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Review article

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The physico-chemical landscape of extracellular vesicles

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Abstract

Extracellular vesicles are defined as nanosized to microsized particles, delimited by a lipid bilayer membrane, that seem to be released by all cell types and that can transport bioactive molecules between cells, tissues, organs and organisms. Therefore, extracellular vesicles are being explored as biomaterials, drug delivery nanocarriers, therapeutics and multiplexed biomarkers. However, the isolation, characterization and large-scale manufacturing of extracellular vesicles remains challenging. In this Review, we delineate the landscape of the physico-chemical properties of extracellular vesicles, originating from their composition and conformational ensemble, and outline how this landscape is defined by the heterogeneity of their size, composition, membrane structure, surface interactions, cargo and co-isolates. We explore the implications of this heterogeneity on the definition of the purity, identity and function of extracellular vesicles. The comparison of this landscape with the landscape of antibody therapeutics and viral vectors can identify lessons to be learned from the bioprocessing of these products, and the distinct challenges associated with the manufacturing and isolation of extracellular vesicles from biofluids, which will require new concepts and technologies. We highlight the importance of a thorough understanding of the physico-chemical properties of extracellular vesicles for their clinical translation. This includes the development of bioprocessing approaches, assignment of product quality attributes, consistency of extracellular vesicle products and manufacturing at scale.

Sections

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Key points

- The complexity of the physico-chemical landscape of extracellular vesicles exceeds that of protein biologics and viral vectors, thus complicating the definition of extracellular vesicle identity and purity.
- Extracellular vesicle populations may be viewed as a continuum of vesicle structures.
- The physico-chemical landscape of extracellular vesicles can inform approaches for their isolation, characterization and large-scale manufacturing.
- New and standardized technologies are required for the clinical translation of extracellular vesicles as marketable products.

Introduction

Natural therapeutic compounds, such as monoclonal antibodies (mAbs)[1] and RNAs[2,3], are often difficult to transform into drugs owing to their limited stability in biological fluids. In addition, targeted delivery of such compounds to specific organs and cells remains challenging but is required to minimize off-target toxic effects. Accordingly, approximately 40% of new drugs do not pass clinical validation, owing to inadequate targeted cell delivery following systemic administration[4]. To address these challenges, several nanocarriers (biomaterials in the nanometre size range) have been proposed[5]. These nanocarriers include synthetic materials, such as polymer micelles, dendrimers, lipid nanoparticles and liposomes[6], as well as nanocarriers of biological origin, such as viral vectors, adenovirus and adeno-associated virus (AAVs)[7].

Extracellular vesicles (including exosomes) are lipid-based biological nanoparticles produced by cells. These vesicles are delimited by a lipid bilayer and cannot replicate on their own[8]. Extracellular vesicles evolved to transport various bioactive molecules (proteins, RNAs, lipids and metabolites) between cells, tissues, organs and organisms[9]. Their high delivery efficiency may be related to their natural or acquired tropism, which allows them to cross tissue and cellular barriers, resulting in fewer off-target effects when compared with synthetic nanoparticles[10,11]. Owing to these inherent natural abilities, extracellular vesicles are being investigated as therapeutics and nanocarriers[8–17]. Bexsero is the first extracellular-vesicle-based therapeutic product that has received marketing authorization. This product is based on outer membrane vesicles derived from *Neisseria meningitidis* and is approved as a vaccine for meningitis[18].

The therapeutic potential of allogeneic extracellular vesicles originating from human cells, such as mesenchymal stem cells and cells derived from adipose tissue or bone marrow, has also been preclinically and clinically tested for various applications, including inflammatory disorders (such as graft-versus-host disease), neurological diseases, tissue injuries, cancer and coronavirus disease 2019 (COVID-19)-induced acute respiratory syndrome distress[18–21]. Such studies may further elucidate the safety, efficacy and mechanisms of action of extracellular-vesicle-based therapies. However, most such products remain at preclinical stages[22,23], and therefore more insight into the mechanisms and functions of extracellular vesicles is needed, including their physico-chemical properties, structure, isolation and biological functions in different in vitro and in vivo systems[8,10,12,14,15].

In this Review, we delineate the physico-chemical landscape of extracellular vesicles, which refers to the interconnected physico-chemical properties that originate from their composition and conformational ensemble. In particular, we explore the physico-chemical landscape to identify the intrinsic features of extracellular vesicles that differentiate them from current viral vectors and mAb products (Fig. 1). This comparison highlights lessons that can be learnt from the bioprocessing of these products and that can be translated to extracellular vesicles, such as continuous manufacturing, digitalization and microfluidic technology, as well as new concepts and technologies required for the study and manufacturing of extracellular vesicles.

The physico-chemical landscape

Establishing proteins and viral vectors as approved drugs requires their stability and a consistency in their physico-chemical properties from batch to batch. For example, antibody-based therapeutics benefit from high efficacy when compared with small molecules. However, this advantage requires the strict control of antibody properties. These properties are indicated as the 'developability' of the antibody, and include conformational and colloidal stability[24], prevention of non-specific interactions[25] and control of micro-heterogeneity originating from post-translational modifications[26].

Product heterogeneity becomes even more prominent for viral vectors as multiple scales are involved, from self-assembling components to the colloidal structure[27]. For example, the genome of AAVs (non-enveloped viruses used in gene therapies)[28,29] encodes several key proteins. These include the three capsid proteins VP1, VP2 and VP3, which self-assemble into heterogeneous structures with different compositions[30]. For enveloped lentiviral vectors, which are also used in gene therapies, variability is manifested in both composition and size. Enveloped lentiviral vectors have a broader size distribution[31] than AAVs, which show a more uniform size distribution with a diameter of approximately 25 nm (ref. 32).

The heterogeneity of these multidimensional properties further increases with extracellular vesicles (Fig. 1a), which can adopt different sizes, lipid and membrane protein compositions, surface molecules and cargos (such as nucleic acids, proteins and small molecules)[33]. Moreover, various molecules can adsorb on to the surface of extracellular vesicles in vitro and in vivo, forming the biomolecular corona. The biomolecular corona is a concept originally introduced in the context of synthetic nanoparticles[34,35], and then extended to extracellular vesicles and other biological nanoparticles[33]. The heterogeneity of extracellular vesicle conformations may be seen in analogy to protein folding, in which proteins of the same primary sequence can fold into various 3D structures[36]. This scenario can be captured by the protein folding landscape, which describes the energy levels of the different structural configurations, including quaternary structures and aggregates, such as amyloid fibrils[37,38]. A similar conceptual tool may be applied to extracellular vesicles; here, lipids, proteins, RNAs and other components can assemble into multiple structures with a variety of sizes and compositions, which determines their physico-chemical landscape (Fig. 1b–d). The free energy difference between alternative configurations may be small so that the landscape can be represented by a continuum of vesicle structures[39,40], rather than a series of discrete subpopulations (Fig. 1d and Box 1). This continuum of entities, which is analogous to the concept of fuzziness in protein interactions[41,42], provides a selective advantage for extracellular vesicles to be involved in multiple functions, including targeted delivery. This concept of

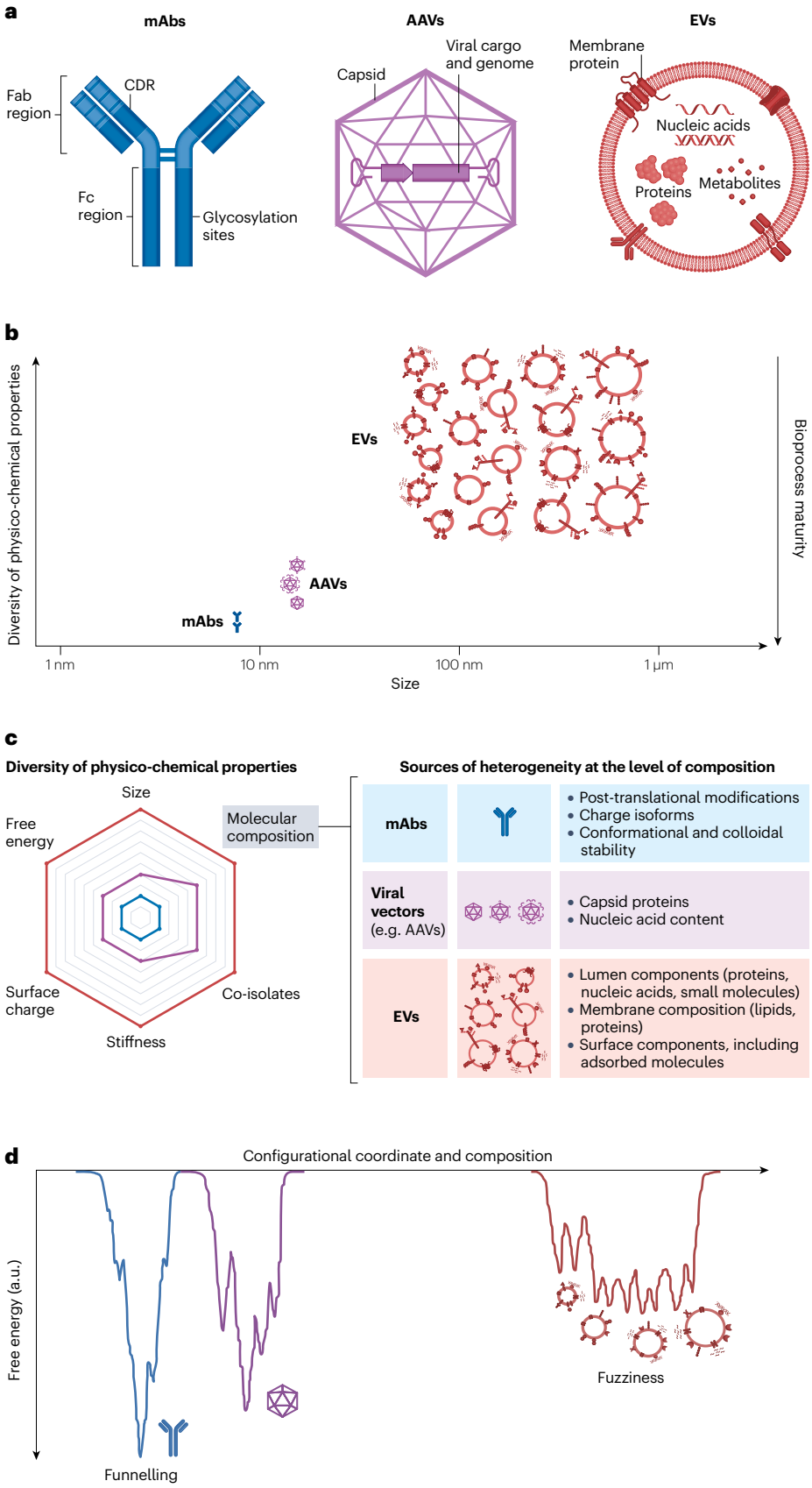


Fig. 1 | Landscape of physico-chemical properties of antibodies, adeno-associated viruses and extracellular vesicles. a, Structural comparison of monoclonal antibodies (mAbs), adeno-associated viruses (AAVs) and extracellular vesicles (EVs). **b**, Size, diversity of physico-chemical properties and maturity of bioprocesses for mAbs, AAVs and EVs. **c**, Physico-chemical properties involve size, molecular composition, surface charge, stiffness, number of co-isolates and free energy. **d**, The physico-chemical free energy landscape of extracellular vesicles in analogy to the energy landscape of protein folding, showing the continuum of vesicle structures, as compared with the landscape of mAbs and AAVs. CDR, complementarity-determining region.

Box 1 | The physico-chemical landscape

The free energy difference between alternative extracellular vesicle configurations can be considered so small that the extracellular vesicle landscape can be represented by a continuum of vesicle structures. This feature determines a peculiar difference between the landscape of proteins and that of extracellular vesicles. Protein conformations are determined by various intramolecular interactions with competing free energy contributions (referred to as frustration). Extracellular vesicles feature more multiscale compositional and structural heterogeneity than proteins, resulting in a higher amount of frustration. In the protein folding problem, frustration is resolved through a hierarchical and selective path of thermodynamically favourable conformations (referred to as funnelling), which may be assisted by external chaperones. For extracellular vesicles, the complexity is intrinsically built in to the heterogeneity of their chemical, physical and organizational properties to allow a multiplicity of interactions. This continuum of entities is analogous to the concept of fuzziness in protein interactions, or the conformational selection of intrinsically disordered proteins[41,42], and provides a selective advantage for extracellular vesicles to be involved in various functions, including targeted delivery.

The multiple spatially correlated and time-resolved interactions between extracellular vesicles and the molecular nanoscale and microscale components of the biological environment determine their identity and recognition[209]. Viewing extracellular vesicles through the lens of a rough free energy landscape suggests that definitions of their identity and purity go beyond composition, but rather encompass their mesoscale properties and co-isolates. These concepts should be revisited in the more complex landscape of physico-chemical properties.

a continuum extracellular vesicle structures can also be applied to discuss similarities and differences between extracellular vesicles and viruses. From a structural point of view, extracellular vesicles share properties with membrane-enveloped viruses, but are not infectious[40]. Extracellular vesicles may even be seen as enveloped viruses that have lost the ability to replicate, or viruses may have originated from extracellular vesicles that have evolved to become infectious[40]. These considerations highlight how relatively small changes in composition can lead to considerable differences in biological function.

In addition to their range of compositions and structures, extracellular vesicles cohabit biological fluids with individual and aggregated proteins, as well as with other extracellular nanoparticles (such as lipoproteins in blood)[43]. These extracellular biomolecules and nanoparticles can interact with extracellular vesicles and remain present as co-isolates in extracellular vesicle preparations, posing conceptual and technical challenges for extracellular vesicle purification. In particular, the biomolecular corona (which is a result of dynamic interactions between the surface of extracellular vesicles, proteins and other extracellular nanoparticles in biological fluids) can mask molecules on the extracellular vesicle surface, thereby redefining their surface properties and possibly altering their functions[33,44–54] (Box 2). In addition, the energy of unspecific binding of proteins on to the extracellular vesicle surface is of the same order of magnitude as the energy involved in the formation of extracellular vesicles, and therefore proteins acquired through corona formation may become components of the extracellular vesicles (Box 2).

At physiological temperature, the lipid bilayer of the extracellular vesicle membrane is typically in the fluid phase, despite its organized structure, and therefore membrane components are not static. This property contributes to the functionality and adaptability of cells. For example, regions of the plasma membrane characterized by a liquid-ordered membrane structure, such as lipid rafts, tetraspanin-enriched microdomains and glycolipid-enriched membrane microdomains, may play a role in extracellular vesicle biogenesis[55–57]. Therefore, the membrane of extracellular vesicles can exhibit heterogeneous structural arrangements[58] and differs from its parent cell lipid bilayer. This is indicated by a distinctively rigid lipid bilayer composition, which is a consequence of the selective enrichment in sphingolipids, cholesterol, glycerophospholipids, ceramide, tetraspanins and other membrane proteins associated with membrane rafts and integrins[59]. Accordingly, the extracellular vesicle membrane possesses distinct characteristics, demonstrating resilience against freeze–thaw cycles and high saline concentrations[60].

Implications for extracellular vesicle biomaterials

Definition of purity

Purity is a key quality attribute of a biological product and strongly connected to dosing. Purity, different from homogeneity, is defined by the kind and identity of desired components in the preparation. Therefore, determination of purity allows the identification and quantification of the amount of co-isolates (also referred to as impurities) present in the preparation. The definition of the kind and identity of a biomolecule may be straightforward, as it is based on the molecular sequence and structure. However, this definition is more challenging for extracellular vesicles, because they are heterogeneous in terms of composition, structure and activity[61] (Fig. 1) and because they represent a ‘mixture in a mixture’, with a range of co-isolates (in particular, lipoproteins) which are often difficult or impossible to distinguish or separate from extracellular vesicles. These co-isolates may be intrinsic to the starting material or arise during the manufacturing process and can contribute to the biological activity of extracellular vesicles.

Physico-chemical properties can serve as quantitative purity descriptors to grade extracellular vesicle preparations. However, homogeneity in size and/or density may be a poor description of purity for extracellular vesicles in the presence of other extracellular nanoparticles, which may have sizes and/or densities similar to those of extracellular vesicles[62] or which are part of the biomolecular corona (Box 2). For example, extracellular vesicle–lipoprotein complexes can form in different physiological conditions[48,49] and on manipulation[52], including the fusion of lipoproteins with extracellular vesicles[47]. Instead, purity can be quantified by specific extracellular vesicle stoichiometry, such as the ratio of volumetric number density of vesicles to the volumetric mass density of proteins, lipids or other extracellular vesicle molecules[8,63–66]. Expected contaminants may be defined based on the source of the extracellular vesicles (for example lipoproteins and serum-derived materials in extracellular vesicle preparations from mammalian blood).

Biomechanical and optical properties (such as nanoparticle stiffness and refractive index) can be assessed to profile mixtures of extracellular vesicles, lipoproteins and other nanoparticle co-isolates, thereby providing a purity parameter. For example, the stiffness of extracellular vesicle preparations can be screened at high throughput using atomic force microscopy (AFM)[52,67–70]. The refractive index of

nanoparticles, which is closely related to their density and composition, can be measured together with their size by interferometric nanoparticle tracking analysis[71,72]. In addition, interactions of extracellular vesicles with synthetic nanoparticles can be used to obtain purity indexes. For example, the clustering of gold nanoparticles on extracellular vesicles has been used to create a naked-eye colorimetric assay to test the presence of exogenous single and aggregated proteins in extracellular vesicle preparations[64,73] (of note, this assay detects proteins in solution, and not proteins adsorbed on extracellular vesicles). Similarly, lipoproteins have highly specific interaction signatures with gold nanoparticles[74]. The strength of these interactions is therefore specific to individual particles, and their quantification using a colorimetric assay may serve as a nanoruler to probe extracellular vesicle stiffness[75]. Finally, vibrational spectroscopy fingerprints contain information specific to the molecular and mesoscale components of a mixture[76–78] and can thus be applied for grading the purity of extracellular vesicle preparations[79,80].

Extracellular vesicle function

Although the exact mechanisms of how extracellular vesicles function in vivo remain to be fully elucidated[81–84], their features can be exploited for extracellular vesicle-based therapeutics[20,23,85]. However, how the physico-chemical properties of extracellular vesicles affect their biological function remains to be thoroughly investigated (Fig. 2). Extracellular vesicle thermodynamics and the biomolecular corona may provide a quantitative frame to the dynamic association of soluble proteins, extracellular vesicles and lipoproteins, and their relation to the purity of extracellular vesicle preparations (Box 2). In contrast to small molecules and mAbs, a straightforward correlation between purity and functionality may not apply to extracellular vesicles, because of the functional effects of extracellular material interacting with extracellular vesicles upon separation[86]. For example, extracellular vesicles derived from cancer cell types that are prone to metastasize to the brain induce binding and aggregation of lipoproteins, affecting their uptake by target cells[48]. In addition, the biomolecular corona that forms around extracellular vesicles in blood plasma[45] contributes to their cellular uptake and in vivo biodistribution[53,54]. Extracellular vesicles isolated from human placental stromal cells can enhance angiogenesis in Matrigel and promote skin regeneration and immunomodulation in mice[46].

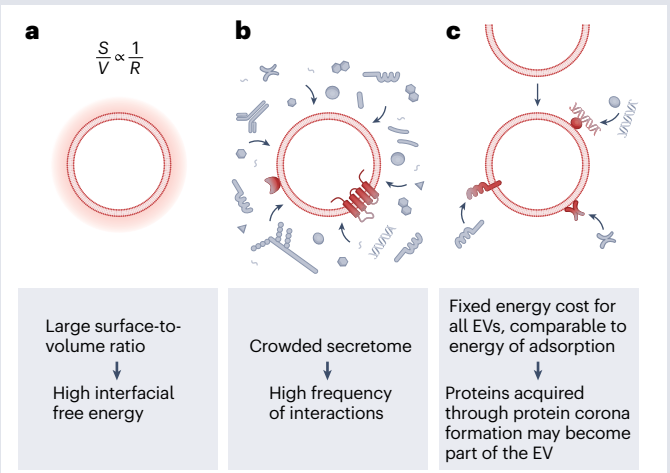
Stability. Extracellular vesicles are typically stable in vitro and can be frozen and thawed without compromising their structure. Depending on the extracellular vesicle type, they may also be stable in harsh environments, for example at low pH[87] or low ionic strength[88], and may

Box 2 | Thermodynamics and the biomolecular corona

Extracellular vesicles (EVs) are part of the nanostructured secretome of cells, a colloidal solution of biogenic nanoparticles that also includes protein aggregates, exomeres, lipoproteins and midbody remnants[43,210,211]. As a nanostructured system, the secretome has a high surface-to-volume ratio, which is thermodynamically mirrored by an increase in the surface free energy by several orders of magnitude with respect to the bulk system[212,213] (see the figure, panel **a**). The spontaneous tendency to relieve excess free energy drives dynamic interactions between components, which are further favoured by crowding. At physiological temperature, these dynamic interactions can result in the formation of protein coronas, EV–lipoprotein aggregates and EV–lipoprotein fusion (which may together be referred to as biomolecular corona)[33,44–54]. This biomolecular corona can be considered a constitutive trait of biological fluids that contain EVs (see the figure, panel **b**). From a manufacturing perspective, this thermodynamic argument suggests that it may be impossible to isolate EVs without a range of co-isolates, challenging the definition of purity in EV formulations[128].

Rule-of-thumb thermodynamics can provide further insight into the protein corona of the EV. The generation of a vesicle requires bending a flat membrane into a sphere. According to the Canham–Helfrich theory, the (elastic) energy associated with this bending is approximately $4\pi K_B$, where K_B is the bending modulus[214]. This implies that there is a fixed energy cost to generate EVs, independent of their size. This intrinsic energy cost is accounted for by incorporating proteins and lipid distributions in the membrane leaflets that favour the curved state. The K_B for EVs has been measured as approximately $10–20 k_B T$, where k_B is the Boltzmann constant and T the absolute temperature[215,216]; therefore, the free energy to form an EV is approximately $125–250 k_B T$. At physiological temperature (310 K), this corresponds to 300–600 kJ mol^{-1}, which matches the value of the energy of adsorption (unspecific binding) of a few tens of proteins. Thus, the energy of EV formation (from the perspective of elastic energy) and the energy of protein corona formation are of the same order of magnitude (see the figure, panel **c**).

In addition, not only the energy but also the types of intermolecular interactions that mediate protein adsorption are similar to those responsible for vesicle assembly. Therefore, it is energetically challenging to entirely remove the biomolecular corona from an EV without altering or destroying the vesicle. These arguments suggest that proteins acquired during corona formation might be regarded as components of the EV and could potentially be exploited as biomarkers[49,208,217].



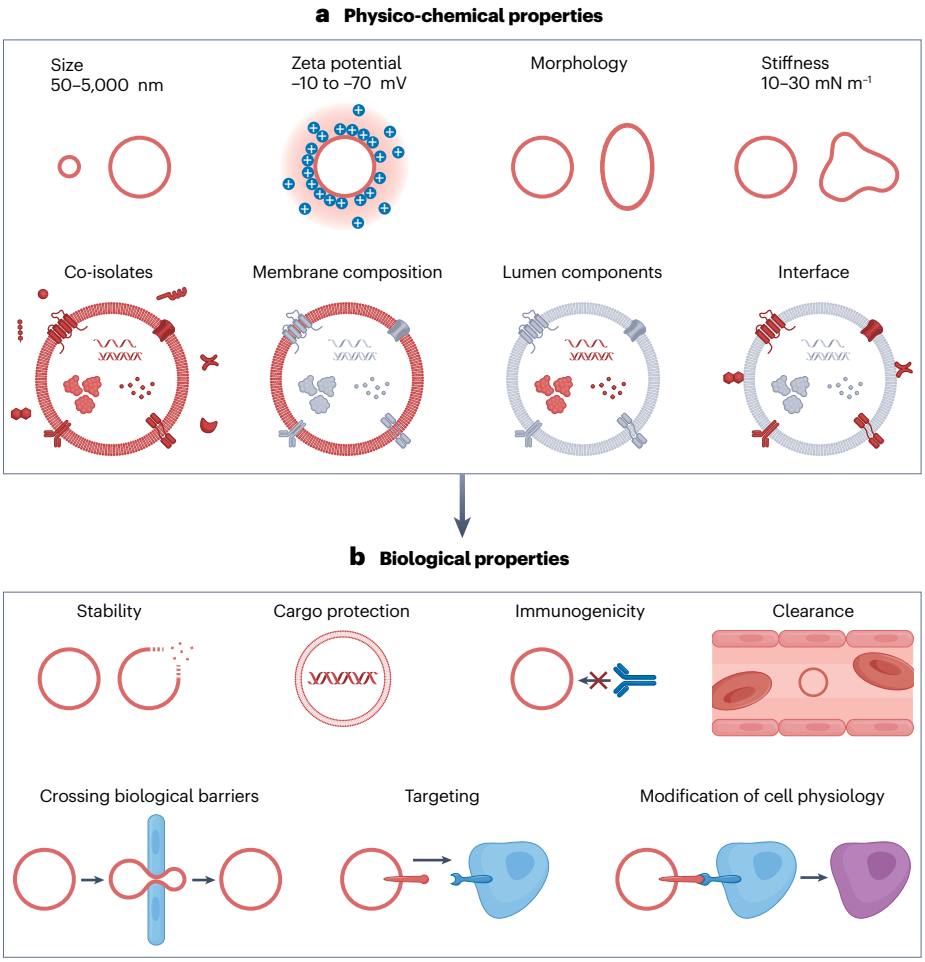


Fig. 2 | The physico-chemical and biological properties of extracellular vesicles. a,**b**, A set of physico-chemical properties (**a**) underlies the various biological properties (**b**) and functions of extracellular vesicles.

withstand harsh procedures, such as lyophilization[89]. Therefore, extracellular vesicles may remain stable for long-term storage[90]. Although stability is likely to be influenced by stiffness and co-isolates, the effects of the various physico-chemical properties of extracellular vesicles on stability remain to be fully investigated in order to generate recommendations for their storage. In addition, stability may depend on the isolation procedure and sample, such as the type of biofluid (blood, semen or urine)[90].

Cargo protection. Bioactive molecules encapsulated in extracellular vesicles are protected from the environment by a lipid membrane. This protection is particularly important for molecules that are prone to degradation, such as RNA and cytokines[91,92]. Similar to the increased stability and resistance to degradation observed when various proteins are immobilized on surfaces, adsorption of proteins to extracellular vesicle membranes (forming a corona) may protect them from degradation.

Immunogenicity. Extracellular vesicles are built of lipids and proteins derived from their organism of origin, and therefore autologous vesicles should not trigger an immune response. Autologous extracellular vesicles have been explored for use in personalized therapy, as testified by early-stage clinical trials[21,93]. However, unintended immunogenicity of autologous or xenogeneic extracellular vesicles, and how this may be affected by their physico-chemical properties, remains to be determined. In cell therapy, the level of cell differentiation is a key factor in determining immunogenicity. Extracellular vesicles from mesenchymal stem cells, which are minimally immunogenic, demonstrate strong therapeutic potential in disease models, including in a model of ischaemia-reperfusion injury[94]. Regardless of their cellular origin, the immunogenicity of extracellular vesicles, like that of synthetic nanoparticles, can be influenced by several factors, including size, endogenous or exogenous content, dosage, and the composition of the biomolecular corona[95].

Clearance. For therapeutic applications, extracellular vesicles should reach their target cells before they are cleared from the body. The pharmacokinetics of extracellular vesicles have mainly been investigated in mouse models, and have shown that clearance of injected extracellular vesicles from the plasma occurs within minutes[96,97]. However, extracellular vesicles may also be retained in organs for longer[98]. In non-human primates, the residence time of injected extracellular vesicles in blood is more than four times longer than in mice[99]. Importantly, plasma clearance does not increase on repeated injections of extracellular vesicles, because the second dose of injected extracellular vesicles is cleared more quickly than the first[99].

The charge and size of extracellular vesicles and their surface molecules may affect clearance. For example, decorating extracellular vesicles with anti-phagocytic molecules increases their circulation half-life[100]. In addition, proteins in the biomolecular corona may modulate the circulation half-life of extracellular vesicles[54]. Similar to synthetic nanoparticles, extracellular vesicles are less stable in phosphate-buffered saline than in serum, because the presence of a biomolecular corona in serum may aid in preserving their integrity[101]. This may suggest further strategies to increase the efficiency of extracellular vesicles in therapeutic applications.

Crossing biological barriers. How the cellular microenvironment can trigger the release of particular extracellular vesicles that are capable of crossing barriers, such as the endothelial and blood–brain barrier[102], and the mechanisms of crossing remain largely unknown[103]. Investigations into these mechanisms, such as endocytosis and transcytosis, have mainly been performed in in vitro model systems[103,104]. Beyond size and charge, other physico-chemical properties may be responsible for barrier crossing, namely the structure and composition of both the corona and the lipid bilayer.

Targeting. The binding of extracellular vesicles to specific cells has mainly been investigated in vitro, and results from these studies suggest that specific molecules on the surface of extracellular vesicles may enable cell targeting. These molecules include cytokines[91], glycans[105], integrins[106], tetraspanins[107] and other adhesion proteins[108]. The cell targeting properties of extracellular vesicles can be tailored and augmented through engineering, for example, by using endogenous methods based on molecular engineering of the cell sources or exogenous methods of modification of already isolated vesicles[109–113]. In the latter case, the potential influence of the biomolecular corona 'variable' should also be considered[50].

Modification of cell physiology. Extracellular vesicles have the ability to influence cell physiology through multiple mechanisms, including through surface interaction and signal transduction. Extracellular vesicles carry bioactive molecules on their surface that can interact with specific receptors on the surface of target cells. In analogy with hormones or antibodies, this binding can trigger a signalling cascade within the target cell and lead to changes in cell behaviour or function. Signalling can occur without internalization of the extracellular vesicles by the cell. In this case, extracellular vesicles act similarly to extracellular signalling molecules, by initiating a response from the outside of the cell[114].

In addition, extracellular vesicles can locally release their cargo on binding to the cell surface. The cargo is released near the target cell receptors, which allows even small amounts of these molecules to effectively interact with their targets and potentially trigger substantial cellular responses. This localized release mechanism ensures that the bioactive molecules are delivered precisely where they are needed, enhancing their impact[91].

Another mechanism is based on internalization and intracellular delivery, in which extracellular vesicles can be internalized by target cells through processes such as phagocytosis, endocytosis or macropinocytosis. Once inside the cell, the extracellular vesicles release their cargo into the intracellular environment, directly influencing cellular functions. This mechanism allows the direct delivery of regulatory molecules, such as microRNAs, proteins or lipids, into the cytoplasm or other intracellular compartments, where they can exert their effects[92].

Extracellular vesicles can also affect cellular physiology by fusing directly with the cell membrane, a process similar to the entrance mechanisms of certain viruses[40]. Fusion allows the content of extracellular vesicles to be delivered directly into the cytoplasm of the recipient cell, bypassing the endocytic pathway. This method of delivery can be particularly effective for altering cell physiology rapidly, as the cargo is immediately available for interaction with intracellular components[115,116].

Finally, extracellular vesicles can affect physiology in specific contexts. For example, extracellular vesicles released by cancer cells can alter the behaviour of specific liver cells, preparing the organ for tumour metastasis by changing its environment[117]. Specifically, the fatty acid cargo of tumour-extracellular vesicles triggers the secretion of tumour necrosis factor by Kupffer cells. This process creates a pro-inflammatory microenvironment, inhibiting fatty acid metabolism and oxidative phosphorylation, and fostering the development of fatty liver[117].

Despite these insights, the full range of mechanisms through which extracellular vesicles interact with cells, and how these are affected by their physico-chemical parameters, remains an active area of research.

Clinical translation of extracellular vesicles

Activity versus purity

The translation of extracellular vesicles into commercial products will require their consistent activity, which should be prioritized over purity and the precise definition of their identity. Moreover, the mechanism of action of co-isolates that cannot be removed without altering the functionality of the extracellular vesicle product should be evaluated, in addition to their batch-to-batch consistency. Importantly, extracellular vesicles need to be consistently active and safe, as consistency plays a key role in ensuring accurate dosing[118] and potency[119]. However, achieving consistency in extracellular vesicle formulations based on the separation of extracellular vesicles from biofluids[120–122] and their large-scale production in bioreactors[123,124] remains challenging. Indeed, extracellular vesicles are 'products by process' and their physico-chemical properties are strongly dependent on the specific processes used during their production and isolation.

Bioprocessing of extracellular vesicles

When compared with the large-scale production of viral vectors and antibodies, bioprocesses for the large-scale production of extracellular vesicles remain limited[124–129] (Fig. 3a). Extracellular vesicle production typically starts with the cultivation of cells in flasks or medium-sized bioreactors, often involving stem[130], dendritic or cancer cells[124]. Harvesting of the medium is followed by a concentration step, typically filtration, which can also partially separate extracellular vesicles. In particular, tangential flow filtration[131] benefits from ease of scaling and limited fouling of the membrane. Alternatively, extracellular vesicles can be concentrated by non-specific polyethylene glycol-induced precipitation[132]. The extracellular vesicles are then further purified by ultracentrifugation, which isolates vesicles of similar densities but also different sizes and compositions, or by size exclusion chromatography[133,134], which separates vesicles according to their size but not their density. As a consequence, ultracentrifugation and size exclusion chromatography both provide good separation yield but are limited in terms of specificity. Therefore, impurities with similar physical properties as extracellular vesicles may be co-isolated.

Alternatively, orthogonal methods which are based on two independent physico-chemical properties may be combined, such

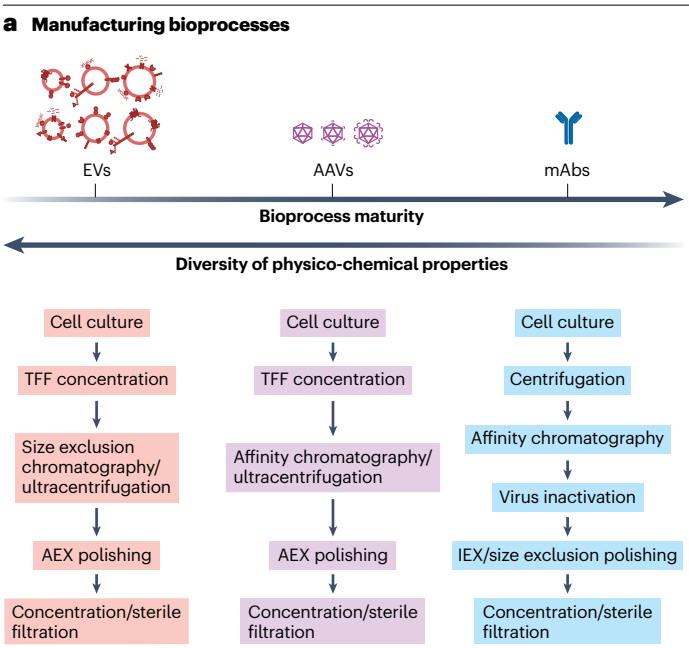


Fig. 3 | Manufacturing of extracellular vesicles. a, The major operation units of bioprocesses for extracellular vesicles (EVs), monoclonal antibodies (mAbs) and adeno-associated viruses (AAVs) include cell culture, centrifugation and concentration, and isolation and purification. **b**, Similarities and differences between AAV and EV manufacturing. AEX, anion exchange; IEX, ion exchange; TFF, tangential flow filtration.

as surface charge and size, as proposed for AAVs[135]. For example, by performing anion exchange chromatography in series with size exclusion chromatography (Fig. 3a), the resolution of separation can be increased at the cost of overall separation yield. Moreover, the surface charge properties of extracellular vesicles can be exploited to isolate and capture them from biofluids, for example using charge-based precipitation[136] and nanowire-induced charge-based capture[137]. In addition, asymmetric-flow field-flow fractionation can be applied to isolate extracellular vesicles on the basis of their diffusion coefficient (and therefore size)[138,139], resulting in higher separation resolution in the size range of extracellular vesicles (hundreds of nanometres), when compared with chromatography. However, this approach has mainly been applied for small-scale analysis and isolation thus far.

The bioprocessing of extracellular vesicles shares similarities with the manufacturing of AAVs (Fig. 3b). Following vector engineering, AAVs are typically expressed by transfected cells, which can either be adherent or in suspension grown in bioreactors[28], followed by clarification via filtration to eliminate cell debris and concentration steps to reduce volume. As in extracellular vesicle bioprocessing, further purification steps involve density gradient ultracentrifugation or affinity chromatography[28,140]. In the former approach, caesium chloride (CsCl) or iodixanol are introduced to form a linear density gradient under ultracentrifugation, which can separate empty and full capsids by exploiting their different density. Both CsCl and iodixanol protocols involve multiple steps, including cell lysis, ultracentrifugation and concentration, and can thus be time-consuming[141]. In affinity chromatography, the resin is functionalized with a ligand that binds to AAVs[142,143], providing vectors with high purity and good recovery. However, this method cannot separate empty from full capsids.

The scale-up of both chromatography and centrifugation remains challenging owing to the design, operation and costs of larger columns and larger centrifuges. Moreover, extracellular vesicle manufacturing would benefit from the development of large-scale separation methods that are based on specific interactions. For example, large-scale affinity methods used for AAVs[142–144] may also be developed for extracellular vesicles. However, affinity capture of extracellular vesicles, for example using magnetic beads or microfluidic and microarray platforms based on antibodies[145–147], peptides[148] or aptamers[149], has mainly been performed at a small scale thus far.

Product quality attributes

When compared with mAbs, analytical approaches tailored to AAVs[150] and extracellular vesicles remain scarce. Moreover, in contrast to antibodies, the absence of a well-established history in the bioprocessing of AAVs[150] and extracellular vesicles introduces uncertainty regarding the essential product quality attributes, that is, the physical, chemical and biological features that satisfy fitness-for-use, including safety and performance. For antibodies, quality attributes include product variants (for example fragments, charge-related and oxidation-related variants and glycosylated variants), process-related and product-related impurities (for example, protein particles) and composition of the product (for example, protein concentration, buffer composition and pH)[151].

For AAVs and extracellular vesicles, several properties (in particular size and composition) would need to be considered as quality attributes. For example, the ratio of empty capsids to capsids loaded with genetic material is a key quality attribute for AAV products, as variations in capsid composition can reduce biological potency[152]. However, assessing this quality attribute at high throughput remains challenging[29]. In addition, genome sequencing, evaluation of the expression of the gene of interest and its activity, determination of vector genome titre and measurement of infectious titre[153] should be performed for AAV products. Accordingly, regulatory assessment of AAVs entails a comprehensive evaluation of these attributes to ensure product safety, concentration, purity, potency and stability[153]. Moreover, as numerous co-isolates can be present in AAV preparations, separation efficiency needs to be demonstrated. This should include assessment of process-related impurities, such as residual host-cell DNA and proteins, helper virus DNA and proteins, and intact helper viruses. Therefore, quality attributes of AAV products include vector size, morphology, ratio of full versus empty capsids, genome copies and thermal stability[154].

Extracellular vesicle products require similar measurements of quality attributes to AAVs. However, owing to their physico-chemical landscape, assessing these attributes is more challenging. In particular, defining the identity of extracellular vesicles is more difficult when compared with antibodies and AAVs. This is due to their complex composition, which encompasses small molecules, proteins and nucleic acids. Moreover, unlike AAVs, the shell of extracellular vesicles is not

composed of only a few capsid proteins but contains a lipid membrane with numerous molecules. Furthermore, refractive index[71] and mechanical properties[67] should be regarded as quality attributes.

Opportunities from established bioprocesses

Established approaches for bioprocessing protein-based drugs can inform strategies to improve the consistency of extracellular vesicle products[124] (Table 1).

Continuous bioreactors. The yield and consistency of extracellular vesicles can be optimized by an appropriate bioreactor design. In particular, perfusion bioreactors have provided a viable alternative to batch operation in the commercial production of biologics, such as mAbs[126,155–158]. These bioreactors offer a continuous supply of nutrients, enabling higher cell densities and, consequently, increased productivity when compared with batch cultures, while maintaining cell viability. Sustained cell culture conditions throughout the entire process ensure

Table 1 | Opportunities for extracellular vesicle manufacturing

Manufacturing approach	Technologies	Features	Advantages	Refs.
Perfusion bioreactors	Hollow fibre reactors Batch–refeed cultures	High cell density and high viability Constant environment Short product residence time	Consistent product qualities Limited product degradation	126,159–163
Process analytical technology	Microfluidics	Miniaturization Parallelization Modularity Portability Control of environment and external fields Compatible with optical and fluorescence techniques	Multiple properties in parallel Quantitative information Process control	147,178,182
Machine learning and digitalization	Bayesian optimization	Reducing the number of experiments and extending the range of explored conditions Transfer of knowledge across experimental campaigns	Reduced time and effort to design the process Improved multidimensional optimization of the process	187
	Hybrid models	Identify unknown correlations between parameters		184–186
Renewable extracellular vesicle sources	Plants, bacteria, microalgae, milk	Natural Renewable Scalable	Contain large amounts of extracellular vesicles	88,89,122, 188–191
Isolation technologies	Microfluidics	Well-defined fluid dynamics and external fields Continuous operation Functionalization of surfaces	Rapid and consistent separation	146,147, 171–177, 179–181
	Coacervates from phase separation	Gentle procedure Applicable at different scales Based on different properties (affinity and charge) Simultaneous separation and concentration of the product	Scalable separation with high yield and specificity, while maintaining intact product	196
Analytical tools	Single extracellular vesicle techniques (for example, interferometric imaging[145], high-throughput AFM nanomechanical imaging[67], interferometric detection of scattering with nanoparticle tracking analysis[72] and super-resolution microscopy[197])	Single vesicle resolution, providing full distribution of measured physico-chemical property	Quantification of physico-chemical properties and product quality attributes Process control and design	67,68,72, 145,197
	Bulk methods with orthogonal read-outs reporting on two or more physico-chemical properties (for example, SEC coupled with light scattering and fluorescence)	High throughput Rapid analysis Compromise between information and effort required		203
Standardization and reference materials	Reference synthetic extracellular vesicles	Well-defined physico-chemical properties that mimic those of extracellular vesicles Consistent preparations based on bottom-up approaches	Structure–function studies Assist development of analytics Measurement of purity	204–206

AFM, atomic force microscopy; SEC, size exclusion chromatography.

consistent product quality attributes. Furthermore, the risk of product degradation is minimized by continuous harvesting, which reduces the residence time of the product in the bioreactor. Such continuous manufacturing can also be applied to extracellular vesicles, for example, using hollow fibre bioreactors[159–162] and batch–refeed cultures[163].

Microfluidic technology. Perfusion processes require analytical methods for in-line characterization, referred to as process analytical technology[164]. In this context, microfluidic devices offer considerable advantages for the analysis of biological components in complex biofluids[165,166], including miniaturization, parallelization, modularity, portability, flexibility in design, fast measurement, limited amounts of required sample and high throughput. Importantly, flow patterns, interfaces, mixing, temperature and external fields can be finely controlled. Various microfluidic devices have been developed to measure quality attributes of antibody formulations. For example, differential scanning fluorimetry can be applied to measure the thermal stability of antibodies by spatiotemporally controlling the temperature at the micrometre scale[167]; viscosity and self-association can be measured in solutions with high protein concentrations without the need for dilution, which may compromise the analysis of weak interactions[168]; and protein aggregates can be detected, which may compromise drug activity and safety[168–170]. Such devices may also be incorporated in upstream and downstream operation units to monitor properties on-line.

Microfluidic technology can also be applied for the isolation, quantification and characterization of extracellular vesicles[171,172]. These operations have been achieved with various strategies, including: affinity capture devices[146,173,174]; active methods using different field-based approaches, such as acoustic[175,176], electric[177] and magnetic fields[178]; and passive techniques based on, for example, viscoelastic microfluidics[179,180] or nanopillar arrays[181]. Microfluidic devices are further compatible with optical and fluorescence techniques, enabling multidimensional read-outs. This approach could be used, for example, to characterize and optimize the multiple physico-chemical properties of extracellular vesicles, including vesicle concentration and vesicle size; determine the amount of specific subpopulations characterized by certain biomarkers; and determine the content and composition of lipids, proteins and RNA molecules. For example, measurement of diffusion can be coupled with multidimensional fluorescence detection in a microfluidic device to measure various extracellular vesicle properties[182].

Digitalization and machine learning. Digitalization and machine learning tools can be applied to optimize the design and improve process understanding of bioprocesses for biological products[183,184]. For example, hybrid models have been developed based on combinations of mechanistic and data-driven models[185]. In particular, mechanistic models can describe the physico-chemical processes involved in the bioprocess using parameters with physical relevance[185]. However, these models often contain parameters related to the kinetics, thermodynamics and mass transport phenomena occurring in bioreactors and separation units, which are not known a priori. Parameters can be estimated using data-driven models based on machine learning. Such hybrid approaches combine the power of mechanistic models in prediction, extrapolation and process understanding with the versatility and practical utility of data-driven models, as shown for chromatographic steps of protein-based drugs[185].

In addition, Bayesian optimization tools can be used to accelerate the design of experiments[186]. Bayesian optimization is typically applied to 'black-box' functions, for which the underlying relationship between the input and output is unknown, and points in the input–output space can only be determined experimentally. In contrast to the static design of experiments, Bayesian optimization applies a sequential procedure, in which a surrogate model of the actual system suggests the next experiment(s) based on acquired data. The suggestion of the experiment(s) is determined by optimizing the trade-off between exploration and exploitation. First, the algorithm samples combinations of parameters that have not been explored before and are distant from experiments that have already been performed. Second, the algorithm focuses on the region that has already proven interesting behaviour based on previous experiments. Based on adaptively sampling locations, the algorithm can achieve optimal conditions faster and with a reduced number of experiments when compared with traditional experimental design. Bayesian optimization is particularly powerful for the multidimensional optimization of bioprocesses for the production of extracellular vesicles, as they involve multiple process parameters and product qualities[187].

Actions for extracellular vesicle manufacturing

In addition to lessons from current bioprocesses, the manufacturing of extracellular vesicles requires new developments that are tightly linked to their physico-chemical properties (Table 1). In particular, the scaled-up expansion of mammalian cells, which is required for the large-scale production of extracellular vesicles, remains challenging. This affects the reproducibility of extracellular vesicle manufacturing. Scale-up can, however, be achieved using alternative sources that are sustainable and contain large amounts of extracellular vesicles, such as plants[188], milk[189,190], bacteria[191] or microalgae[87,88,120]. The choice of the source should consider the intended application and biological function of extracellular vesicles, which is tightly connected to their physico-chemical properties and their parent cell, as well as to safety issues or regulatory requirements.

In terms of separation, size exclusion chromatography and tangential flow filtration are typically applied for single molecules rather than for multimolecular complexes, such as extracellular vesicles, and are difficult to scale up[192,193]. Therefore, alternative separation processes would be required that are tailored to the characteristics of extracellular vesicles and viral vectors. For example, methods based on precipitation and phase separation, which have been developed for the purification of antibodies[194,195], may also be applied for the separation of extracellular vesicles, as recently shown with coacervates formed by phase separation of zwitterionic polymers[196]. This method benefits from the use of a liquid-like phase that guarantees product stability, speed and scalability[196].

The heterogeneity and complex nature of extracellular vesicles requires the application of a large number of different biophysical assays[87] for their characterization. Importantly, the heterogeneity of the population should be resolved at the single vesicle level. Various methods have been developed to analyse the physico-chemical properties of individual extracellular vesicles[197–199], such as digital single extracellular vesicle flow cytometry[200], interferometric imaging[145], high-throughput AFM nanomechanical imaging[67], interferometric detection of scattering with nanoparticle tracking analysis[72] and super-resolution microscopy[197]. However, although the throughput of these methods is constantly increasing, this high-resolution analysis can slow down the development of new bioprocesses, which typically require the screening of many operative parameters. Therefore, in parallel with single-particle analytical methods, new approaches

should be developed that offer a compromise between the information provided and the time and effort required by the analysis. Ensemble methods based on orthogonal read-outs are particularly promising in this regard[182]. For example, in the analysis of antibodies, chromatography is typically used in conjunction with light scattering and fluorescence detection[201]. This approach has also been used for AAVs[202] and, more recently, extracellular vesicles[203].

Importantly, analytical and separation methods should be standardized for extracellular vesicles[204]. In particular, synthetic reference extracellular vesicles that mimic their key physico-chemical properties[205,206] may enable the development of new analytical methods, improve measurements of purity, and shed light on the correlation between physico-chemical properties and biological modes of action.

Outlook

Extracellular vesicle preparations typically encompass diverse vesicle types with partially overlapping energies but substantial variations in physico-chemical properties, including size, composition, surface charge, membrane stiffness and co-isolates. Rather than viewing these as discrete subpopulations, they may be referred to as a continuum of vesicle structures. In addition, the biomolecular corona (the dynamic phenomenon that forms on extracellular vesicles in biofluids) may be considered a component of extracellular vesicles, depending on the interaction energy between the biomolecules in biofluids and the extracellular vesicle membrane. The multidimensionality of composition and conformational ensembles presents challenges in defining the identity and purity of extracellular vesicles, and makes the isolation of homogeneous subpopulations difficult. However, mapping the physico-chemical landscape of extracellular vesicles in comparison to other biologics, such as AAVs and antibodies, may inform strategies to develop bioprocesses for their manufacturing. For example, approaches applied for the manufacturing of antibodies, such as continuous bioprocessing, microfluidics and digitalization, may also improve the manufacturing of extracellular vesicles. Nevertheless, owing to their complex physico-chemical landscape, new separation and quantitative analytical methods are required that can be performed at high throughput to ensure purity and quality of extracellular vesicle products. Isolation technologies will be required to obtain extracellular vesicles with homogenous physico-chemical parameters (for example, size, composition and stiffness).

Although the complexity and heterogeneity of extracellular vesicles may pose a challenge for their manufacturing at scale, it offers opportunities for their application as biomaterials. Rationalizing this complexity in the context of the physico-chemical landscape will unlock possibilities in bioengineering. In particular, a distinct advantage provided by the complexity of extracellular vesicles is their versatility in biological functions. For example, specific populations of extracellular vesicles can block viral entry by competing for binding to cellular phosphatidylserine receptors. This function is mediated by phosphatidylserines, which are a common physico-chemical trait of the surface of extracellular vesicles in semen, saliva and breast milk, but not in blood (that is, a feature in the physico-chemical landscape connecting the extracellular vesicles found in semen, saliva and breast milk, but not those in blood). This gives a plausible explanation of why many viruses are transmitted by blood rather than by other body fluids[190,191].

The complexity of extracellular vesicles also holds great potential for diagnostic applications, such as liquid biopsies, as they can contain biomarkers specific to their parent cells[207]. In addition, the biomolecular corona could be exploited for biomarker discovery[208]. However, to use extracellular vesicles for diagnostics, the correlation between the physico-chemical properties of extracellular vesicles from different sources, and their relation to pathologies, needs to be determined.

A better characterization and understanding of the complex physico-chemical landscape will also assist in moving extracellular vesicles into clinical applications — in particular, understanding the composition of the biomolecular corona and the different affinities of surface molecules for the extracellular vesicle membrane. Moreover, it remains unclear how cells control the release of extracellular vesicles, and how release mechanisms relate to their physico-chemical properties, and, subsequently, biological functions and modes of action. The surface molecules directing extracellular vesicles to their targets need to be identified, and the impact of co-isolates and surface molecules on their biological functions should be assessed. Finally, the key advantages and limitations of the complexity of extracellular vesicles should be established in each specific context, in comparison with other state-of-the-art materials.

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