexity.

Cationic synthetic systems also led to interesting *in vitro* efficiencies (depending on target cells), but are associated with well-known limitations for *in vivo* use, as encountered when vectorising nucleic acids [110-112]: toxicity, allergic reactions, lack of specificity. To be used *in vivo*, cationic systems have to be optimised, and only a small number of formulation combines small size and stability with *in vivo* efficiency, such as smart ionizable LNP [90]. LNPs have been reported to activate the complement system, and could therefore induce a hypersensitivity reaction known as complement activation related pseudoallergy (CARPA) [113, 114]. Moreover, as evocated, LNP require have to be PEGylated to increase their physico-chemical as well as their *in vivo* stability. But is is now well described, that PEG can activate splenic B cells to produce anti-PEG antibodies. These anti-PEG antibodies could both induce antibody mediated anaphylactic responses upon secondary exposure, but also trigger the so-called accelerated blood clearance effect (ABC) [115-117]. In contrast, EVs produced endogenously are likely less immunogenic and less toxic. It should nevertheless be noted that despite first intuitions concerning their *in vivo* stability, when administered in a non-autologous host, EVs are quickly eliminated ($t_{1/2}=4\text{min}$) and found in elimination organs like most synthetic vectors [118-121]. Surface stabilisation could therefore be a supplementary step to consider for allogeneic applications. Nevertheless, in contrast to what has been encountered with liposomes, EVs have not been shown to induce hypersensitivity reactions, often linked to PEG presence in the formulation [122, 123]. Several phase 1 studies with autologous exosomes were completed in the 2000s [124-126], establishing GMP (Good Manufacturing Practices) to produce pharmaceutical-grade EVs: no grade II toxicity and no maximal tolerated dose were achieved, indicating the safety of EV administration. After injection, EVs were shown to present low toxicity [127, 128], and numerous studies are now ongoing in the clinic [129].

One other advantage of EVs compared to synthetic vectors lies in their ability to interact with target cells. For example, PC:Cholesterol liposomes were internalised to a lesser extent than EVs *in vitro* as well as *in vivo*, when delivered intratumorally [118]. Multiple other teams obtained similar results with EVs and PC or PC:Cholesterol liposomes [36, 130, 131]. We recently reported that murine mesenchymal stem cells-derived EVs (mMSC-EVs) were internalised to a greater extent into target cells compared to HSPC:Cholesterol liposomes, and that these nanoparticles followed different endocytosis pathways [132]. Several adhesion molecules (including integrins, immunoglobulins, and proteoglycans) as well as tetraspanins were reported to be involved in EV interactions with cells [133]. Implication of filopodia was also reported to help EV internalisation, a mechanism known to be used by viruses or other pathogens for cell infection [134]. Such surfing is supposed to allow moving along the plasma membrane to avoid zones of dense cortical actin skeleton which are difficult to penetrate and thus reach endocytic hot spots [135]. Different endocytosis pathways have been proposed for EVs internalisation depending on EV cell source (immune cell vs. non immune cells), vesicle type, targeted cells but also experiment conditions (quantity, incubation time, EV marker used, etc.) [109, 134, 136-138]. Clathrin-dependent endocytosis, caveole-dependent endocytosis, lipid-raft mediated endocytosis, macropinocytosis, phagocytosis and in some cases fusion with plasma membranes have been reported [109, 134, 136-142]. It was also observed that the efficiency of EVs internalisation was directly correlated with micro environmental and intracellular acidity [141]. After internalisation, EVs mainly follow the endolysosomal pathway and reach the multivesicular endosomes (MVEs), which are most often directed to lysosomes [134, 137]. Various hypotheses have been put forward regarding EVs capacity to escape endosomes. The acidity of late endosomes which would increase the fusogenic properties of these vesicles with endosomal membranes [141] [109], as well as the composition of late endosome or intraluminal vesicles (rich in anionic lipid lysobisphosphatidic acid (LPBA), implying ALIX and ESCRT-III complexes) [143, 144] have been reported to play a role in such escape, by a back-fusion process, allowing EV cargo release in the cytoplasm. Interaction of the 'kiss and run' type with endoplasmic reticulum (ER) have also been reported, suggesting molecules exchange, such as miRNA or mRNA, as ER was recently evidenced to be a central nucleation site of siRNA-mediated RNA silencing [145]. Nevertheless, whatever the involved pathways may be, it is clearly established that EVs are able to efficiently deliver, and in picomolar quantities, several types of biomolecules capable of inducing an effect in target cells [22, 24, 146, 147]. This is a crucial added value of EVs in regards to their synthetic counterparts. The latter sometimes require highly complex formulations to allow the cytoplasmic delivery of biomolecules, such as the used of ionizable lipids able to destabilise endosomal membranes [90, 110, 148, 149].

While exposing several advantages, EVs also exposed some well identified drawbacks, already mentioned. One of the main challenges is large-scale, rapid and efficient EV production. Historical protocols, relying mostly on ultracentrifugation, are usually long (~4-5 days in total including 4-6h of ultracentrifugation [150]) compared to the formulation of synthetic vectors and production yields could be variable and low [128, 151]. Nevertheless, protocols have been improved in the past years, and numerous studies describing production of clinical grade EVs are now available, establishing a base line for a rational therapeutic development [152-155]. Focusing on protein loading into EVs, looking at biological loading, the requirement of transfection steps and multiple biotechnological modifications would be supplementary limitations. The loading process should be rendered as simple and rational as possible,

maybe by leaning towards physico-chemical loading methods, which could be easier to control, more appropriate for large scale production and exhibiting better encapsulation performance (Table 1-2).

Thus, although only few studies have been published so far, they have established the benefit of using EVs, and exposed results equivalent or superior to similar liposome-based protein delivery formulations. EVs therefore represent great therapeutic potential, in a context in which no reference exists for intracellular protein delivery. Further investigations should manage to consolidate the effectiveness and promise of these natural carriers as intracellular protein delivery systems.

Competing interests statement

The authors have no competing interests to declare.

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FIGURE

Figure 1

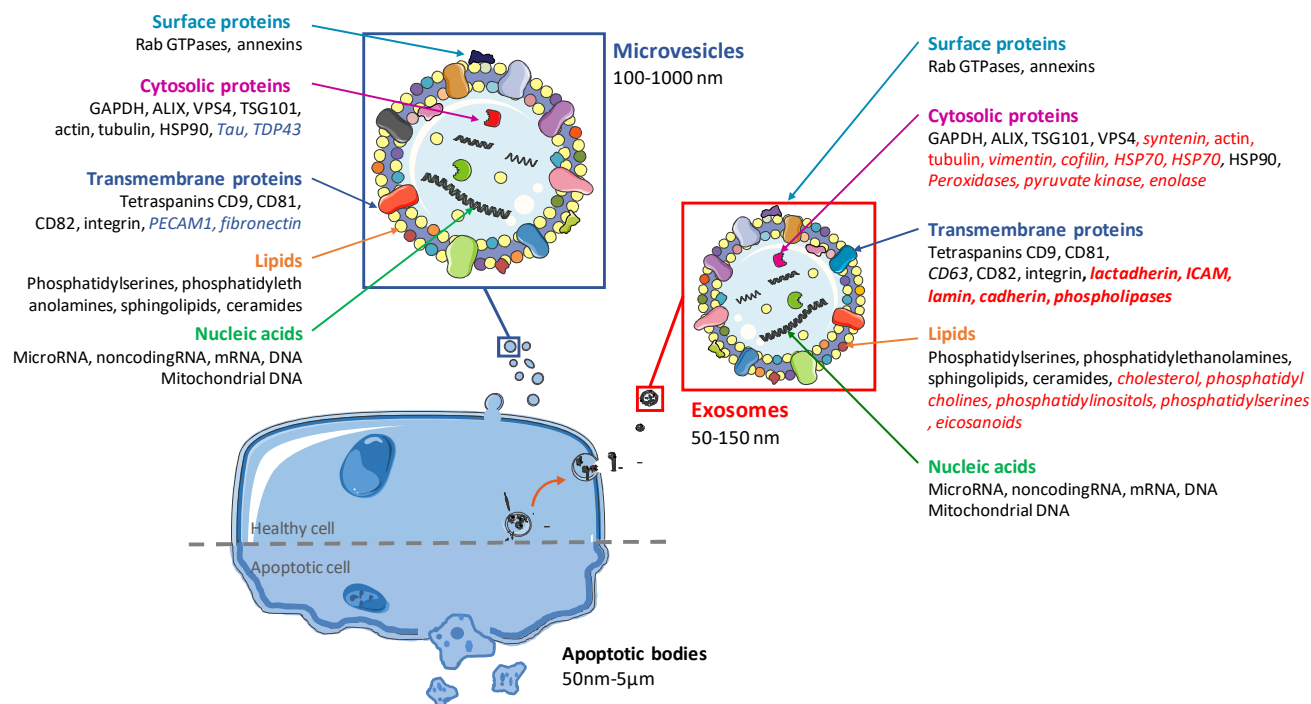


Figure 1. EV subtypes and composition. The elements detailed are those common to microvesicles (MVs) and exosomes, referred to EVs in the review – apoptosomes have not been used for protein delivery. Drawings are not to scale.

Figure 2

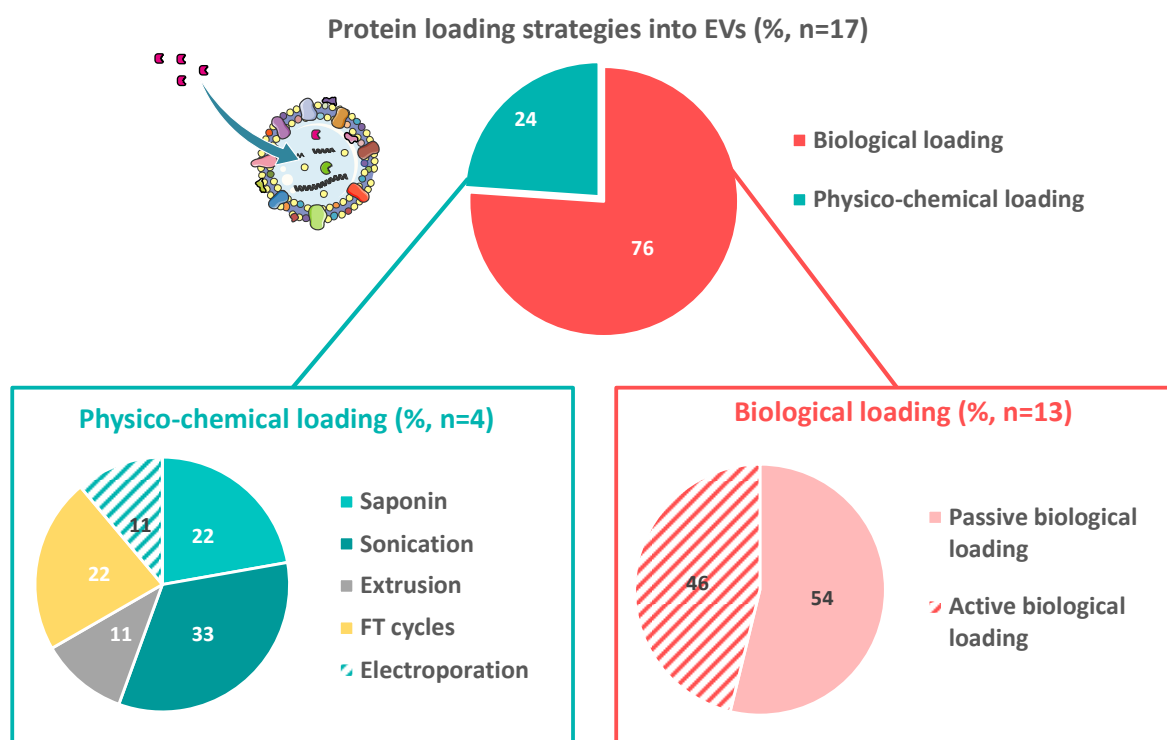
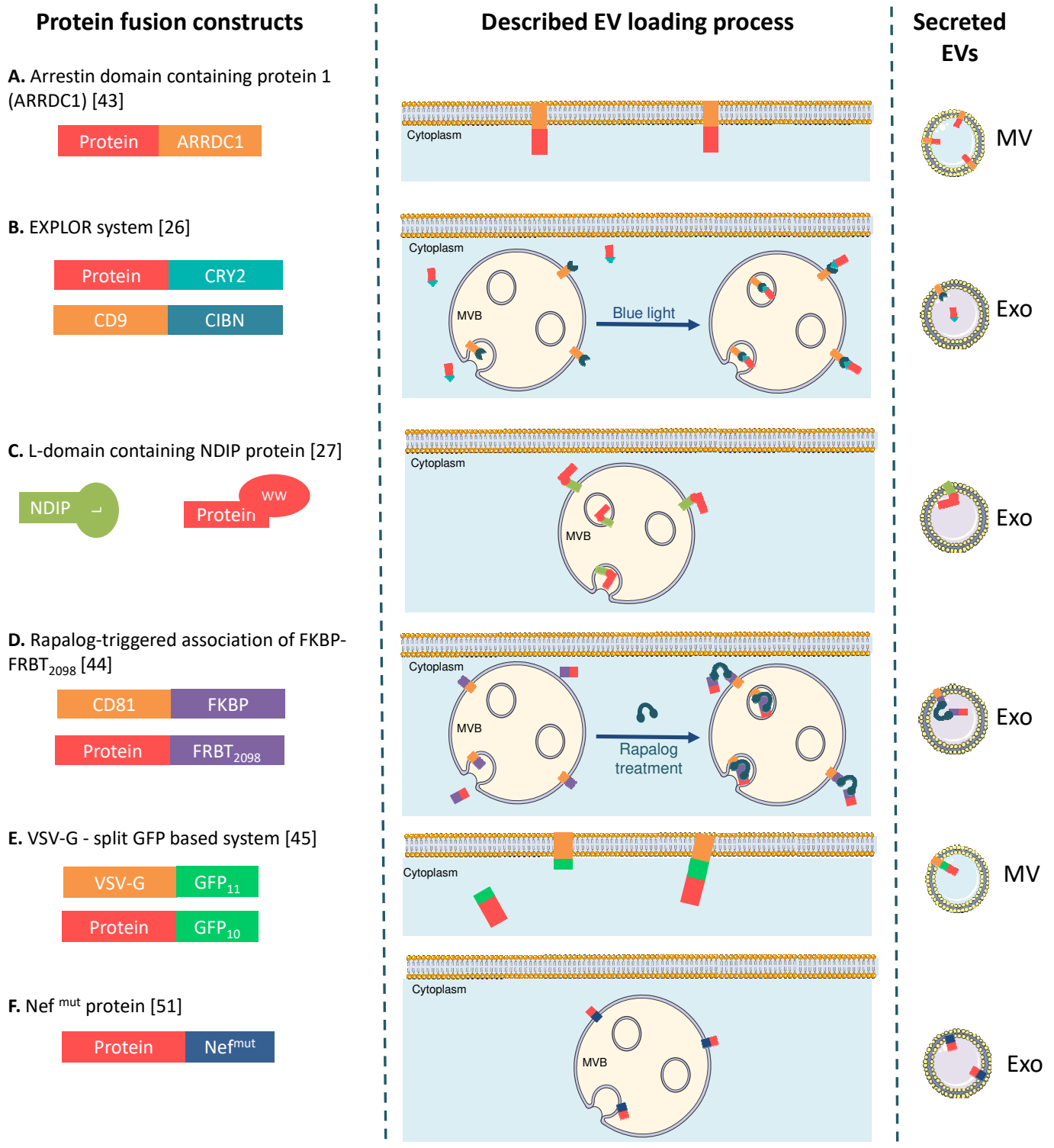


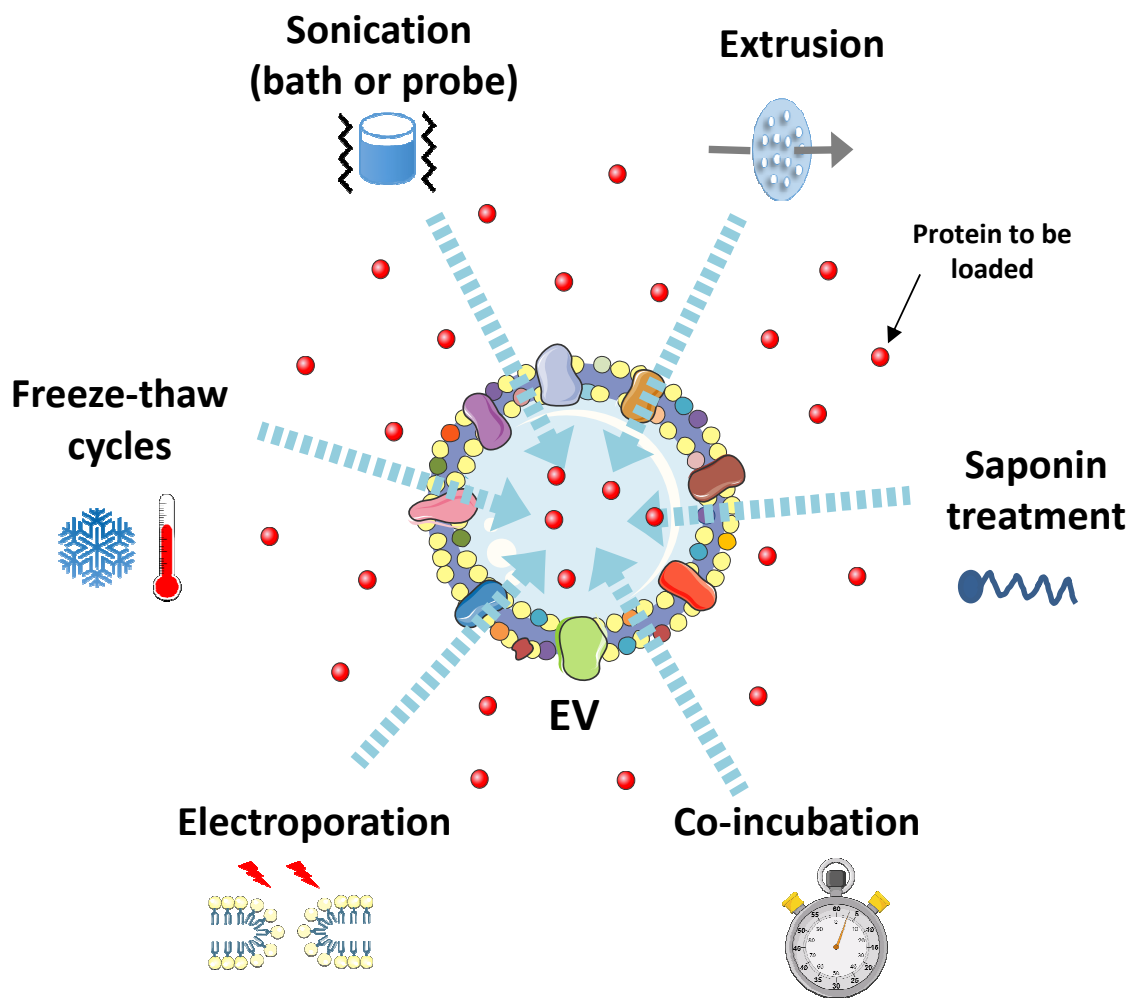
Figure 2. Charts on studies using EV for intracellular protein delivery. A. Loading methods (among 17 studies including biologic (n= 13) and physico-chemical (n=4)). B. Type of biological methods (Passive (n=7) vs. Active (n=6)), C. Loading methods used in the 4 studies relying on physico-chemical tools (Saponin (n=3); Sonication (n=2); Extrusion (n=1); F-T cycle (n=2); Electroporation (n=1). Abbreviations. F-T: freeze-thaw.

1 **Figure 3**



1 **Figure 4**

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7 **Figure 4: Schematic representation physico-chemical loading procedures used to associate therapeutic protein to**
8 **EVs.**

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TABLES

EV source	Transfection agent	EV production and enrichment method	EV size (nm)	Protein (size)	Free protein separation	Loaded amount**	Ref.
Melanoma YUSAC 2 cells	Lipofectin®	24h in serum free medium dC-UC sucrose gradient (cushion)	NS	Survivin-T34A (15 kDa)	Sucrose gradient (cushion)	NS	[30]
HEK293T cells	Lipofectamine® 2000	72h in media containing 5% MV-free FBS dC-UC	104-159	Cytosine deaminase (CD) (84 kDa)	Sucrose gradient UC	NS	[156]
HEK293T cells	Lipofectamine® 2000	72h in media containing 5% MV-free FBS dC-UC	88	Cytosine deaminase (CD) (84 kDa)	-	NS	[157]
Lung cancer A549 cells	Adenoviral vectors	72h in media containing EV free FBS dC-UC	65 - 320	CFTR glycoprotein* (200 kDa)	-	NS	[158]
Human Mesenchymal stem cells	Adenoviral vectors	Low glucose DMEM -10% EV depleted FBS Ultrafiltration (100Kda)- sucrose cushion	100	Akt (56 kDa)	Sucrose gradient UC	NS	[32]
IC21 macrophages	GenePORTER® 3000	5 days in 10% EV-depleted FBS DMEM dC-UC*	133.8 ± 4.1	Lysosomal enzyme TPP1 (61 kDa)	SEC (Sepharose CL-4B)	987 molecules/EV 1,0E-10 µg / EV	[28]
HEK293T cells	Effectene®	48h in FBS free or 10% EV depleted FBS DMEM Precipitation (ExoQuick TC) or one UC (120 000g,2h)	161-187	Bax (21 kDa) Super repressor sriKB (NS)	-	1-2 molecules/EV	[26]
Glioma LN18 and HEK293T cells	TurboFect®	72h production in NS media dC-UC (without 10 000g UC step)	146	WW - Cre recombinase (38 kDa)	-	NS	[106]
HEK293T cells	Fugene 6®	72h in 10% EV depleted FBS media dC-UC / sucrose gradient	90-100	p53 (47.3 kDa) WW- Cas9 (163 kDa)	Sucrose gradient	540 cargo protein/ ARRM	[43]
HEK293T cells	PEI	24h in 10% EV depleted FBS media + transfection reagents dC-UC	140 - 1000	SpCas9 (163 kDa)	-	10 µg of isolated EVs contained 100 ng of the Cas9 protein	[42]
Mouse muscular cells	Naked pDNA	dUC*	NS		-		[51]
HEK293T cells	Lipofectamine 2000	48-72h in 5% EV depleted FBS media dC-UC*	NS	anti-HPV16-E7 scFv (~30 kDa)	-	NS	[52]

Expi293F™	PEI max	48h in Expi293F media dC-UC*	~150	Cre recombinase (38 kDa)	-	8.9 - 34.1 Cre/vesicles	[44]
HEK293T cells	PEI	48h in DMEM or Freestyle 293 expression media dC-UC*	185 -200	Cre recombinase (38 kDa) Ago2 (97 kDa) SaCas9 (160 kDa)	SEC (qEV, Izon) + immunocapture (retaining VSV-G positive EVs)	~933 Cre molecules per vesicles	[45]

Table 1. EV-based protein delivery systems: biological methods.

Abbreviations. **CFTR**: cystic fibrosis transmembrane conductance regulator; **GFP**: green fluorescent protein; **HPV-E7**: Human Papilloma Virus E7 protein; **mCAT-1**: murine cationic amino acid transporter-1; **RFP**: red fluorescent protein; **TPP1**: tripeptidyl peptidase-1; **NS**: not specified, **UC**: ultracentrifugation; **dC-UC**: differential centrifugation-ultracentrifugation.*dC-UC following [53] standards. ** Reported values are formatted according to the information given by the authors. Formatting in the same unit format would have been preferable, but this was unfortunately not possible in regard to available data”.

EV source	EV production and enrichment method	EV size (nm)	Protein (size)	Loading Method	EV size after loading process (nm)	Free protein separation	Loaded amount**	Ref.
HeLa cells	72h in 10% EV depleted FBS αMEM media dC-UC*	102 ± 17	Saporin (34 kDa)	Electroporation	105 ± 25	PBS washing + Ultrafiltration (100kDa)	NS	[31]
Raw 264.7 macrophages	4-5 days in 10% EV depleted FBS dC-UC*	100	Catalase (60 kDa)	Incubation RT	-	SEC (Sephacrose CL-6B)	109 molecules/EV	[60]
				F-T cycles	~157		437 molecules/EV	
				Sonication probe	~155		940 molecules/EV	
				Extrusion	~140		809 molecules/EV	
IC21 macrophages	4-5 days in 10% EV depleted FBS dC-UC*	106 ± 9	Lysosomal enzyme TPP1 (61 kDa)	Sonication bath	130 ± 8	SEC (Sephacrose CL-4B)	6908 molecules/EV	[28]
				Saponin	135 ± 12		4934 molecules/EV	
HEK293T	48h in 10% EV-depleted FBS DMEM dC-UC; OptiPREP gradient	105 ± 16	Cas9-RNP	Sonication probe	152 ± 22	UC (100,000g; 70min)	15%	[61]
				F-T cycles	130 ± 12		37%	

Table 2. EV-based protein delivery systems: physico-chemical methods.

Abbreviations. **ArgCPP:** arginine-rich cell-penetrating peptide (used for surface functionalization); **TPP1:** tripeptidyl peptidase-1; **F/T:** freeze-thaw; **NS:** not specified. **dC-UC:** differential centrifugation-ultracentrifugation*:dC-UC following [53]standards. ** Reported values are formatted according to the information given by the authors. Formatting in the same unit format would have been preferable, but this was unfortunately not possible in regard to available data”.

Vesicle type	EV source/liposome formulation	Protein	Loaded amount	Nanoparticle size (nm)	Target Cells	Efficiency <i>In vitro</i> : % decrease in cell viability	Ref.
EVs	Melanoma YUSAC 2 cells	Survivin-T34A (~15 kDa)	NS	NS	MIA PaCa-2 human pancreatic adenocarcinoma	<i>In vitro</i> : 30%	[30]
	HEK293T cells	Cytosine deaminase (84 kDa)	NS	100-160	HEI-193 human schwannoma cell line	<i>In vitro</i> : 80% <i>In vivo</i> : total tumor growth inhibition	[156]
			NS	88	U87-MG, U251-MG and E98 glioblastoma cell lines	<i>In vitro</i> : 30% <i>In vivo</i> : 70% tumor growth inhibition	[157]
	HEK293T cells	Bax (21 kDa) Super repressor srikB (NS)	1.4 mol. / EV	160-190	HeLa cells (cervix adenocarcinoma)	<i>In vitro</i> : no apoptosis evaluation, cytochrome c release observed	[26]
	HeLa cells	Saporin (34 kDa)	NS	90-120	CHO-K1 cells	<i>In vitro</i> : 83%	[31]
Lipid nanoparticles	TFA-DODAPL:DOPE (2:1) (BioPORTER)	caspase-3 (31 kDa) caspase 8 (55 kDa) granzyme B (28 kDa)	~100%	NS	Human leukemic T cell lymphoblast Jurkat cells human prostate epithelial Ki-Ras 267β1 cells	<i>In vitro</i> : 40-60%	[85]
	TFA-DODAPL:DOPE (2:1) (BioPORTER)	Dm-dNK (~30 kDa)	NS	NS	TK1-deficient human osteosarcoma cell line	<i>In vitro</i> : 95% with a concomitant treatment with nucleoside analogue ganciclovir	[86]
	DOTAP:DOPE (2:1 M), APOA-I and DSPE-PEG-anisamide	cytochrome c (12 kDa)	64-75%	20-30	NCI-H460 non-small cell lung cancer	<i>In vitro</i> : 24% <i>In vivo</i> : ~73% tumor growth inhibition	[98]
	DOPC, DOPE, DMPA, cholesterol (40:20:20:20,M)	VDAC (31 kDa) Bak (19 kDa)	NS	~200	GL26 mouse glioblastoma cells	<i>In vitro</i> : 20-40%	[92]
	Lipidoid EC16-1, cholesterol, DOPE, ceramideC16-PEG ₂₀₀₀ -methoxy DSPE-PEG ₂₀₀₀ -Biotin	Aco-Rnase A (14 kDa) Saporin (30 kDa)	NS	110 - 140	B16F10 melanoma cells 4T1 murine breast cancer model	<i>In vitro</i> : 80-90% (RNase A and saporin) <i>In vivo (iv)</i> : 80% tumor volume reduction with saporin	[81]
		Saporin (30 kDa)	NS	NS	SW620 human colorectal adenocarcinoma NCI-H460 non-small cell lung cancer cell line	<i>In vitro</i> : ~80-90% <i>In vivo (iv)</i> : decrease in tumor weight (~50%) in both tumor models	[80]
	Lipidoid EC16-80	NBC-RNase A (14 kDa)	NS	150-155	B16F10 PC-3 HeLa MDA-MB-231	<i>In vitro</i> : ~40%	[101]

Lipidoid EC16-80	PTEN (47 kDa)	80,5 ± 7%	573 ± 74	PC3 cells LNCaP	<i>In vitro</i> : ~80%	[82]
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Table 3. Comparison of EV- and liposome-based protein delivery systems: cancer applications. Abbreviations. **GFP:** green fluorescent protein; **DOPE:** dioleoyl phosphatidylethanolamine; **TFA-DODAPL:** trifluoroacetylated lipopolyamine; **DOTAP:** 1,2-dioleoyl-3-trimethylammonium-propane; **APOA-I:** apolipoprotein A1; **DSPE:** 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; **PEG:** polyethylene glycol; **PG:** phosphatidylglycerol; **SQDG:** sulfoquinovosyl diacylglycerol; **MGDG:** monogalactosyldiacylglycerol; **Chol:** cholesterol; **nls:** nuclear localisation sequence; **EggPC:** egg phosphatidylcholine; **NS:** not specified; **Aco** : Cisaconitic anhydride modified protein. **NBC** :4-nitrophenyl 4- (4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate; **PTEN** :phosphatase and tensin homolog deleted on chromosome 10; **Dm-dNK** : Drosophila melanogaster multisubstrate deoxyribonucleoside kinase; **VDAC:** Voltage-dependent anion channel. **DOPC:** 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine; **DMPA:** 1,2-Dimyristoyl-sn-Glycero-3 Phosphate; **iv** : intravenous;

Vesicle type	EV source/liposome formulation	Protein (size)	Vesicle size (nm)	Therapeutic strategy	Target cells	Efficiency (% restoration of iodide efflux)	Ref.
EVs	Lung cancer A549 cells	CFTR (200 kDa)	65-320	Delivery of non-mutated WT CFTR	CF15 cells (nasal epithelial cells)	25%	92
							93
Liposomes	DOSP:MM27	anti-K8 antibody (54 kDa)	100-200	Delivery of anti-K8 antibody to disrupt K8-F508del-CFTR interaction and subsequent entrapment of CFTR	Genetically modified HeLa cells (cervical cancer cells)	75%	94
							[158]
							95
							96
							[99]
							97
							98

Table 4. Comparison of EV- and liposome-based protein delivery systems: cystic fibrosis application. Abbreviations. CFTR: cystic fibrosis transmembrane conductance regulator; DOSP: aminoglycoside lipid dioleoyl succinyl paromomycin; MM27: imidazole-based helper lipid; K8: cytokeratin8; NS: not specified

Vesicle type	EV source/liposome formulation	Protein (size)	Evaluation	Efficiency	Ref.
EVs	Glioma LN18 and HEK293T cells	ww-Cre (38 kDa)	<i>In vivo</i>	<i>In vivo (nasal)</i> : ~30% recombination	[106]
	Expi293F™ cells	Cre (38 kDa)	<i>In vitro</i>	40-50% recombination (endosomal disruptive molecules required)	[44]
	HEK293T cells	Cre (38 kDa)	<i>In vitro</i>	~ 67% recombination	[45]
		Cas9-RNP (160 kDa)	<i>In vitro / in vivo</i>	<i>In vitro</i> : 40% KO of targeted gene (PKN1) <i>In vivo (iv)</i> : ~1.5 fold decrease in targeted gene	
	HEK293T cells	Cre (38 kDa)	<i>In vitro/in vivo</i>	<i>In vitro</i> : 95% recombination <i>In vivo (local)</i> : functional recombination in neurons (no quantification)	[159]
	HEK293T cells	Cas9-RNP (160 kDa)	<i>In vitro/ in vivo</i>	<i>in vitro</i> : ~10% indel <i>in vivo (iv)</i> : ~10% indel <i>in vivo (ip)</i> : 50% KO targeted gene (eGFP)	[42]
		dCas9-VPR (205 kDa)	<i>In vitro/ in vivo</i>	<i>in vitro</i> : sgRNALuc:dCas9-VPR = + 470 fold increase in Luciferase activity <i>In vivo (hyd.)</i> : sgRNA-HGF:dCas9-VPR increase HGF expression (~1.5fold)	
Liposomes/ Lipid nanoparticles	Lipofectamine® RNAiMAX and 2000	(-30)GFP-Cre (~70 kDa)	<i>In vitro</i>	50-75% recombination	[18]
		(-30)GFP -Transcription Activator Like Protein (GFP-TALE-VP64 ; ~110 kDa)	<i>In vitro</i>	3.5 fold increase in target gene expression	
		Cas9-RNP	<i>In vitro /in vivo</i>	<i>In vitro</i> : 60-80% gene KO (eGFP) 8 - 11 % of HDR (eGFP repair reporter) <i>In vivo (local)</i> : 13% KO (eGFP)	
	Lipofectamine® CRISPRMAX	Cas9-RNP	<i>In vitro</i>	<i>In vitro</i> : 8% (MCF-7 cells) to 85% (HEK293) % indels HDR in HEK293 : 17% HDR	[89]
	Cationic bioeducible lipids	(-27)GFP-Cre	<i>In vitro/in vivo</i>	<i>In vitro</i> : 80% recombination <i>In vivo (local)</i> : functional recombination (no quantification)	[83]
		Cas9-RNP	<i>In vitro</i>	70% KO targeted gene (eGFP)	
	Chalcogen containing lipidoids	(-30)GFP-Cre	<i>In vitro</i>	45% recombination	[84]
		Cas9-RNP	<i>In vitro</i>	50% KO targeted gene (eGFP)	
	Ionisable LNPs (5A2-SC8 /DOPE/Chol/DMG-PEG/DOTAP)	Cas9-RNP	<i>In vitro/in vivo</i>	<i>In vitro</i> : 80% indels <i>In vivo (im)</i> : restoration of 4.2% of dystrophin (WB) in DMD ΔExon 44 mice model <i>In vivo (iv)</i> : PCSK9 decrease in liver/plasma (5-7.8% indel at gene loci)	[90]
		Cas9-RNP	<i>In vitro</i>	15% KO targeted gene (eGFP)	
	Non cationic lipid based	Cas9-RNP	<i>In vitro</i>		[91]

Lipid-containing
oligoaminoamides

Cas9-RNP

In vitro

40%-30% KO targeted gene (eGFP; folate receptor 1)

[97]

Table 5. EV and lipid nanoparticle based systems for gene engineering protein delivery. Abbreviations. **NS:** not specified; **Cre** : Cre recombinase; **WW motif** : proline rich domain; **Cas9-RNP** : Caspase 9-sgRNA Ribonucleoprotein complex; **(-30)GFP**: negatively charged Green Fluorescent Protein ; **TALE** : Transcription Activator Like Effector; **Cas9-VPR**: composite tripartite activator domain VP64-p65-Rta (VPR) to activate gene expression; **PKN1**: Protein Kinase N1; **iv** : intravenous; **ip** : intraperitoneal; **hyd** : hydrodynamic injection. **LNP**: lipid nanoparticle; **5A2-SC8**: dendrimer based ionisable lipid; **DOPE**: dioleoyl phosphatidylethanolamine; **Chol**: cholesterol; **DMG-PEG**: Dimyristoyl glycerol - poly (ethylene glycol); **DOTAP**: 1,2-dioleoyl-3-trimethylammonium-propane. **DMD**: Duchene Muscular Distrophy; **ΔEx44 DMD mice**: DMD exon 44 deletion mouse model; **PCSK9**: Proprotein convertase subtilisin/kexin type 9.

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Graphical abstract

