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Extracellular vesicles as a next-generation drug delivery platform

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Abstract

Extracellular vesicle (EV)-based cell-to-cell communication is conserved across all kingdoms of life. There is compelling evidence that EVs are involved in major (patho)physiological processes, including cellular homeostasis, infection propagation, cancer development, and cardiovascular diseases. Various studies suggest that EVs have several advantages over conventional synthetic carriers, opening new frontiers for modern drug delivery. Despite extensive research, clinical translation of EV-based therapies remains challenging. Here, we discuss the uniqueness of EVs along with critical design and development steps required to exploit their full potential as drug carriers, including loading methods, in-depth characterisation, and large-scale manufacturing. We compare the prospects of EVs to those of well-established liposomes and provide guidelines to direct the process of developing vesicle-based drug delivery systems.

As the field of targeted drug delivery has expanded, nanotechnology has contributed substantially to the development of smart carriers in recent decades.[1] In particular, lipid-based nanocarriers offer a versatile platform for drug encapsulation, which has led to clinical translation of several formulations. In addition to synthetic nanocarriers, cell-derived extracellular vesicle (EV)-based carrier systems have attracted considerable interest.[2]

EVs are a heterogeneous group of small, lipid-bound nanoparticles acting as key mediators of many (patho)physiological processes.[3] They are also being explored for the delivery of therapeutic payloads to specific cells or tissues, harnessing their intrinsic tissue homing capabilities.[4] From a drug delivery perspective, EVs are comparable to liposomes, given that both are phospholipid-based. However, EVs are assembled from a complex mixture of various lipids and surface and membrane proteins; some of these components aid tissue targeting, while others ensure minimal non-specific interactions.[5,6] These unique protein-decorated phospholipid vesicles have been postulated to contain the specific barcodes needed to find their target both locally and at distant sites. Despite extensive research, the superiority of EV-based drug delivery over delivery *via* engineered nanocarriers, such as liposomes, and the associated risk-benefit ratio remain matter of debate.[7]

Here, we critically discuss the prospects of EVs as drug delivery vehicles and as next-generation therapeutics. We outline the advantages of EVs over standard delivery methods, discuss current obstacles related to their clinical and industrial translation and highlight synergies with other emerging fields, such as cell therapeutics (EVs are sometimes considered 'cell-free cell therapeutics'). We also propose a colour code guideline regarding experimental requirements and scientific needs to facilitate the development of EVs as drug carriers to evaluate their delivery efficacy and allow benchmarking against alternatives.

Uniqueness of extracellular vesicles biology and function

Composition of EVs. EV secretion appears to be an evolutionarily conserved process present throughout all kingdoms of life.[8] Regarding fundamental biology, EV research focuses on understanding the biogenesis and release of these natural carriers and their fate upon interaction with

target cells. This also comprise the genotypic and phenotypic responses that EVs induce and the mechanisms by which EVs mediate cell-to-cell communication.[5,9]

Several subtypes of EVs, including exosomes, ectosomes, microvesicles, membrane vesicles, and apoptotic bodies, have been identified.[10] These EVs have been isolated from various sources, including mammalian and prokaryotic cell cultures, blood plasma, bovine milk, and plants.[8] Each EV subpopulation may be derived *via* distinct biogenesis pathways, and because their precise biogenic origin is impossible to ascertain in most cases, a comprehensive characterisation of the vesicles is crucial. In addition, different EV formulations may have substantially different size distributions; thus, standardised characterisation is challenging.[11] The general recommendation in the field is to use 'EVs' as a general term. Importantly, the general concept of '*the* EV' does not exist—currently, the term 'EV' comprises a heterogeneous population, as indicated in the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines.[12] Proteomic evidence suggests that an EV core protein signature (*e.g.,* CD63, CD9, or CD81) of highly expressed vesicular proteins is commonly shared between EVs of diverse parent cell origins.[13] Various tetraspanins are commonly used as molecular markers of EVs. In contrast to the previous MISEV guidelines, there are no typical EV markers that need to be identified on EVs, but careful discrimination of EVs from contaminants, such as protein aggregates and viruses, is important. To add an additional layer of complexity, vesicles still carry parent cell-specific signatures, which are crucial components permitting target cell interactions in distinctly different manners.[14] In addition to the core signature of highly expressed and highly enriched vesicular proteins, other typically low-abundance and less-enriched protein components are present; these proteins reflect the specific parent cell origin of the EVs and may also vary depending on the nature and biogenesis of different EV subpopulations.[15] From a drug delivery perspective, this complexity needs to be understood via comprehensive (multi)omics studies[16] and addressed in all characterisation and production processes (**Fig. 1**). Here, we elucidate the assembly of EVs on the cellular/molecular level and their mediation of selective intercellular signalling activities to extend biomedical research.[17]

Uptake and biological role of EVs Under physiological conditions, EVs are signal carriers involved in the homeostasis of several processes and of events during cell development, *e.g.*, cell differentiation.[18]

EV-mediated cross-talk may occur unidirectionally or reciprocally, *i.e.*, one cell sends information to another with or without reciprocal signal transmission from the recipient cell, respectively, or even *via* systemic communication, during which EVs traffic to various tissues and organs. This interaction may involve not only the release and delivery of EV cargo but also cell surface interactions and target cell modulation, such as immune cell activation by major histocompatibility complex-peptide interactions. The mechanisms by which EVs are taken up by their target cells are still poorly understood, and examples from the literature are often specific for a certain type of vesicle.[5] Currently known cellular entry routes of EVs range from receptor-mediated endocytosis, lipid raft interactions, clathrin interactions, phagocytosis, macropinocytosis, and possibly direct fusion.[9] Like many other nanocarriers, EVs are taken up into endosomes need to escape the endosome to release their cargo into the cytosol. Endosomal escape is associated with degradation in acidic compartments of the lysosomal pathway, which could impair the integrity of EV cargoes.[19] Although EVs were initially postulated to be an unprecedented route for direct cell membrane fusion and cytosolic delivery,[20] vesicle uptake has been confirmed to be a very complex mechanism, which requires more in-depth evaluation exploring subcellular analyses based on high-resolution microscopy or novel live-cell reporters.[21] On the other hand, the biological effects induced by EVs are currently well known. During oncogenesis, tumour cells increase their yield of EVs, allowing not only the modulation of surrounding healthy cells, immune cell dysregulation, and tumour proliferation but also communication with distant tissues, *e.g.*, during angiogenesis.[22] Glioblastoma cells were shown to secrete EVs capable of immunosuppression by blocking T cell activation and receptor stimulation.[23] Moreover, widely used cytotoxic drugs, such as taxanes, may also induce shedding of EVs with pro-metastatic properties.[24] Although the role of EVs in tumour biology has been investigated extensively, the development of new tools for treatment and diagnostics is still hampered by the absence of tumour-specific EV markers.

A comparable modulatory role of EVs has been observed in the progression of resistance to infections. In the context of viral infections, some EVs may carry viral proteins from infected cells and follow comparable biogenesis pathways.[25] Furthermore, bacteria utilise EVs for the transmission of resistance genes and virulence factors,[26] which has sparked interest in the development of bacterial vesicles for vaccination applications.[27] Bacterial EVs from non-pathogenic or probiotic bacterial sources may also

be harnessed as potential EV-based delivery carriers, and their production may be readily scalable by cultivation of EV-producing bacteria in small fermenters.[28,29] This is a promising avenue for the manufacturing of EVs with novel functionalities and in conjunction with biomaterials.[30,31] However, immunogenicity requires more detailed evaluation for bacterial vesicles than for mammalian EVs owing to the potential presence of lipopolysaccharides, as recently discussed in detail.[32]

Extracellular vesicle-based drug carriers

Development. With the development of new analytical tool, it has been found that many previously applied isolation techniques are not specific for EVs and lead to the inclusion of contaminants. Methods are constantly refined, but they often expose the limitations in the field, making it difficult for new researchers to follow progress in the state-of-the-art methods. For every drug nanocarrier, a comprehensive physico-chemical characterisation and its interactions in biological environments must be investigated for therapeutic development. While liposomes have been extensively evaluated for efficacy and biocompatibility both *in vitro* and *in vivo*, methodologies well adapted to the considerably more complex EVs are lacking. These natural vesicles are assembled and packaged in a cell-specific manner, *e.g.*, cancer-derived EVs, carry molecular information distinct from that carried by stem cell- or blood cell-derived EVs. While challenging from the perspective of drug carrier development, these properties make EVs a promising biomarker for liquid biopsies in several applications.[33]

In regenerative medicine, EVs derived from mesenchymal stem cells (MSCs) are already under clinical assessment[34] for future use in nanodelivery (**Table 1**). Stem cell-derived EVs can induce immune cells to undergo modulation from an activated inflammatory state to a tolerant regulatory state. Some of the strategies used to stimulate EV shedding and enhance yield can also be used for MSCs. N-methyldopamine and norepinephrine induced an increase in MSC-derived EV production without altering their modulatory capacity.[35] Other approaches apply physical stimuli such as pH variations or low oxygen conditions, but their long-term effect on the physiological properties of EVs needs to be evaluated. In a murine wound healing model, MSC-EVs were associated with secretion of an interleukin-1 receptor antagonist and induced rapid gingival healing.[36] Comparable effects have been shown for systemic application of MSC-EVs in patient trials, which has unfortunately led to the

use of ‘exosome’ products in unapproved applications. The Food and Drug Administration recently stated that serious adverse effects were experienced by patients in Nebraska treated with unapproved products marketed as containing exosomes[37]. The agency emphasised that there are currently no regulatory approved EV products and that some clinics “deceive patients with unsubstantiated claims about the potential for these products to prevent, treat or cure various diseases or conditions”. Importantly, any therapeutic application of EVs requires transparent reporting of data on vesicle manufacturing and characterisation, suitable quality control provisions, preclinical safety, and efficacy.[38] Moreover, a rational clinical trial design and regulatory monitoring are important to ensure patient safety, as recently indicated by the international societies on stem cells and EVs.[39] To support the use of MSC-EVs, functional assays that allow *in vitro-in vivo* correlation of the therapeutic potency of different stem cell preparations must be developed.[40] Despite these caveats, ongoing efforts to produce EVs from MSCs under good manufacturing process-like conditions[41,42] and to design upscaling approaches[43] will be instrumental for their development as drug carriers.

166 **Table 1. Ongoing clinical trials with extracellular vesicles**

#	Name*	Status	Condition	Type of extracellular vesicles§	Location	NCT number
	Stem cell-derived extracellular vesicles					
1	A Clinical Study of Mesenchymal Progenitor Cell Exosomes Nebulizer for the Treatment of Pulmonary Infections	Recruiting; Phase 1/2	Drug-resistant infections	Mesenchymal stem/progenitor cell-derived exosomes	Shanghai, China	NCT04544215
2	Effect of Microvesicles and Exosomes Therapy on β-Cell Mass in Type I Diabetes Mellitus (T1DM)	Unknown status	Diabetes mellitus type 1	Mesenchymal stem cell-derived exosomes	Sahel, Egypt	NCT04213248
3	Evaluation of Safety and Efficiency of Exosome Inhalation in SARS-CoV-2 Associated Pneumonia.	Enrolling by invitation; Phase 1/2	SARS-CoV-2 pneumonia	Mesenchymal stem cell-derived exosomes	Samara, Russia	NCT04213248
4	A Pilot Clinical Study on Inhalation of Mesenchymal Stem Cells Exosomes for the Treatment of Severe Novel Coronavirus Pneumonia	Completed; Phase 1	SARS-CoV-2 pneumonia	Mesenchymal stem cell-derived exosomes	Shanghai, China	NCT04213248
5	Safety and Efficiency of Method of Exosome Inhalation in COVID-19 Associated Pneumonia	Enrolling by invitation; Phase 2	SARS-CoV-2 pneumonia	Mesenchymal stem cell-derived exosomes	Samara, Russia	NCT04602442
6	Effect of UMSCs Derived Exosomes on Dry Eye in Patients With cGVHD	Recruiting; Phase 1/2	Dry eye	Umbilical mesenchymal stem cell-derived exosomes	Guangzhou, China	NCT04213248
7	MSC-Exos Promote Healing of MHs	Recruiting; early Phase 1	Macular holes	Mesenchymal stem cell-derived exosomes	Tianjin, China	NCT04213248
8	A Tolerance Clinical Study on Aerosol Inhalation of Mesenchymal Stem Cells Exosomes in Healthy Volunteers	Recruiting; Phase 1	Safety and tolerance studies	Mesenchymal stem cell-derived exosomes	Shanghai, China	NCT04213248
9	Allogenic Mesenchymal Stem Cell Derived Exosome in Patients with Acute Ischemic Stroke	Completed; Phase 1/2	Cerebrovascular disorders	Mesenchymal stromal cell-derived exosomes	Tehran, Iran	NCT03384433
10	Evaluation of Adipose Derived Stem Cells Exosomes in Treatment of Periodontitis	Recruiting; early Phase 1	Periodontitis	Adipose-derived stem cell-derived exosomes	Cairo, Egypt	NCT04213248
	Allogenic and autologous extracellular vesicles					

Table 1. Continued

#	Name*	Status	Condition	Type of extracellular vesicles§	Location	NCT number
11	Safety and Efficacy Evaluation of Allogenic Adipose MSC-Exosomes in Patients with Alzheimer's Disease	Recruiting; Phase 1/2	Alzheimer's disease	Allogenic adipose mesenchymal stem cell-derived exosomes	Shanghai, China	NCT04213248
12	A Clinical Study of Mesenchymal Stem Cell Exosomes Nebulizer for the Treatment of ARDS	Not yet recruiting; Phase 1/2	Acute respiratory distress syndrome	Allogeneic human mesenchymal stem cell-derived exosomes	Ruijin, China	NCT04602104
13	MSC extracellular vesicles in Dystrophic Epidermolysis Bullosa	Not yet recruiting; Phase 1	Dystrophic epidermolysis bullosa	Allogeneic mesenchymal stem cell-derived extracellular vesicles	Aegle Therapeutics	NCT04173650
14	Effect of Plasma Derived Exosomes on Cutaneous Wound Healing	Enrolling by invitation; early Phase 1	Ulcer	Autologous exosome-rich plasma	Kumamoto, Japan	NCT04213248
	Other cells or extracellular vesicle sources					
15	COVID-19 Specific T Cell Derived Exosomes	Active; Phase 1	SARS-CoV-2 pneumonia	T cell-derived exosomes	Kayseri, Turkey	NCT04213248
16	Extracellular Vesicle Infusion Therapy for Severe COVID-19	Not yet recruiting; Phase 2	SARS-CoV-2 pneumonia, acute respiratory distress syndrome	Bone marrow derived extracellular vesicles	Direct Biologics	NCT04493242
17	Edible Plant Exosome Ability to Prevent Oral Mucositis Associated with Chemoradiation Treatment of Head and Neck Cancer	Active; Phase 1	Head and neck cancer, oral mucositis	Grape exosomes and fentanyl patch	Louisville, USA	NCT04213248
	Drug loaded extracellular vesicles					
18	iExosomes in Treating Participants with Metastatic Pancreas Cancer with KrasG12D Mutation	Not yet recruiting; Phase 1	Metastatic pancreatic adenocarcinoma, pancreatic ductal adenocarcinoma	Mesenchymal stromal cell-derived exosomes loaded with siRNA against KrasG12D	Houston, USA	NCT04213248
19	Study Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Colon Cancer Tissue	Active; Phase 1	Colon cancer	Plant exosomes loaded with curcumin	Louisville, USA	NCT04213248
20	Trial of a Vaccination with Tumor Antigen-loaded Dendritic Cell-derived Exosomes	Completed; Phase 2	Non-small cell lung cancer	Dendritic cell-derived exosomes loaded with antigen	Villejuif, France	NCT04213248

*Trials currently listed on clinicaltrials.gov; for consistency, trial names are indicated as listed on clinicaltrials.gov.

§The type and/or source of extracellular vesicles are indicated as given by the study sponsor and were not verified regarding suitable characterisation (*e.g.,* exosomes and microvesicles).

Characterisation as a prerequisite for meaningful safety and efficacy studies. A comprehensive characterisation of EVs and their interaction with cells and tissues is essential for the use of EVs in drug delivery applications. While safety and efficacy characterisation is pivotal for the clinical advancement of EVs, insights into the mode of action of EVs may open new frontiers in drug carrier engineering. The identification of critical attributes sufficient to achieve long-distance targeting is crucial to mitigate the risks associated with the high complexity of this system. However, the virus-like size and the increased complexity of EVs compared to synthetic delivery systems (*e.g.*, liposomes), which partially contribute to the superior drug delivery capacity of EVs, render comprehensive characterisation and quality assurance challenging.[42] Purity and identity issues pose major challenges for analytical techniques, and the inability to characterise the entire system results in significant risks; these considerations need to be interpreted in the context that EVs constitute a cell-free cell therapy. Standard characterisation techniques, *e.g.*, nanoparticle tracking analysis, imaging flow cytometry, and detection of components by biochemical means (including imaging,[44] flow cytometry, and western blotting), involve size measurements. Recently, EVs have also been used as a platform to visualise and study enriched membrane proteins by cryoelectron transmission microscopy.[45] High-throughput technologies such as next-generation sequencing and mass spectrometry[46] (proteomics, lipidomics, and transcriptomics), along with cryoelectron microscopy, contribute greatly to the evaluation of the molecular composition and structure of EVs.

The systematic investigation of efficacy and safety of EVs requires determination of their identity and purity. For this, the International Society for EVs (ISEV) initiated the standardisation of EV isolation and characterisation techniques in 2014. The MISEV guidelines were updated in 2018 by 382 researchers.[12] **Box 1** summarises the most fundamental characteristics that should be evaluated when working with EVs. To further enhance rigour and reproducibility, the EV-Track platform was created in 2017.[47] EV-Track is a crowdsourcing knowledgebase that allows authors to deposit their isolation and characterisation protocols prior to publication and receive recommendations on potential shortcomings of the experimental design. More recently, additional advice on the optimal reference material for use during EV characterisation has been proposed.[48]

Targeting capabilities and clearance. Liposomes deliver their drug cargo mostly through passive accumulation in certain tissues unless they carry additional surface ligands. EVs may have an inherent targeting ability and the potential to deliver functional RNA to other cells[49] and across certain biological barriers, such as the blood-brain barrier.[50] For some combinations of parent and target cells, superior tissue homing capabilities have been identified; for example, unidirectional synaptic transfer of miRNA from T cells to antigen-presenting cells.[51] While synthetic drug delivery systems have shown significantly lower targeting efficacy than natural drug delivery systems, EVs may constitute a natural route for efficient transport.[52] Indeed, different mammalian tumour EVs were shown to preferentially target healthy cells in the predicted tissue, *e.g.*, epithelial cells and lung fibroblasts, depending on the integrin expression pattern of the parent cells.[53] Similar results have been obtained for EVs from sarcoma cells, which showed preferential tumour homing.[54] For safety reasons, such cancer EVs are not suitable as drug carriers because they may negatively influence tumour invasion or epithelial-mesenchymal transition, or they may carry tumour resistance genes.[55] A comparative evaluation of EVs derived from different cell lines and their biodistribution pattern showed that although EVs accumulated primarily in the liver, lung, spleen, and gastrointestinal tract, the vesicle source and administration route significantly influenced the biodistribution. While dendritic cell-derived EVs were preferentially taken up by the spleen, melanoma cell-derived EVs accumulated more prominently in the liver.[56] Many studies indicate that similar to administration of liposomes, systemic EV administration leads to non-specific accumulation in the liver, spleen, gastrointestinal tract, and lung.[56,57] Interestingly, native EVs also showed substantial accumulation in tumour tissue,[56,57] an effect further enhanced by addition of a specific targeting ligand. However, the half-life of EVs is considerably shorter than that of liposomes. Even when stealth properties were implemented *via* polyethylene glycol, the terminal half-life of EVs was at most 60 minutes,[7] while that of modified liposomes was up to several hours.[58] Notably, these studies used fluorescent dyes to label EVs and radionuclides to label liposomes, a difference that may affect comparability. Therefore, more comparative biodistribution studies are required, especially with non-cancer cell-derived EVs. A head-to-head assessment comparing the delivery efficacy of vesicles and liposomes would also require optimisation of the liposomal comparator system in addition to EV engineering.[59] Another important yet underestimated parameter indicating the efficacy of EV nanocarriers is the mechanical stiffness of

228 the target cell environment. A recent approach using extracellular matrix-simulating hydrogels showed
229 that EVs were superior to liposomes in escaping stress-relaxing environments,[60] indicating the
230 influence of vesicle surface proteins.
231 *Immune responses and potential toxicity.* Owing to the occurrence of adverse immunological reactions
232 to nanomedicines, such as anaphylaxis, cytokine release syndrome, neutralisation of biological
233 activity, cross-reactivity with endogenous protein counterparts, and non-acute immune reactions, the
234 production of biologicals has been terminated.[61] EVs have been widely claimed to be biocompatible
235 based on their mammalian cell origin and 'physiological' composition, but such generalisation should
236 be avoided. Intravenous administration of EVs purified from bovine milk to mice induced no adverse
237 events and only moderate cytokine release.[62] Similarly, a wealth of safety data are available on
238 administration of blood cell-derived EVs during blood transfusions; in most cases without notable
239 adverse effects , even though platelet-derived EVs have most recently been associated with
240 transfusion-related acute lung injury.[63] In contrast, the potential oncogenic activity of EVs—especially
241 stem cell-derived EVs with angiogenic activity, which have potential tumour-promoting activity in
242 pre-existing tumours that are dormant due to the lack of angiogenic activity—remains a major
243 concern.[64] EVs can carry tumour or pathogenic peptides presented by major histocompatibility class
244 receptors to elicit interactions with immune system components, a technique used, for example, in
245 cancer immunotherapy.[65,66] For carrier development, EVs should have low immunogenicity and be
246 derived from healthy human cells. Intravenous and intraperitoneal administration of human embryonic
247 kidney cell-derived EVs to mice for three weeks did not show toxic effects.[67] This finding provides an
248 important indication for the potential use of EVs as drug carriers, and almost all data from non-human
249 primate studies are similarly reassuring. In summary, the immunogenicity and biocompatibility of
250 each individual EV formulation must be evaluated, as is the process for liposomal carriers and
251 biologics. Specifically, studies in suitably complex *in vitro* models, including advanced 2D and 3D
252 models,[68] and their translation to rodent and non-primate animal models should be evaluated. In
253 addition to organ distribution studies and careful selection of EV labelling methods,[69] studies
254 evaluating repeated dosing under various regimes are required to allow further clinical investigations.
255 The use of EVs in an autologous manner by transferring patient cells into culture medium and
256 isolating vesicles for re-administration to the patient has been proposed. Although EVs could be used

in this manner for certain applications, several issues limit this strategy, particularly for acute diseases such as infections or cardiovascular incidents.[70] The autologous use of EVs is feasible under certain conditions, including those for which a) the use of autologous EVs is important, *e.g.,* for EV-mediated transfer to mitochondria to maintain genetic compatibility,[71] and b) a source of autologous EVs is readily available, *e.g.*, for the use of blood- or plasma-derived EVs for autologous purposes, such as when vesicles from cancer patients are used in an autologous transplantation protocol aimed at delivering therapeutics to tumour tissues[72]. However, most applications are likely to use well-established non-autologous EVs primarily owing to need, safety considerations, and the regulatory/commercial desirability of a streamlined, exceptionally well-qualified product. This approach is preferred because non-engineered, non-autologous EVs have been administered to human subjects in numerous clinical studies with good safety outcomes.[38] Currently, this avenue is being pursued with MSC-EVs for regenerative medicine and EVs derived from dendritic cells for vaccine delivery, as these vesicles were found to be safe in several phase I clinical trials.[73]

Clinical translation of drug-loaded extracellular vesicles

While scientists and engineers have attempted to harness the unique properties of EVs to develop smart drug delivery systems that exhibit substantial benefits in targeting, safety, and pharmacokinetics compared to those of synthetic nanocarriers, clinical translation of EVs remains challenging.[74] Owing to the inherent complexity of the EVs themselves, size heterogeneity, and natural (batch-to-batch) variations encountered during their production, the intrinsic risks of the production process are higher than those of purely synthetic production systems.

By appropriate selection and/or engineering of the cells from which EVs are derived, various platforms for loading EVs and conjugating targeting moieties have been developed.[2] These can be divided into three groups: (i) natural EVs, which are native or obtained from genetically engineered cells; (ii) hybrid EVs, which are post-modified with drugs or surface ligands; and (iii) EV-inspired liposomes (**Fig. 2**). The active substance in EV-based therapeutics determines their pharmaceutical classification.[38] From a regulatory perspective, the aforementioned groups likely fall into the pharmaceutical category of biologicals (termed biological medicines, biologicals, or

biopharmaceuticals, depending on regional practices),[38] which includes medicines that contain one or more active substances made by or derived from biological cells. Generally, the active substances in biologicals are more complex than those in non-biological medicines. Currently, substances and constructs with this level of complexity can be produced only by living organisms. However, such production methods are intrinsically faced with a degree of inherent biological variability, which may result in product heterogeneity. From a process design perspective, this heterogeneity is influenced both by biological processes inside the cells used to express the biologicals (upstream processing) and by the manufacturing process used to produce the biologicals (downstream processing).[75] Notably, culture conditions, such as the cell passage, cell density, and frequency of EV harvesting, strongly influence aspects of product quality, including yield, EV composition, and EV bioactivity.[76] In contrast to mammalian cells produced for cell therapy, for which such deviations may be at least partially compensated by phenotypic adaptations and *de novo* synthesis, EVs are relatively static products; thus, they are not expected to change significantly after harvesting (with the exception of degradation). Quality-by-design approaches have been proposed for cell-based products. The key tenet is that quality should be built in by design.[77] While this approach requires an in-depth understanding of the process and process parameters that need to be evaluated for their association and impact, an analogous approach is appealing for the production of EVs. However, changes in product quality are more challenging to detect and assess in EV production processes than in protein production processes. Overall, because minor changes may have considerable impact on product quality and activity, process stability for EV manufacturing is considered to be lower than that for cell and antibody production. Despite this limitation, EV manufacturing can build on these processes.[78] While significant parts of good manufacturing practices can be adapted from the existing fields of biologics, liposomes and cell-based therapies, the size and the compositional and structural complexity of EVs are unique and therefore require additional in-process controls. These in-process controls include measures to ensure that the final product meets the previously identified critical quality attributes and may include measurement of size and concentration, exclusion of contaminants, identification of functional markers, and evaluation of drug loading to assess the therapeutic activity per number of vesicles. Due to the size and complexity of EVs, technological limits may hamper the detailed physicochemical, immunochemical, or functional characterisation of the EV product from an

analytical standpoint, specifically, from a production and quality control perspective. The currently limited ability for product characterisation also has implications for the mechanistic understanding of EV drug delivery products and may render the translational process cumbersome and risky, similar to that of other nanoparticle-based products. However, despite the recent controversy about the potential of nanomedicine,[79] nanosized formulations permit biodistributions that are different from those possible for free drugs and hence can confer considerable benefits.

Current state. Manufacturers of biologicals are asked to identify all substances in a drug that cause a certain pharmacological, immunological, or metabolic action and are responsible for the biological (therapeutic) effects, indicating the mode or mechanism of action of the drug. Additionally, non-active components (termed excipients) used in the final formulation should be declared. Interestingly, in a recent review by the ISEV, it was postulated that if the whole therapeutic effect could be ascribed to the loaded molecules and not to the EVs in systems with drug-loaded EVs, the EVs would be considered excipients. In this case, only the safety profile and not the mode of action would be requested for the EVs. However, the suitability of conventional toxicity testing approaches for biopharmaceuticals and EVs is limited[38] owing to the unique structural and biological properties of these products, such as species specificity, immunogenicity, and (unpredicted) pleiotropic activities. Individualised risk analysis-centred approaches, such as those applied to human cell-based products, may be more applicable, as recently suggested by the ISEV community.[38]

Scale-up and manufacturing. While EV manufacturing is expected to profit from knowledge in other fields, including the vast experience in protein manufacturing and increasing expertise in cell therapy, the following unit operations are unique to EV production and therefore deserve special attention (**Fig. 3**).

Key Unit Operations

Upstream

Cell culture and characterisation of EV sources. EV manufacturing can profit from developments in classical biologics (*i.e.*, antibody and protein production) and cell therapy. Host cell selection and culture conditions constitute critical upstream process steps.[80] Currently, there is no consensus on the optimal technology for EV production. Parent cells need to be selected based on the activity and tissue

homing properties of EVs and on potential immunogenic and oncogenic considerations. Additionally, genetic stability, host cell impurities (such as pathogens, and especially viruses) and EV yields play a major role in the parent cell selection process.[81] Once selected, these host cells are cultured to efficiently yield EVs with the appropriate phenotype. Suggested methods include multi-layered culture flasks, bioreactors, and hollow fibre cartridges. For small-scale manufacturing, cells can be expanded in shake flasks, spinners, roller bottles, wave bags, or bioreactors. For large-scale cell culture, cells can be grown in stainless steel bioreactors (up to 20,000 L scale), platform-rocker wave bags (up to 500 L scale), or even in disposable bioreactors (up to 2,000 L scale).[81] From a process safety perspective, closed systems are preferred; however, these systems are more difficult to monitor than open systems.[42] Genetic drift and contamination need to be monitored closely, following protocols used in the plants producing biologicals and cell therapies. Accumulating evidence indicates that bovine milk-derived EVs may be a valid alternative source for obtaining large amounts of biocompatible vesicles. Although feasibility studies using milk EVs as drug carriers are underway,[82] large-scale isolation of pure vesicles from complex milk stills needs optimisation.[62]

Optional endogenous drug loading. Parent cells may be engineered to boost EV production and/or yield EVs with enhanced properties. Several methods to load EVs with different molecules have been experimentally evaluated.[83] These methods include (i) endogenous techniques in which the EV-producing cells also equip vesicles with drug cargo or modified structural protein/RNA components or (ii) exogenous approaches in which drugs are loaded into EVs post isolation. The endogenous loading technique has a lower degree of complexity if the producer cells directly shed EVs containing the desired molecule.[84] Endogenous loading approaches have also been used for the encapsulation of nanoparticle-based drugs into EVs.[85] Because the loading efficiency is typically limited, cells may be genetically engineered to produce EVs containing the desired active components. However, this approach is limited to biologically accessible drugs, such as nucleic acid and protein drugs. Recently, an optogenetically engineered exosome system that integrates a blue-light responsive membrane module for controllable protein–protein interactions to encapsulate large quantities of anti-inflammatory proteins into EVs was presented.[86] While highly interesting, genetic engineering of EV-producing cells results in more complex production upscaling than isolation of vesicles from naïve cells and is less elaborate than post-isolation engineering.

373

374 ***EV Harvesting and Engineering***

375 *Product separation and characterisation of prepared EVs.* For EV isolation, the primary separation of
376 products from cells can be accomplished by well-established procedures for the isolation of biologics,
377 including centrifugation, depth filtration (mechanical sieving and adsorption) or tangential flow (cross
378 flow) filtration . While several strategies for EV isolation and purification, such as differential
379 ultracentrifugation (dUC), precipitation, size exclusion chromatography , affinity chromatography, and
380 tangential flow filtration, have been evaluated, there is no consensus on an appropriate EV isolation
381 technique for large-scale manufacturing.[87] This lack of consensus is mostly because some procedures
382 may negatively affect the integrity and quality of EVs. Additionally, yield and product purity vary
383 among methods and reports, and the overall EV purity generally appears to be low.[88] Moreover, for
384 heterogeneous EV populations, the isolation method may lead to selective isolation of one specific
385 subpopulation with higher or lower biological activity than the total population. Density gradient
386 centrifugation and dUC offer a decent compromise in terms of yield and purity. dUC is broadly
387 accepted as a common method to obtain 'pure' EVs, especially when using chemically defined media
388 without foetal bovine serum. However, careful evaluation of artefacts introduced by the culture
389 medium is pivotal, as it was recently shown that co-precipitation of nucleic acid impurities is possible
390 even under these conditions.[89] In fact, recent studies suggest that ultrafiltration and size exclusion
391 chromatography (alone or in combination) outperform dUC in terms of both yield and purity.[90,91]
392 However, depending on the required EV purity, orthogonal methods that target different EV
393 characteristics may need to be integrated at the expense of yield.[89] Another intrinsic problem arises
394 because most of the currently used purification techniques were originally developed for the
395 purification of viruses,[42,92] which are amongst the most critical potential contaminants of EVs.
396 Recently, Barone *et al*. comprehensively analysed the risks, costs, and implications of viral
397 contamination in biological manufacturing.[93] Virus risk mitigation strategies for general
398 biopharmaceutical manufacturing are based on three tenets: (i) prevention of viral entry by selecting
399 low-risk starting and raw materials and using manufacturing controls; (ii) testing of in-process
400 materials to ensure they are free of virus and enable lot rejection; and (iii) clearance of viral
401 contaminants (*via* inactivation and/or removal) from the product. While both careful selection of cells

and raw materials and testing (based on polymerase chain reaction or *in vitro* virus assays) can reduce risk, the unique size of EVs makes the inactivation and/or removal of viral contaminants even more challenging for EVs than for other biopharmaceutical products. Current strategies are based on affinity rather than size exclusion. Extensive multi-step downstream processing may, however, critically force manufacturing costs to increase. In addition to the challenges associated with the complete characterisation of contents (*including* contaminants), the spatial and conformational organisation of the constituents is extremely challenging to assess with existing analytical techniques. Even small changes in composition or architecture can significantly affect efficacy and safety. The use of functional activity assays is of utmost importance because isolation methods and the presence of other EV populations or contaminants may decisively influence product activity and off-target effects.

Exogenous drug loading. For clinical EV translation, reproducible and technologically accessible methods are needed to load them with the desired drugs.[94] While loading methods for liposomes have been optimised and applied in industrial production, such settings for EVs are still lacking. Exogenous loading methods work passively by the association of drugs with the lipid bilayer membrane after incubation,[83] by attaching therapeutics to the EV surface,[95] and by mechanical or chemical techniques to transiently open the EV membrane to allow diffusion of compounds into the vesicle. The most common approaches to temporarily permeabilize the membrane include sonication, electroporation, saponin treatment, and passive incubation.[96] The advantages and disadvantages of each approach depend on the experimental settings, the types of drugs and source of EVs, and they can be scaled (**Fig. 4c**). Passive incubation is a very simple method in which purified EVs are incubated with drugs to allow incorporation into the vesicle membrane. Many early loading protocols followed this method because it exhibits excellent performance for incorporation of hydrophobic compounds such as curcumin.[97] However, the stability of drugs loaded by passive incorporation across the EV membrane is still unclear. For hydrophilic compounds, loading may be enhanced by the addition of saponin, which has been shown to be effective for large proteins.[98] Saponins are mild surfactants that induce transient membrane destabilisation and may also affect biomolecules; thus, careful purification is needed when saponins are used on a large scale. Mechanical methods for permeabilising the EV membrane, such as electroporation or sonication, have been shown to be successful for both small molecules and macromolecules.[50] Even though these methods can be scaled up, their potential

influence on protein and nucleic acid drugs requires careful consideration.[99] In addition to concerns with maintaining the stability of biomacromolecules, the size of the drugs poses another challenge during EV-loading procedures. Large enzymes >200 kDa have been successfully loaded into EVs using saponin pretreatment.[98,100] As the size of nucleic acids that may be encapsulated exogenously into EVs is also limited, a cell nanoporation method for large-scale production of functional EVs has been developed.[101] EV yield and mRNA loading were enhanced by this method; however, the required additional steps of transfection and electrical stimulation render its industrial adoption relatively difficult.

Recently, an alternative method based on liposome fusion has been proposed.[102] Liposomes containing fusogenic lipids were incubated with EVs, and the cargo of the synthetic liposomes was merged with that from the EVs. Such an approach may pave the way for efficient loading of larger molecules without compromising the EV membrane.[95]

Downstream

Purification. In addition to the EV isolation and purification steps prior to drug loading, additional purification steps may be necessary to remove the free drug and exclude the potential contaminants introduced during post-processing (**Fig. 4b and d**). Magnetic immunoaffinity purification has gained increasing attention owing to the high purity of the obtained products. However, low yields are generally obtained, although theoretical yields may be underestimated due to contaminants.

Quality control—(minimal) characterisation of engineered EVs. For routine manufacturing and product monitoring, critical quality attributes need to be defined. Methods to assess these attributes may include evaluations of parent cell properties (*e.g.*, evaluation of viability and surface marker expression to assess the phenotype), EV characteristics (*e.g.*, evaluation of quantity, size, and surface marker expression), assessment of microbial contamination (*e.g.*, detection of endotoxin and mycoplasma) and (application-specific) functional activity.[42] For assessment of batch-to-batch variations, we suggest the use of a concept similar to that applicable to biosimilars—the analytical characteristics of the products should be highly similar to those of the reference product. While non-cell culture methods (such as isolation from plasma or milk) offers interesting alternatives to cell culture and access to potentially large amounts of EVs, these sources contain EVs originating from

460 many cell types[103] that cannot be separated easily. Therefore, characterisation of the functional activity
461 of EVs is even more crucial in quantifying on-target and off-target effects.
462
463 *Formulation and shelf life.* Recent studies have focused on the formulation and storage conditions
464 (*e.g.*, –80 °C vs. 4 °C) of EVs by assessing the size, charge, and number of EVs, but strong
465 correlations have not been found.[95,104] Storage at 4 °C has been shown to cause aggregation and
466 damage to the EV structure.[105] Moreover, even though the size and number of EVs remained
467 unchanged at −80 °C, alterations in biological activity were detected.[106] Lyophilisation has been
468 investigated as an alternative for long-term storage; however, its impact on vesicle integrity during
469 reconstitution depends on the use of cryoprotectants.[105] Although storage at −80 °C is recommended, it
470 is logistically most challenging and costly method (**Fig. 4e**).
471 **Safety by design and process de-risking.** While a few years ago, the mammalian cell origin of EVs
472 was a major hurdle to their clinical translation, considerable advances have been made in cell-based
473 therapeutics. Regarding safety, EVs derived from autologous cells are associated with lower risks than
474 EVs derived from heterologous cells (including cell lines). However, the time needed to produce
475 autologous EVs is often incompatible with the time available for initiating treatment. During the time
476 required for the manufacturing and quality control of patient-specific EVs, the clinical condition of the
477 patient may worsen, making it impossible to administer the personalised product. Despite the several
478 examples of autologous products developed and commercialised by pharmaceutical companies, the
479 current frameworks seem to be predominantly suited for small-scale academic production rather than
480 for large-scale pharmaceutical production, and production costs may be prohibitive. While the use of
481 allogenic EVs appears generally feasible, the selection of parent cells, assessment of immunologic and
482 oncogenic effects, and risk of viral contamination need to be minimised by continuous monitoring.
483 Selection of assays for monitoring, particularly their sensitivity, is a key challenge in determining the
484 time required for clinical translation of EV-based drug carriers. Regulators have yet to release
485 guidance on how the safety and potency of these EVs should be tested. Currently, EVs are tested batch
486 by batch, with each laboratory and company using different assays.[107]
487

Perspectives

EVs may be used as carrier systems for various drug delivery applications. Compared to standard delivery methods, EVs have been shown to deliver functional cargo with decreased immune clearance when administered systemically to rodents. However, more evaluation in clinically relevant systems and direct, quantitative comparison with liposome-based alternatives are required to comprehensively assess the risk/benefit ratio.[59] Successful translation of EVs depends on the availability of cost-effective large-scale production, isolation, and characterisation methods with high sensitivity to assess batch-to-batch variations (and their biological consequences), and the availability of widely applicable methods for loading drugs (**Fig. 4f**). The increasing availability of new analytical techniques is expected to provide new insights into the uniqueness of EVs and may inspire the engineering of next-generation synthetic systems. The production of artificial EVs or EV mimics can overcome challenges related to sterility, mass production and regulation. Exciting new avenues, including the fusion of drug-loaded liposomes with EVs to improve drug loading capabilities, are already being explored.[102] Notably, the production of designer EVs by implanted cells has recently been reported. This technique offers a new route for *in vivo* production of engineered exosomes inside the body.[108] Despite these promising results, more insights into the mechanisms that make EVs so effective at infiltrating cells and evading immune detection are needed to unlock their full potential.

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Annotations:

7 Kooijmans
This original work presented one of the first examples for modifying EVs with polyethylene glycol and the influence on *in vivo* biodistribution of vesicles.

38 Lener
A seminal position paper from an international consortium of EV scientists on the regulatory needs when studying vesicles in clinical trials.

42 Rhode
This perspective provides a roadmap for the development of EV-based therapeutics in a very early stage of manufacturing as well as during early clinical safety and proof-of-concept testing.

48 Welsh
This paper provides guidelines on the standardization of commonly used analysis platforms for characterizing EV refractive index, epitope abundance, size and concentration

60 Lenzini
In this recent work, the influence of mechanical properties of EVs on the diffusion from extracellular matrix simulating environments was evaluated.

83 Fuhrmann
One of the first comprehensive examples of comparing different loading methods for various types of EVs using a set of model compounds with varying water solubility.

102 Piffoux
This work gives a well-studied example of controlled fusion of EVs with liposomes to enhance loading and surface functionalisation of the vesicles.

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Figure Captions

Figure 1. *Illustration of extracellular vesicle (EV)-mediated cell cross-talk, clearance mechanisms, and immune responses. EVs are produced as heterogeneous mixtures of different subpopulations, and they may participate in proximal and distal communication between cells. After entering the systemic circulation, they must avoid elimination organs, such as the liver, lungs, and kidneys, as well as immune cells. Their target tissue efficiency depends on the degree of functionalisation and target cell interaction.*

Figure 2. *EVs can be grouped based on the origin of their constituents into native EVs, EVs originating from (genetically) engineered cells, post-modified EVs, or EV-inspired liposomes. As EVs are of cellular origin, they are intrinsically more complex than synthetic carrier systems. DLS:*

Dynamic Light Scattering; NTA: Nanoparticle Tracking Analysis; FC: Flow Cytometry; WB: Western Blot Analysis.

Figure 3. *Process steps and key unit operations in the production process of drug-loaded EVs. While upstream processing can adapt safety and quality concepts used in the production of cells for cell therapies, downstream processing can partially adapt concepts used in the production of biologicals. EV isolation and purification are unique and intrinsically prone to viral contamination (sterility issues during upstream and downstream processing are indicated by exclamation marks), which is difficult to remove due to the similar colloidal properties of EVs and viruses and because most purification processes were developed for the purification of viruses.*

Figure 4. *Illustration of technological manufacturing options. (a) Consideration of the cell source and associated risks. (b) Isolation methods as a function of EV yield and purity. Abbreviations: TFF - tangential flow (cross flow) filtration, DGC - density gradient centrifugation, SEC - size exclusion chromatography, dUC - differential ultracentrifugation.. (c) Available drug loading methods as a function of loading efficacy and costs for large-scale production.[109] (d) Sizes and densities of potential contaminants and impurities,[46] which are to be quantitatively detected and removed. (e) Methods for formulation and storage of final EV products and the effects of these methods on EV quality.[110] (f) Colour-coded guidelines for successful EV-mediated drug delivery. EV development for drug delivery involves the selection of large-scale isolation and characterisation, assessment in immunogenicity and biodistribution models, drug loading and functionalisation, selection of liposomal comparators, formulation/storage, and assessment of batch-to-batch variation. The current state of technology and literary evidence regarding the considerations in each section supporting potential preclinical development is indicated by colour. On the relative scale, high implies comprehensive examples in the literature and substantial technological development, medium indicates the need for further studies based on ongoing efforts, and low indicates that more fundamental and comparative studies are required. This diagram indicates that research in no section has reached the highest level and that additional scientific assessments are still required before more standardised and large-scale methods can be developed to bring EV-based carriers closer to clinical translation.*

Boxes

Box 1. **Summary of the minimal information for studies of extracellular vesicles (MISEV) guidelines defined in 2018**.

Parameters to be determined	*Readout and relevance*
Size distribution should be analysed using techniques such as nanoparticle tracking analysis and tuneable resistive pulse sensing.	Size and yield
Single EVs should be visualised at high resolution, *e.g.*, using electron microscopy or single-particle analysers.	Morphology and presence of a bilayer
No broadly applicable molecular markers were proposed. However, at least one transmembrane or cytosolic protein should be analysed to demonstrate the characteristics of EVs.	Differentiation from cell debris
No recommendations were made for universal negative markers. However, contaminants and major constituents of non-EV structures	Degree of purity of the EV formulation

should be carefully depleted.	
The generalised term ‘extracellular vesicles’ should be used when a clear identification of the subpopulation is lacking.	Avoiding misuse of nomenclature

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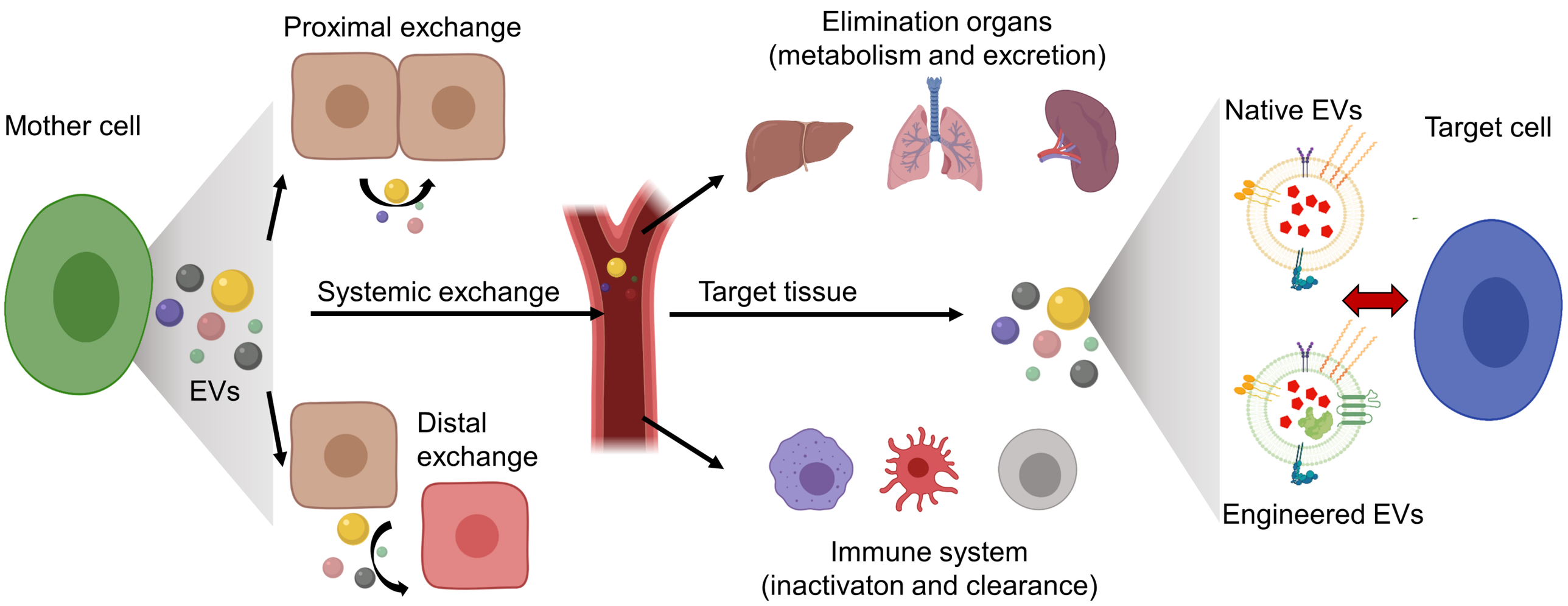

Proximal exchange
Elimination organs
(metabolism and excretion)
Mother cell
Native EVs
Target cell
Systemic exchange
Target tissue
EVs
Distal
exchange
Immune system
(inactivaton and clearance)
Engineered EVs

Cells

Native EVs

EVs from genetically engineered cells

Post-modified EVs
(drug loaded, surface modified)

EV-inspired liposomes

Liposomes

Characteristics

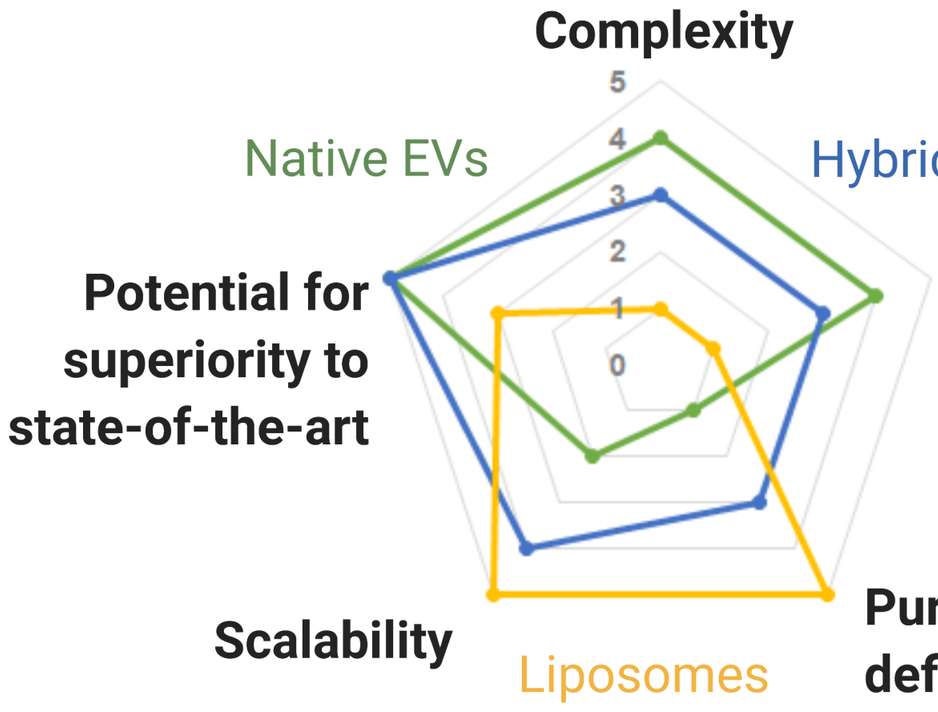
native — hybrids — synthetic/engineered

Size distribution
DLS, NTA, etc.

Composition
FC, WB, proteomics, etc.

Structure /Visualisation
Electron Microscopy

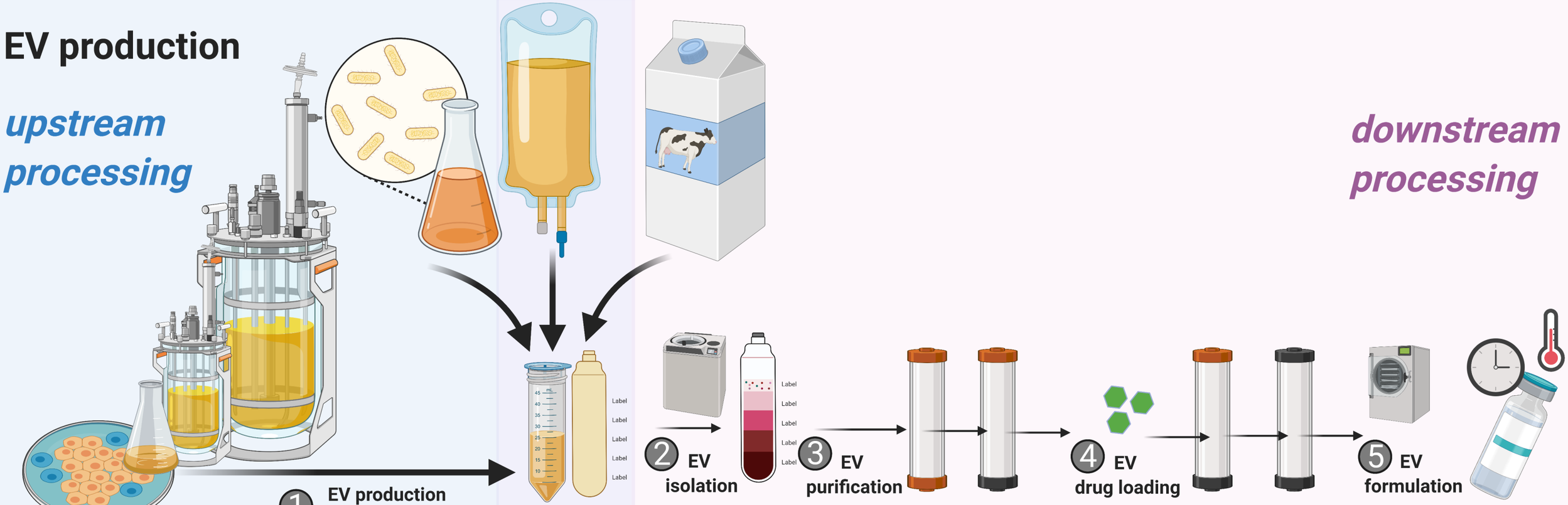
Activity
Functional assays



EV production

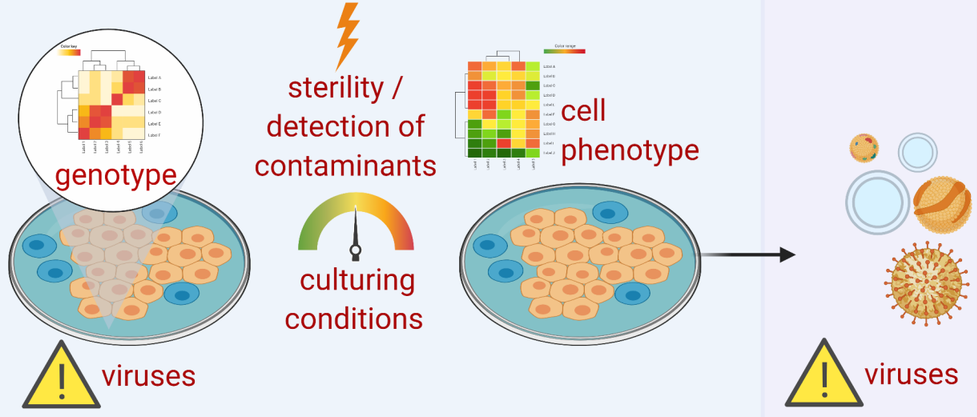
upstream processing

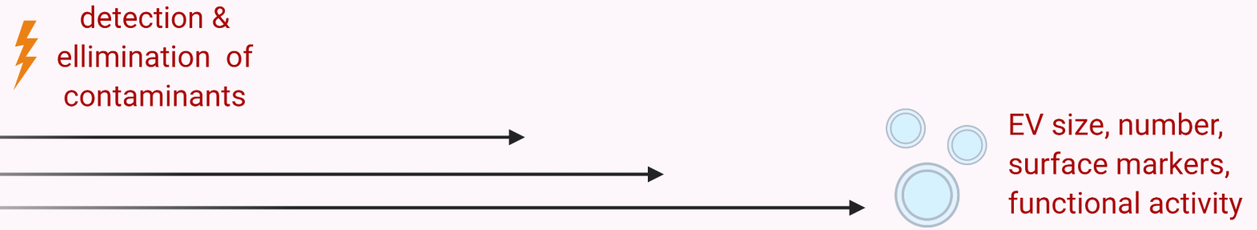
downstream processing



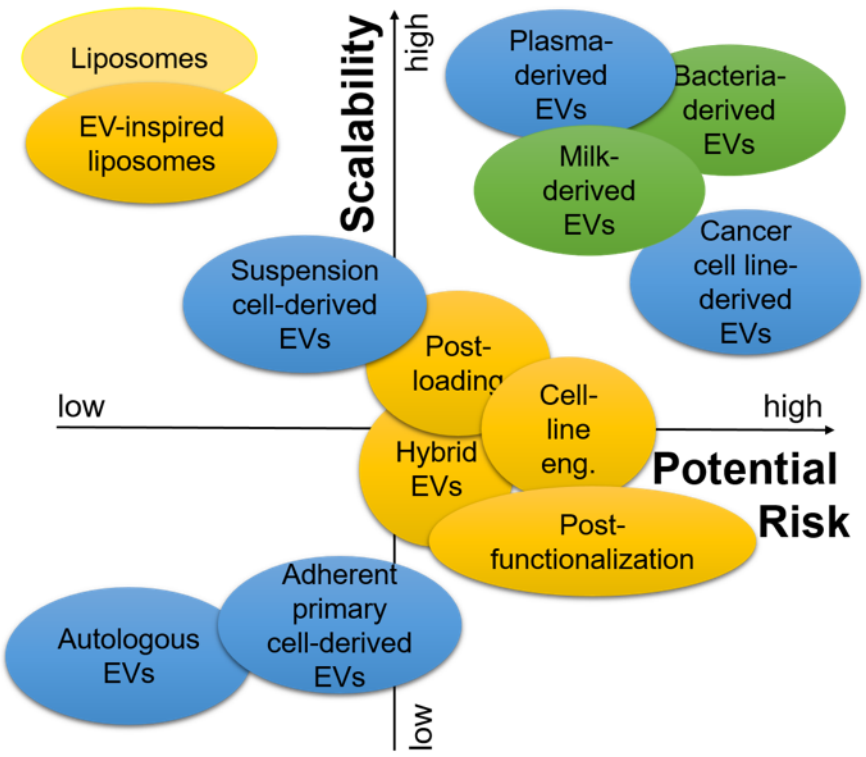
upstream | **downstream**

cell therapies | EV specific | biologicals

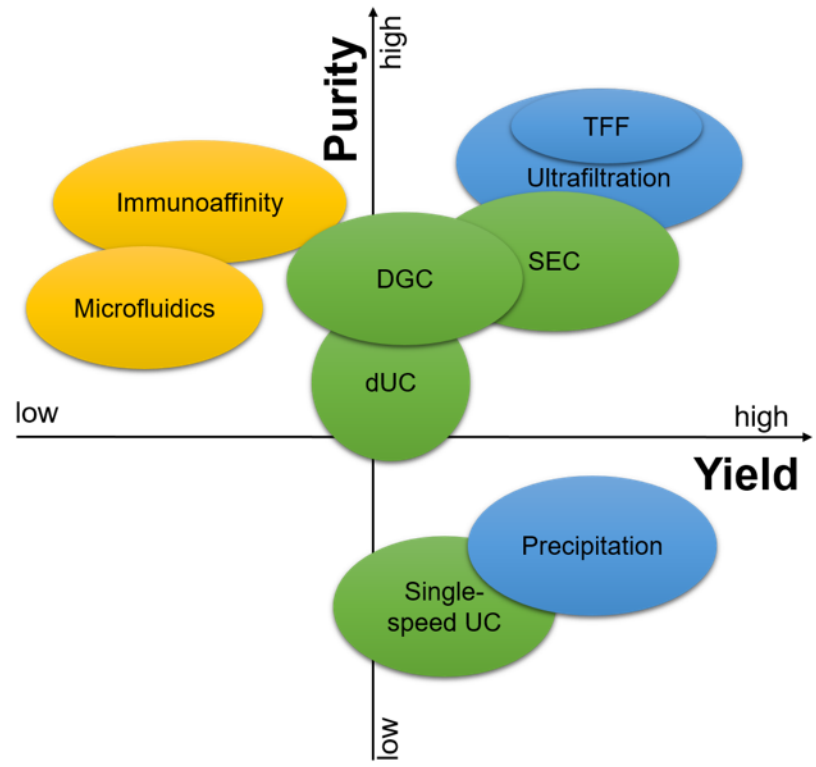




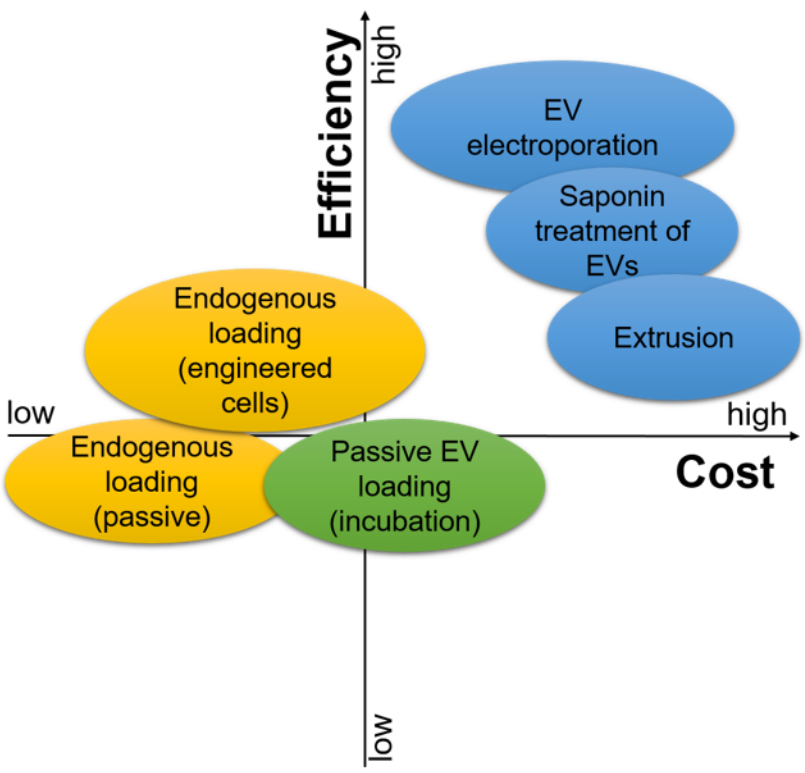
a Selection of Parent Cells



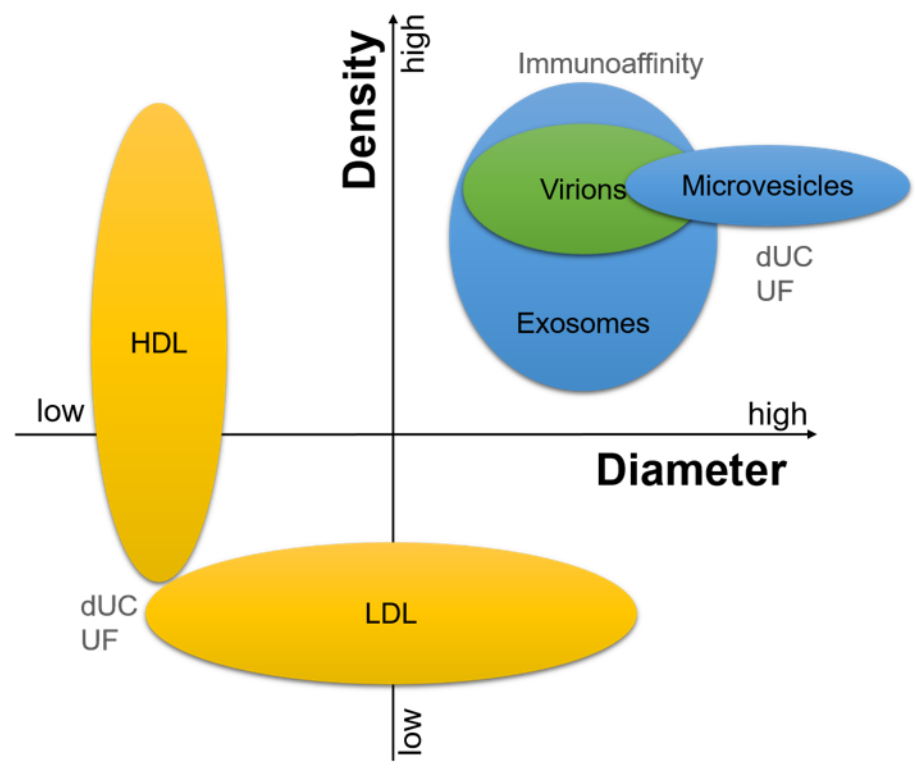
b Primary EV Isolation



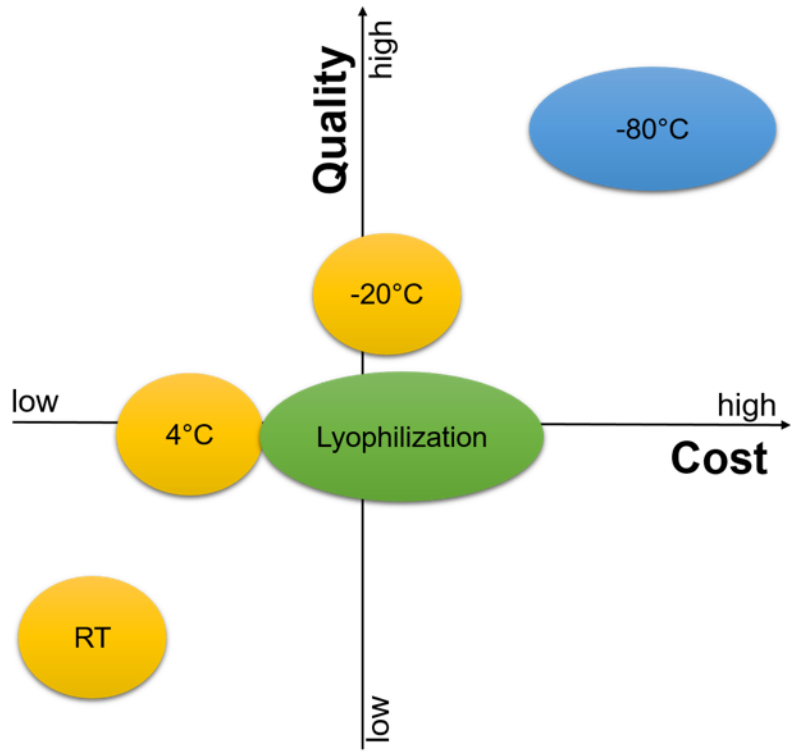
c EV Drug Loading



d EV Purification & Contaminants



e EV Formulation & Storage



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