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Extracellular vesicles as delivery systems at nano-/micro-scale

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Abstract

Extracellular vesicles (EVs) have shown significant promises as nano-/micro-size carriers in drug delivery and bioimaging. With more characteristics of EVs explored through tremendous research efforts, their unmatched physicochemical properties, biological features, and mechanical aspects make them unique vehicles, owning exceptional pharmacokinetics, circulatory metabolism and biodistribution pattern when delivering theranostic cargoes. In this review we firstly analyzed pros and cons of the EVs as a delivery platform. Secondly, compared to engineered nanoparticle delivery systems, such as biocompatible di-block co-polymers, rational design to improve EVs (exosomes in particular) were elaborated. Lastly, different pharmaceutical Loading approaches into EVs were compared, reaching a conclusion on how to construct a clinically available and effective nano-/micro-carrier for a satisfactory medical mission.

Graphical abstract

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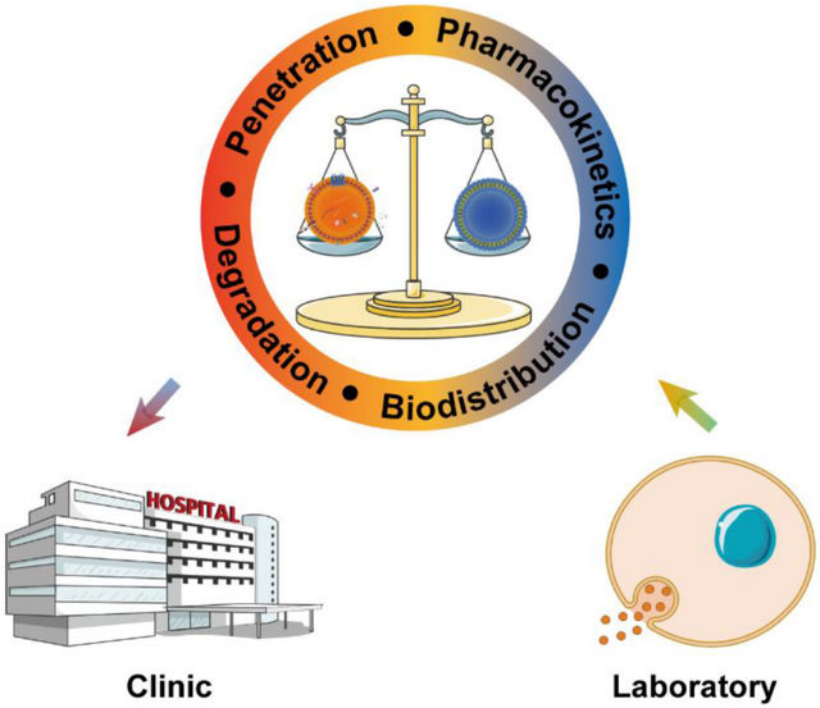

Schematic illustrations of natural extracellular vesicles versus artificial particles *en route* to a next-generation drug delivery system.

Keywords

Nanomaterials; Extracellular vesicles; Exosomes; Drug delivery

1. Introduction

Since their discovery, extracellular vesicles (EVs), nano- and micro-scaled biogenic particles, have emerged as versatile communicators among cells, weaving a regulatory network of cell signaling[1]. The biogenesis of EVs has not been fully explained, but commonly recognized pathways produce three major EVs, including (i) exosomes (30–200 nm): vesicles first formed in the multivesicular bodies (MVBs) and then released from MVBs when fusing with the plasma membrane; (ii) microvesicles (MVs) (200–2000 nm): outward germination of plasma membrane and direct formation of vesicles; (iii) apoptotic bodies (>1000 nm): vesicles released by senescent or apoptotic cells[2]. In fact, the determination of the exact type of EVs is difficult, and needs specific equipment to identify with a series of standardized characterizations established by the International Society for Extracellular Vesicles (ISEV)[3]. Simultaneously, exosome had commonly been used as a generic alternative to EV before ISEV issued the guidelines. Although we agree to the nomenclature recommended by ISEV, in this review we continue to use the terms mentioned in the original publications as the exact EV dealt with is unknown[4].

EVs have been demonstrated to contain plenty of endogenous biomolecules, such as proteins, nucleic acids and lipids[5]. Especially, exosomes have worked as a powerful tool to reverse the pathological states in many diseases in lieu of cell therapy[6]. With rapid development in their separation and purification methods[7], exosomes are further divided into three types based on their hydrodynamic radii: large exosome (Exo-L, 90-120 nm), small exosome (Exo-S, 60-80 nm), and exomeres (<50 nm), respectively. Recently, rapid and accurate detection of disease-related exosomes has enabled early diagnoses of many latent and malignant diseases in precision medicine[8, 9]. Among all subtypes of EVs, exosomes that fall in a comparable size range to engineered nanomaterials have been widely used as delivery vehicles in the upsurging nanomedicine research[10]. For this reason, modified exosomes with appropriate surfaces, structures and contents become a hot pick for targeted drug/biomolecular delivery[11].

However, several challenges remain. Firstly, EVs with different cell origins have significant heterogeneity. For this reason, EVs possess a variety of biological functions and inherit distinctive physicochemical traits from their parental cells. Therefore, for each specific EV to be applied as the delivery platform, this requires an overall understanding about its cell origin and biochemical profile. Secondly, current separation techniques that enrich EVs from tissue/cell cultures or *ex vivo* samples have a mixture of effectiveness, so state-of-the-art methods of rapid particle enrichment with the technical reproducibility are greatly needed. Thirdly, given the specific biogenesis, the customized modifications of EVs with minimal alteration to improve their physical, chemical and biological characters to accomplish a desired pharmacokinetics and biodistribution are preferred [12, 13].

Here we provide a concise review at recent advances in EV-based drug/biomolecular delivery, with a focus on a diversity of technical approaches to carry pharmaceutical or imaging cargoes in a variety of biological systems. Through this review the major routes to acquire biogenic EVs or engineered nanoparticles as respective delivery systems are summarized before we compare those natural and artificial nanomaterials to rationalize the design of engineered EVs for an enhanced delivery system. The purpose of this rational design is for a next-generation delivery platform for clinical translation and approved application. In this review, while we discuss all types of EVs, we keep a focus on exosomes.

2. Biogenesis and destination of EVs

Insofar our understanding towards the life cycle of EVs remains preliminary as many fundamental questions await to be answered. In this part, the biogenesis and destination of EVs are discussed (Fig.1), along with the important physicochemical properties of EVs, including their size, morphology, surface biomarker and mechanical properties.

Apoptosis is a gene-regulated program of cell suicide, through which the harmful and senescent cells are eliminated to maintain the stability of the normal cell population in the tissue[14]. As one of three EVs, apoptotic bodies are merely released during the apoptotic death of cells, when they carry molecular signals of ‘find-me’ and ‘eat-me’, to be further cleared by the macrophages or adjacent epithelial cells[15]. Alternatively, the exact machinery of MV biogenesis remains unknown, but with no doubt it starts from the

formation of lipid microdomains in the cell membrane, followed by sorting of biomolecular cargo into MVs lumen[16]. During this process lipid translocation and lipid flipping may occur on the cell membrane, indicating the formation of MVs[17]. Regulated by signaling molecules, including small GTPase ADP-ribosylation factor 6 (ARF6), Rab GTPase11 (RAB11) and rho-associated protein kinase (ROCK), MVs can directly be unleashed from the plasma membrane to the outside of the cell via budding[18].

Shuttling between cells, exosomes are generated and degraded inside the cytoplasm, as biological components found in the exosomes are derived from the Golgi apparatus or plasma membrane. At first, cells produce early endosomes through endocytosis mediated by lipid rafts, gradually evolving into late endosomes when the endosomal membrane buds inward and engulfs nucleic acids, proteins and others to form the intraluminal vesicle (ILV). When a sufficient amount of ILVs is formed in the endosome, it transits into MVB[19]. In this duration, endosomal sorting complex required for transport (ESCRT) is a driving force for membrane shaping and scission, leading to the formation of MVBs[20]. Nevertheless, many recent studies showed that both ESCRT-dependent and ESCRT-independent pathways play their indispensable roles in the MVB formation[21]. After maturation in the cytoplasm, MVBs become either fused with the plasma membrane to release exosomes or merged by the lysosome to degrade. Hence, the biogenesis and intracellular release of EVs are a result of inter-cellular exchange regulated by a network of cell signaling.

The uptake of exosomes by receiving cells is energy-consuming, and both time-dependent and dose-dependent, in which exosomes interact with their target cells through multiple mechanisms, relying on the specific cell types in both origin and destination. Here four different uptakes are explained as follows: (i) Phagocytosis mainly occurs if exosomes are internalized by cells with significant phagocytic ability. (ii) Micropinocytosis is a pathway that generates pseudopodia to wrap exosomes into cells. (iii) Endocytosis includes receptor-mediated endocytosis, caveolae or clathrin-dependent/-independent endocytosis, and lipid raft-dependent endocytosis. Typically, through a receptor-mediated uptake, specific ligands enriched on the surface of exosomes would interact with homologous receptors on the plasma membrane of recipient cells. (iv) Fusion, particularly occurs in acidic conditions, such as tumor microenvironment. Current evidence suggests that fusion may not be the main pathway for exocrine entry, but it takes place under low pH condition[22].

It is in the know that the size[23], shape[10] and surface charge[24] could affect the internalization of nanomaterials by cells. The rigidity of nano-/micro-materials, whether natural or engineered, is also an important parameter for cell engulfment [25]. Nature has endowed exosomes with many unique properties, including minimized dimension, enriched surface proteins and equipped molecular contents, that suggest their capability of being excellent carriers for drug delivery (Fig.2). Next, we consider how biophysical aspects impact the performance of EVs in content delivery.

3. Biophysical and mechanical properties of EVs

The mechanics of EVs plays important roles in at least two critical aspects of drug delivery: 1) uptake by cells and 2) transport through tissues. Consideration of their mechanical

properties and physical interactions with the biological systems can help optimize the application of EVs for drug delivery.

Various studies have shown that biophysical properties impact cell-nanoparticle interactions. The size, shape, and elasticity of micro-/nano-scaled particles influence their ability to be delivered into cells (Fig.3). The endocytosis rate and the total amount of uptaken nanoparticles are dependent on particle size in a non-monotonic manner, with ~50 nm diameter being close to optimal for maximum uptake for spherical gold nanoparticles[26]. Simultaneously, high aspect ratio (the ratio of length over width) in their morphology reduce uptake efficiency of nanoparticles[26]. Moreover, recent work has shown that the mechanical properties of nanoparticles are related to uptake efficiency. Soft nanoparticles have been demonstrated to be more effectively internalized by tumor and non-tumor cells than stiff nanoparticles[27]. This study utilized nanolipogels (NLGs) that are nanosized particles, consisting of a lipid bilayer encapsulating alginate with tunable elasticity (45 kPa to 19 MPa). The alginate interior can be crosslinked or uncrosslinked resulting in stiffer or softer NLGs, respectively. The proposed mechanism is that softer NLGs enter cells via fusion, while stiffer NLGs enter cells through endocytosis. However, another study, using lipid-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles when elasticity was varied by different water inclusion (0.76-1.20 GPa), exhibited an opposite trend, where increased cellular uptake was associated with the stiffer PLGA-based nanoparticles[28]. This points to a possible machinery of cellular uptake governed by particle elasticity, where a biphasic response may emerge. That is, fusion pathway is restricted to particles at low stiffness regime (~MPa), whereas particles at high stiffness regime (~GPa) depend on endocytosis.

Here, the size and lipid bilayer structure of NLGs resemble natural EVs. It was reported that EVs from human neural stem cells possessed a value of elastic modulus = 24.9 ± 21.2 MPa[29]. Furthermore, a variety of EVs with different origins have been measured with a range of Young's moduli from <1 MPa to >1000 MPa[30]. Intriguingly, exosomes derived from non-malignant, metastatic malignant to non-metastatic malignant cells displayed significantly decreasing stiffness, corresponding to their increasing endothelial disruption and transendothelial penetration[31]. This may be explained by the findings that EVs secreted by different parent cells can distinguish themselves by their lipid and metabolite compositions, therefore owning characteristic mechanical properties[32]. In addition, physical manipulation of EVs, such as ultracentrifugation and sonication, can modify their elasticity to some extent [29]. Also, surface modification (e.g., through polymer or lipid functionalization) might adjust mechanical properties of EVs as did for synthetic particles, such as changing the polymer type, length, density/coverage[33, 34]or varying the phospholipid composition/phase behavior[35]. Hence, similar to engineered nanoparticles whose mechanical properties would be determined by a series of physicochemical features, including particle size, shape, chemical composition, and surface ligand[36, 37], natural EVs can be modified in many ways to alter their mechanical properties, so tuning their delivery efficacy.

In order to reach their target cells, EVs must physically penetrate into regions of interest. Delivery of cargo deep inside tissue typically requires properties that facilitate transport through dense, extracellular matrix (ECM)-rich microenvironments with small pore sizes.

EVs can often be larger than these pores, thus presenting an obstacle in the length scale. Several considerations are important toward addressing this. Tissue microenvironments are non-elastic, capable of storing and dissipating energy and being remodeled under applied force[38]. A recent study showed that in purely elastic gels with small pores, EVs become physically confined, whereas in stress-relaxing gels EVs can rapidly diffuse through them, as pores can enlarge due to stress-mediated relaxation[39]. In addition to pores enlarging, EVs can shrink in size due to water efflux if proper membrane channels are present (e.g., aquaporins). Aquaporins are cell membrane channel proteins that mediate fluid exchange, while suppression of AQP1 (aquaporin-1) expression significantly decreases EV diffusion through stress-relaxing gels[39], suggesting that EV deformation (with volume reduction) mediated by fluid expulsion is an important interstitial EV transport mechanism. Thus, consideration of the non-elasticity of the target tissue ECM and the membrane channel expression on EVs is important in optimizing for their delivery through dense tissues with small pores.

While rapid diffusive transport facilitates small EV dissemination, this mode may be limited for large, micron-scaled EVs. However, certain cell fragments whose sizes are even larger than exosomes and MVs, known also as microplasts, are shed during cell spreading and migration[40-43]. These giant version of EVs contain an active cytoskeleton, complete with the actomyosin and adhesion machinery, that enables them to exhibit active and persistent motility, in a manner similar to cell migration[40-43]. Whole cells are able to navigate through dense ECMs with pore sizes smaller than the cell diameter[44]. In addition to matrix degradation by proteases, cells can also migrate in these confining microenvironments via MMP-independent mechanisms, notably by cytoskeletal force-driven processes leading to cell and matrix deformations[44-46]. Cells can physically squeeze through tight spaces smaller than the cell nucleus through active mechano-chemical processes[47, 48]. Cells can also mechanically remodel the ECM, which has viscoplastic properties, via dynamic cell protrusion-contraction activities[49, 50]. These abilities are mediated by mechanical forces and conferred by the cytoskeleton. Microplasts, with active cytoskeletal components, have the basic contractile and protrusion machinery, as demonstrated by their migratory capabilities, in addition to being much smaller than cells. Those findings confirmed that the viscoelasticity of the ECM and the deformability of EVs govern physical transport through dense tissues. It is highlighted that mechanical properties are important considerations when designing and optimizing EVs for pharmaceutical delivery.

Mechanical properties of EVs can be measured by atomic force microscopy (AFM)[51, 52]. Those properties, including size and stiffness, can be extracted[30, 53-56]. Nanoindentation driven by a piezoelectric stage is applied by the AFM tip onto individual vesicles, and force-distance curves (FDCs) are measured as the tip compresses the vesicle. The slope of the FDC is related to the EV stiffness, which is often reported at the small indentation, linear regime. At larger indentation, nonlinear effects and discontinuities occur. Discontinuities are putatively due to penetration through the lipid bilayer. FDCs demonstrate hysteresis after large indentations. Additionally, some vesicles recover and some do not after large deformations, as measured by repeated indentations[56]. Intrinsic mechanical properties (e.g. Young’s modulus or bending modulus) which are independent of vesicle size, can be extracted via different models, based on appropriate assumptions. The Hertz Model

assumes an isotropic elastic material, the Thin Shell Theory assumes a hollow shell, and the Canham-Helfrich Theory assumes a fluid membrane. More details and assumptions of each model are discussed in prior work[30]. The Canham-Helfrich model appears to describe red blood cell (RBC) vesicles reasonably well, and a bending modulus on the order of around 15kBT has been computed for these vesicles[56]. It has also been shown that diseased RBC vesicles, from patients with hereditary spherocytosis, are softer than those from normal donors[56].

The above descriptions are primarily for small EVs, but AFM can be used to measure both small and large EVs. For larger EVs, particularly microplasts, with key cytoskeletal components such as actin, myosin II, and their associated binding and interacting proteins, the mechanical properties most likely resemble those of cells, but without their nuclei. The cytoskeleton is active when supplied with ATP, with many dynamic processes such as actin turnover and myosin II contractions (which are regulated by intracellular signaling and extracellular cues) that can impact its mechanical properties[57-62]. F-actin gels, which is a network of filaments, is known to exhibit nonlinear strain-stiffening elasticity based on rheometry[63]. Intact and live cells demonstrate poroelastic, viscoelastic, and nonlinear properties as measured by AFM[64, 65]. Thus, cytoskeletal content, concentration, and activities within large EVs significantly impact their mechanical properties. Fluid-dominant EVs and cytoskeleton-filled EVs are expected to exhibit fundamentally distinct mechanical behaviors. Furthermore, microplasts can have mechanical properties that change over time, as the cytoskeleton undergoes active remodeling and as the supply of ATP is gradually reduced.

4. The life cycle of EVs

Artificial drug delivery system (e.g., liposomes, polymers, biomimetic particles) or sustained release systems (e.g., hydrogels, artificial cells) have been actively studied for biological properties *in vivo*[66, 67]. Insights into EV's *in vivo* journey will help to enhance our understanding of EV biology as well as to improve our perception to modify EVs for a better construction of exceptional delivery vehicle. like many other nano-/micro-sized materials after administration[68], EV-based delivery systems undergo a process as follows (Fig.4): (i) flow through blood circulation when intercepted by reticuloendothelial system (RES, mainly including liver and spleen); (ii) cross the vascular endothelial barrier and extracellular matrix (ECM) to reach the disease sites (e.g., tumors); (iii) uptake by target cell and escape from lysosome degradation to intracellular or intranuclear locations; (iv) end up in component degradation or exocytosis[69]. Among them, whether a particle can deceive or break through the interruption of the RES *in vivo* and accomplish subcellular transfer is an essential index to evaluate its cargo delivery potential.

After administration, artificial nanoparticles readily interact with a diversity of biological components in the circulatory system, including cells and proteins[70]. For instance, micron-size liposomes can easily be swallowed by white blood cells, while lipid nanoparticles (LNPs) reach red blood cell (RBC) core without a problem[71]. When delivery by LNPs was compared to that by EVs, it was found out that EVs induced a much milder immune response than LNPs after being injected intravenously, because the synthetic lipids of LNPs

were ionizable and toxic, producing much higher levels of pro-inflammatory cytokines[72]. Immunoglobulins and complement proteins on the surface of nanoparticles, which formed a protein corona, can trigger more phagocytosis by binding to membrane receptors on Kupffer cells[73]. Oppositely, PEGylation has been utilized as 'camouflaged cloak' to reduce the formation of the protein corona and mitigate non-specific adsorption and accumulation of the macrophages[74]. However, this PEGylation strategy may also impair the target binding[75] or enhances phagocytosis by human neutrophils[76].

To deal with the same problem in EV-based drug delivery systems, transportation of EVs through the bloodstream is first explored, when different chemical or biochemical trackers have been used in murine models, including fluorescent proteins[77], lipophilic dyes[78, 79], conjugated probes[80, 81], and engineered particles[82, 83] to obtain pharmacokinetics of EVs. As a result, the half-lives of different EVs exhibit a biphasic profile, where the half-life of distribution (α phase) in mice is ~1.5-4 min[84, 85] but the half-life of elimination (β phase) varies greatly. The organ-level distribution of EVs spanned from liver, spleen, kidney to lung, is estimated to be ~3-4 hours after intravenous injection[86, 87]. Intriguingly, one study revealed that exosomes could stay at tumors longer than LNPs of comparable sizes[88]. Compared to artificial particles with up to 90% of injection captured by liver, exosomes could minimize their clearance by RES up to 23% [89, 90]. It is mainly because that CD47 (SIRPα) expressed on surface of exosomes can be recognized as 'do-not-eat-me' signal, obviating phagocytosis by macrophages. Taking advantage of this feature, the membrane of EVs with CD47 has been extracted and used to wrap biomimetic vesicles, and this new approach has proven effective to avoid phagocytosis of particles by macrophages and so to prolong their circulation in the bloodstream[91].

Engineered nanoparticles as drug delivery systems have shown excessive retentions in the liver trap, being a primary barrier that prohibits the nanoparticles from potential clinical therapeutics, where Kupffer cells and sinusoidal endothelial cells are the main components to intercept the nanoparticles in the liver. However, the specific homing affinity of EVs for the organ they were originally derived from help counteract the liver trap[92]. For instance, the amount of HT1080 exosomes absorbed by HT1080 cells (a fibrosarcoma cell line) *in vitro* was approximately twice than that of Hela exosomes, while the accumulated HT1080 exosomes exceeded three times that of Hela exosomes at fibrosarcoma tumor site *in vivo*. In addition, some tumor cell lines with strong metastatic ability, such as breast cancer (MDA-MB-231) cell, generated EVs with specific and formidable metastatic organo-tropism to lung[93]. Simultaneously, melanoma exosomes were found to preferentially flock to bone marrow[94], whereas EVs from vascular endothelial cells also demonstrated a clear affinity to bones[95]. These homing migrations make the EVs somehow detour the liver traps.

The second barrier to blockade transportation of micro/nano-sized particles comprises of vascular endothelium and tissue matrix. For example, a tight junction between the adjacent capillary endothelial cells at the blood-brain barrier (BBB) forms a continuous non-fenestrated barrier, while the pericytes and tissue-specific supporting cells (astrocytes) in the tissue matrix make it hardly passable. For this reason, most chemotherapeutic drugs cannot penetrate the blood-brain barrier, which significantly compromises the brain tumor treatment[96]. Being rational drug delivery platforms to the brain, artificial nanomaterials

need to be extremely small in size and well dispersed and conjugated to equip with targeting/guiding moieties for a possible penetration through BBB, yet showing questionable efficiencies[97]. Oppositely, exosomes have been reported to pass through the BBB with a decent efficiency [83], and they have been widely used in drug delivery to the central nervous system[98]. EVs show better ability to shuttle through the biological barrier including BBB, blood retinal barrier (BRB) and gastrointestinal (GI) barrier[99]. The main mechanism behind this transcellular transport have been demonstrated to be transcytosis where the endothelial recycling endocytic pathway is engaged[100]. Besides, the exosomes of Hela cells triggered the endoplasmic reticulum (ER) stress of vascular endothelial cells and eventually destroyed the barrier function of endothelial cells[101]. As a result, EVs accumulated inside tumors far more than that of similarly-sized liposomes[88]. As the enhanced retention and permeability (EPR) effect has been recently questioned not to be the only way for solid tumor extravasation[102], EVs possess more active transcytosis pathways than other engineered micro-/nano-sized particles when serving as tumor drug carriers[102].

The autophagic-lysosomal pathway constitutes the third barrier, which results in intracellular degradation of internalized substances, thereby only a residual number of cargos being unleashed into the cytoplasm. The engulfed particles into cells first reach the early sorting endosome, either to be directly degraded by lysosome or to develop into late endosomes in the cytoplasm. Contained substances in the late sorting endosome turn into MVB, most fused with lysosomes to be further destroyed by lysosomal hydrolases within, while a relatively small portion of late endosomes finally releases cargoes[103]. Contrary to artificial materials, EVs own a unique biochemical composition in their membrane rich of sphingomyelin, cholesterol and di-saturated lipids, and these contents are usually higher than those in cell plasma membrane, empowering them with sufficient rigidity to resist lysosomal degradation and triggering the lysosome-mediated endosomal permeabilization to transfer cargo molecules into the cytoplasm[104]. The mechanism regarding lysosome-mediated endosomal permeabilization may lie in many folds. Among them, one is due to the membrane fusion between endosomes and exosomes in close proximity of the endosomal lumen; other could be owing to the unstable nature in the lysosomal membrane integrity that requires ESCRT-dependent reparation, leading to exosomal escape[105].

Exogenous particles injected intravenously are mainly cleared from the body via renal or hepatobiliary elimination. Substances with hydrodynamic diameters <6 nm are usually removed by renal filtration through glomeruli, while larger sized particles could accumulate in the mononuclear phagocyte system (MPS) [106]. MPS contains a heterogeneous group of immunocytophagic cells that reside in a diversity of tissues, including Kupffer cells of the liver, and macrophages in the lymph nodes and intestines. liver nonparenchymal cells, namely Kupffer cells and liver sinusoidal endothelial cells, prefer to sequestering particles of sizes larger than sinusoidal endothelial fenestrae (species-dependent, ~150 nm in general). In comparison, smaller particles penetrate into the perisinusoidal space, where hepatocytes absorb them and relay to the bile tubules, being excreted into the intestines and finally in the feces[107]. In fact, the elimination of engineered particles could be much complicated by many other factors than size dependency, such as influence of surface charge, morphology and/or biodegradability. In addition to renal or hepatobiliary elimination where non-biodegradable particles follow, biogenic EVs can be fully degraded and recycled

in vivo, a bonus feature of EVs as delivery vehicles. Assuredly, elimination pathways of EVs can be bypassed via strategic modification. For example, oncogenic exosomes were hijacked by mesoporous silica nanoparticles (~70 nm in diameter) with targeting moiety in the bloodstream and hauled cross the hepatic sinusoid to be removed through fecal excrement[108]. Therefore, an adjustable elimination pathway will guide the design of purposeful EVs and vice versa.

5. Engineered EVs as delivery platforms for disease treatment

Pharmaceutically active biomolecules include hormones, peptides, cytokines, proteins (e.g., tissue growth factors, monoclonal antibodies) and therapeutic oligonucleotides. Basically, the physicochemical characters of drug carrier and the route of administration greatly affect their biological safety and therapeutic efficacy. Liposomes and LNPs are commercially available delivery systems and have been proven to be efficacious for small chemotherapeutic molecules as well as nucleic acid analogues[109]. Currently, among 23 nanomedicines approved by FDA, liposome, polymeric micelles, and nanocrystals are the main components[110]. As of December 2020 at Clinicaltrials.gov, 162 clinical trials examining nanoparticle have been completed, most of which are concentrated in the field of cancer treatment. Besides, there are 41 active clinical trials using nanoparticles, where two of them are related to the vaccine against coronavirus disease 2019 (COVID-19) entered Phase I/II (i.e., NCT 04283461, NCT 04368988). However, artificially made nanoparticle delivery systems had shown many setbacks in clinical applications, due to various reasons, such as undetermined toxicity and undefined component[111]. In contrast, 94 clinical trials (25 under title of EV and 69 exosome) are currently listed at Clinicaltrials.gov and most of them employ EVs in liquid biopsy for diagnoses of chronic/acute disorders and malignant cancers. Particularly, due to broad source and low immunogenicity, MSC-derived EVs have attracted many clinical attentions for therapeutic outcomes (e.g., NCT 02565264/NCT 02138331)[112]. EVs (especially exosomes) as natural biologics rather than synthetic materials, have been put on the stage as an important figure in smart delivery platforms (Fig.5).

5.1 Peptide/protein delivery

To carry monoclonal antibodies or antibody fragments for treatment, EV usually serves as a ‘display platform’ instead of a loading vehicle. In a recent report, exosomes were reprogrammed with monoclonal antibodies expressed on exosomal surface which could simultaneously bind to T-cell surface CD3 and epidermal growth factor receptor (EGFR) on the triple negative breast cancer (TNBC) cells[113]. Therefore, those engineered exosomes, like a hinge, rebuilt the contact between cancer cells and immune cells, so to promote anti-tumor immunity of the host in recognition of cancers. Other engineered EVs that stably expressed angiotensin converting enzyme II (ACE2) receptor on their surface, competitively bound to SARS-CoV-2 virus against host cells, so as to protect the host from virus invasion[114]. Peptide drugs are common therapeutic biomolecules whose molecular weight is lower than that of monoclonal antibodies. Although there are few studies on EV-based peptide delivery, small functional peptides such as arginylglycylaspartic acid (RGD), and

the receptor for advanced glycation end product (RAGE)-binding peptide (RBP), were expressed on the surface of EVs for enhancement of their targeting[115, 116].

5.2 Nucleic acid delivery

Compared to protein or peptide drugs, therapeutic nucleic acid molecules based on complementary sequences can be designed specifically for newly-emerging mutations in recurrent cancers or influenza viruses, keeping treatment synchronized with mutation rates[117]. In several global health issues, especially for those fatal diseases such as COVID-19 with yet valid treatment, clinical trials using nucleic acid therapies or vaccines have shown initial success[118, 119]. Technical inclusion of exogenous nucleic acids into EVs and their delivery for therapeutic purpose have long been studied. Nucleic acids including siRNA, shRNA, mRNA, miRNA, DNA and CRISPR/Cas9[120], own therapeutic effects in many diseases by silencing pathological genes, editing defective genes and/or expressing remedial proteins[118]. Currently, nucleic acids or genetic materials are mainly delivered using viral vectors of different types and with varying capacities. For example, the average diameter of adenoviral and lentiviral vectors is approximately 150 nm, while their maximum allowable genome capacity are 37 kb and 14 kb, respectively[121, 122]. In contrast, adeno-associated virus (AAV) has been regarded as the most established gene delivery vector for liver diseases, but its small packaging capacity of only ~5 kb remains a limit[123]. Furthermore, although viral vectors are relatively efficient in nucleic acid delivery, the safety issue of applying viral vectors in clinical settings has raised concerns and been in hot debate[124].

To load nucleic acids into EVs, a variety of techniques including room temperature incubation, saponin penetration, freeze-thaw cycle, sonication, and extrusion have been reported, together with their individual stability, release profiles, cellular uptake and bioavailability[125]. Different nucleic acids via a diversity of encapsulations into EVs are summarized in Table 1. Among them, parental cell modification and direct EVs electroporation are the most adopted methods, where exosomes are the most studied EVs to deliver target genes, possibly due to their characteristic nano-/micro-size. For notable applications, exosomes are engineered with exogenous nucleic acid materials to deliver through tumor microenvironment to treat cancers or through BBB to modulate neurodegenerative disorders. Meanwhile, it is worth mentioning that exosome-based nucleic acid delivery has shown great promise in the treatment of hereditary diseases, in part restoring sensory functions, such as hearing and vision. Using exosome-associated AAV derived from culture media of transfected HEK-293T cells, it can efficiently deliver gene drugs into cochlear and vestibular hair cells and partially recover hearing in a mouse model of hereditary deafness[126]. Exosome-associated AAV also outcompeted AAV in intravitreal gene transfer to the mouse retina, effectively reaching the inner nuclear and outer plexiform, which paves a new avenue for eye treatment[127]. Moreover, refractory viral infections, such as human papillomavirus and hepatitis B virus that put patients at high risks for cancer development, can be potentially treated using exosome-encapsulated clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease (Cas) systems. HPV or HBV-specific gRNAs together with Cas9 proteins were loaded into exosomes, protecting cargoes from degradation, and delivering them into infected cells to

disintegrate the viral DNA in the host genome[128]. Therefore, EVs possess an infinite potential of delivering a variety of genetic materials safe and sound to remedy ineradicable diseases.

5.3 Chemotherapy drug delivery

Two primitive methods to pack EVs with small molecule chemotherapeutic drugs have been reported: (1) use drugs to treat parental cells to obtain the drug-loaded EVs [129]; (2) direct incubation of EVs with chemical drugs for substantial loading [130]. For example, following the first approach, since the drug-resistant cells could easily flush out the drugs, researchers collected the supernatant of paclitaxel-treated drug-resistant cells and centrifuged to acquire paclitaxel-loaded EVs to simply complete the drug loading [131]. Alternatively, Table 2 demonstrated the advantages and challenges of different loading methods of drugs into EVs for a comparison. Without delivering aid, many traditional anticancer drugs, such as paclitaxel, doxorubicin, and curcumin, have suffered their low water-solubility, short half-life, and poor stability in clinical applications, which require frequent and high-dose administration and result in serious systemic toxicity. loading drugs into EVs can increase drug stability and bioavailability and maximize their retention in target lesions[132]. Compared to the free drug injection, engineered exosomes carrying drugs for tumor treatment substantially prolonged drug release time and significantly shrank tumor volume *in vivo*[133, 134].

Hydrophobic drugs are first dissolved in water-miscible solvent and further dispersed in aqueous solutions to incubate with EVs. As such, drug molecules insert into the membrane bilayer by self-assembly between the hydrophobic tail of phospholipid molecules [135]. This direct incubation of therapeutics with EVs is the simplest way to load drug but the efficiency is relatively low. To enhance, electroporation or electroosmosis is a process of forming hydrophilic pores due to an external electric field, which increases the membrane permeability and allows more cargoes to cross the biological barrier. Electroporation can ramp up the loading efficiency with no obvious damage to the biological performance of the membrane structure[136]. Nonetheless, the loading efficiency of drugs and the structure integrity of EV membranes highly depend on a variety of parameters covering the electroporation (e.g., voltage and pulse frequency), the biochemical composition of EVs, and the type of cargoes to be loaded[137]. Therefore, unoptimized settings may cause the inefficient loading or/and the membrane rupture.

Alternatively, the low frequency ultrasonication can generate cavitation bubbles, which produces small instantaneous pores in the biological membrane, promoting the cargo transfer. It has been proven that sonication is 5.3 and 19.6 times higher in efficiency than electroporation and incubation, respectively, to load PTX into EVs[138]. By applying 1 MHz ultrasonic at low sound pressure from 0.05 to 0.3 MPa and examining the holes produced by sound perforation through scanning electron microscopy, researchers found out that the size of the pores formed on the cell membrane ranged from 100 nm to 1.25 μm, not suitable for hole formation in the EVs[139]. On the cell membrane, the size of the hole formed by sonication is in positive correlation with sound pressure or treatment time[140].

Accordingly, new parameters need to be adjusted to be directly applied on the EVs to figure out variables regarding the hole size formed.

6. EVs *en route* to a next-generation drug delivery system

Drug delivery systems based on engineered nanomaterials have shown great effectiveness in many biomedical studies, exemplified by excellent loading capacity, high biocompatibility and bioavailability, and tunable pharmacokinetics. However, despite of persistent research heat over the last decade, there have been few nano-drug systems applied in the clinical practice. Biogenic natural nanoparticles like EVs could be a game changer in drug delivery research. Many modified exosomes have entered the stage of clinical trials, most of which elect to use tumor cell-derived or MSC-derived exosomes[141]. The typical cargoes carried by these exosomes are small RNAs and chemotherapy drugs. One of those clinical trials showed their preliminary exciting results, where autologous tumor EVs loaded with methotrexate were administered via intrapleural infusion, and this therapeutics improved symptoms in patients with lung cancer of malignant pleural effusion, typified by decreased volume of pleural effusion possibly through the infiltration of activated cytotoxic T lymphocytes into the tumor microenvironment[142]. These clinical trials have testified the short-term safety and therapeutic feasibility of EVs. However, different from synthetic materials made from controlled processes, EVs have unresolved biogenic machinery and unclarified endogenous contents. Furthermore, the loading or expression of exogenous cargoes in the EVs is probability-dependent and hardly manipulable. Strategies of chemical modifications used for surface functionalization of nanomaterials might be borrowed to design and manufacture engineered EVs for a better delivery platform[143]. To this end research efforts have been continuously made for EV modification to attain the upscaled bioproduction, quality control, effective package, and controlled release (Fig. 6).

As aforementioned, EVs are excellent candidates for the next-generation high-quality drug delivery carrier. But the major problems that hinder the EV therapy from laboratory to clinic are their low-efficient production and purification. Researches have been conducted to explore how to scale up the bioproduction of EVs[144, 145]. The biogenic amount of EVs depends on an array of cellular stress responses such as starvation, hypoxia, and heat; however, reproducible technology that enables the large-scale production of clinical-grade EVs has not been developed. Being most therapeutically interesting EVs, exosomes are produced from parent cell cultures, where disturbances in environmental parameters (e.g., cell confluency, shear stress) could sway the proliferation capacity and differentiation potential (if exosomes are derived from stem cells), thus changing the biologics of secreted exosomes[146]. To improve, new dynamic methods including stirring tank bioreaction and perfusion-based production have been employed for large-scale exosome harvesting[147]. lately, a pilot study was reported, using bioreactor-based production of large quantities of MSC-derived exosomes and high-scale electroporation of siRNA into exosomes at clinical grade, to successfully downregulate $Kras^{G12D}$ mutations in a patient-derived xenograft mouse model of pancreatic cancer[148]. large-scale exosomes (a magnitude of 10^{12} per harvest) from bone marrow-derived MSCs were engineered by electroporation of $siRNA^{G12D}$ into exosomes. The loaded exosomes possessed a shelf-life time of 3-6 months at −80 °C and survived freeze-thaw cycles before intraperitoneal injection into $Kras^{G12D+}$

pancreatic cancer models, where oncogenic Kras expression was significantly suppressed, metastatic tumor burden was reduced, and inflammations were found minimal in major organs, in association with prolonged survival[148]. Using such prepared exosomes for intravenous injection, a clinical trial at Phase I has been initiated to treat patients with metastatic pancreatic cancer with $Kras^{G12D+}$ mutation, to identify its maximum tolerated dose and dose-limiting toxicity (NCT 03608631). This research set foot in clinical use of EVs, providing a unique niche of exosome therapy in cancer treatment.

Moreover, upon production, current isolation of exosomes remains a bottleneck for the commercial manufacturing. For example, ultracentrifugation requires high cost and intensive labor with a return of low efficiency, while polymer sedimentation where typically polyethylene glycol is added to precipitate exosomes can be easily contaminated with other unwanted substances, such as residual polymers[149]. Instead, emerging industry-compatible approaches are being adopted to enhance the separation, especially physical techniques such as size-exclusion chromatography and tangential-flow filtration, and immunoaffinity capture with high specificity[146].

The next task falls on the quality control of EV products. In stem cell-based therapy, it is necessary to formulate detailed specifications for the acquisition, sorting, identification, transportation, and storage of donor cells. In parallel, to establish a reliable purity threshold is essential in order to prevent contamination of microorganisms, endotoxins, and other types of cells. Besides, long-term clinical safety monitoring and rigorous tracking of cells in the body are also indispensable after treatment[150]. Similarly, the standardized characterization and quality control of pharmaceutical EVs are of critical importance for clinical testing. In scenario of the United States, to initiate the clinical trial, an investigational new drug (IND) application needs to be filed with Federal Drug Administration (FDA) with guidelines of its quality controls being established. Characteristic criteria and analytic methods for multimodal parameters of EVs from umbilical cord derived mesenchymal stromal cells (UC-MSC) have been released, including parental cell count and viability, EV particle size, morphology and surface markers, the impurities and biological function assessment [151]. At the same time, storage conditions, such as low-binding packing materials, serum-free media and appropriate temperatures for preservation of well suspended EVs during logistics to obviate the aggregation and degradation, are also necessitated[152].

Effective package depends on proper loading and adequate dosage. loading efficiency mainly relies on the affinity between the cargo and the carriers. Computational studies have been employed to predict the molecular interactions between different poly[(rac-lactide)-co-glycolide](PLGA) and drugs to be loaded[153]. However, due to the complex component, EV-based carriers are not suitable for computational design or prediction analysis for optimized drug loading. Moreover, the determination of EV dosage for drug delivery is also problematic. Currently, the dosage of EVs is quantitatively defined by the total proteins or the number of particles contained, but the effective dosage could be varying a bit if the progenitors of EVs are varied. Consequently, the overdosage of EVs is impractical to obviate and the outcome can be detrimental. For example, EVs are mainly distributed in liver and spleen after intravenous injection, but the excessive dosage of injected EVs led to their

accumulation in the lung, resulting in asphyxia in mice[154]. Therefore, profound studies on EV pharmacokinetics and its pharmacodynamics within a diversity of biological systems are prerequisites to be further applied in clinical settings.

Controlled release of therapeutic or diagnostic agents triggered by stimuli responsiveness has been widely practiced in nanomaterial delivery systems, showing advantages in spatial and temporal control of drug dumping. For instance, it could localize subcellular delivering sites and achieve the delivering accuracy at even femtosecond level[155]. In this manner, artificial or biomimetic nanoparticles could be modified in response to different external or internal stimuli, including physical (e.g., magnetic field, mechanical pressure, thermo switch, and light irradiation)[156, 157], chemical (e.g., pH, ROS, glucose)[156, 158, 159], and/or biological signals (e.g., proteins)[160]. Nonetheless, stimuli-responsive EV-based delivery systems remain little explored[161]. In addition, studies are necessitated to investigate the actual release rate of loaded cargoes in the EVs, and to delineate the translation efficiency of pharmaceutical genes, if released in a controlled manner. EV therapeutics would be otherwise unfeasible to be evaluated for its safety and potency.

7. Conclusion and perspective

To date, EV therapy in replacement of cell therapy has been widely recognized and practiced in regenerative medicine, tissue repair and disease treatment, attributed to its minimized immunoresistance and tumorigenic risk, and maximized inherent biological activities from progenitors. As discussed in this review, methods to characterize EVs and to evaluate their safety and performance in preclinical studies have been established and optimized over decades. As a result, therapeutic success of EV-based delivery systems has been expected and accepted, setting hopes for next clinical applications. In parallel, to initiate good manufacturing practice (GMP) campaign and meet GMP standards, intensive research efforts on pharmaceutical EVs have been made to take steps forward down the drug development pipeline, from scalable production and isolation of clinical-grade EVs, their accurate characterization and assessment, controllable drug loading and release profiling, to systematic acquisition on the relevant pharmacological and toxicological data.

Previously, allogeneic exosomes obtained from immune cells (such as dendritic cell and natural killer cells) or tumors, enabled antigen presentation and T cell stimulation, and provoked anti-tumor activities of $CD8^+$ T cells[162]. With an idea resembling chimeric antigen receptor T (CAR-T) cell therapy, another perspective can be envisaged where patient-derived autologous EVs can be collected, modified, and applied to treat their own diseases. With more encouraging results believed to come, autologous EV delivery and therapy are warranted in the near future.

Put together, the employment of immunomodulatory EVs in clinical settings offers a versatile next-generation therapeutical delivery platform and transforms the conventional disease treatment into a new era of cell-free therapy. With no doubt it adds a significant value to the development and practice of precision medicine when preventive or therapeutic interventions can be tailored to those who will benefit the most.

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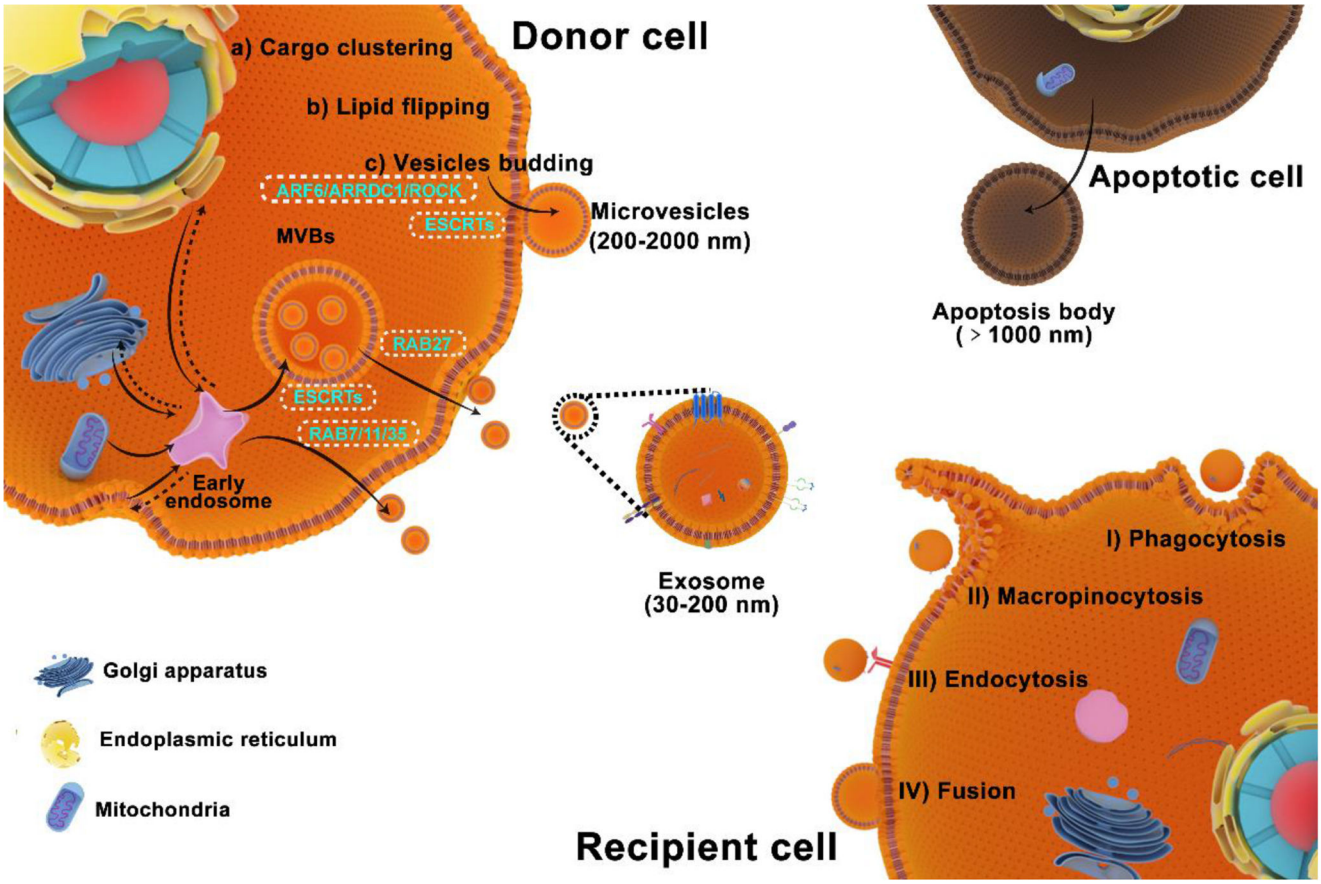

Figure 1.

Biogenesis and destination of EVs. Different types of EVs have distinct biogenic and disposal mechanisms as discussed in the text.

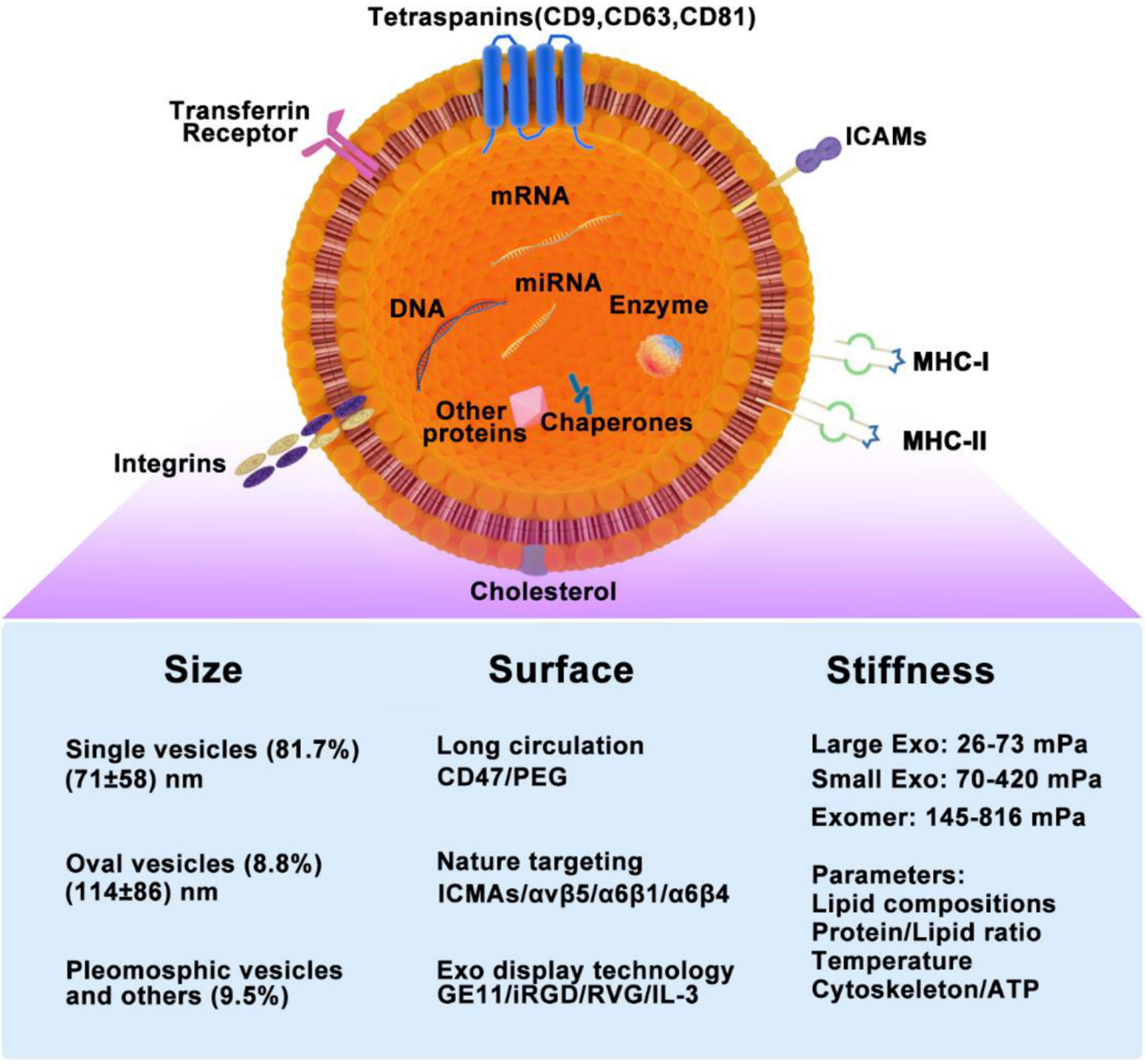

Figure 2.

Structure, contents, and biophysical properties of exosomes. Exosomes have lipid bilayer membrane, heterogeneous components, highly expressed tetraspanin proteins (CD9, CD81, and CD63) on its surface, plentiful tetraspanin-associated proteins ICMAs, integrins and so forth. A large number of DNAs, RNAs, enzymes, and other functional proteins are encapsulated within.

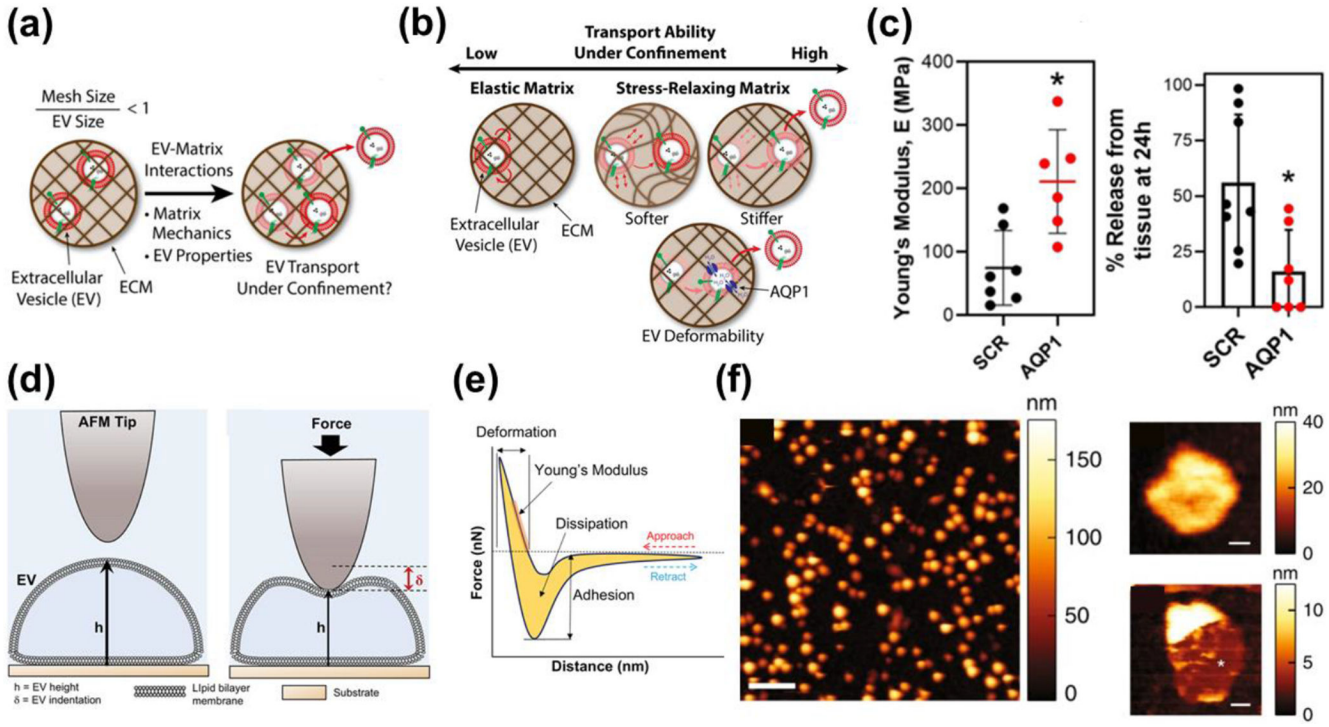

Figure 3.

(a-b) Schematic illustration regarding the EV transport through elastic matrix where the mesh size is smaller than the size of EVs. Aquaporin-1 expression on the EVs can increase its deformability. (c) AQP1-depleted EVs (AQP1) exhibited a significantly higher stiffness than control EVs (SCR). Reprinted with permission from Ref[39]. (d) Schematic representation of EVs' mechanical properties measurement by atomic force microscopy (AFM). (e) A typical force-distance curve (FDCs) recorded on the EV surface and several common mechanical parameters related to the EV stiffness. Reprinted with permission from Ref[30]. (f) AFM image of RBC EVs. Reprinted with permission from Ref[56].

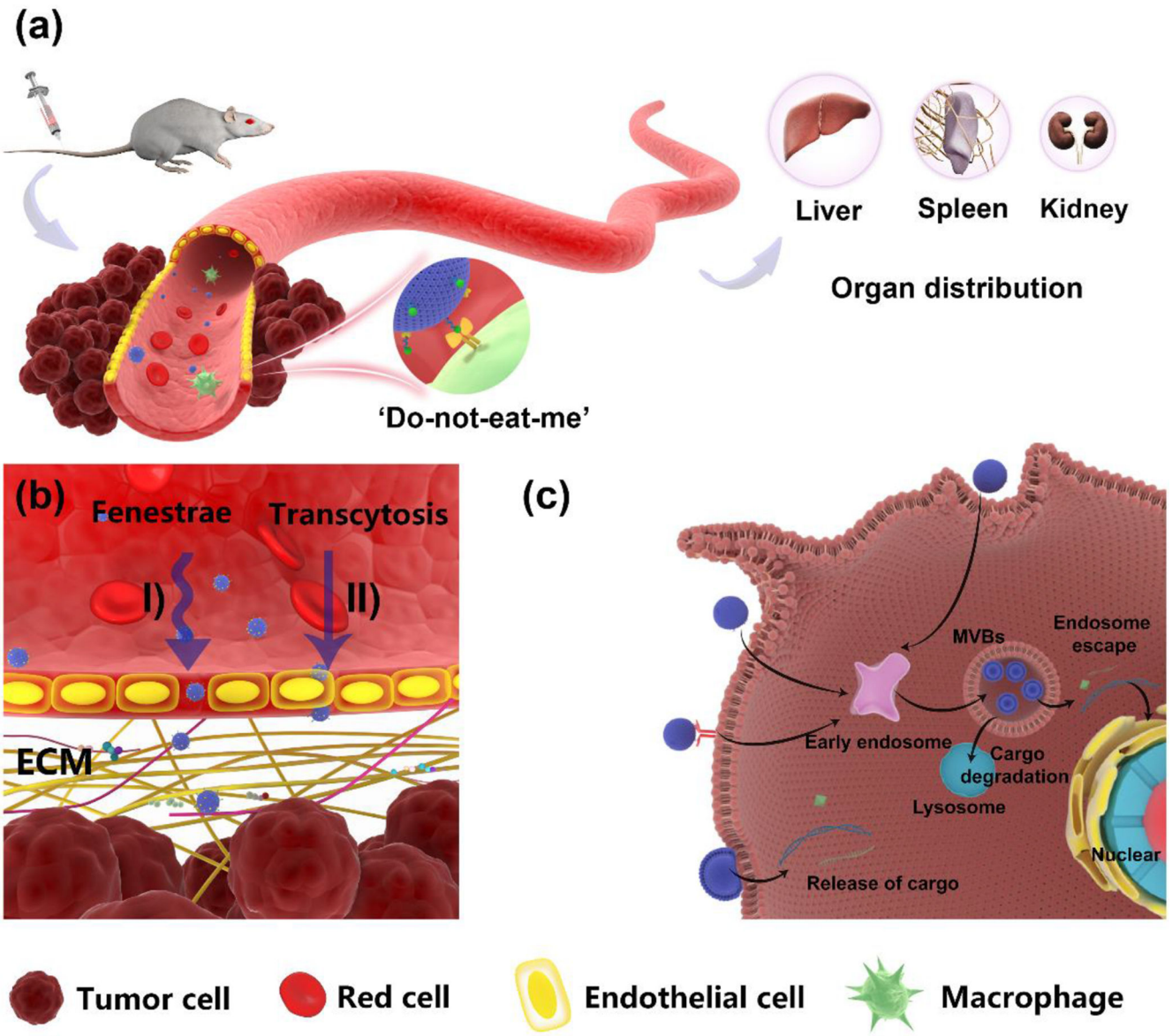

Figure 4.

The life cycle of EVs during their tumor drug delivery. (a) EVs mainly accumulate in the liver, spleen, and kidney after injected into mice through the tail vein. CD47 expression on its surface helps resist phagocytosis by macrophages. (b) EVs penetrate through vascular endothelial cells via two routes: 1) deformability of EVs allows them to passively extravasate through the inter-endothelial fenestrae; 2) transcytosis is the active uptake of EVs by endothelial cells, then releasing cargo through exocytosis. ECM, extracellular matrix. (c) Cellular internalization of EVs via membrane fusion can directly release cargo.

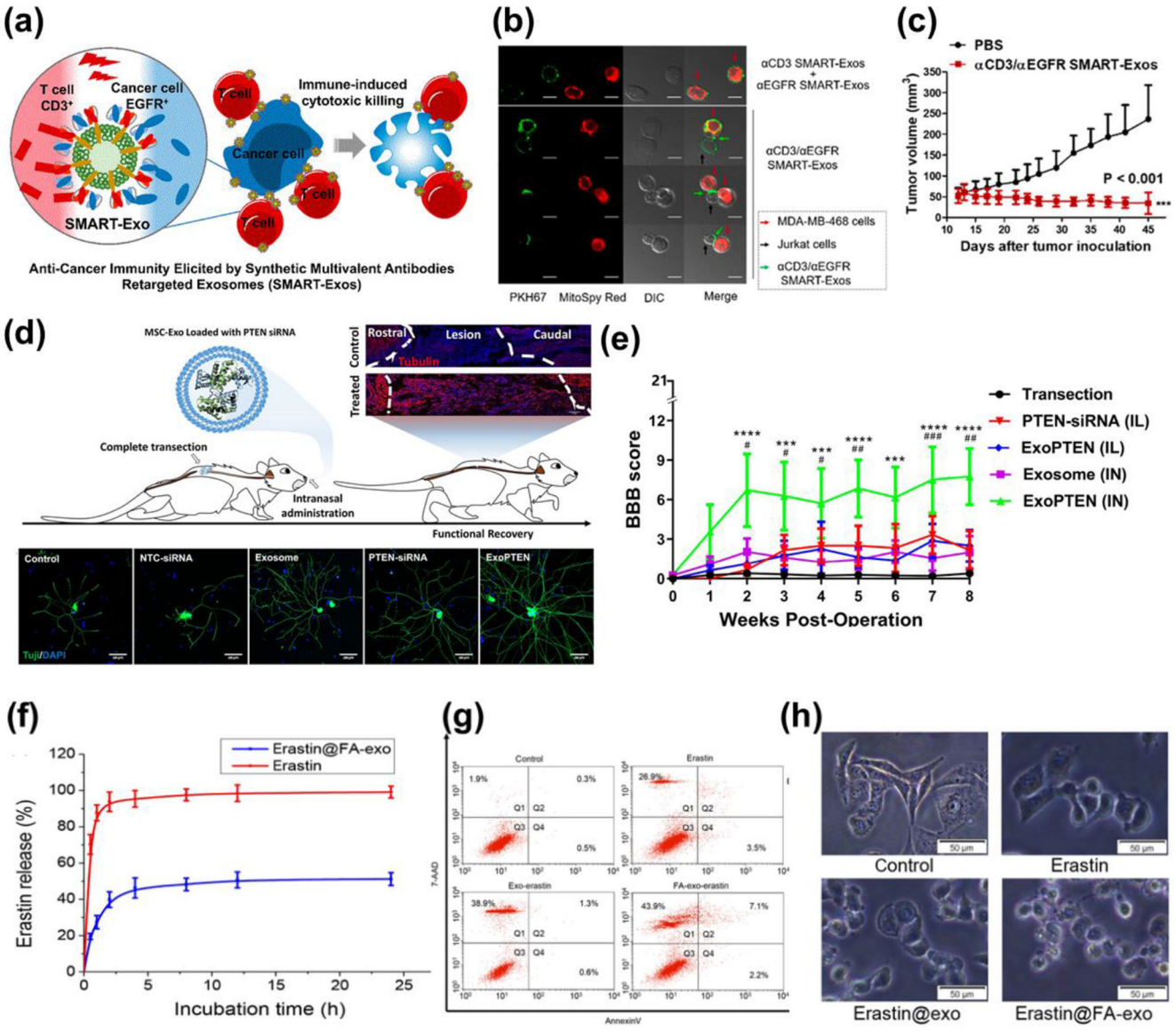

Figure 5.

(a) Schematic illustration of synthetic multivalent antibodies retargeted exosomes (SMART-Exos). (b) Confocal imaging of αCD3/αEGFR SMART-Exos (green) participating in cross-linking of MDA-MB-468 (red) and Jurkat (no fluorescent label) cells. A mixture of αCD3 SMART-Exos and αEGFR SMART-Exos was used as a control. Scale bars: 10 μm. (c) αCD3/αEGFR SMART-Exos can significantly inhibit tumor growth. Reprinted with permission from Ref[113]. (d-e) MSC-Exo loaded with phosphatase and tensin homolog small interfering RNA (PTEN-siRNA) enhanced axonal growth and elicited functional recovery. Reprinted with permission from Ref[178]. (f) *In vitro* erastin@FA-exo could delay drug release compared with free erastin in pH 7.4. (g-h) Erastin@FA-exo induced more apoptosis (Annexin V/7-AAD) and ferroptosis of MDA-MB-231 cell. Reprinted with permission from Ref[186].

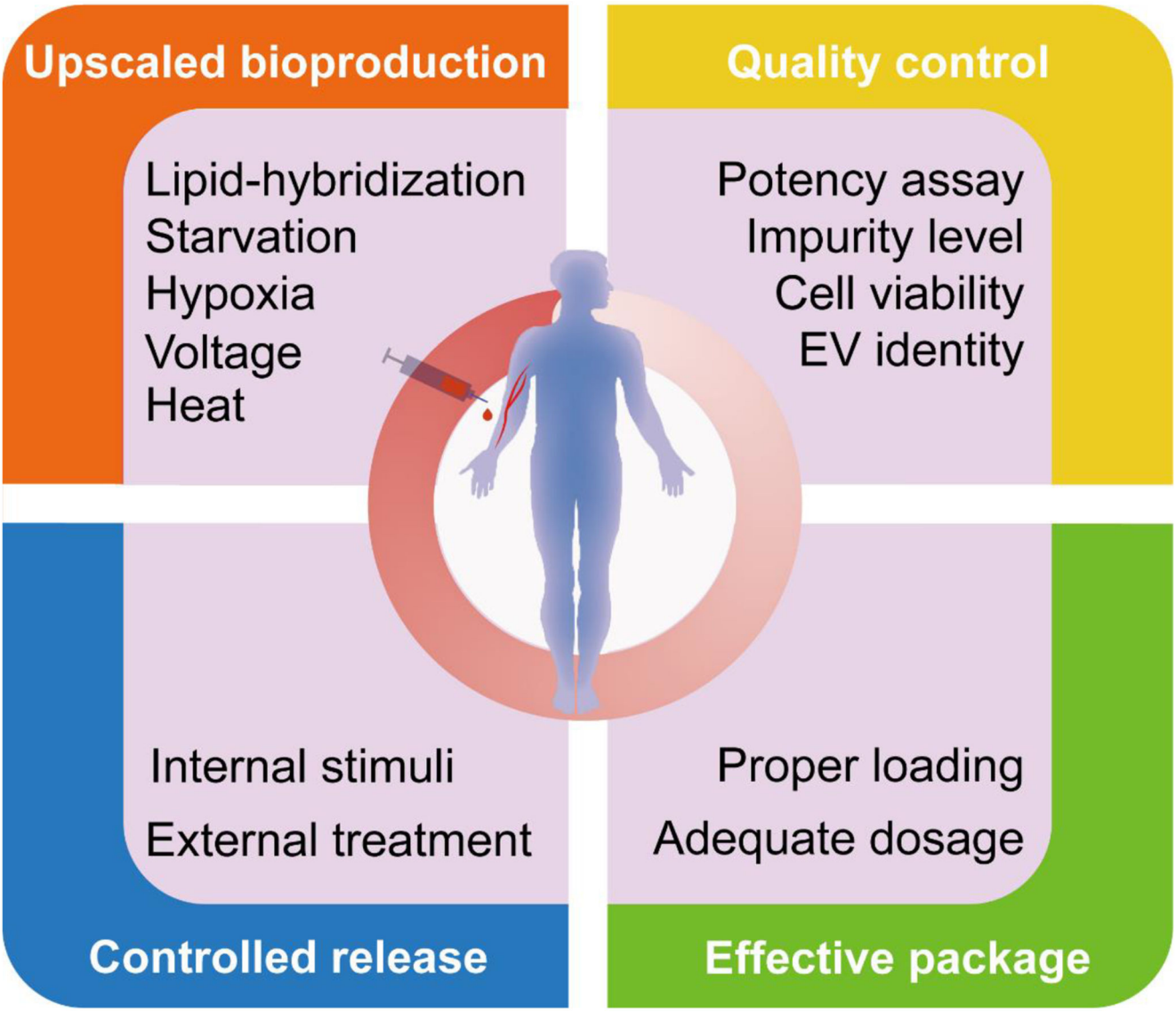

Figure 6.
The challenges for EVs *en route* to a next-generation drug delivery system.

Table 1.

Summary of the methods applied when loading nucleic acids into EVs

Method	Cargoes	Method evaluation	References
Parent cell treatment	minicircle DNA, siRNA, mRNA, miRNA, DNA	Low loading efficiency, decided by the efficiencies of genes entering cells and being further sorted into EVs.	[163-169]
Electroporation	shRNA, siRNA, miRNA, anti-miRNA	Simple operation, high load efficiency, but pH and heat generated may be damaging.	[145, 170-176]
Incubation	siRNA, anti-miRNA	Low load efficiency but facile method, suitable for hydrophobic compounds encapsulation.	[177-179]
Extrusion	siRNA	High load efficiency, complex pre-processing.	[180]
Sonication	miRNA, siRNA, anti-miRNA	High load efficiency, but the redundant heat may undermine membrane integrity .	[179, 181]
Auxiliary reagent	siRNA, shRNA	Difficult to judge whether EVs or chemical transfection reagents work.	[182, 183]

Table 2.

Summary of the methods applied when loading small molecule chemotherapeutics into EVs.

Cargo	Properties	Loading	Efficiency	Administration	Reference
Paclitaxel	Poor bioavailability, low aqueous solubility	Electroporation or incubation	33% or 8%	Intravenous or oral	[138, 184]
Gemcitabine	Poor cellular uptake, short half-life	Incubation or electroporation	2.8% or 11.7%	Intravenous	[136]
Doxorubicin	Rapid clearance, evident cardiotoxicity	Incubation	0.8%	Intravenous	[185]
Erastin	Low aqueous solubility, renal toxicity	Sonication	N/A	Intravenous	[186]
Imperialine	Short half-life, unfavorable biodistribution	Micelle-aided method, incubation or sonication	24.9%	Intravenous	[133]
Curcumin	Low aqueous solubility, instability, and low bioavailability	Incubation	N/A	Intravenous	[143, 187]
Cucurbitacinl	Low aqueous solubility	Incubation	N/A	Intranasal	[188]
BAY55-9837	Short half-life, poor stability	Electroporation	N/A	Intravenous	[189]