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Review

Exosomes and exosome-inspired vesicles for targeted drug delivery

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Abstract: The similarities between exosomes and liposomes, together with the high organotropism of several types of exosomes, have recently prompted the development of engineered-exosomes or exosome-mimetics, which may be artificial (liposomal) or cell-derived vesicles, as advanced platforms for targeted drug delivery. Here we provide the current state-of-the-art of using exosome or exosome-inspired systems for drug delivery. We review the various approaches investigated and the shortcomings of each approach. Finally the challenges identified up-to-date in this field are summarized.

Keywords: liposomes, exosomes, extracellular vesicles, drug delivery, drug targeting, bioinspired systems, engineered systems.

Contents

1. Introduction

The most recent discoveries in the field of extracellular vesicles are unravelling exciting concepts on intercellular communication pathways [1, 2]. The fast growing literature in this field is showing that nano to micron sized vesicles budding from cells and named exosomes (EXs) (which are one of the types of extracellular vesicles (EVs)), display specific organotropic behavior as part of their active

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role in cell-to-cell communication and material (protein and/or nucleic acid- cargo) transfer pathways. Besides local cell-to-cell communication, in some cases the secreted factors play a key role in the interactions between cells located far apart from each other [3-6]. The tremendously high and specific organotropism of some types of EXs, is one of the major goals of all the types of nano-based drug delivery systems (DDSs) that wave been screened up-to-date, which remains unmet [7-10]. By providing important insights on the key elements that dictate the biological fate of vesicles and their ability to interact and be taken up by specific cells, the novel and fast growing field of EXs is now inspiring the design of ex-novo nanovesicles as carriers for targeted drug delivery applications. The exploitation of various types of EXs or EX-mimetics for targeted drug delivery is indeed a novel research area which is currently under intensive research [11-17].

In this review we provide the current state-of-the-art on the development of EXs as well as artificial nanoparticulate systems that aim to mimic their properties (EX-mimetics), as innovative nanocarriers with high drug targeting efficiency.

Due to the many similarities between liposomes and exosomes (which will be mentioned analytically in a separate part below), the application of liposome engineering technologies to engineer EXs has been proposed as a way to overcome EX limitations. The many similarities between the two systems, is also the reason why liposomes are the nanoparticle types which are preferentially used for construction of artificial EXs or EX-mimetics as drug carriers. We will focus on such approaches since they may accelerate the development of specific roadmaps to construct engineered-EXs or, the more realistic approach of developing EX-mimetics or else EX-inspired liposomes as advanced targeted drug and/or imaging agent carriers in theragnostics.

1.1 Definition, Biogenesis and main functions of Extracelular vesicles and Exosomes.

In order to understand why EXs can be used as targeted drug carriers we need to initially clearly define what they are, and additionally review their basic functions. EXs are one of the types of a broader category of cell-derived vesicles characterized as *Extracellular Vesicles* (EVs) [18, 19].

EVs are membrane-contained small vesicles, secreted by all types of pro- and eukaryotic cells [20,21]. The three main categories of EVs are (**Scheme 1**):

(i) Apoptotic Bodies (ABs),
(ii) shedding Micro Vesicles (MVs) and
(iii) EXosomes (EXs).

In more detail:

(i) ABs are released during cell death (apoptosis) and are heterogeneously shaped vesicles with sizes between 50–5000 nm. ABs are formed from the plasma membrane and they contain DNA, RNA, histones and signalling molecules [22]. They usually have high amounts of phosphatidylserine (PS) in their membranes since the outer membrane of apoptotic cells is enriched in PS.

(ii) MVs are formed by blebbing of the cell membrane with concurrent incorporation of cytosolic proteins and their sizes range between 20 –1000 nm, depending on the origin cells and the method applied for their isolation from cell media [23]. Their formation can be triggered through Ca2+ influx, phorbol esters, ATP, etc [24]. Some common biochemical characteristics have been identified between MVs from different cells, such as their high membrane levels of PS, and some common surface markers (CD40 ligands, integrins and selectins) [12] [**Error! Bookmark not defined.**].

(iii) Finally, EXs include a more homogeneous (in shape and size) population of vesicles compared to MVs, with sizes that range from 50nm up to 120nm. Their biogenesis is initiated by inward budding of the plasma membrane which results in the formation of intermediate endosome-vesicles, the multivesicular bodies (MVBs). After that, depending on their composition, MVBs are either degraded after being fused with lysosomes, or they form EXs after fusion with the plasma membrane and release of EXs from the cells [25-28]. EXs contain surface proteins unique to the

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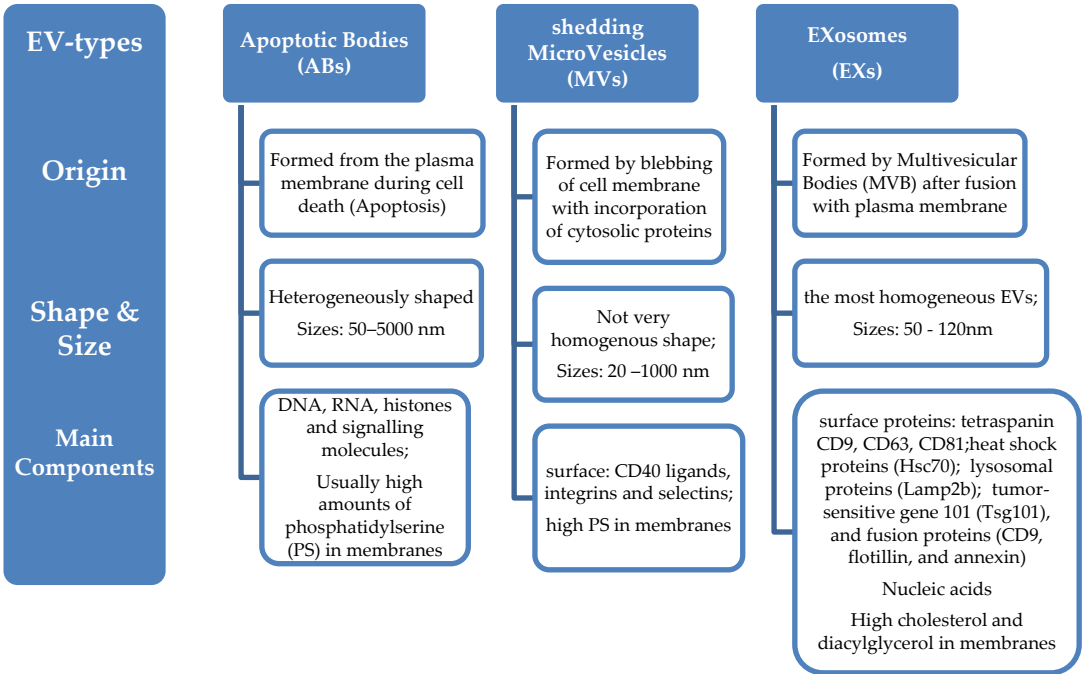
endosomal pathway, which are generally used to characterize exosomes and distinguish them from microvesicles (MVs), apoptotic bodies, and other vesicles, such as tetraspanin CD9, CD63, CD81 [29], heat shock proteins (Hsc70), lysosomal proteins (Lamp2b), the tumor-sensitive gene 101 (Tsg101), and fusion proteins (CD9, flotillin, and annexin) [30] and incorporate nucleic acids, cytosolic proteins and receptors. Their lipid composition differs from other extracellular vesicle types, since they are rich in cholesterol and diacylglycerol. EXs are generally considered as transporters of miRNA that regulate specific intracellular mRNA activity [31].



Scheme 1. Types of Extracellular Vesicles (EVs) and characteristics. i) Origin; ii) Shape and Size distribution; iii) Main components.

As concluded from the definitions given above, MVs and EXs are smaller compared to ABs. Additionally they differ from ABs in their content, since they rarely contain DNA [32]. The main function of MVs and EXs is the intercellular transfer of lipids, RNA, and cytosolic proteins; however it has been recently suggested that the two types of vesicles have different reporter molecule transfer functions [33, 34].

Although these two vesicle-types, MVs and EXs are separate classes of vesicles, due to the fact that they overlap in size, and since the commonly used non-specific protocols for EX isolation and purification rely solely on the vesicle size differences (as described below), it is a fact that in many of the reports published, the EX preparations used are impure (since they probably include MVs and large protein aggregates). Because of this, it has been proposed to use the term EVs as a general term for all small vesicles/particles which include both vesicle types, EXs and MVs, excluding only the ABs [22] [**Error! Bookmark not defined.**].

According to the later definition, one can state that EVs are shed by most cells constitutively as well as in response to endogenous and exogenous triggers, and are involved in intercellular communication under both, physiological and pathological conditions [32] [**Error! Bookmark not defined.**]. They mediate intercellular communication as signaling organelles (transmitting specific information from their origin-cell to their target-cells). In physiological conditions, EVs are important mediators for cell-to-cell and inter-tissue communication and exhibit important functions in regulating homeostasis as well as in a variety of other conditions [23, 33, 35]. In pathological conditions, the information transferred by EVs, mainly cancer cell EVs, may have detrimental effects; EVs have been demonstrated to contribute to various pathologies such as, tumorigenesis and metastasis [36], inflammation [37], and immune system activation [38].

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As a result of the above-mentioned functions, EVs may serve as novel tools for various therapeutic and diagnostic applications, such as (a) anti-tumour therapy, (b) pathogen vaccination, (c) immune-modulatory and regenerative therapies and (d) drug delivery.

Herein we will focus on the applications of EVs in Drug delivery. As we will present analytically, the attempts that have been made to use EXs (or better EVs) for drug delivery [**Error! Bookmark not defined.**]. Indeed in the last 5 years, EVs were proven to have high targeting potential for specific cell types but in most of the cases, following systemic administration, they failed to demonstrate the anticipated therapeutic results [**Error! Bookmark not defined.**]. The later failed attempts revealed a number of shortcomings in the methodologies employed for the use EVs as targeted-DDSs. It is well-known today that the main pre-requisites to use any type of vesicle for the targeted delivery of drugs are that: (i) they can be loaded with a sufficient amount of drug, in order to elucidate a therapeutic response. (ii) They are stable (retain their size, structure and drug load) during circulation in the blood stream before reaching their therapeutic target in the body. (iii) They can avoid uptake by the macrophages and circulate for prolonged time periods so that they can reach their cellular targets (iv) They are biocompatible, or else non-toxic and non-immunogenic [39]. In accordance to these requirements, the main problems identified (and still remaining to be solved), for successful translation of EVs into targeted drug carriers are the following: (i) Drug loading and/or retention of drugs in EXs is not sufficient. (ii) The poor pharmacokinetics of natural EVs when loaded with bioactive agents, and (iii) the fact that it still remains a big problem for such systems to be included in industrial roadmaps, giving the very low yield of isolation from biological media or cell cultures (see paragraph 3, below).

Due to the above mentioned shortcoming, other approaches are currently being exploited, such as the use of EX- or EV-mimetics which are in most cases liposomes constructed after appropriate selection of their components to mimic the composition (and hopefully) the structure of organotropic EVs.

Before ending this introductory part on EVs, we should mention that some attempts have been made up-to-date; to classify the various types of EVs and some general databases such as Vesiclepedia (http://microvesicles.org/) and Exocarta (http://www.exocarta.org/) have been developed. Nevertheless, as mentioned already, due to technical difficulties to purify and clearly identify each class of vesicles, is not clear in many of the past studies which specific kind of vesicles are actually used and the use of the terms MVs and EXs in many published studies is not consistent.

1.2 Current bottlenecks in nanoparticle-assisted targeted drug delivery and Liposomes

Several types of nano-based drug delivery systems (DDSs) have been and are currently being considered for drug targeting applications over the last 4 decades. Among all the nano-based DDSs, liposomes (LIPs) - that first reached approval as drug delivery systems- are the most biocompatible and least toxic artificial systems, which is logical since they are constructed by phospholipids and cholesterol, the main components of cell membranes [**Error! Bookmark not defined.**].

Liposomes have made great impact on therapeutics due to their advantages as DDSs; Indeed in addition to their non-toxic and biocompatible nature that can accommodate high payloads of drugs, they have the capability to load multiple drugs, to provide protection of drugs from degradation, and to enhance drug endocytosis into cells. It is well known that Liposome (LIP)-assisted drug delivery has a major impact on many biomedical areas, and that several LIP formulations are proven to stabilize therapeutic compounds, overcome cellular/tissue uptake obstacles, and improve biodistribution, enabling thus the effective delivery of encapsulated drugs to target sites and minimizing their systemic toxicity [40]. Liposomes are especially potent in the treatment of some types of cancer, in which they can achieve passive targeting via the leaky tumor vasculature, by the enhanced permeability and retention (EPR) effect. As a result of all the LIP advantages as DDSs mentioned above, several liposomal drug products are currently available in the market, while others

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are under clinical testing. However, despite all the efforts to increase the active targeting of drugs to diseased sites by incorporation into ligand-targeted-liposome formulations [41-43] none have been successfully translated into products [**Error! Bookmark not defined.**], a bottleneck that has been limiting the broader applicability of liposomal drug formulations in therapeutics.

In the last 30 years, the use of numerous targeting ligands of different types (monoclonal antibodies, proteins, peptides, small molecules, carbohydrates, aptamers etc.), to target different receptors which are overexposed on the membrane of the target cells (such as HER2, VEGF, e-selectine, nucleolin, tranferrin, folate and EGR receptors, V3 integrin, SSTR2, ASGPR, etc.), has been investigated with the aim of further increasing the delivered fraction of LIP-encapsulated cargo to diseased sites, compared to the amount delivered via (control) non-ligand-targeted liposome formulations [44]. However, despite improved biodistribution and therapeutic outcomes in preclinical studies, the translation of such ligand-targeted liposome formulations into the clinic has not been successful. Several possible reasons, which perhaps have not been adequately considered, have been proposed to explain the former discrepancy, such as: (i) disease-dependent anatomical barriers, (ii) target accessibility and expression, (iii) formulation stability, and (iv) the targeting-ligand density on the LIP surface [**Error! Bookmark not defined.**].

Currently, the need for superior targeted-drug carriers is further upgraded due to the fact that the tremendous therapeutic potential of biopharmaceuticals (BPs) will become available only after formulation and delivery issues are resolved. BPs (therapeutic peptides, proteins, and nucleic acids), demonstrate exquisite specificity which enables successful treatment of serious unmet medical needs and therefore they represent a growing fraction of the current drug development pipelines [45].

Additionally, the development of drugs that act on the central nervous system is presently severely hampered by the lack of efficient methods to deliver the drugs (especially BPs) into the brain. It is well known today that only a very small fraction (<1%) of injected antibodies enter the brain by passive diffusion, while other large molecule-drugs (e.g. peptides) can be only administered by peripheral injection or invasive intra-cranial procedures, approaches that failed in the clinic. The extremely challenging issue of blood-brain-barrier (BBB) crossing is an urgent need, taking into consideration the worldwide rise in neurodegenerative disorders, as well as the yet unsolved problem of treating brain cancers, both situations having at present no recognized therapeutic solution [46].

In addition to the approaches mentioned above, other advanced methodologies have been exploited with the aim to further increase the targeting potential of ligand-targeted-liposomes [**Error! Bookmark not defined.**, 47]. In this context, phage display methodologies have been utilized for the selection of high affinity ligands [48]. Additionally multi-targeted liposomes have been constructed, which can target two of more receptors simultaneously, with the aim to increase their targeting efficiency [49]. Other approaches employ the use of physical methods as stimuli, to further increase the targeting efficiency of ligand-targeted-liposomes, such as magnetic or ultrasound enhanced targeting [50-52]. Nevertheless, in addition to other potential problems, the last multifunctional systems may be perhaps too complicated for translation into drug products, a factor that should also be seriously accounted for when searching for solutions in the problem to realize actively targeted liposomes, or any other type of nanoparticles (NPs).

Along this line, the recent findings demonstrating the potential of Engineered EVs (EEVs) towards successful delivery of bioactive agents to selected body targets, open new horizons towards the development of liposomes with compositions inspired from EVs (EV-inspired LIP) that will hopefully demonstrate enhanced targeting efficiencies, compared to the ligand-targeting-LIPs exploited up-to-date.

2. Similarities and Differences between EXs and Liposomes

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As reported before [**Error! Bookmark not defined.**, 53], EXs have many similarities with small unilamellar vesicle-(SUV)-type liposomes (Fig.1), which explains why most types of artificial EX-mimetics are mainly based on liposomes. These similarities could allow researchers to engineer EXs or EVs (in order to load drugs into them or/and attach different molecules on their surface), using the methodologies developed in the liposome technology field. Indeed, both EXs and SUV-LIP, are vesicular structures consisted of one lipid bilayer and having a mean diameter ranging from around 50nm up to 120nm. The **major difference** being SUV-liposomes and EXs **is** the complex surface composition of EXs, and more specifically, the characteristic array of membrane proteins such as tetraspanins, present on the membrane of EVs; whereas SUV-liposomes do not usually have proteins in or on their lipid bilayer. These proteins are required on EXs, in order to facilitate the efficient targeting and cellular uptake of the EXs by their recipient cells. However, one should not only focus on the protein components of the EX surface, ignoring their unique lipid composition, since this may also play an important role in the *incorporation and functional conformation* of the proteins in their membrane, as well as on the specific interactions between EXs and serum proteins which will dictate the in vivo fate of the vesicles.

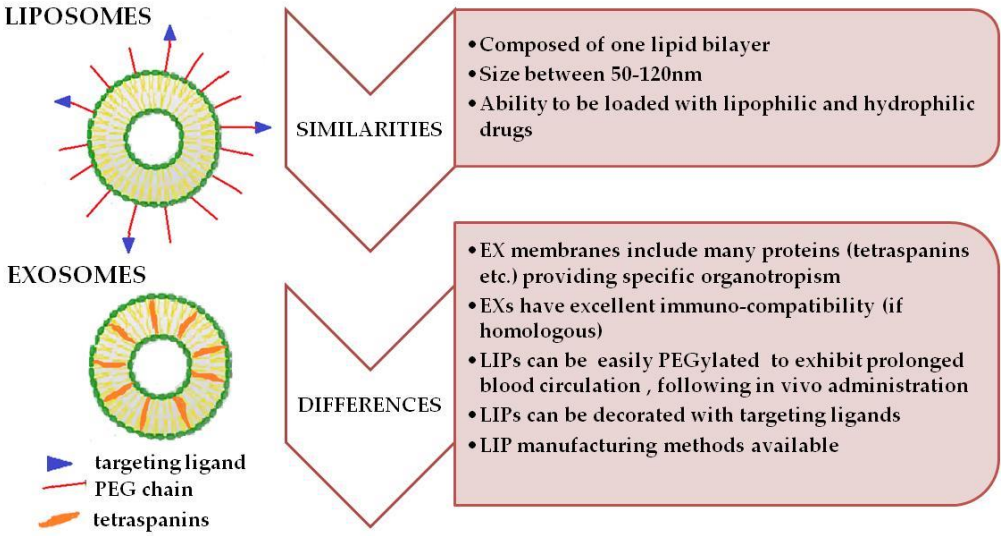

Figure 1. The basic structural characteristics, as well as the main similarities and differences of Liposomes and Exosomes are presented.

When comparing the advantages and disadvantages of EXs and liposomes, in respect to their applicability as targeted-DDSs it becomes evident that the two systems are complimentary, since the advantages of the one system are disadvantages of the other and vice-versa (**Scheme 2**). Thereby, the incorporation of the advantageous features of the two vesicle types into one hybrid vesicle, if of course possible, would most possibly result in the realization of an advanced system for drug targeting applications. Such approaches have indeed been initiated [54], and will be described in detail below.

Due to their similarities with EXs, SUV-liposomes are usually engaged as control vesicle formulation for comparison, to detect the superiority of exosomes for drug delivery applications. However, recently some previously reported head-to-head comparisons of EVs versus liposomes have been weakened due to the inadequate choice of liposome formulation types that were used as controls. Researchers are thus encouraged to implement more appropriate controls in order to prove any potential superiority of EVs over other synthetic nanoparticles [55].

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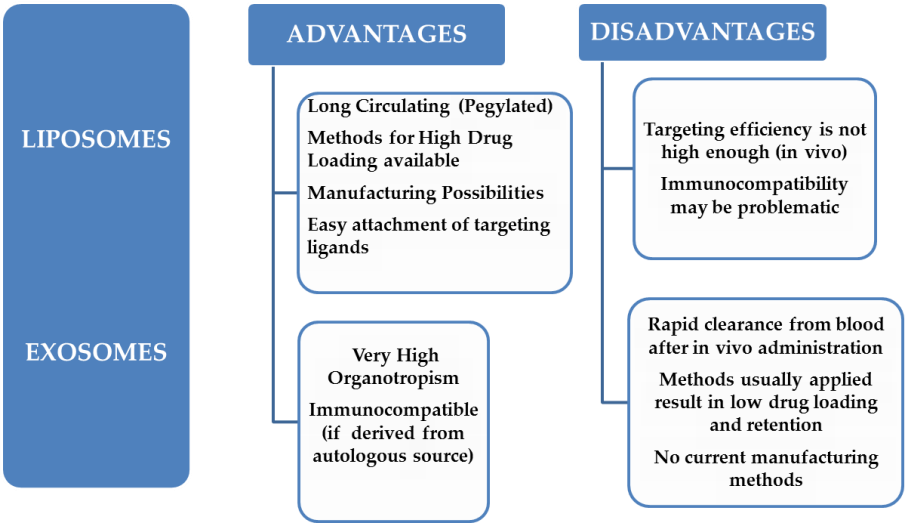

Scheme 2. Advantages and Disadvantages of Liposomes and Exosomes

3. Sources, Methods of Isolation and *in vivo* Clearance of unmodified Exosomes

In this part, a brief reference to the sources of EVs and EXs will be made, since the parental cells from which the vesicles are extracted from, highly determine their structural components and thereby play the most important role on their functions and potential in vivo fate. Additionally, the methods used currently for isolation and purification of EXs will be described, as well as the basic findings from studies that have been conducted up-to-date for exploitation of the in vivo kinetics and clearance of EXs from the blood circulation following in vivo administration, via various administration routes.

3.1 Sources of Exosomes

Most cell types such as dendritic cells (DCs), epithelial cells, macrophages, reticulocytes, mast cells, platelets, neurons, B cells, T cells, oligodendrocytes, tumour cells and Schwann cells have been demonstrated to be able to release exosomes [29, 31-33, 56-58]. Most of the in vivo detected circulating exosomes (about 80%) are derived from platelets [**Error! Bookmark not defined.**,***Error! Bookmark not defined.***].

Mesenchymal stem cell (MSC) derived EXs, are currently being exploited for numerous applications *since* they have been demonstrated to play a role in cell-free therapy of many diseases, including myocardial infarction, drug addiction, and status epilepticus [59]. They are also thought to be able to ameliorate liver injury, inflammation -induced preterm brain injury, and various types of cancer.

In addition to the above mentioned cells, EXs are also found in many physiological fluids such as plasma, saliva, urine, lymph, breast milk, semen, amniotic fluid ascites fluid, bronochoalveolar lavage fluid, cerebrospinal fluid, bile, and malignant pleural effusion, etc. [33, 60-67].

The cellular origin of EXs highly determines their contents, and as a consequence to that, also their functions. EXs which are produced by B lymphocytes contain functional MHCI, MHCII, and T cell co-stimulatory molecules, and can thus stimulate T cell proliferation [68]. Alternatively, EXs from cancer cells contain cell adhesion related molecules, such as gelatinolytic enzymes, and thus they have the ability to enhance the progression and metastasis of tumors [69]. Cancer cell-derived EXs are actively incorporated by mesenchymal stem cells (MSCs) (in vitro and in vivo), since the transfer

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of exosomal proteins and miRNAs depend on the physical and functional characteristics of tumor-supporting fibroblasts [70, 71].

For further information about signal transmission pathways and molecular contents of EXs, which are out of the scope of this review articles, interested readers may refer to ExoCarta (http://www.exocarta.org) or EVpedia (http://evpedia.info), as well as the American Society for Exosomes and Microvesicles (http://www.asemv.org).

In reference to the current efforts to use EXs as drug delivery systems, it should be highlighted that despite the identified very high organotropism of EXs derived from cancer cells, a shift to safer alternatives such as EXs produced from foods, has been recently made in this research field. From all the relevant sources of this category, bovine milk- and fruit-derived EXs are probably the most investigated for the purpose [72]. Indeed, bovine milk has been proposed as a scalable source of exosomes that can act as a carrier for chemotherapeutic/chemopreventive agents [73]. In addition to bovine milk, exosomes from milk of other sources such as porcine and murine milk have also been considered [74].

3.2. Isolation Methods

Several methods have been developed to efficiently isolate EXs from cells and biological fluids [75]. Each method exploits a specific trait of EXs, such as their size, shape, density and surface antigens to aid isolation. However, the most challenging task of all methods employed is to specifically purify EXs from the wide spectrum of EVs, cellular debris and interfering molecular components [76, 77]. For this reason, the quality of isolated EXs should be examined each time before any further application is persued. Several techniques have been used to measure EX size, size distribution, morphology, quantity as well as protein and RNA composition [78].

In all EX isolation methods described in the literature, the first and general step is to culture parental cells in serum-free media and allow the cells to condition the media. Conditioned media is then collected and processed in different ways based on the method applied. For bodily or other types of fluids, the principle for EX isolation is the same as when starting from cell culture conditioned medium, but because of the vicosity of some fluids it is necessary to dilute them and in some cases to pre-clean them from large bioparticles as well as to spike them with protease inhibitors to prevent the degradation of the EX proteins [***Error! Bookmark not defined.***].

3.2.1.Ultracentrifugation

Ultracentrifugation (U/C)-based EV isolation is considered as the gold standard and is one of the most commonly used and reported techniques for EXs isolation [***Error! Bookmark not defined.***-***Error! Bookmark not defined.***]. There are two types of methodologies, based on the principles of separation: Differential and density gradient U/C. Differential U/C involves serial stepwise centrifugation in order to isolate EXs based on their density and size differences from other components in the sample. Density gradient U/C segregates EXs to specific layers according to their size, mass and density in a gradient material, such as sucrose. Usually, at the final step, small EXs are isolated and usually resuspended in phosphate buffer saline (PBS), as it is the most commonly used solvent in all applications [***Error! Bookmark not defined.***- ***Error! Bookmark not defined.***]. As U/C is mostly based on vesicle size the purification of EXs from other EV-types, and mainly MVs that have overlapping size disstributions with EXs, is questionable.

3.2.2. Immunoaffinity

Immunoaffinity capture-based techniques exploit interactions between antibodies and selective surface proteins of the EXs in order to isolate them [**Error! Bookmark not defined.**,**Error!**

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Bookmark not defined.]. Higginbotham et al recently demonstrated the feasibility of using fluorescence-activated vesicle sorting to analyze and sort individual EXs from DiFi cells (human colorectal cancer cells). Indeed, the EGFR and the exosomal marker CD9, were detected on the surfaces of these DiFi EXs by mAb conjugation [79]. Ideally, the EX tags for immunoisolation should be membrane- bound, lacking soluble counterparts, and should be of course, highly expressed on the surface of the EXs produced by the specific biological source selected. According to the applied immunoaffinity procedure, antibodies which are specific for the surface proteins of the EXs to be isolated (e.g. CD63,CD81,CD93 etc.), could be immobilized on a variety of support media, which may be magnetic beads, chromatography matrices, elisa plates as well as microfluidic devices [**Error! Bookmark not defined.**, 80].

Even though it finally results in a comparable production yield when compared to that of U/C methods, immunoaffinity uses much less sample-volume and extracts larger amounts of purified EXs, regardless of the vesicles size differences.

3.2.3. Other Size-based isolation methods

The most popular size-based EX isolation technique is ultrafiltration. Based on their size EXs can be isolated with sequential filtration using membrane filters with defined size-exclusion limits (specific membrane pore sizes) [81].

Another size-based separation technique is size-exclusion chromatography that uses a column packed with beads with pores smaller than those of the EXs. Fractions are eluted sequentially in the order of decreasing sizes, and EXs can be thus isolated, from larger and smaller particles.

Size-exclusion procedures are less time-consuming compared to U/C methods, and furthermore do not require special equipment (ultracentrifuge). However, biologic fluids and cell culture media contain a large number of NPs in the same size range with that of EXs [***Error! Bookmark not defined.***], and thereby, such size exclusion methodologies could only be applied when the aim is to obtain mixed EV samples, rather than pure EXs.

3.2.4. Precipitation

EXs can be isolated from biological fluids by altering their solubility. Various methods use polymers that can precipitate EXs according to their surface characteristics. Specifically, by binding with water molecules, polymers force less soluble EXs out of solution, and the EXs can be then collected by low speed centrifugation [***Error! Bookmark not defined.***,***Error! Bookmark not defined.***]. In several biologic fluids, precipitation seems to realize higher EX recovery compared to U/C. However, a big disadvantage of this method is the co-precipitation of other non-EX contaminates, including the polymeric material used [***Error! Bookmark not defined.***, 82].

3.2.5. Yield of common Isolation methodologies

Despite the impressive progress that has been made for EX isolation in the recent years, it has been proven quite challenging to rapidly and efficiently isolate EXs, due to the heterogeneity of EVs, the complexity of the biological fluids used, and the potential contamination of the starting material from other extracellular components with similar size and biochemical characteristics. Many isolation methods are currently available as analyzed above. A commnon aspect among these methods is that they all involve multistep procedures and thereby finally provide a low production yield and/or a low purity of EXs. The U/C method for instance, which is the currently most classical and reliable isolation methodology, can isolate only a small portion of EVs (~ 20-25%). On the other hand, immunoaffinity methods require expensive antibodies and matrices, but finally lead to similar yields with those acquired when using U/C. Size-exclusion methods are often used in combination with U/C or other techniques, but the complex-methods finally realize low yields due to the fact that a large fraction of the sample EVs is lost due to its adhesion to gel materials of filters. Polymeric precipitation

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instead might achieve a higher yield than the other methods but cannot purify EXs from the polymeric material used [83, 83]. Altogether, the very low production yield of EXs imposes a tremendous impediment to their utility in research, delaying thus their potential translation to the clinic, for amy potential application.

Several strategies have been empirically employed and reported in the literature to circumvent the poor EX production yield. These include prolonging incubation times to increase the secretion of EXs from cells, applying higher initial sample density or changing the composition of the culture medium. In a recent report, heat stress applied to MCF-7 cells, was demonstrated to increase the number of EXs produced by the cells (compared to the same number of non-stressed cells) [85]. However, none of these approaches has resulted in any significant increase of production yield or purity.Thus, a novel approach to substantially augment the production yield of EVs is urgently needed.

3.2.6. Microfluidic methods for EX/EV purification

The use of **microfluidics** has been recently explored in the search for improved technologies for EX isolation and purification. Wu et al developed a unique lab-on-chip technology that integrates acoustics and microfluidics to isolate EVs directly from biological fluids, such as whole blood, with high purity and yield. This acoustofluidic device consists of two modules that are intergrated in a chip: a microscale cell-removal module that first removes larger components, followed by an EX-isolation module that separates the different types of EVs. It is an automated EX isolation method with short processing time that offers the advantage of the preservation of the structure, characteristics and functions of the EXs [86]. Another label-free passive microfluidic approach recently implemented for isolating EXs is viscoelastic microfluidics, where particle separation is determined by elastic lift forces acting on particles of different sizes in a viscoelastic medium [87]. The microfluidic chip uses a viscoelastic sheath fluid to align the EV sample along the sidewalls of the channel and then EXs can be isolated from two-sided outlets. This device achieves >90% purity and >80% recovery of EVs from cell culture media.

Another interesting method utilizes a three-dimensional **nanostructured microfluidic chip** to isolate EVs in a more efficient way. This device consists of ciliated micropillars, forming a porous silicon nanowire-on-micropillar structure. It was demonstrated that this microfluidic prototype device can preferentially trap EVs, while simultaneously filtering out proteins and cell debris. Trapped EVs can be recovered intact by dissolving the porous nanowires in PBS buffer [88]. In another study an alternative microfluidic–based device has been developed for the isolation of EVs from biological fluids. Specifically the developed device was an integrated double-filtration microfluidic platform that was shown to efficienlty isolate, enrich and characterize EVs from urine samples of healthy and cancer patients. This method openes up new avenues in clinical diagnosis and therapeutic outcome monitoring, since the EVs isolated from the biological fluids could be used for cancer diagnosis and for monitoring the efficiency of different treatments [89].

The recent technological progress in EX isolation with microfluidic-based techniques has increased the efficiency of EX isolation. However, the above described microfluidic devices need further validation and standardization before they can be translated into devices for clinical diagnosis and treatment .

3.3 In vivo Clearance of unmodified Exosomes

As briefly mentioned above, one of the initial shortcomings identified for using EXs as carriers for drug targeting applications is their rapid clearance form circulation and very short residence time in the blood following in vivo administration. Indeed several types of EVs from different sources have been administered in vivo in order to follow their in vivo fate.

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In one study unmodified tumor-derived EXs isolated from the supernatant of 4T1, MCF-7, and PC3 cells together with PC/Chol liposomes (control) and also liposomes (EX-mimetics) formulated with the lipid extracts of the EXs, were found to demonstrate a comparable rapid clearance and minimal tumor accumulation following iv injection. These findings indicate that the unique protein and lipid composition of the EXs does not appreciably alter their clearance rate, or their biodistribution, following i.v administration [90]. However it was interesting that when delivered intratumorally, the EXs remained associated with tumor tissue to a significantly greater extent compared to the PC/Chol liposomes [**Error! Bookmark not defined.**].

In another pharmacokinetic study, it was found that most of the administered EX-dose did not reach the target tissue but was sequestered in other tissues, while the spleen was found to have a major role in the clearance of EXs [91]. Sun et al. found that 1 h post- intraperitoneal injection of curcumin-loaded EXs in mice, EXs accumulated in the liver, lungs and spleen [92]. A similar high accumulation in the liver and spleen was also observed by Hwang et al. [93] with 99m Tc-hexamethylpropyleneamine oxime (HMPAO)-labeled EXs. Takahashi et al observed that luciferase-expressing EXs (produced from transfected B16 cells) rapidly disappeared from the blood (half-life of 2 min) after i.v. injection in mice, because they were sequestered in the lungs and the spleen [94]. Morishita et al, measured the tissue biodistribution of 125 l-labeled EXs isolated from transfected B16-BL6 cells following their i.v. injection in mice, and found that 4 h post-injection, 28%, 1.6% and 7% of the radioactivity was detected in the liver, spleen and lung, respectively; while it was additionally proven by other studies, that EXs are mainly captured by macrophages [95, 96].

Phagocytosis has been identified as the principal mechanism of exosome internalization, and different mechanisms for EX endocytosis in macrophages have been described [97, 98]. Integrins and lactadherin that binds to α vβ3/β5 integrins which are expressed in EXs from dendritic cells, seem to have an important role in EX capture [99]. Additionally the deposition of fragments of the complement protein C3 on the surface of EXs as well as the lectin galectin-5 found on red cell-derived EXs, seem to be involved in macrophagic capture. Convincing evidence was also reported about the capture of EXs by a specific subpopulation of macrophages, the CD169+ macrophages, via their interaction with the sialylated protein receptor CD169 [**Error! Bookmark not defined.**]. The spleen has also been identified as a key anatomical station for EX sequestration, and a clarification of the mechanisms involved in this process will be required in order to develop modified versions of EXs that will be able to escape splenic capture and demonstrate increased biological activity [**Error! Bookmark not defined.**].

Oppositely, some types of allogenic exosomes have been demonstrated to realize a privileged immune status, and demonstrate decreased clearance by MPS. Specifically, EXs derived from immunocytes, express the CD47 receptor [100] that interacts with signal regulatory protein α (SIRPα) resulting in the generation of a signal (characterized as the "don't eat me" signal), that blocks their uptake by phagocytes [101]. EXs released by primary fibroblast-like mesenchymal cells can also bypass immune clearance by monocytes and macrophages utilizing a similar mechanism, and demonstrate prolonged blood circulation [102]. Thereby, the source of EXs seems to be particularly important conserning EX clearance kinetics and mechanisms, and as a consequence probably also EX biodistribution.

In any case the problem of rapid clearance could be solved by the introduction of polyethylene glycol (PEG) molecules to coat the surface of the vesicles, as successfully applied for many years in the liposome field. In fact Kooijmans et al have recently shown that the introduction of PEG to EXs, with a method which is similar to that used for liposomes (post-formation incubation with PEG-lipid micelles), results in stealth properties, which significantly increases their circulation time in mice [103].

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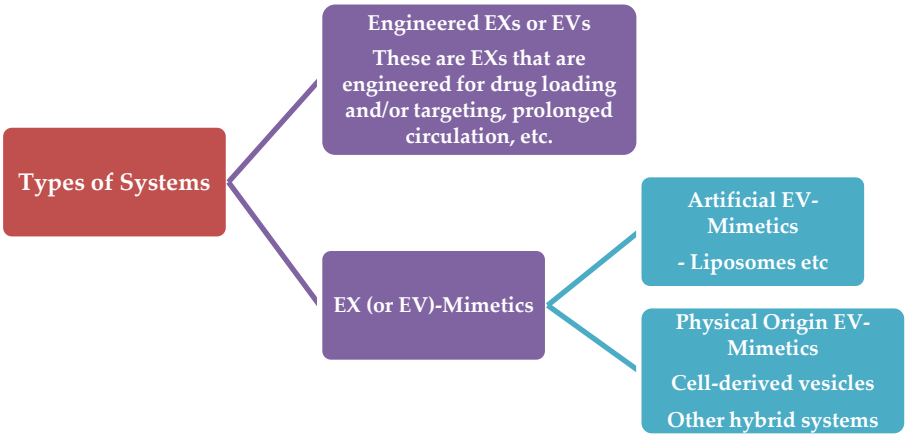
In conclusion, the currently available knowledge concerning the in vivo disposition and blood circulation of unmodified EXs, reveals that in most cases (depending of EX source), the need for ways to engineer their surface characteristics in order to increase their circulation time, may be required (depending of course on the specific application and administration route). Such approaches, may increase the potential applications of EXs for drug targeting applications, and will be discussed in the next part.

4. Types of Systems

In this review we will use the following categorization and naming for the various types of EX-like systems tested up-to-date as drug carriers (**Scheme 3**): The two main categories of EX-evolving-vesicles being currently exploited for drug delivery will be defined as:



Scheme 3. Classification of EV-like vesicles used for Drug Delivery applications

1. ***Engineered EXs or EVs***. This first category will include vesicles derived from isolated and purified EXs or EVs. In order to be used for drug delivery applications, EXs are initially engineered for:

(i) Efficient loading of drugs in the EXs and/or

(ii) Surface-modification/attachment of molecules on their surfaces; such modification may be required when the in vivo fate (stability and/or pharmacokinetic/biodistribution profile) of the EXs is not considered to be relevant for the specific drug delivery and/or targeting application. In fact, as analyzed above, in most cases EXs the clearance of unmodified EXs following in vivo administration (especially if iv-injection is used), is rapid, posing a problem for their application as targeted drug carriers.

2. ***EX or EV- mimetics***. This second category of vesicles includes all the vesicle-types that are not formed using EXs or EVs as their **starting material**. Such approaches have been explored in order to overcome the low yield of EV isolation/purification methodologies, or to develop drug carriers for broader applicability (since the use of non-autologous cellular materials or material of tumour origin could trigger numerous types of toxicities due to immunological responses or other reasons). Thereby, EV-mimetics are further divided in sub categories, based primarily on the origin of the starting materials used for their preparation; Here we distinguish two sub-categories of EV-mimetics:

a. ***Artificial EV***-mimetics, when the starting material is of synthetic or semi-synthetic origin (this category also includes lipids extracted from natural sources, such as cells or EVs). In most cases artificial EV-mimetics are actually liposomes with or without specific proteins in their membrane (inspired from specific types of EVs or EXs with high organotropism).

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b. ***Physical-origin EV-mimetics***, when the starting material may be derived from other types of cellular components excluding EVs (such as whole cells [in this case the vesicles are names "**cellular vesicles**"]). In this subcategory again the starting material is engineered as mentioned above for engineered EVs, and the same methodologies apply.

In this part, we will present examples of the various types of systems, categorized as mentioned above, and we will additionally describe the methods used up to know to load drugs and modulate the surface of the various types of vesicles.

4.1 Engineered EXs or EVs

Several studies have exploited the potential use of EVs for drug delivery/targeting or in general theranostic applications, after being engineered for drug loading, or surface modification. Some recent examples are mentioned here.

Pascucci *et al* prepared paclitaxel (PTX)-encapsulating EXs that demonstrated strong anti-proliferative activity against CFPAC-1 human pancreatic cells *in vitro* [104]. The feasibility of PTX EXs as a potent chemotherapeutic system to treat MDR cancer was also assessed, and PTX-EXs demonstrated 50 times higher cytotoxicity towards drug resistant MDCKMDR1 (Pgp+) cells (compared to control formulations). In another study, a potent anticancer effect by EXs was observed in a model of murine Lewis lung carcinoma pulmonary metastases [105]. Lv *et al.* reported that EVs isolated from resistant anticancer drug-treated HepG2 cells conferred superior immunogenicity in inducing HSP-specific NK cell responses, a finding that could lead to the development of an efficient vaccine for hepatocellular carcinoma immunotherapy [106]. Sun *et al* investigate the potential to deliver curcumin, a polyphenol with anti-inflammatory and antineoplastic activity, by mouse lymphoma-derived (EL-4) EXs [**Error! Bookmark not defined.**]. In another study, curcumin-loaded EL-4 derived EXs were efficiently delivered -noninvasively- to microglia cells via the *intranasal* route [107]. Haney *et al* successfully used RAW264.7 cell-EXs to deliver catalase (a tetramer protein of 250 kDa), and the engineered-EXs were readily taken up by neuronal cells in vitro, while a considerable amount of the same catalase-loaded-EXs were detected in the brain of a Parkinson's disease mouse model after *intranasal* administration, and realized a significant neuroprotective effect [108]. In another study, EXs were engineered for both: Doxorubicin (DOX) loading and for RGD peptide attachment on their surface. These dually engineered EXs presented highly efficient targeting of DOX to αv integrin-positive breast cancer cells in vitro, while they were found efficient for targeted delivery of DOX to tumors after i.v. injection, leading to a significant inhibition of tumor growth without demonstrating any measurable toxicity [109]. **The later example proves the potential of specific EX types to target tumors even after i.v. injection** although as presented above unmodified EXs are usually rapidly cleared from blood circulation.

Nakase and Futaki encapsulated a dextran macromolecule (70 kDa) into EXs derived from CD63-GFP-HeLa cells. The EXs were engineered to combine a cationic lipid (Lipofectamine) and a pH-sensitive fusogenic peptide (GALA) that would induce the fusion of endosomal and exosomal membranes for improved uptake by cells. Indeed, the cellular uptake of dextran by these engineered EXs was significantly enhanced by the combined treatment [110]. Fuhrmann *et al* engineered EXs to encapsulate different types of porphyrins (with differing hydrophobicities), and such EVs induced a stronger phototoxic effect in a cancer cell model compared to the free drugs [111]. The delivery of small RNAs using engineered EXs has been studied by several groups [112-116]. In more detail:

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-Alvarez-Erviti *et al* engineered EXs from dendritic cells to be loaded with exogenous siRNA and have RVG peptide on their surface. After iv-injection these RVG-targeted EXs delivered siRNA specifically to neurons, microglia and oligodendrocytes in the brain [**Error! Bookmark not defined.**], proving again the targeting ability of surface modulated-EXs after i.v. administration.
-Peripheral blood plasma derived EXs were used to transport exogenous siRNA to human blood cells, and siRNA was successfully delivered into monocytes and lymphocytes, causing selective gene silencing of mitogen-activated protein kinase 1 [**Error! Bookmark not defined.**].
-EVs loaded with siRNA were taken up by recipient cells and significantly reduced HER2 protein expression [**Error! Bookmark not defined.**].
-Didiot et al demonstrated that EXs that were loaded with hsiRNAs targeting *Huntingtin* mRNA were efficiently internalized by mouse primary cortical neurons and promoted dose-dependent silencing of *Huntingtin* mRNA and protein [**Error! Bookmark not defined.**].
Nevertheless engineered EXs have also been found to perform worst compared to some liposome types. Indeed, exogenous cholesterol-conjugated siRNAs (Chol-siRNA) and endogenous miRNA were inserted in EXs derived from both a melanoma and a monocyte/dendritic cell (DC) line and their delivery potential in distinct target cells was assessed. In more detail, the delivery of siRNA by the engineered-EXs and also by anionic fusogenic liposomes (prepared by employing the same loading approach, as control formulations), was tested and the results showed that the EXs were unable to functionally deliver the associated small RNAs. In contrast, the anionic fusogenic liposomes induced a marked siRNA-mediated gene knockdown under identical experimental conditions [117].

Recently, macrophage-derived EXs were engineered to attach a PEG-conjugated ligand to target the sigma receptor on their surface, and they were additionally loaded with PTX; they were found to exhibit superior in vitro and in vivo results against a pulmonary metastases model, compared to the control formulations [118].

4.2. EX (or EV) - Mimetic's

As mentioned above, there are two types of EV-mimetic systems: (a) Artificial EV-mimetic's and (b) Physical-origin EV-mimetic's. The main theoretical basis and some examples of the potential applications of the two different types for drug delivery will be presented below.

4.2.1 Artificial EV-mimetic's

While pure populations of EXs can be isolated from EX-secreting cell lines, these EXs, unlike those released from autologous primary cells, have immunogenic and oncogenic potential, inhibiting their broad use as drug delivery systems. Moreover, EVs play multifaceted roles in health and disease, including the intercellular transfer of pathogens and disease-associated proteins [119, 120], introducing major barriers for the translation of naturally secreted EXs to the clinic. EV- mimetics may help circumvent these barriers [121].

Artificial EV-mimetics are based on the idea that not all components in natural EXs are essential for specific and efficient delivery. Thus, assembling lipids into a bilayer structure (which resembles the membrane of the EX) and functionalizing the vesicle surface with proteins, or modulating their surface by the transport of a message through direct contact with target cell receptors, or by attaching hydrophilic molecules to increase their blood circulation, is considered as an artificial EV-mimetic. As mentioned above most of the artificial EV-mimetics proposed or studied up-to-date are actually liposomes. Theoretically by using the knowledge acquired by appropriate analysis of the surface characteristics of organotropic EV-types about their composition, one may be able to develop artificial liposomal systems with the desired targeting properties. Proteomic and lipidomic analysis may be helpful to identify the most important EV components that determine their high targeting potential and elucidate their structure in order to make it possible to develop liposomes as artificial EV-

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mimetics. Importantly, only small unilamellar vesicles (SUVs) are ideal precursors for the preparation of vesicle that can mimic EXs due to their similarities to natural exosomes (size range and membrane disposition). Thus, by applying classical techniques used for preparation of SUV liposomes (e.g. thin-film hydration method, reverse-phase evaporation method, ethanol injection method, ether injection method, microfluidic-based methods, extrusion techniques, etc.), liposomes with a size range similar to that of natural EXs can be easily obtained.

Some examples of such artificial EX-mimetics developed for drug delivery applications follow:

Very recently, EX-mimicking liposomes (formulated by copying the lipid composition of EXs as a starting point) were formulated for the delivery of VEGF siRNA to A549 cancer cells and HUVECs. These EX-mimetics had lower cytotoxicity compared to Lipo-2000 and DOTAP liposomes, and higher storage stability and physical stability (reduced aggregation) in serum. They also appeared to be able to be endocytosed into A549 cells and HUVECs. Notably, these EX-mimicking liposomes exhibited significantly higher cellular uptake and silencing efficiency compared to PC/Chol liposomes. However, their oligonucleotide delivery efficiency was still very low compared to that of cationic lipids, such as Lipo 2000 and DOTAP [122].

The following examples are not directly related with artificial-EX as drug delivery systems but as therapeutics, however they are of interest since the results prove that artificial EXs can target specific cell types.

In one study targeted and *in vivo* traceable artificial EXs were developed to mimic dendritic-cell-derived EXs. The theoretical background is that dendritic-cell-derived EXs are known to mediate and modulate immune responses in vivo by semi-direct T cell activation, and that T cells can eradicate primary, metastatic and relapsed tumors and ameliorate otherwise fatal viral infections. Taking this into account, the EX-mimetic-liposomes were coated with an optimized number of MHC Class I/peptide complexes and a selected specific range of ligands for adhesion, early activation, late activation, and survival T cell receptors. It was finally shown that that these artificial-EXs activated and expanded the functional antigen specific T cells at sufficient levels [123].

The group of Martinez-Lostao et al. observed that T lymphocytes, which were present in synovial fluid (SF) of rheumatoid arthritis (RA) patients, were sensitive to APO2L/TRAIL. In addition, there was a drastic decrease in the amount of bioactive APO2L/TRAIL associated with EXs in SF from RA patients [124]. Taking these results into account, the group conjugated APO2L/TRAIL in artificial lipid vesicles resembling natural EXs and used these artificial-EXs as a treatment in a rabbit model of antigen-induced arthritis (AIA). These artificial-EXs increased APO2L/TRAIL bioactivity and resulted in a more effective treatment of AIA compared with soluble, un-conjugated APO2L/TRAIL; reduced synovial hyperplasia and inflammation in rabbit knee joints were noticed [125]. Subsequently, they generated liposomes coated with bioactive Apo2L/TRAIL, to analyze their apoptosis-inducing ability on cell lines from hematological tumors. These liposomes (LUVs-Apo2L/TRAIL) greatly improved Apo2L/TRAIL activity [126].

Deng et al prepared artificial-EXs using lipids that were derived from intestinal, mucus-derived, EX-like nanoparticles (IDENs). In brief, the liposomes were formed by extracted IDEN lipids which were hydrated, bath sonicated and probe sonicated. It was found that these artificial-EXs possessed similar NK T-cell inhibitory activity as the original ones [127].

4.2.2 Physical-origin EV-mimetics

Due to the low yield of EV isolation from cell media or other sources, EV mimetics have been also composed by using other types of physical-origin media as starting material. Most of the physical-origin EV-mimetics studied up-to-date are derived from whole cells. Some other hybrid type EV-mimetic systems have been found in the literature and will be mentioned separately.

4.2.2.1 Cellular Vesicles (CVs)

Cell-derived vesicles (CVs) are a new and rapidly evolving class of biological drug delivery systems. They are inspired from EVs but represent a totally different type of endogenous nanocarriers. CVs retain the surface characteristics of their parental cells and are thus highly biocompatible with efficient intrinsic targeting ability (providing, of course, that they are of

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autologous origin); additionally they don't need any further surface functionalization. This offers a clear advantage over other (synthetic) drug delivery systems. Moreover, the fact that CVs are derived from natural sources helps for a higher retention in the circulation and for a reduced clearance rate, increasing the circulation time of their therapeutic cargo in the body, without the fear of any stimulated systemic toxicity. These stable and long-circulating endogenous nanocarriers provide protection of the drug cargo from degradation and increase drug delivery to targeted tissues [128. 129]. Although the broader use of non-autologous CVs, as also EXs, could not be envisioned for the same safety reasons mentioned in the case of (engineered) EXs, the substantially higher production yield of CVs could be the key difference that will perhaps enable the development of a road-map for rapid manufacturing of CV-loaded drugs from autologous cells.

CVs are obtained by subjecting cells to a physical process producing vesicles of nano-dimensions. The very small size of CVs (<200nm) facilitates the cellular uptake of the delivered drug, due to the increased permeability and retention effect. However, the greater advantage of CVs is that they escape from innate immune recognition, avoiding the rapid degradation and clearance by the body's immune defense systems. Indeed, synthetic nanoparticles are known to interact with the innate immune system, including the complement system, and these interactions with the immune system impose significant clinical limitations. CVs, as natural carriers, diminish complement activation responses and evade blood clearance by the immune system, enabling long circulation of their cargo, being thus, ideal candidates of clinically approved nanocarriers.

CVs are derived from the cell plasma membrane, are composed of a lipid bilayer, and have a final size between 50 and 200nm. CVs are closed vesicles that incorporate many cellular contents from the parental cells, such as membrane proteins, intracellular proteins and RNAs. Beside the different methods utilized for their production and isolation, CVs and EVs present many similarities in terms of their size (nano-scale), morphology, key membrane proteins, and lipid composition [130]. As far as vesicle content is concerned, cryo-TEM and western blot analysis of isolated CVs showed that they include all the membrane proteins of their parental cells and also preserve their topology, which is logical since the lipid bilayer membrane of the CVs is directly derived from the plasma membrane of the cell. They also preserve the characteristic intracellular proteins of the original cells; therefore CVs contain both membrane proteins and intracellular proteins from the parental cell membrane. In addition, RNA analysis with RT-PCR revealed that CVs enclose intracellular RNAs from parental cells. After RNA profiling of CVs the presence of intracellular miRNA, rRNA and tRNA was verified, Indicating that CVs can be used as vehicles for RNA delivery.

Beside protein and RNA analysis of CVs, their lipid composition was also investigated in one study [**Error! Bookmark not defined.**]. Specifically, it was demonstrated that CVs are more similar to EXs than parental cells (U937 cells in this case), in regards to their major lipid constituents (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and lysophosphatidylcholine). However, some differences were detected in the relative lipid amounts, which may imply different properties for each vesicle type. Interestingly, the lipidic profile of CVs has a strong resemblance to that of erythrocytes, a fact that could explain the high cellular uptake and long circulation time in the body of some CV-types [**Error! Bookmark not defined.**].

CVs are produced by subjecting cells to a physical process (different procedures have been utilized) resulting in vesicles of nano-scale sizes. Many methods have been used and new methods are continuously emerging in the literature, including passing cells through an extruder, through microchannels or through custom-made devices inside a centrifuge. The most commonly used technique involve serial extrusions of the cells through polycarbonate membrane filters of decreasing pore sizes, usually starting form 10-μm, then 5-μm, 1-μm, and lower. The CVs are finally obtained by performing density gradient ultracentrifugation or size-exclusion column chromatography in order to realize sizes between 100 and 200 nm, which are required to take advantage of the enhanced cellular uptake and retention (EPR) effect and avoid premature clearance from the body [**Error! Bookmark not defined.**, 131].

Another CV fabrication technique described in the literature is the cell-slicing system to generate vesicles from living cells. This method actually exploits the ability of the cellular membrane lipid bilayer to self-assemble into vesicles. Specifically, living cells are flowing inside a *microfluidic device*

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and are serially sliced by 500nm-thick silicon nitride blades while passing through microchannels lined with the blades. When cells are sliced, a free-standing piece of plasma membrane with intact lipid bilayer structure forms a vesicle that contains the cellular contents of the parent cells [132].

The above described methods generate a high yield of CVs from living cells over a relatively short period of time. They are simple, rapid and inexpensive methods that require only common laboratory equipment. Furthermore, when compared to EV isolation procedures, CV fabrication methods are far more superior as they produce larger quantities of nanovesicles over a shorter time period. Specifically, the yield of isolated CVs is more than 100 times higher compared to that of EVs [133, 134]. EV isolation and production methods usually requires up to 4 days, whereas CV production can be completed in a single day. The difference in processing time is mainly due to the time needed for cells in culture to secrete enough EVs for isolation. This step is unnecessary in CV fabrication as CVs are produced immediately after the cells in culture reach confluency [**Error! Bookmark not defined.**].

CVs, like EVs, shuttle endogenous biological cargo such as mRNAs, RNAs and proteins that can be efficiently taken up by targeted tissues. Moreover, some recent reports showed that CVs can efficiently encapsulate and deliver exogenous biomolecules to targeted diseased tissues [**Error! Bookmark not defined.**, **Error! Bookmark not defined.**]. Specifically, Yoon and colleagues demonstrated by using the cell-slicing system that during self-assembly, the plasma membrane fragments envelop exogenous materials from the buffer solution. About 30% of material can be encapsulated in CVs, and the generated CVs can efficiently deliver the exogenous cargo across the plasma membrane of recipient cells [**Error! Bookmark not defined.**, **Error! Bookmark not defined.**]. On the other hand, Jang et al [**Error! Bookmark not defined.**] showed that by subjecting cells of different origin to serial extrusions through filters with decreasing pore sizes after the cells had been loaded with chemotherapeutic agents, high quantities of drug-loaded CVs are generated.

Recent reports present CVs as viable nanocarriers for drugs in the treatment of various diseases. For cancer treatment, CVs seems to have an advantage as they achieve passive targeting via enhanced permeability and retention effect on leaky vasculature of tumors. Indeed, CVs were demonstrated to successfully deliver different chemotherapeutic substances, such as doxorubicin, at high amounts to tumor tissues and significantly reduced tumor growth without the adverse effects observed with equipotent free drug [**Error! Bookmark not defined.**, **Error! Bookmark not defined.**]. In another study researchers utilized CV carriers in liver regeneration. CVs were produced by serial extrusions of primary hepatocytes and were then injected intravenously in mice. It was clearly shown that CVs contained the protein content of the parental cells and that they efficiently promoted hepatocyte proliferation in vitro and liver regeneration in vivo [**Error! Bookmark not defined.**]. Strategies inspired by this study could lead to the usage of CVs in tissue repair and regeneration, but also in drug delivery.

4.2.3 Other types of EV- mimetic systems

Several other types of EV-mimetic systems can be found in the literature, which are actually hybrid systems aiming to combine the advantages of EXs with those of other systems. Actually the hybrid systems produced after fusion between liposomes and EXs [**Error! Bookmark not defined.**] (mentioned above), can be included in this category instead of the category of engineered-EXs.

As an example of other hybrid systems (that will not be discussed more herein), we mention a previous case where non-enveloped viruses were associated with EVs to form virus-EV hybrid particles that combine the respective advantages of EVs (low immunogenicity, targeting) and viruses (stable gene expression). Indeed, hepatitis A virus released by cells was cloaked in host-derived membranes that resemble EXs and this hybrid system was found to be fully infectious [135].

5. Methods of Preparation and Engineering of Engineered EXs and EX-mimetics

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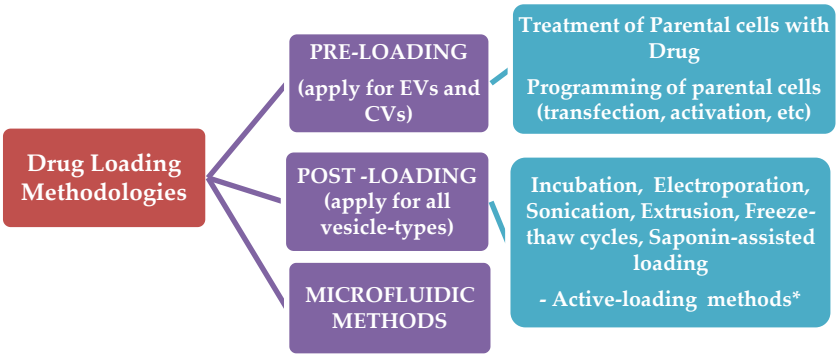
In the next part we will focus on the methodologies used up-to-date for drug loading and surface modification of various types of EVs. **The same methodologies to load drugs also apply for EV-mimetics.**

5.1 Drug loading methodologies

The drug loading methodologies applied up-to-date in the case of EXs, and their physical mimetics (CVs), are categorized in two main groups, the pre-loading methods and the post-loading methods. The recently developed microfluidic approaches are discussed separately [Scheme 4].



Scheme 4. Categories of methods used for loading drugs into EXs and EX-like vesicles

In pre-loading methods, the drug is initially produced or loaded in the parental cells, and thus the EVs or CVs isolated or produced by them, are already pre-loaded with the desired drug. Such methodologies are particularly useful when oligonucleotides or proteins are to be loaded in the vesicles, in which case the cells can be programmed to produce "a-la-carte" EVs or CVs (after applying particular cell engineering techniques).

In post-loading methods, the drug is loaded in the EVs after their isolation. In the last years scientists are trying to apply high yield drug loading methods that have been used for liposome engineering, and in some cases, the loading efficiencies acquired are significantly improved, when compared to the simple methods used in earlier reports (such as electroporation and simple incubation). Nevertheless, we have not been able to detect any reports about the potential to use special loading methods that have been very successful for efficient loading of amphiphilic drugs into liposomes, such as the "active or remote" loading methods for amphiphilic drugs, as Doxorubicin etc. [136], which can be developed as a "one-step" manufacturing methods on a microfluidic setup, as recently proven[137]. The only relevant report found was that of Zhang t al. [138] who tested the remote loading method in Red Blood cell (RBC) ghosts that were reconstructed into vesicles. It was found that the membranes needed to be enriched with cholesterol in order to retain a fluorescent dye, and up to 10% cholesterol was tested, while the fluorescence signal reached a plateau after 5% cholesterol. The 5% cholesterol enriched vesicles were remotely loaded using an ammonium sulfate/PBS gradient; the maximum loading reported was only 10% for DOX, and 40% for vancomycin. The authors explain the difference by the higher MW of vancomycin, implying that the vesicles were still not stable enough to retain low-molecular weight molecules. The fact that DOX was shown to be precipitated in the vesicles, does not comply with this explanation. Furthermore, the group did not try to optimize the method, by perhaps adding more cholesterol in the vesicles, or by other means. More recently the same group tested remote loading for platelet vesicles, using identical protocols for vesicle engineering and loading, and testing the same drugs; they found that 5% cholesterol-enriched platelets could be loaded with up to 11% DOX and 33% Vancomycin, giving the same explanation for the difference, while again DOX was verified to be entrapped in the vesicles

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in a crystalline form [139]. Thereby, remote loading in such EX-like vesicle types, could be further exploited for optimization.

In the next part we will present the most common methods applied up to-date and their results, and then discuss potential future challenges. In order to avoid misunderstanding, we should mention at this point that many previously applied EX-loading methodologies which are known in the liposome field as "passive" loading methods since the drug is entrapped in the liposomes passively due to a concentration gradient (incubation of vesicles with drug solutions) or because the liposomes are formed in the drug solution (thin film method, sonication, extrusion, etc.), have been characterized in several previous review articles as "active loading methods". We clarify here that in liposome technology we use the term "active loading" or "remote loading" only for techniques such as the ion or pH trans-membrane gradient methods, as mentioned above [**Error! Bookmark not defined.**,**Error! Bookmark not defined.**].

5.1.1. Pre-loading methods

5.1.1.1. Treatment of parental cells with drugs

In this type of methods, the parental cells are treated with a drug and the cells subsequently secrete pre-loaded with drug EXs or EVs. Although it is impossible to control the loading efficiency with this type of methods, several studies, which are summarized below, have been conducted using such techniques, owing to their simplicity.

Pascucci *et al* encapsulated paclitaxel (PTX) into murine SR4987 mesenchymal stroma cells, after culturing the cells with a low dose of PTX for 24 h and subsequently washing and reseeding them with fresh medium. After 48 h PTX-loaded-EXs primed with high dose of PTX were collected. The PTX-loaded EXs showed strong anti-proliferative activity against CFPAC-1 human pancreatic cells in vitro, as compared with control EXs which were isolated from untreated cells [**Error! Bookmark not defined.**].

Lv *et al.* isolated EVs from resistant hepatocellular carcinoma cells (HepG2) cells treated with different drugs (paclitaxel, carboplatin, etoposide and irinotecan hydrochloride). After exposure to the anticancer drugs, membrane microvesicles confirmed by TEM and Western Blot to be EXs, were actively released by the cells. The later EXs differed in their ability to present heat shock proteins (HSPs) on the cell surface. Their size ranged between 30 and 100 nm with the majority of the vesicles being around 70–90 nm [**Error! Bookmark not defined.**].

Another approach is the one developed by Lee *et al.,* a method of liposome-mediated EV engineering for anticancer drug-loading. Synthetic fusogenic liposomes which were loaded with hydrophobic drugs were efficiently incorporated into the host cell membrane and the drugs were thus subsequently loaded into the membranes of the cell-secreted EVs [140]. The same group used this method to introduce lipid-azides in the membrane of EVs which were further conjugated with targeting moieties and loaded with Paclitaxel (PTX) and tirapazamine (TPZ) [141].

5.1.1.2 Parental cell engineering

In the last decade an increasing number of reports in the literature describe methods for successful loading of therapeutic cargo in EXs, by modulation of the EX-producing parental cells. The most common cell engineering techniques involve transfection or activation of parental cells.

Transfection is the most widely used and the most efficient method for loading of therapeutic proteins or oligonucleotides into EXs. RNA (miRNA, siRNA and mRNA) and protein sequences are easily transfected as synthetic oligonucleotides or expressed from a plasmid backbone. Cell transfections are carried out by the calcium phosphate method or by commercially available lipid transfection reagents such as Lipofectamine, or others. With such methods, biological cargos can be packaged into EXs to promote or silence gene expression or regulate transcription in recipient cells.

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Studies have shown that transfection can also be used to overexpress a specific protein on the surface membrane of the EXs, or the proteins could be packaged into the EX lumen. Indeed, in addition to modifying EX membranes through genetic engineering of their parent cell, the therapeutic cargo of EXs may also be manipulated by altering various aspects of their regulated biogenesis.

miRNAs were successfully introduced into EXs in several studies by using miRNA expression vectors. Akao, et al., showed that transfecting modified miR-143 in THP-1 macrophage cells led to the successful loading of the modified miRNA into the EXs. This work exhibited that when overexpressing a miRNA into parental cells this effectively leads to the passive load of miRNA into EXs [142]. Ohno, et al., exhibited that the miRNA-loaded EXs could also be targeted effectively to recipient cells after engineering their surface with targeting peptides, and demonstrated that intravenously injected EXs accumulated in the tumor site and reduced the tumor growth [143].

Interestingly, ***cell activation*** has been used as an approach for loading functional cargo in EXs. While it is not the most appropriate method for EX loading it shed light on some aspects of EXs physiology and function. Cell activation as a methodology for EX loading was, however, only studied a few times. THP-1 monocytes stimulated with different inflammatory stimulants showed increased levels of miR-150 in the resulting EXs with subsequent increased endothelial cell migration [144]. Furthermore, brain extracts from rats undergoing middle cerebral artery occlusion induced increased expression of miR-133b in multipotent mesenchymal stromal cells (MSC) EXs after co-culture of MSCs with neurons and astrocytes [145].

Another strategy for loading EXs with nucleic acids exploits viral packaging mechanisms. During the production of adeno-associated virus (AAV) vectors, a fraction of the vectors was associated with EXs and outperformed conventionally purified AAV vectors in transduction efficiency [146].

5.1.2 Post-loading Methods

In this section the post-loading methods used up-to-date for drug loading into engineered-EVs of artificial-EVs are described starting from the simplest methods and continuing with the most complicated ones (usually applied in liposome technology).

5.1.2.1 Incubation with drug

The simplest way to incorporate any cargo into EXs is their co-incubation, simply by mixing the isolated vesicles with the drug. The driving force for the loading is the different concentration of the drug in and out of the vesicle membrane. Hydrophobic drugs interact with the vesicle's lipid layers and the drugs diffuse into the EX cavity along the concentration gradient. The low loading capacity is considered as the main drawback of this method. The loading efficiency of co-incubation method depends on the lipophilic properties of the drug, as well as the concentration gradient [**Error! Bookmark not defined.**]. Some examples of drug loading into EXs by their incubation with the drug of interest are presented below:

Sun *et al* incubated curcumin with EXs in PBS at 22 °C for 5 min and the EXs were subsequently purified [**Error! Bookmark not defined.**]. Curcumin was thus self-assembled into the lipid bilayer of EXs via hydroscopic interactions, resulting in increased drug stability. Using the same method, Zhuang *et al* encapsulated both curcumin (Exo-Cur) and a signal transducer and activator of transcription 3 (Stat3) inhibitor (Exo-JSI124) by mixing curcumin or JSI124 with EL-4 cell derived EXs in PBS. After incubation at 22 °C for 5 minutes drug-loaded-EXs (Exo-cur or Exo-JSI124) were

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subsequently isolated [**Error! Bookmark not defined.**]. Didiot *et al.* developed a robust, efficient, and highly reproducible method for loading therapeutic RNA into EXs upon co-incubation of hydrophobically modified small interfering RNAs (hsiRNAs) and EXs, without affecting the vesicle size nor their integrity [**Error! Bookmark not defined.**]. Fuhrmann *et al* reported the loading of porphyrins of different hydrophobicities intro EVs by incubation with the drug at RT for 10 min [**Error! Bookmark not defined.**], while Haney *et al* successfully loaded the enzyme catalase (a tetramer protein of 250 kDa) into RAW264.7 cell-extracted EXs in PBS at RT for 18 h [**Error! Bookmark not defined.**].

5.1.2.2 Electroporation

By this technique, an electrical field disturbs the phospholipid bilayer of vesicles (such as EVs or EXs or cells) creating small pores in their membrane, and allowing thus, the passage of the drug into the vesicles. The integrity of the vesicle membrane is then recovered resulting in the formation of drug-loaded vesicles.

Electroporation has been used for loading siRNA into EXs [**Error! Bookmark not defined.**]. The idea is that siRNA molecules, which are relatively large and cannot diffuse into EXs spontaneously, due to their hydrophilic nature, could be assisted by electroporation in order to diffuse into EXs. Wahlgren et al proved that electroporation leads to superior loading of siRNA over chemical transfection [113, 115]. In more detail, in order to overcome the toxicity related with the use of cationic liposomes for the delivery of nucleic acids (due to electrostatic complexation of the negatively charged nucleic acids with cationic lipids), this group used EXs which could prevent the electrostatic complexation between nucleic acids and cationic lipids, due to the negative surface charge of EXs. For this, two different strategies, electroporation and chemical transfection, were applied to introduce a double-stranded siRNA into the EXs. For the chemical transfection, a siRNA against MAPK1 was mixed with the transfection reagent to allow the formation of transfection complexes (siRNA embedded in lipid micelles). The complex was then incubated with the EXs and vesicles were subsequently purified. However, since it was impossible to separate the EXs from the complex, chemical transfection was found to be inapplicable as an EX-loading method. On the other hand, electroporation was found to result in efficient exogenous siRNA loading into the same EXs [**Error! Bookmark not defined.**]. Nevertheless, the use of electroporation as a method to load siRNA into EXs has been criticized. In fact, Kooijmans et al stated that electroporation disrupts Exs integrity and is "far less efficient than previously described". They showed that electroporation of EXs in presence of siRNA is accompanied by extensive siRNA aggregation, which may cause overestimation of the amount of siRNA actually loaded into the EXs [147]. In an effort to overcome this issue, Johnsen *et al* reported that when electroporation is carried out in an optimized buffer such as trehalose disaccharide, the buffer can aid the structural integrity and inhibit the aggregation of EXs extracted from adipose-derived stem cells (ASCs). In more detail, when EXs were resuspended in electroporation buffers, such as cytomix electroporation buffer or trehalose pulse medium, and electroporation was performed at 400 V and 125 μF, morphological changes and aggregation of EXs were observed; Oppositely, when the trehalose-containing buffer system was used during electroporation, the structural integrity of the EXs was preserved [148]. In the same direction, Lamichhane et al. used electroporation to load small RNAs (siRNA, miRNA or single stranded DNA (ssDNA)) into EXs isolated from two different cell lines, HEK293T and MCF-7, by alleviating nucleic acid aggregation. They mixed EXs with nucleic acids in electroporation buffer and electroporation

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was carried out at 400V and 125μF with two pulses. All samples were purified and subsequently transferred into tubes where EDTA was added to alleviate nucleic acid aggregation. Samples were then incubated at room temperature for 15min and centrifuged at 5000×*g* at 4°C for 5min to remove buffer and unincorporated cargo. [**Error! Bookmark not defined.**].

Despite oligonucleotides, electroporation has been successively used to encapsulate small hydrophilic drugs into EXs. Fuhrmann *et al* encapsulated TMP (5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (*p*-toluenesulfonate)) which is used for its photodynamic effects. Electroporation was performed at 200Ω, 500μF, 200mV and a pulse time of 20–30 ms [**Error! Bookmark not defined.**]. Other examples of drug loading into EXs by electroporation include DOX loading into mouse immature dendritic cells (engineered to express Lamp2b (an exosomal membrane protein) fused to αv integrin-specific iRGD peptide (CRGDKGPDC)) derived EXs. The purified EXs were efficiently loaded with DOX via electroporation. [**Error! Bookmark not defined.**]

Nakase and Futaki used the electroporation technique to encapsulate a dextran macromolecule (70 kDa) into EXs which were isolated from CD63-GFP-HeLa cells. The EXs combined a cationic lipid (Lipofectamine) and a pH-sensitive fusogenic peptide (GALA) for the fusion of endosomal and exosomal membranes inside cells. [**Error! Bookmark not defined.**].

5.1.2.3 Sonication

In this method, EXs derived from donor cells are mixed with drugs and subsequently sonicated by a probe sonicator which allows the drug to flow into the EXs due to the sonication-induced deformation of their membrane. In principle, reformation of exosomal membranes under sonication may enable the passage of drugs across relatively tight lipid bilayers [**Error! Bookmark not defined.**]. Some examples of drug loading with sonication are presented below:

Kim *et al* tested sonication as a method to load paclitaxel (PTX) into macrophage released EXs, and the method resulted in high loading efficiency and sustained drug release, while it did not significantly affect the protein and/or the lipid contents of the EXs [**Error! Bookmark not defined.**].

Haney et al effectively loaded catalase into exosomes. The catalase-EX mixture was sonicated (500V, 2 kHz, 20% power, 6 cycles by 4 sec pulse /2 sec pause), cooled down on ice for 2 min, and then sonicated again [**Error! Bookmark not defined.**].

Sonication was also used as a method to load small RNAs (siRNA and miRNA) in EXs (exosomes and microvesicles). However, the application of EXs for therapeutic RNA delivery may be limited by such loading approaches that may induce cargo aggregation or degradation. Lamichhane et al loaded functional small RNAs (siRNA, miRNA or single stranded DNA (ssDNA)) into EXs isolated from two different cell lines, HEK293T and MCF-7, by the use of sonication. For this, the nucleic acids were incubated with the EXs at RT for 30min followed by sonication in a bath sonicator at 35kHz for 30sec. Mixtures were then placed on ice for 1min and sonicated again for the same time period. These conditions led to minimal detectable aggregation [**Error! Bookmark not defined.**, 149], probably due to the mild sonication conditions applied.

5.1.2.4 Extrusion

In this method, EXs are mixed with a drug, and the mixture is loaded into a syringe-based lipid extruder and extruded through membranes with 100–400 nm porous size, at controlled temperature. During the extrusion, the EX membrane is disrupted and vigorously mixed with the drug, resulting in drug loading into the EXs.

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Fuhrmann *et al* loaded porphyrins of different hydrophobicities into EXs by extrusion. The procedure was conducted at 42 °C using a syringe-based hand-held mini-extruder equipped with a heating block, and polycarbonate membranes of 400 nm pore size. Each sample was extruded 31 times. It was found that the extrusion method resulted in alteration of the zeta potential of the vesicles, possibly due to modulation of the constitution of the lipid membrane of original EXs. The extruded EXs were demonstrated to cause cytotoxicity, whereas EXs loaded with the same porphyrin but prepared by other methods did not show significant cytotoxicity [**Error! Bookmark not defined.**].

Catalase has also been loaded into RAW264.7 macrophage-derived EXs by extrusion. For this, the catalase-EX mixture was efficiently extruded (x10 times) through 200 nm-pore diameter filters [**Error! Bookmark not defined.**].

5.1.2.5 Freeze/thaw cycle method

In this method, drugs are mixed and incubated with EXs at RT and the mixture is subsequently frozen at -80 °C or in liquid nitrogen and re-thawed at RT. This process is repeated for at least 3 cycles to ensure drug encapsulation [**Error! Bookmark not defined.**]. However, by this method EXs may aggregate, while the drug loading efficiency is generally lower than that of sonication or extrusion.

The freeze-thaw method has been used for catalase incorporation into EXs. The catalase solution was mixed with the EXs, incubated for 30 min, then rapidly freezed at −80° C, and thawed at RT. The freeze-thaw cycle was repeated three times [**Error! Bookmark not defined.**].

Sato *et al* proposed a new approach to prepare engineered hybrid EXs by fusing the membranes of EXs with those of liposomes, using a freeze-thaw method for this. Raw 264.7 cell-derived EXs were mixed with fluorescently labelled liposomes composed of phospholipids, pegylated-phospholipids and fluorescently labeled phospholipids. The mixtures were frozen in liquid nitrogen and thawed at RT for 15 min. To further examine the applicability of the membrane fusion technique, they investigated the fusion behavior between EXs bearing a specific membrane protein (HER2) and liposomes. HER2 and phosphorylated HER2 were detected in the EX-liposome mixtures, indicating that EX-liposome hybrids carrying specific proteins can be obtained by freeze-thaw methods. Cellular uptake studies performed using the hybrid EXs revealed that the interactions between the developed EXs and cells could be modified by changing the lipid composition, and consequently also the properties of liposomes [**Error! Bookmark not defined.**].

5.1.2.6 Saponin-assisted loading

Saponin is named from the Latin “sapo” which meaning “soap” and it is a surfactant molecule that upon incubation with EXs generates pores in their membrane through interaction with cholesterol, leading to increased EX-membrane permeability. Saponins can be composed of one to three straight or branched sugar chains, and are most often composed of D-glucose, L-rhamnose, D-galactose, D-glucuronic acid, L-arabinose, D-xylose or D-fucose. The sugar chain can contain from one to several monosaccharide residues, and is usually attached at C-3 [150]. The surface activity as well as some other biological functions of saponins (including their haemolytic activity), are attributed to their characteristic structural features and their amphiphilic nature which results from the presence of a hydrophilic sugar moiety and a hydrophobic genin (called sapogenin) [**Error! Bookmark not defined.**].

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Haney *et al* loaded catalase into EXs, ex vivo, using different methods. For the loading by the saponin-assisted method, a mixture of catalase and EXs (from Raw 264.7 macrophages) was supplemented with 0.2% saponin and placed on a shaker for 20 min at RT. This method resulted in high loading efficiency, sustained release and catalase preservation against proteases degradation. Moreover, although saponin is a surface-active agent, it did not degrade catalase, whose activity was preserved. [**Error! Bookmark not defined.**].

Saponin can also assist in loading other hydrophilic molecules into EXs. For saponin-assisted drug loading of hydrophilic porphyrins, EXs and drug were incubated with 0.1 mg/mL saponin at RT for 10 min. High drug loading was assessed by this method [**Error! Bookmark not defined.**].

However, there are concerns regarding the *in vivo* hemolytic activity of saponin. Haemolysis of red blood cells seems to result from the ability of saponin to form complexes with the cholesterol of the cell membrane, leading to pore formation, cell membrane permeabilization and alterations in the negatively charged carbohydrate portions on the cell surface; however, the exact mechanism of the haemolytic activity of saponins is not clear. In any case, when used as a method to assist drug-loading into EXs, the concentration of saponin should be limited to the minimum, and the EXs should be washed immediately after incubation with saponin [**Error! Bookmark not defined.**].

5.1.3. Comparison of different loading Methods

Table 1 summarizes the advantages/disadvantages of the aforementioned pre- and post-loading methods. In some reports, several methods were used for loading drugs into the same EXs, and thus the methods could be accurately compared:

Haney *et al.* used various methods in order to encapsulate catalase (a 240 kD protein) into EXs (exoCAT) for Parkinson's disease therapy. In fact they used: (a) incubation at RT, (b) saponin-assisted loading, (c) freeze-thaw cycles, (d) sonication and extrusion. In all cases, the size of the obtained catalase-loaded EXs was in the range between 100nm and 200nm. Sonication and extrusion or saponin-assisted loading resulted in stable EXs with high loading efficiency, sustained release, preservation against proteases degradation and proven (*in vitro* and *in vivo)* capability for targeted delivery [**Error! Bookmark not defined.**].

Fuhrmann *et al* applied several techniques for the loading of porphyrins with different hydrophobicities into EXs. They particularly used: (a) incubation of drug with EXs at RT, (b) electroporation, (c) saponin-assisted loading, (d) extrusion and (e) Hypotonic dialysis. Details for the exosome preparation according to methods (a) - (d) have already been discussed above. Hypotonic dialysis (method e) was performed by transferring the EX-drug mixture into dialysis membranes (cellulose ester, MW cut-off of 100 – 500 Da) placed in 10mM PBS (pH 7.4) and stirred at RT for 4 h. The loading efficiencies acquired were dramatically higher when co-incubation with 0.01% (w/v) saponin or hypotonic dialysis was used. For both of the latter two methods the loading of the drugs was up to 11-fold higher, compared with the efficiencies acquired by the other methods tested (incubation, electroporation and extrusion) [**Error! Bookmark not defined.**].

Kim *et al* tested different methods to load PTX into macrophage-derived EXs. The methods investigated were: a) incubation at RT, b) electroporation and c) sonication. The amount of PTX loaded into EXs was found to increase as follows: incubation at RT < electroporation << sonication. It was also found that reformation of the exosomal membrane upon sonication resulted in high loading

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efficiency and sustained drug release. The authors also proved that the mild sonication utilized did not significantly affect the protein and/or lipid content of exosomes [**Error! Bookmark not defined.**].

Goh et al used 4 different methods to load doxorubicin (DOX) into CVs: (a) Incubation at 37°C for 5 min, (b) incubation with 0.2% saponin for 5 min, (c) Incubation for 24-h at RT, (d) Freeze-thaw cycles (3 cycles). DOX loading by addition of 0.2% saponin resulted in the highest loading efficiency, but also resulted in increased CV size. When incubation at RT for 24 h was used, low loading efficiency and a large increase in size were realized, suggesting that aggregation of CVs occurred with Dox acting as an intercalating agent. Incubation of DOX with CVs at 37°C seemed to yield a balance between size and loading, and 40% of the drug was released after 36 h (in vitro). A much slower release (20% of the loaded-DOX after 36 h) was observed in the case of the saponin-assisted DOX-loaded CVs [**Error! Bookmark not defined.**].

Lamichhane et al. reported the use of sonication and electroporation as methods to load siRNA into EXs. They suggested that sonication may be a suitable alternative to electroporation for small nucleic acid loading in EVs. They also reported that the use of sonication may be superior to electroporation in respect to loading efficiency and siRNA aggregation [**Error! Bookmark not defined.**].

Table 1. Comparison of the early methods applied for drug loading into engineered EXs or EVs.

Method	Advantages	Disadvantages	Drug loaded / application
Treatment of parental cells with drug	- Relatively simple - Does not require addition of drug into the system	- Low loading efficiency - Drugs may be cytotoxic to cells	- Paclitaxel (Ptx) [**Error! Bookmark not defined.**]. - hydrophobic sensitizers (model drug) [141].
Incubation with drug	-Simplest method - Do not require extra equipement	- Low loading efficiency	Curcumin [**Error! Bookmark not defined.**,**Error! Bookmark not defined.**]; si RNAs [**Error! Bookmark not defined.**]; Porphyrins [**Error! Bookmark not defined.**]; Catalase [**Error! Bookmark not defined.**]; PTX [**Error! Bookmark not defined.**]; DOX [**Error! Bookmark not defined.**].
Electroporation	- Loading with large molecules possible	-Disrupts EX integrity - siRNA aggregation - Low loading efficiency (hydrophobic drugs)	siRNA[**Error! Bookmark not defined.**;**Error! Bookmark not defined.**]; Porphyrins [**Error! Bookmark not defined.**]; DOX [**Error! Bookmark not defined.**]; Dextran macromolecules [**Error! Bookmark not defined.**]; PTX [**Error! Bookmark not defined.**]
Sonication	- Increased loading efficiency (compared to other methods) - Applicable for small RNAs	-Potential deformation of membrane -Not efficient for hydrophobic drugs	PTX [**Error! Bookmark not defined.**]; Catalase [**Error! Bookmark not defined.**]; siRNA, miRNA, ssDNA [**Error!**

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			Bookmark not defined.;Error! Bookmark not defined.]
Extrusion	- High drug loading efficiency	-Potential deformation of membrane	Porphyrins [**Error! Bookmark not defined.**] Catalase [**Error! Bookmark not defined.**]
Freeze/thaw method	- Medium loading - Fusion of membranes possible [**Error! Bookmark not defined.**]	- Exosomes may aggregate - Low loading Efficiency	*Catalase* [**Error! Bookmark not defined.**]; DOX [**Error! Bookmark not defined.**]
Saponin-assisted loading	- High drug loading, compared to the other methods used in early reports	- Generates pores in EXs -Haemolysis/Toxicity concerns [**Error! Bookmark not defined.**] - Saponin conc. Control & Washing required	*Catalase* [**Error! Bookmark not defined.**]; Porphyrins [**Error! Bookmark not defined.**]; DOX [**Error! Bookmark not defined.**]

5.2 Surface Modification Methods

The proteins expressed on the surface of EXs compose an integral variable in EX biodistribution and cell-targeting capabilities. Modification of surface proteins aims to improve the targeting efficiency of EXs to tissues and cells types of interest. EX components can be engineered at the cellular level or they can be modified after their isolation [151].

At the cellular level, one approach is the use of cellular transgene expression to develop a modified EX membrane protein with specific signaling or homing properties. This can be done by inserting the coding sequence of the ligand of interest in frame to the coding sequences of the signal peptide and the amino-terminus of the peptide of the specific membrane protein. By using a gene transfer vector, such as retroviral or lentiviral vectors, this fusion cassette is expressed in parental cells and consequently the transduced cells generate EXs expressing the peptide of interest on their surface. The most commonly modified transmembrane proteins include tetraspanins (CD63, CD9, CD81), lysosome-associated membrane glycoprotein 2b (Lamp-2b), glycosyl-phosphatidyl- inositol (GPI), platelet-derived growth-factor receptors (PDGFRs), and lactadhein (C1C2 domain) [152].

Alvarez-Erviti et al. [**Error! Bookmark not defined.**] fused the rabies viral glycoprotein (RVG) with Lamp-2b to specifically deliver EXs to neurons and glia. Targeted EXs successfully crossed the blood-brain barrier and delivered their silencing RNA cargo, resulting in BACE1 knockdown. In a similar way, Tian et al. managed to modify immature dendritic cells to express Lamp2b fused to αv integrin-specific iRGD peptide [**Error! Bookmark not defined.**]. Another study by Liu, et al., showed that engineering the membrane surface of EXs to express the RVG peptide effectively delivers opioid receptor mu siRNA into the brain [153].

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Several examples of methods used to modify the surface of EXs, after their isolation are available. Nevertheless, the reaction conditions for EX surface modification/functionalization must be strictly specified in order to avoid EX disruption and aggregation due to inappropriate temperature, pressure and/or osmotic stress. Among the methods that are available for cross-linking reactions, the copper-catalyzed azide-alkyne cycloaddition (CCAAC), known as click chemistry, has several advantages. Click reaction is achieved by the reaction of an alkyne chemical group and an azide chemical group to form a triazole linkage. The reaction works both in organic and aqueous media, making it ideal for EXs (or liposome) surface modification, while it is also rapid, efficient and offers chemo-selectivity over the conjugation site, compared to other traditional cross-linking reactions [154-156]. Some examples of click chemistry modification of EXs are summarized below.

Smyth *et al* attached fluorescent molecules to the surface of mouse 4T1 breast cancer exosomes, by efficiently functionalizing exosomes with terminal alkyne groups. In order to do this, the amine groups of the exosomal proteins (or the exosomal membrane lipid phosphatidylethanolamine) were cross-linked with the carboxyl group of 4-pentynoic acid using carbodiimide activation. The alkyne-functionalized EVs were conjugated with azide-fluor 545 using click chemistry [**Error! Bookmark not defined.**]. No differences in morphological and functional properties were found, suggesting that modification by click chemistry does not alter EX characteristics, allowing the incorporation of exogenous molecules on the surface of EX. In order to investigate the extent of exosomal protein modification with alkyne groups, they used liposomes with surface alkyne groups of a similar size and concentration to EXs and estimated that approximately 1.5 alkyne groups were present for every 150 kDa of exosomal protein.

Based on previous reports of Lakrishnan et al., Lee *et al* used membrane fusogenic liposomes (MFLs) to insert azide-lipids in the plasma membrane [157]. Lipid-azide was efficiently delivered to the plasma membrane via MFLs and subsequently loaded into the membrane of EXs that were formed. More specifically, cancer cells were treated with liposomes bearing lipid-azide (azide-MFLs) and the EXs produced from the cells showed significantly marked fluorescence when reacted with DBCO-carboxyrhodamine, verifuing that EXs containing azide-lipids through liposome-based cellular engineering, could be decorated easily with various functional moieties by using copper-free click chemistry. EXs bearing azide groups were further decorated with targeting moieties in order to allow their specific delivery to cancer cells. EXs were also packaged with chemotherapeutics through liposome-based cellular engineering. Paclitaxel (PTX) (a hydrophobic anti-cancer drug) and tirapazamine (TPZ) (a hydrophilic agent that becomes cytotoxic selectively in hypoxic conditions) were loaded in EXs and delivered to target cells [**Error! Bookmark not defined.**].

Besides covalent bonding on EXs-surface, some non-covalent strategies have additionally been used in order to provide stable modification of the EX surface. In general, multivalent electrostatic interactions and receptor-ligand binding are commonly used for surface modification of EXs.

Multivalent electrostatic approaches typically involve a highly cationic species adhering to negatively-charged functional groups present on biological membranes. Nakase and Futaki used electrostatic interactions to bind cationic lipids to the surface of EXs. Such interactions produced EXs with a positively-charged surface potential that enhanced binding and uptake into recipient cells. [**Error! Bookmark not defined.**]. However, there are concerns that certain cationic nanomaterials can cause cytotoxicity [158]. Moreover, cationic nanomaterials are typically taken up into the cell *via* endocytosis, leading to lysosomal degradation and poor EX loading [**Error! Bookmark not defined.**].

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Receptor-ligand binding was achieved by Qi et al. who developed a dual-functional EX-based superparamagnetic nanoparticle cluster as a targeted drug delivery vehicle for cancer therapy. They efficiently conjugated transferrin-superparamagnetic nanoparticles to the surface of blood derived EXs, by targeting the tranferrin receptors on the EX surface [159].
An alternate approach to attach proteins on EXs, relies on the trafficking of oligomeric membrane-anchored proteins to EXs. The exogenous protein is fused with two domains that mediate aggregation and membrane localization, e.g., by adding a myristoyl- moiety. This approach was validated using a GFP reporter protein [160]. However, the potential interference of the protein oligomer formation with the functionality of the proteins in the target cells remains of concern [161].

Very recently, a holistic approach was proposed as a method to facilitate the isolation together with loading and surface modification (for improved targeting efficiency) of EXs. Based on the fact that loading and targeting of patient-derived EXs should be carried out without altering the EX surface Gao et al. demonstrated that a phage display identified peptide (CP05) may enable targeting, cargo loading, and isolation/purification of diverse-origin-EXs (including patient-derived EXs), through binding to CD63—an exosomal surface protein. Indeed, the systemic administration of EXs which were loaded with CP05-modified, dystrophin splice–correcting phosphorodiamidate morpholino oligomer (EXOPMO), resulted in 18-fold increased dystrophin protein in dystrophin-deficient mdx mice compared to the control. Loading CP05-muscle– targeting peptide on the previous EXs further increased dystrophin expression in muscle with functional improvement and without any detectable toxicity. Thus Gao et al. demonstrated that an exosomal anchor peptide can be used as a tool for holistic EX engineering, probing gene function in vivo, and targeted therapeutic drug delivery [162].

5.3. Microfluidic methods for Engineering of EX and EX-mimetics

As mentioned above, microfluidic technologies and lab-on-chip methods have been recently applied in some cases for (i) the isolation/ purification of EXs form cell media or other sources/biological fluids [**Error! Bookmark not defined.-Error! Bookmark not defined.**] and (ii) for the preparation of CVs from whole cells [**Error! Bookmark not defined.**].

It is also known today that microfluidic methods are applied by many labs for the preparation of liposomes and other types of nanoparticles which are used for drug delivery. In fact it has been proven that even ligand-targeted liposomes can indeed be prepared in such “one step” fully automated, controllable and scalable microfluidic systems [163-164]. Such technologies being scalable, easily automated and highly reliable can indeed assist the development of roadmaps for manufacturing of artificial EX-mimetics, accelerating their translation into pharmaceutical products. Whether such methods can also assist efforts to increase the yield of EX isolation, this remains to be explored.

6. Potential clinical applications of EXs and EXs-mimetics

6.1 Current status

Up-to-date, most of the reports related with the use of EXs-like vesicles for drug delivery concern the use of EXs derived from cells, such as cancer cells, dendritic cells, MSC, from biological fluids

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[27,29,53-64] or from other types of sources such as milk of fruits [69,70,**Error! Bookmark not defined.**]. CVs and totally artificial EXs (or EX-inspired liposomes) have been studied in a limited number of cases as drug delivery systems, since this specific research topic has been initiated very recently. Most of the studies are actually early preclinical proof-of-concept studies, to prove: (i) the possibility of EX-like vesicles to be loaded with sufficient amount of drugs; (ii) their capability to retain the drug under in vivo simulating conditions; and (iii) their potential to deliver the drugs in a functional state to the target cells of interest, at higher amounts compared to the free drug or other types of nanocarriers. In several cases, in vivo studies have also been carried out in appropriate disease models, verifying the in vitro findings.

The pre-clinical studies performed up-to-date concerning the usage of EX-like vesicles for drug delivery are aiming to treat several potential diseases such as different types of cancers [165-167], cardiovascular diseases [168, 169], Parkinson's and Alzheimer's disease [170] as well as other neurodegenerative diseases [171, 172], musculoskeletal diseases [173], kidney and diabetes-related pathologies [174], and others.

The potential of such EX-like vesicles to overcoming the BBB has been also under investigation [175, 176]. In addition to some examples mentioned before, in which EXs were engineered to attach brain homing peptides or antibodies that target the brain endothelium in order to enhance their brain targeting efficiency, in a recent study it was demonstrated that there is no need for such modification to penetrate the BBB. Indeed it was proposed that naïve macrophage EXs can utilize, the integrin lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1), and, additionally the carbohydrate-binding C-type lectin receptors, to interact with the BBB microvessel endothelial cells. It was demonstrated in vivo that naïve macrophage-EXs, cross the BBB and deliver a cargo protein, the brain derived neurotrophic factor (BDNF), to the brain following i.v. injection. Furthermore, the delivery is enhanced in the presence of brain inflammation, a condition which is often present in CNS diseases. Thereby, naïve macrophage-derived EXs can function as nanocarriers for brain delivery of therapeutic proteins to treat CNS diseases [177].

In terms of the current clinical studies involving EX-like materials, a search in the US-NIH clinical trial database (https://clinicaltrials.gov/), using the word "exosomes" resulted in 98 clinical studies, most of which being related with biomarker identification and diagnosis / prognosis or therapy of various types of cancers. 10 of the therapeutic studies are listed in **Table 2**, as examples of cases that are related with their potential use for the delivery of drugs. From these examples only one is directly involved with the application of engineered EXs as a DDS, specifically the "Study Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Colon Cancer Tissue"which involves the use of plant-derived EXs for the delivery of curcumin (Table 2). The background of this study is related with the work from the James Graham Brown Cancer Center that suggests the use exosomes as a delivery vehicle to overcome all the major obstacles of using curcumin as an anti-inflammatory agent, providing increased stability, solubility, and bioavailability of curcumin [178]. The work was further extended to define the resource that can supply a large quantity of EXs with a maximum binding capacity of curcumin, and emerging data indicated that fruits release exosome-like particles, that could strongly bind to many hydrophobic drugs including curcumin, and are taken up by the intestine cells as well as the immune cells in the intestine. These results suggest that these fruit-derived exosomes are potentially used as a delivery vehicle to treat intestinal disorders. Moreover, both fruit EXs and curcumin should not generate any side-effects since they are consumed by humans daily. In this clinical trial:" the effect of exosomally delivered curcumin on the immune modulation, cellular metabolism, and phospholipid profile of

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normal and malignant colon cells in subjects who are undergoing surgery for newly diagnosed colon cancer will be characterized. In selected subjects, the effect of exosomally delivered curcumin on the production of cytokines, the changes of immune cells, and glucose metabolism by administration of 13C-glucose prior to surgical resection will also be characterized". Although the study is active it has not yet started recruiting, thereby no details or results are listed.

Table 2. List of the clinical studies involving the usage of EXs as DDs, or related studies. Study Titles are in **bold** for the most relevant studies.

Status	Study Title	Conditions	Interventions	Phase	NCT Number
Not yet recruiting	Allogenic Mesenchymal Stem Cell Derived Exosome in Patients With Acute Ischemic Stroke	Cerebrovascular Disorders	Biological: exosome	Phase 1 Phase 2	03384433
Enrolling by invitation	Effect of Plasma Derived Exosomes on Cutaneous Wound Healing	Ulcer	Other: plasma-derived exosomes	Early Phase 1	02565264
Active, not recruiting	**Study Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Colon Cancer Tissue**	Colon Cancer	curcumin Curcumin conjugated with plant exosomes	Phase 1	01294072
Recruiting	Dendritic Cells-Derived Exosomes in Human Sepsis	Sepsis	Drug: Antibiotics		02957279
Not yet recruiting	Plant Exosomes and Patients Diagnosed With Polycystic Ovary Syndrome (PCOS) 17	Polycystic Ovary Syndrome	Ginger exosomes Aloe exosomes Placebo	Not Applicable	03493984
Unknown †	Effect of Microvesicles and Exosomes Therapy on β-cell Mass in Type I Diabetes Mellitus (T1DM)	Diabetes Mellitus Type 1	Biological: MSC exosomes.	Phase 2 Phase 3	02138331
Not yet recruiting	**iExosomes in Treating Participants With Metastatic Pancreas Cancer With KrasG12D Mutation**	KRAS NP_004976.2:p.G12D Metastatic Pancreatic Adenocarcinoma	Drug: Mesenchymal Stromal Cells-derived Exosomes with KRAS G12D siRNA	Phase 1	03608631
Recruiting	MSC-Exos Promote Healing of MHs	Macular Holes	Biological: exosomes derived from mesenchymal stem cells (MSC-Exo)	Early Phase 1	03437759
Active, not recruiting	Edible Plant Exosome Ability to Prevent Oral Mucositis Associated With Chemoradiation Treatment of Head and Neck Cancer	Head and Neck Cancer Oral Mucositis	Dietary Supplement: Grape extract Drug: Lortab, Fentanyl patch, mouthwash	Phase 1	01668849

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Completed	**Trial of a Vaccination With Tumor Antigen-loaded Dendritic Cell-derived Exosomes**	Non Small Cell Lung Cancer	Biological: Dex2	Phase 2	01159288

From the other studies listed in Table 2, the most relevant with the scope of this review include:

A phase I trial entitled "iExosomes in Treating Participants With Metastatic Pancreas Cancer With KrasG12D Mutation", will study the best dose and side effects of **mesenchymal stromal cell-derived exosomes loaded with KrasG12D siRNA (iExosomes)** for treating participants with pancreatic cancer which have the KrasG12D mutation. This is a Phase I study, that has not yet staring to recruit patients.

Gustave Roussy and Curie institutes have developed an immunotherapy involving metronomic cyclophosphamide (mCTX) followed by vaccinations with tumor antigen-loaded dendritic cell-derived exosomes (Dex). Phase I trials showed the safety and feasibility of Dex vaccines but no induction of T cells could be monitored in patients. Since 2007, a new process for isolation of second generation Dex with improved immune stimulatory capacities was validated, thereby the group proposed a phase II maintenance immunotherapy study in advanced unresectable NSCLC patients responding or stabilized after induction chemotherapy with Dex-based treatment, in order to improve the median progression-free survival (PFS) rate at 4 months in these patients.

It is worth emphasizing that all of the products currently under clinical evaluation involve engineered EXs, and no other types of EX-like vesicles is currently in the clinic.

Nevertheless, although most of the initial clinical trials are ongoing, the use of EVs for therapeutic or drug delivery applications may be limited due to the potential for "undesired off-target effects" and also "dilution effects" which may occur following systemic administration, and affect their ability to reach their intended target sites. Recently in an effort to overcome such off-target effects and exploit EV therapeutic potential, EVs were embedded into implantable biomaterials, for local delivery of enzyme prodrug therapy-type (EPT) therapeutics. In other words EVs were used as smart carriers to stabilize the enzymes in a hydrogel where local controlled conversion of a prodrug to the active compound would take place. In the specific case an anti-inflammatory product was studied and EVs were demonstrated to confer comparable or superior antiinflammatory activity to that of synthetic carriers[179].

6.2 Challenges and Future Perspectives

Summarizing the previous parts, the main challenges towards unlocking the potential of EX-like vesicles, regardless of their type (engineered-EXs or EX-mimetics [CVs or artificial liposomes]), towards the development of novel targeted drug delivery systems with enhanced targeted efficiency, are related to the following factors : (i) the abundance of starting material for their construction and their preparation yield; (ii) the loading efficiency of drugs; (iii) the blood circulation time, assuming that this determines their targeting efficiency; (iv) the inherit targeting efficiency of the system selected and how this may be affected by various engineering methodologies; (v) methods/roadmaps for scalable, repeatable manufacturing.

For *EXs* and *CVs*, a first and very important question is whether the use of such vesicles from allogenic sources will initiate immune responses and/or if they will be safe. Providing answers to the

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later questions are particularly urgent for cancer-cell-derived EXs and CVs, especially since they are probably the vesicles with the highest organotropism. Despite the recent findings about the preferable immune properties of macrophage-derived and other types of EXs [100-102], it is not yet clear which types of EXs can be safely used as DDSs with broad applicability. A key point in this direction will be to classify the vesicles derived from different sources (different types of cells, biological fluids etc), in terms of their organotropism, immunogenicity and biodistribution, following administration by enteric and parenteric routes. Such classifications will be particularly useful for (a) Selection of the optimal type of vesicle source for each specific drug targeting application, and (b) Acknowledgement of the challenges that will be encountered in order to achieve the specific goal. In other words, will the vesicles need to be engineered to increase their blood circulation time, their targeting efficiency, or perhaps both?

For ***Engineered EXs*** a challenge remains today to increase their production yield. The low yield of isolation of EXs from cells, together with safety issues is the main reason for the exploitation of safer and more abundant EX sources, such as fruit and milk. In the later cases, it is not clear if there are any advantages of such systems compared to targeted-liposomes, especially if i.v. administration will be used. In engineered-EXs drug loading also needs to be improved, by deeper exploitation of more efficient and perhaps scalable and reproducible microfluidic methods, which have been already explored as methods to increase the yield of EX-isolation [180]. The development of future lab-on-chip approaches that will include parts for microfluidic isolation of EXs, and separation by molecular profiling, followed by parts for drug loading by microfluidic mixing may be the solution for high-yield production of drug-loaded EXs. However it remains to be verified if the organotropic EX-surfaces will be preserved after such manipulations.

Between EXs and CVs, CVs seems to have several advantages for clinical application, the most important if which are: (i) the high yield and (ii) the simple purification processes required for their production. These two advantages may be the ones that will perhaps facilitate the construction of a roadmap for the rapid manufacturing of engineered CVs from autologous sources as drug targeting systems. This may be technically possible, providing that the engineering methodologies will be such that the important structural components of the parental cells will be retained on the vesicles produced, for preservation of organotropism, and that at the same time any components that may result in detrimental biological effects (e.g. oncogenesis, etc., if the parental cells are tumor cells) will be extracted from the final CVs produced. In such cases, as suggested also before [121] perhaps the high organotropism of such CVs will prevail and the required amount of drug for a sustainable therapeutic effect will rapidly reach the target sites, regardless of a potentially low circulation time in the blood. Thereby, no further manipulation of the CVs will be required for increase of their blood circulation time, providing the additional advantage of avoiding any potential side effects caused by hydrophilic coatings which are used in the liposome field (such as PEG).

The development of ***artificial EXs*** or ***EX-inspired liposomes*** may be preferable, in order to avoid potential safety problems related with the use of allogenic vesicles from any source, and especially from cancer cells, as well as the low yield and time required for producing vesicles from autologous sources. Additionally, the manufacturing of such systems is already well studied and microfluidic-based approaches have been already optimized and ready to use GMP-compatible equipment for scale up production is available. However, although in theory the identification of the protein and lipid components of EXs may be possible today, by the use of advanced proteomic and lipidomic tools, it remains to be verified if such approaches will lead to the development of liposomes with high targeting efficiency and also if their circulation time will be sufficient for reaching their in vivo targets. Additionally, it is not currently clear how the specific components that are responsible for the high organotropism of EXs can be identified; also it has never been studied if perhaps CVs can be used for the same purpose. In fact, perhaps the use of CVs (and not EXs) may be preferential, since they are thought to contain the most organotropic-important components of the cell-membrane [133]. Another issue that needs to be explored is if the proteins are embedded in the membranes with specific conformation, and if this is important for their organotropism.

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Finally, several other issues have not been considered up-to-date, such as the stability of engineered EXs, CVs, or artificial-EXs, since it is not readily straightforward to predict how stable the protein parts of their membrane will be during storage. Furthermore, nothing is known about the potential to lyophilize such vesicle dispersions, as done with liposomes.

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