Mastering the Tools: Natural vs. Artificial Vesicles in Nanomedicine

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Keywords: extracellular vesicles, exosomes, artificial vesicles, liposomes, polymersomes, nanomaterials, nanomedicine, nanotherapeutics

Naturally occurring extracellular vesicles and artificially made vesicles represent important tools in nanomedicine for the efficient delivery of biomolecules and drugs. Since its first appearance in the literature 50 years ago, the research on vesicles has been progressing at fast pace, with the main goal of developing carriers able to protect cargoes from degradation, as well as to deliver them in a time- and space-controlled fashion. While natural occurring vesicles have the advantage to be fully compatible with their host, artificial vesicles can be easily synthetized and functionalized according to the target to reach. The future research is striving to merge all the advantages of natural and artificial vesicles, in order to provide a new generation of highly performing vesicles, useful to ameliorate the therapeutic index of the transported molecules. This Review summarizes all the up-to-date manufacturing techniques used to produce both natural and artificial vesicles, exploring the promises and pitfalls of the

different production processes. Finally, pros and cons of natural vs. artificial vesicles are discussed and compared, with special regard towards the current applications of both kinds of vesicles in the healthcare field.

1. Introduction

The clustering of independent molecular reactions at sub-cellular scales is possible thanks to the separation of the liquid compartments inside the cells.[1] In fact, the spatial compartmentalization allows to confine biomolecules (e.g. proteins, nucleic acids) and ions within selected sub-cellular compartments, delimitated by membranous layers, with the final outcome of tuning the kinetic reactions, altering the flux through specific pathways and protecting each living cell from detrimental effects, including uncontrolled proteolysis, inappropriate covalent modifications and pH lowering.[2,3]

Along with the intracellular compartmentalization described above, the self-assembly ability of the cellular membranes is used by cells for storing, transporting and signaling, at both intra- and intercellular levels. Indeed, lipid bilayers may form small compartments, with a size of 100 nm or less, and volumes included in the attoliter scale. This group of lipidic units includes synaptic vesicles, intracellular and extracellular vesicles (EVs). In particular, EVs are important cellular carriers with high stability conferred by a specific subset of lipids in their membranes (e.g. cholesterol, sphingomyelin glycosphingolipids and phosphatidylserine).[4] They are able to quickly diffuse through the extracellular medium, transporting a vast variety of bioactive molecules, and therefore resulting in an efficient delivery of molecular mediators to neighboring cells.[5,6]

The first evidence of their presence in the human blood is dated back to 1946, when Chargaff and West found that the time of coagulation of plasma increased after ultracentrifugation.[7] They ascribed this effect to the presence of novel subcellular sized procoagulant factors. Subsequently, these factors were identified as small lipid-rich vesicles, with a diameter of 20–

50 nm, and referred as "platelet dust" by Wolf in 1967.[8] Later on, Trams et al.[9] coined the term "exosomes" to define the membrane vesicles shed from the surface of several cell lines (i.e. normal and neoplastic). In 1987, Johnstone et al. identified the exosomes as the vesicles formed in the inward budding of the membrane of early endosomes, and secreted to the extracellular space through the fusion of multivesicular bodies (MVBs) with cell membrane.[10] Ten years later, in 1996, the seminal paper from Raposo et al. was the first to report that exosomes from B lymphocytes may express antigens. This discovery dramatically boosted the interest for EVs in the field of cancer immunology.[11]

After many years of intense research, it has been established that exosomes belong to a wider group of EVs, which includes different types of vesicles released by cells.[12] In fact, depending on their biogenesis, EVs can be classified in three main categories: i) exosomes (30-150 nm) derived from the endocytic compartment;[13] ii) microvesicles (50 nm-2 μm) directly released from plasma membrane through a shedding mechanism; iii) apoptotic bodies (> 50 nm).[14] The size of the different EV subtypes partially overlaps, and to date no available technique can isolate and purify a single kind of EVs from the others. For that reason, researchers are trying to generate new methods for EV preparation, based on potential (but still not clearly identified) class-specific biomarkers and, in turn, able to discriminate among selected subtypes.[15,16]

The last decade witnessed a huge number of findings in the EV field, generating the need to revise their nomenclature. Since its constitution in 2012, the International Society for Extracellular Vesicles (ISEV) published several recommendations and guidelines for all scientists working in the field. As a result of this process, a new EV classification has been proposed, based purely on their dimension and dividing them in small EVs (<200 nm) and medium/large EVs (>200 nm).[17,18] The latest ISEV position statement paper contains important information about EV nomenclature, sources, separation methods and characterization tools, as well as indications for the best functional studies to evaluate EV

biological activity.[17] These guidelines represent a useful "roadmap", since both the basic and, importantly, the clinical research in the EV field are growing at a very high rate.

As mentioned earlier, their importance resides in the crucial role during intercellular communication. When EVs are released in the microenvironment, they may be captured by adjacent cells or conveyed to distant target cells,[19] then representing an inter-organ mean of communication.[20] EVs, in fact, have been found in almost all biological fluids, (including cerebrospinal fluid, blood, saliva, urine, amniotic fluid, malignant ascites or breast milk),[13,21] as well as in the supernatants of cultured cells.[22,23] This important aspect reflects the potential role of EVs as noninvasive molecular diagnostic and prognostic tools for several diseases.[13]

Interestingly, the relevance of EVs is witnessed by their conservation during evolution from bacteria to superior eukaryotes, such as human and plants, and their ability to mediate even the "interkingdom" communication.[24]

Several biomolecules may be carried within the lumen of EVs, such as DNA (both mitochondrial and genomic),[25,26] RNA (including mRNA, miRNA, siRNA and lncRNA),[27–30] lipids and functional proteins (such as active enzymes).[23] These cargoes are protected from degradation by proteinases and nucleases, commonly present in the extracellular environment, allowing for an efficient delivery of active biomolecules to the target cells.[19]

The involvement of EVs in the intercellular signaling is still matter of intense investigation, in order to grasp their full contribution in both physiological and pathological contexts.[31] In fact, several studies have demonstrated how EVs participate in physiological processes, such as the modulation of the immune response,[32] but also in pathological processes, including cancer genesis and progression.[33,34] Moreover, through cell-to-cell communication, EVs may protect adjacent cells from oxidative stress by transferring antioxidant molecules, such as Apolipoprotein D or Peroxiredoxins, both *in vitro* and *in vivo*.[35,36] Due to their important functions in modulating the oxidative stress response,[37] EVs have found to be key carriers during neuronal degeneration and neurodegenerative diseases.[38,39] In this context, EVs may

represent transporters of potential prognostic and diagnostic biomarkers.[40] Particularly, some EV-associated miRNAs gained much attention as possible biomarkers for Alzheimer's disease,[41] multiple sclerosis,[42,43] amyotrophic lateral sclerosis,[44] and Parkinson's disease,[45,46] because of their capability of modulating the expression of specific genes involved in the pathology onset. Moreover, the intrinsic ability of EVs to deliver biomolecules, together with their low immunogenicity and capacity to cross the blood brain barrier (BBB), have been exploited to design biocompatible carriers of specific miRNAs, proteins or conventional drugs for therapeutic purposes.[47] In the Section 3.1, we report some of the most relevant strategies that scientists have been investigating to deliver EV-cargoes towards specific target sites where they can elicit their effects.

The fundamental role played by cell membrane-mediated compartmentalization, and the potential of nanosized vesicles to interact preferentially with some types of cells (see Section 3.2), stimulated nanomedicine research to develop synthetic counterparts, featured with the ability to carry either natural or synthetic molecules.

As a result, micro- to nanoscale synthetic systems, made of different materials (i.e. inorganic molecules, lipids, surfactants and amphiphilic block copolymers) are in constant development. Those systems have been designed to enable the molecular recognition of specific targets and to carry signaling molecules, including nanotherapeutics. Although synthetic nanoparticles and emulsions are matter of continuous researches, they often lack the inner aqueous compartment, able to store and protect the cargoes. Indeed, EVs have the advantage to store and transport water soluble biomolecules towards target cells, in response of changes in the microenvironment.[5] Moreover, cargoes can be encapsulated in hollow particles, such as EVs, with no need of physi-/chemisorption steps, which are essential for molecule loading on synthetic nanoparticles (e.g. metallic colloids).[48,49]

With the aim to mimic this remarkable feature of natural occurring vesicles, research efforts have strived in the fabrication of synthetic counterparts, i.e. artificial vesicles (AVs), constituted

by an aqueous compartment physically separated from the surrounding liquid environment by a membrane. Being assembled by their molecular components, AVs permit to solve the issues of yield and purity, which are typical limitations encountered as natural vesicles are extracted from biological sources.[50] Clearly, the possibility to modify both chemical and physical characteristics of the AVs is of great relevance for a number of possible applications, including: drug delivery, subcellular organelles mimicking, advanced theragnostic approaches and targeted catalysis.[51] Specifically, AVs dimensions generally range from micro- to nanoscale in terms of lateral size, corresponding to nano- to femtoliter volumes. Therefore, the methods used for the synthesis of submicron scale AVs may control the physicochemical processes involved in the molecular self-assembly, in order to define the features of the resulting particles, with particular attention to dimensions, shape, polydispersity, surface chemistry, stability, as well as to the amount and chemical nature of the loaded cargo.[50]

Not surprisingly, liposomes are the first type of AVs investigated. These systems consist in soft hollow particles made of phospholipids, the same constituents of cellular membranes, able to self-assemble in spherical vesicles. Their structure is characterized by an aqueous lumen enclosed by a lipid bilayer, that allows the encapsulation of both hydrophobic and hydrophilic molecules in the membrane and in the aqueous core, respectively. The word “liposome” derives from the *Greek* words: *lipos* ("fat") and *soma* ("body"). Their discovery dates back to 1965, from the pioneeristic studies of the British hematologist Bangham, who characterized lipid phase structures by electron microscopy together with a specific negative staining procedure.[52] Their biocompatibility and in particular the possibility to fuse with natural cellular membranes, lead to a great interest both in academia and in industry, resulting into a growing number of researches and applications, for example as drug delivery vehicles and also in cosmetic formulations.[53] In the past years, many studies have been pursued to increase the stability of such ‘natural’ liposomes. In an attempt to produce a synthetic version of an aqueous compartments, in 1999 Discher et al. utilized for the first time amphiphilic polymers chemically

similar to phospholipids, but with an increased mechanical resistance and stability *in vivo*.[50] Since then, these polymeric versions of AVs, called polymersomes, have been widely implemented. Polymersomes can be defined as hollow artificial particles, whose membrane is formed by amphiphilic synthetic block copolymers. Similarly to liposomes, their size ranges from the nano- to the microscale. They show a versatile functionalization chemistry, several tunable physicochemical properties, and possess the possibility to be loaded with a heterogeneity of molecules, resulting in promising systems to replace the corresponding liposomal formulations. Despite of their intrinsic potentialities, such novel AVs are still poorly present in the market, if compared with the classical liposomes. In fact, polymersomes have still unsolved issues regarding their biocompatibility, as well as their degradation routes, which need to be better investigated.[54] Both EVs and AVs represent innovative carriers for the delivery of nanomedicines, signal molecules, and other functional cargoes within the human body. Importantly, they have the potential to deliver selected molecules in a way that is biocompatible, as well as time and space controlled.

This Review provides a critical comparison between the natural and artificial vesicles. This comparison might seem quite ambitious due to the different molecular composition and properties of AVs with respect to EVs. However, the current state of the art requires a more careful evaluation of the vesicle field, as they are both objects of intense researches. In particular, the rationale behind this critical confrontation lies in the emerging role of synthetic biology concerning the fabrication of artificial biosystems.[55] Such systems can be envisaged as tools that recreate some of the functions of living structures, adding also new properties. For instance, the design of AVs is clearly inspired by the natural vesicle structures and functions but is boosted by a broader chemical composition (polymers, lipids and hydrids), larger responsivity to stimuli (light, pH, and temperature) and wider set of tunable physicochemical properties (mechanical and chemical stability). On the other hand, the development of such

novel physicochemical strategies derived from AVs fabrication can be leveraged to add new functionalities to EVs, [56] finally improving EV theragnostic applications.

All this knowledge is opening up new frontiers in which the AVs and EVs research fields, usually developed separately, start to interact.

The first part of this Review illustrates mechanisms and processes implicated in the EVs and the AVs production. The second part focuses on their application in human health. The third section addresses the actual critical challenges, as well as all the possible future directions and developments of vesicle-focused research. **Figure 1** summarizes the main features of EVs and AVs.

2. Production Routes for Natural and Artificial Vesicles

2.1. Recovery of secreted EVs

As highlighted in the introduction, EVs are natural carriers of biomolecules and in the past 30 years researchers have been engaged in improving their release, as well as their loading efficiency with cargoes of interest, to match the scale-up required for clinical applications. EVs may derive from *ex vivo* cultured tissues, such as patient-derived cells,[57,58] or from cell lines [22,59,60] and also from bacteria [61–63] and plants [64,65]. Among all the cell types evaluated for EV production, mesenchymal stem cells (MSCs) gained great attention for their intrinsic therapeutic potential [66–68] and the possibility to produce EVs at a larger scale.[69,70] A currently ongoing pilot clinical study (NCT04276987) is investigating on human MSC-derived EVs for the treatment of patients with pneumonia induced by SARS-CoV-2, with the aim to evaluate their safety and efficacy on a still “mysterious” disease.

The EV production process starts with cell cultivation and expansion. Considering the low frequency of MSCs in the tissues of origin,[71,72] several strategies have been explored to efficiently isolate cells holding all useful parameters for clinical applications. Normally, MSCs

are cultivated in a standard medium (Dulbecco's modified Eagle's medium - DMEM), supplemented with 10% fetal bovine serum (FBS), which provides nutritional and growth-stimulatory factors.[73,74] However, the FBS contains bovine-derived EVs that may be co-purified together with the MSCs-derived vesicles. The presence of these bovine contaminating EVs may cause immune reactions in receiving patients, or may compromise the efficacy of the EV treatment when used in *in vitro* studies.[75,76] To overcome this issue, several media have been produced to cultivate human MSCs and compatible with the use in patients. These include both serum-free and chemically defined media, eventually supplemented with 5% human thrombocyte lysate.[77] However, it is still controversial if these cells retain the same secretory capabilities in such chemically-defined media, and further investigations are needed.[74]

In order to obtain a higher yield of vesicles, different strategies have been developed at each level of the EV recovery process. It is possible to divide them in two main categories: the first regarding the optimization of the cell culture conditions; and the second on the improvement of the EV isolation and purification techniques. The latter may be accomplished thanks to the development of novel technologies closely related to the classic approaches based on ultracentrifugation, ultrafiltration, size-exclusion chromatography. All these aspects have been extensively reviewed elsewhere.[78,79] Here we will focus specifically on the strategies that aim at: (1) engineering/treating the EV-producer cells to secrete more EVs; (2) increasing the starting number of EV-producer cells. **Figure 2** is a summary of the described approaches.

2.1.1. Improving the EV-producer cells

A strategy to improve the yield of EVs is to ameliorate the efficiency of donor cells (genetically or chemically) in order to increase the number of EVs secreted per single cell. On this line, Kojima et al. in 2018 performed a screening with the goal of identifying the genes involved in exosome production and release, using an embryonic kidney cell line (HEK-293T). In detail, the HEK-293T cells were co-transfected with selected candidate genes together with an EV

reporter construct containing a CD63-nluc-fusion protein, which should localize in the EV lipidic bilayer. By measuring the luminescence produced in the cellular supernatant, the authors identified three genes (i.e. the metallo-reductase STEAP3, the proteoglycansyndecan-4 and a fragment of L-aspartate oxidase) responsible for enhancing exosome biosynthesis and obtaining therefore a reliable method for improving vesicle production.[80]

Another trigger of EV secretion has been individuated by Verderio and collaborators, back in 2005. The group found that the activation of purinergic receptor 2 (P2 receptor) in microglial cells, mediated by ATP, was able to stimulate EV formation and shedding. This process was associated with the activation of acid sphingomyelinase, within the p38 MAPK pathway.[81,82] More recently, in 2018, the same group demonstrated that other cytokines, such as the pro-inflammatory cytokine INF-γ or the anti-inflammatory IL-4, but also IL-23, IL-13, IL-27 and TGF-β, are equally able to induce the release of EVs, independently from P2 receptor and sphingomyelinase activation.[83] Notably, only pro-regenerative microglia-derived EVs were able to induce oligodendrocyte precursor cells (OPC) proliferation, differentiation and myelin deposition.[84] On the other side, when subjected to different stimuli, cells release EVs carrying different patterns of cytokines.[85] These EV-associated cytokines secreted by neural stem/precursor cells (NPCs) have been demonstrated to be able to activate the signal transduction in target cells, as shown by Cossetti and Iraci in 2014.[86]

However, these approaches have the drawback to possibly spread pro-inflammatory cytokines towards the target cells. To overcome this issue novel strategies to enhance the EV production have been explored. For example, Fukuta and colleagues in 2020, successfully applied low level electric treatment (ET - 0.34 mA/cm2) for 60 minutes to both normal and cancer cells, thus improving the yield of EVs collected 24h later. They demonstrated that ET did not impair viability of donor cells and importantly did not affect the quality and functionality of the collected EVs from each kind of donor cell tested.[87] These results are really promising although further studies are needed to evaluate the ET on other cell types. Moreover, the

development of a well-defined methodology to apply ET to a higher number of cells will contribute to the general improvement of this novel technology for EV production. Until then, a good alternative to raise the EV yield consists in the increase of the starting material.

2.1.2. Increasing the EV-producer cell number

As introduced above, bacteria represent an ideal source of EVs, as they can be easily and continuously grown, increasing the yield of EVs produced. Bacteria-derived EVs gained great attention thanks to their key role in the modulation of host immune response. In fact, they are currently under investigation to be used as promising vaccine formulations in clinical trials. For example, in a Phase II clinical trial, in 2010, infants received vaccination for serogroup B *Neisseria meningitidis* (Meningococcus, MenB) through the inoculation of three bacterial antigens (rMenB) encapsulated within bacterial Outer Membrane Vesicles (OMV). The results obtained are promising since rMenB incapsulated in OMV demonstrated greater immunogenicity than rMenB administered alone. Thus, rMenB+OMV have the potential of protecting against MenB infection.[62] Together with their use related to vaccine applications, bacteria-derived EVs are being evaluated as "nanoantibiotics", novel therapeutic delivery systems against bacterial infections. In particular, myxobacteria-derived OMVs retain an intrinsic antimicrobial activity, associated with the presence of cystobactamids, naturally occurring inside bacterial vesicles.[88] Also, bioengineered OMVs have been exploited as siRNAs delivery vehicles able to specifically kill cancer cells.[89]

Plant-derived EVs (PEV) are a time- and cost-efficient alternative to animal-derived EVs, showing an increase in the total amount of vesicles that can be isolated per cell.[64,65] An ongoing Phase I clinical trial (NCT01668849) is currently investigating the ability of grape-derived PEVs to act as anti-inflammatory agents. They are administered to head and neck cancer patients subjected to radiation and chemotherapy to reduce the incidence of oral mucositis. Another Phase I clinical trial (NCT01294072) is investigating the ability of PEV to efficiently

deliver curcumin (constituent of the spice turmeric, with cytostatic effects on cancer cells and immunomodulating proprieties) to patients who are undergoing surgery for newly diagnosed colon cancer. In both studies the edible PEVs are administered safely as dietary supplements.[90]

Another strategy to scale-up the number of donor cells is to culture them into bioreactors, i.e. a device that supports biological active environment. In 2016, Watson and colleagues compared the hollow-fiber bioreactor *vs.* the classical 2D flasks for culturing cancer cells and producing EVs. The authors managed to obtain 40-fold more bioactive EVs per mL of conditioned medium with the use of the bioreactor, compared with the conventional 2D culturing method.[91] The hollow-fiber bioreactor is a 3D culture system where the cells are cultured and expanded in a monitored enclosed system. There are two compartments, one is for nutrient perfusions (intra-capillary space) and the second is an extra-capillary space (ECS) surrounding the polysulfone hollow fibers, where cells adhere. Supernatant can be continuously collected from the ECS for isolating EVs.[92]

Additionally, microcarriers have been used to large-scale cell expansion in a 3D environment. The microcarriers are small beads with a diameter comprised between 100 and 300 µm which provides a higher surface area-to-volume ratio for cellular expansion in the liquid medium. Microcarriers are made of several materials such as dextran, collagen or glass that may influence the cell growth kinetics or the phenotype. All these variables make the selection of microcarriers an important step in the optimization of the production process.[93,94] In 2017 Haraszti et al. demonstrated that cultivating cells in a microcarrier-based 3D system allows to obtain 20-fold more EVs compared with the 2D culture system.[95]

Importantly, a recent work published in the Journal of Extracellular Vesicles demonstrated how the cell culture system impacts the metabolomic profile of prostate cancer cell-derived EVs.[74] The authors found that EVs isolated from the supernatants of cells cultivated in the bioreactors were 100-fold more abundant than the ones isolated from cells cultivated in traditional 2D-flasks. Moreover, they observed that the general culture conditions and growth environment

may influence the cell metabolism and, consequently, the nature of the EV content. Cells, in fact, are cultured continuously without any traditional 2D passaging detachment step, which may influence cellular metabolic activity. All the new information regarding the bioreactor mediated EV production could help to better control all the possible variables, with the final goal of a controlled and larger EV yield.

2.2. Formulation of AVs

The production of AVs is carried out through the use of two different groups of methodologies: (1) wet-chemistry preparation and (2) microfluidics/printing technologies.[96] The wet chemistry approaches are mainly based on the self-assembly of surfactant micelles and it is used, for example, to generate liposomes. This approach is commonly involved in micelles assembly in water, obtained by gradually increasing the surfactant concentration above its critical micelle concentration (CMC). By reaching the CMC, the micelle formation takes place as a consequence of the unfavorable hydrophobic tails interaction with the surrounding water molecules.[97] The wet-chemistry preparation methods have been developed since 1970, thanks to the ease of such experimental approach, thus opening novel possibilities towards the use of vesicles as drug carriers.[96] Although the microfluidic-based methods appeared later in the years (starting from the 2000s), they represent emerging protocols allowing an improved and high-throughput synthesis of AVs.[98] While the AVs chemical preparation is based on the self-assembly processes following the compresence of hydrophilic and hydrophobic moieties (as in the case of common surfactants and amphiphilic block copolymers),[99] the microfluidics mainly relies on the immiscibility of two different liquids, which, in turn, produces almost monodisperse small droplets of variable size (typically between microliters and attoliters).[98,100] The following paragraphs enter in the detail of these methods.

2.2.1. Wet-chemistry preparation

Liposomes

A common strategy to obtain liposomes is the film-hydration method, which consists in the deposition of a phospholipid layer, generally casted from a chloroform solution, followed by an hydration step, through the addition of an aqueous medium.[101] Hollow particles are obtained once the phospholipid film is hydrated and detaches from the support.[102] Through this method, Palchetti et al. in 2019 elaborated a protocol to finely control the composition, rigidity and dimension of ten different liposomes formulations, allowing the so-produced liposomes to safely interact with plasma proteins derived from human serum.[103]

In terms of their structure, liposomes can be classified as multilamellar vesicles (MLVs) if they are composed of several lipid bilayers (their size is in the range 50-1000 nm), or unilamellar vesicles (ULVs) if they are formed by a single lipid bilayer. ULVs have permitted the development of many commercialized drug delivery products.[104] On the other hand, the interest towards MLVs is originated from the possibility to study passive drug transport and sustained drug delivery application with a slower release rate in comparison to ULVs.[105] The ULVs can be, in turn, classified according to their different lateral sizes: 20-100 nm - small unilamellar vesicles (SUVs); 100-1000 nm - large unilamellar vesicles (LUVs); and 1-200 μm - giant unilamellar liposomes/vesicles (GUVs). The MLVs are the result of the direct redispersion of the lipid film in the aqueous medium from a film hydration approach. Their sizes are in the range comprised between 0.05-10μm. In turn, it is possible to obtain SUVs or LUVs by employing sonication or extrusion (i.e. flowing the liposome suspension through polycarbonate membrane filter of defined pore size under high pressure).[106] Finally, GUVs are typically obtained by using electroformation, a technique in which lipids are placed in between two conductive glass substrates (ITO/glass). An alternating potential difference across the electrodes triggers the swelling process of the hydrated lipid layer.[107]

The size and the polydispersity of the liposomes is extremely important for healthcare applications, as the smaller is the size, the longer is the circulation time and the therapeutic

efficacy. In fact, whereas nanoscale liposomes can have circulation times up to 10 hours, larger sizes (>200 nm) cause a decrease down to 4 hours.[108] Polydispersity is also an important parameter to consider. Its range is usually reported from 0 (ideally uniform) to 1 (highly disperse), being values up to 0.3 considered as a upper limit, according to some reports.[109,110] Clearly, size values and distribution are quality factors for liposomes. However, it is very challenging to define optimal values for PDI, as this is highly dependent on the resulting application and on the different routes of bioactive administration, as reported by the FDA's "Guidance for Industry" concerning liposome drug products.[111]

To this aim, it becomes fundamental to shrink the liposome lateral size down to the nanoscale and reducing the polydispersity as much as possible. The conventional methods for shrinking liposomes sizes and reducing size dispersity are based on extrusion or sonication. Usually the extrusion is the favored approach since it permits higher reproducibility, excellent unilamellarity - especially if the extrusion passages are iterated.[112] A recent study by Nele et al. pointed out also the effect of lipid composition and PEGylation for achieving liposome unilamellarity.[113] However, the methods based on extrusion are time-consuming and inefficient,[114] so that new researches have strived to improve the scale-up production of liposomes.

Liposomes composition can be classified into five types, depending on the specific mechanism of intracellular delivery: classical, pH-sensitive, cationic, immune and long circulating. It is straightforward that the phospholipid formulations have to be properly designed in order to control the physicochemical properties of the resulting liposomes. For instance, an important aspect, which strongly depends on the lipid formulation, is the phase behavior of the lipid mixtures which self-assemble during the synthesis process. Remarkably, the lipid formulation influences the packing structure of the resulting self-assembled phospholipids, and not the overall shape of the particles. For example, the cubosomes and hexosomes systems are spherical themselves, but are characterized by cubic or hexagonal lipid packing order as a result of the

self-assembly of surfactant-like lipids, forming nonlamellar phases in the presence of an efficient stabilizer.[115] More specifically, the chemical structures of the phospholipids commonly involved in the liposomes preparation have different spatial features, such as occupied volume, geometry, chains flexibility, and so on. These spatial features allowed to define the critical packing parameter (*p*), that is the ratio of the hydrophobic volume to the product of the head group area and chain length. Such a parameter considers the volume occupied by a single amphiphilic molecule in the phospholipid leaflet structure, permitting to control the morphology of the resulting system. Remarkably, hollow vesicles are formed for *p* ranging from 1/2 to 1. Differently, for *p* outside of this range, the resulting structures are not vesicular, giving rise to a set of different morphologies comprising spherical micelles ($p<1/3$) and cylindrical micelles ($1/3<p<1/2$), planar bilayers ($p=1$), and reverse micelles ($p>1$).[116]

The fluidity of the mentioned supramolecular structures is a further important aspect strongly affected by the chemical composition, since the liposome bilayer flexibility decreases with saturation and length of the phospholipids hydrophobic tails.[116] However, the leaflet fluidity can be tuned by adding molecules able to intercalate in the bilayer structure as well. To this aim, cholesterol is probably the most widely employed additive, due to its ability to improve the biological membranes stability, and to modulate their rigidity.[117] These are fundamentals aspects for the application of liposomal drug formulations in advanced nanomedicine strategies, as vesicles shape and flexibility do influence the liposomes interaction with cell membrane, then the fusion process involved in cargo internalization.[116] The different chemical properties of the liposomes derive from the possibility to change their phospholipid composition used during their assembly.[118] This can be achieved via functionalization with different chemical moieties (e.g. poly(ethylene)-glycol (PEG), antibodies, small molecules, carbohydrates) or with the introduction of charged or pH-sensitive liposomes in the formulation (see **Figure 3A**). Liposomes' size and morphology are usually characterized by employing dynamic light scattering (DLS) and cryogenic-transmission electron microscopy (cryo-TEM).[119] In

particular, the latter permits to characterize their morphology, as reported in Figure 3B. As evident, doxorubicin drug loading (at 2 mg/mL) modifies liposomes' shape, resulting from the formation of solid doxorubicin-sulfate crystal nanorods within the inner compartments, ultimately forcing the elongation of the liposomes into ellipsoids. By increasing the doxorubicin concentration in the range 2-2.75 mg/mL, the resulting ellipsoids axial ratios (i.e. ratio between the major axis to the minor axis) is observed to increase from 1.17 to 2.5.[119]

The researches on liposomes have largely benefited from the vast toolbox of lipids with tunable physicochemical characteristics, introducing systems of natural and synthetic origin.[120] It is well-known that the interplay of lipid length, degree of unsaturation and the percentage of cholesterol can all affect the gel-to-liquid phase transition temperature, bilayer thickness and fluidity. In particular, cholesterol favors conformational order of the fluid lipid phase, while it perturbs the lateral packing order of low-temperature gel phase of lipid bilayers. As a result, a concentration-dependent weakening effect of the gel-to-liquid phase transition temperature is typically observed. In fact, whereas at low cholesterol molar percentage (10-30 mol %), the gel-to-liquid transition is still observed, high cholesterol molar percentage (40-50 mol %), remove the transition temperature.[121]

A fundamental aspect which has been investigated is the final charge of the headgroup (positive, negative or neutral), being all the tails neutral.[122] Commonly neutral liposomes are characterized by the functionalization of the phosphate head group of phosphatidic acid with positively charged molecules, such as choline (2-Hydroxy-*N*,*N*,*N*-trimethylethan-1-aminium) or ethanolamine. These systems are characterized by good biocompatibility, low cellular uptake and propensity to aggregate, ultimately affecting their circulation and clinical efficacy. The presence of an electrostatic charge critically changes the properties. The most common positively charged liposomes are based on the positively charged dimethyl- or trimethyl-ammonium propane headgroups functionalized at the positions 1,2 with hydrophobic fatty acids side chains. These systems strongly interact with cellular membranes (for instance the products

LipofectinTM and TransfectACETM) and have found important applications in gene-transfer technologies. However, their applications in nanomedicine is hampered by the fact that they also strongly interact with serum proteins, resulting in reticuloendothelial system uptake and fast clearance. The negatively charged liposomes are based on the negatively headgroups, such as phosphatic acid, phosphatidylglycerol or phosphatidylserine. In these systems, the head group is functionalized at the positions 1,2 with fatty acids. At the position 3, the phosphate group can be functionalized through a phosphodiester linkage with a phosphoglycerol moiety or with the amino acid serine, resulting in phosphatidylglycerol or phosphatidylserine head groups, respectively. The negative charge stabilizes them against aggregation. Moreover, they can be captured by specific receptors on macrophages, thus facilitating their endocytosis at rates much faster than neutral phospholipids. In fact, negatively or also partially negative charged liposomes are common in many of the FDA-approved liposome formulations, such as in Ambisome® or Exparel®.[104]

The immune liposomes are modified with antigens of interest, inserted into the liposomal moieties or within the interior water core, allowing the enhancement of their immunogenicity. Finally, the long circulating liposomes are modified to increase their stability in the bloodstream, their clearance and their biodistribution. The latter are obtained by inserting phosphatidyl-inositol into the bilayer,[123] or PEG at the vesicles interface [124] with the aim to inhibit the serum protein adsorption *in vivo*, thus resulting in a longer circulation time [125] and a reduction of the mononuclear phagocyte system uptake.

Among the possible routes for improving the intracellular drug delivery of cargo is the introduction of pH-responsive liposomes, especially considering the responsivity to the acidic pH which is typical of the endosomal system or of tumoral environments. To this aim, the lipid dioleoylphosphatidyl ethanolamine (DOPE) is among the most investigated example for the assembly of pH-sensitive liposomes. This system has a cone shape due to the small headgroup which occupies a lower volume in comparison to the hydrocarbon chains, impeding the

formation of a lamellar phase but rather of an inverted hexagonal phase stabilized by the electrostatic interactions between the amine and phosphate groups of the polar headgroups.[126] The intercalation of an acidic amphiphile, such as the cholesteryl hemisuccinate (CHEMS, a succinic acid esterified to the beta-hydroxyl group of cholesterol), reduces the electrostatic interactions favoring the formation of bilayers, thanks to the negative charge of the succinate groups. Under acidic pHs, the protonation of the carboxylic groups destabilizes the liposomal structure [127] and finally permits to release the cargo. There are other worth to mention studies dealing with pH-responsive liposomes which mimic the properties of DOPE. One interesting example has been provided by Shi and coworkers who prepared cationic/anionic lipid formulations based egg phosphatidylcholine, dimethyldioctadecylammonium bromide (DDAB), CHEMS and Tween-80.[128] More recent examples include esterase-catalyzed double smart PEG-lipid derivatives [129] and formulations including egg yolk lecithin (EL) mixed with a lithocholic acid derivative showing pH-triggered orientational that disrupt the membrane.[130] Notably, there are still some issues to be solved before pH-responsive liposomes can be considered for clinical studies and market, especially the control of their stability, the drug release rate.

Polymersomes

As mentioned in the introduction, more advanced methods to obtain micelles and hollow particles involves block copolymers, which are polymeric macromolecules consisting of polymer chains (blocks) covalently bound, often in a linear configuration. Block copolymers are generally obtained through innovative synthetic polymerization reactions (e.g. atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer (RAFT), and ring-opening metathesis polymerization (ROMP)) suitable to prepare several copolymers with controlled molecular weight, low polydispersity index and well-defined composition.[131] In particular, block copolymers are generally designed as structures made out of blocks whose solubility can be individually modified by tuning the physicochemical properties of the solution,

such as pH, redox state, solvent mixture composition. The resulting self-assembly of the polymer chains allows the synthesis of polymeric micelles and hollow polymeric vesicles, namely polymersomes.[132] A representative and widely used chemical structure for diblock copolymers - often employed to assemble vesicles for biological purposes - is constituted by PEGylated-polymethacrylates, which represent stealth and biocompatible systems. Their chemistry can be easily tuned in order to introduce several chemical functionalities, which in turn result in surface engineered particles (see Figure 3C). Similarly to liposomes, also polymersomes are conveniently characterized by TEM and cryo-TEM, as reported in the example from Men et al.[133] who investigated the effect of water amount used in the preparation of these vesicles on the size and shape of PEG_{45}-*b*-PS_{206} polymersomes (see Figure 3D).

There are three strategies to produce polymersomes. Firstly, as for liposome preparation, polymersomes can be obtained through the film-hydration method.[134] Analogously to the phospholipid layer, the thin film of polymer swells and detaches from the solid support to form the desired vesicles. The second approach consists in the self-assembly of the polymer chains into solution to form the hollow particles.[135] In addition, a third recent and promising strategy to obtain polymersomes suspensions consists in the polymerization-induced self-assembly (PISA).[136] The potentiality of the PISA process is related with the direct assembly of polymersomes during the polymerization of the block copolymer, thus reducing the number of synthetic steps. Moreover, in all mentioned synthetic approaches, it is possible to tune the ratio between the hydrophilic/hydrophobic parts constituting the copolymer structure, by controlling the degree of polymerization during the reaction. That permits to obtain colloidal particles with different size and shapes. In fact, for a fixed hydrophilic part, the gradual increase of the hydrophobic section leads to the formation of micelles, then worms, vesicles, and finally tubular structures.[135]

As further discussed in the next Section, an aspect of particular relevance is the application of polymersomes as stimuli-responsive particles, sensitive to physicochemical parameters, such

as pH, temperature, hydrolysis and redox agents,[137] in order to obtain synthetic carriers with an improved space and time controlled release of the transported payload. In fact, several kinds of polymersomes, differing in chemical composition, surface functionalization, dimensions, membrane thickness and permeability, have been reported. Among them, pH-sensitive polymersomes have been prepared introducing a protonable block into the vesicle-forming copolymer. This block exhibits a low solubility in water in its uncharged state, and a higher water affinity as charged after protonation. Such block is commonly an amine-based polyelectrolyte (e.g. poly[2-(diethylamino) ethyl methacrylate, PDEAMA) and it is the main constituent of the inner part of the membrane. So, the vesicles are firstly assembled in solution at a pH above the pK_a of the pH-sensitive block, then, as the pH increases, the particles can swell or disassemble, since the ionization of the chemical groups in the membrane causes an increased hydrophilicity and electrostatic repulsion.[137]

Another important class of polymersomes is constituted by the redox-sensitive particles, which can be destabilized depending on the redox state of the environment. They are made of amphiphilic copolymers, whose block moieties are covalently bound by a disulphide bond. The disulphide bond is stable in the oxidizing extracellular environment, but easily breaks up in the reducing intracellular conditions.[138] Notably, any kind of biocompatible block combination can be selected for the redox-responsive particles design. Hydrolysable polymeric blocks (e.g. poly(caprolactone), PCL) and thermo-sensitive blocks (e.g. poly(N-isopropylacrylamide), PNIPAAm), may offer further relevant alternatives as potential therapeutic delivery strategies.[139,140]

As in the case of liposomes, the role of PEG is noteworthy, since it is the most employed hydrophilic block due to its stability, biocompatibility and water affinity. Furthermore, PEG coronas on the delivery system interface confer stealthiness to the carrier, meaning longer bioavailability *in vivo*.[141]

Alternatively, specific recognition patches characteristic of biomacromolecules - responsible for the formation of nucleic acid and protein complexes in the cells - can be used to obtain protein and DNA containers, forming cages, nanoparticles and, in the case of oligonucleotides, more complex architectures, namely DNA origami.[142] In particular, DNA can assembly into several nanostructures, such as cubes, tetrahedra, octahedra, nanotubes, origami and many others, which have been obtained in a wide plethora of different sizes and shapes, depending on their sequence and length, together with the assembly medium physicochemical features.[143–145]

2.2.2. Microfluidics and printing approaches

Along with the abovementioned processes, instrumental approaches have emerged for colloids preparation. In particular, microfluidic systems and inkjet printing technologies have stood out as the most widely employed tools to prepare high quality dispersions of solid and liquid particles in fluid continuous phases, with a higher control in terms of polydispersity and reproducibility. Microfluidic chips are small fluidic circuits consisting of micrometric channels, able to handle volumes under the microliter scale.[146] Generally, liquid droplets are injected into a stream of a second immiscible liquid phase, and then emulsions of small volume compartment are generated. Notably, this approach is suitable for particles formation in water, especially in the case of liposomes, that have been obtained through injection of ethanol phospholipidic solution in an aqueous continuous flow.[147,148] By properly designing the physicochemical features of the emulsion, any kind of compound - from small molecules to (bio-)polymers, as well as supramolecular complexes - can be confined into the dispersed vesicles to obtain high-throughput dispersions of several microreactors. In this context, chemical reactions, biomolecular interactions and quantitative analytical assays might take place.

To this aim, several approaches have been investigated for the microfluidic production of liposomes. Relevant examples in the field of GUVs include comprising droplet transferring process[149] or droplet jetting[150]. However, as abovementioned, it is important for the healthcare applications, to reduce the liposome size down to the nanoscale. This issue can be solved by leveraging direct fluid mixing under laminar flow of an organic phase containing lipids with a water phase containing buffer ions. Typically, the fabrication process is based on the mixing of two streams (lipid-rich organic phase with a water phase) within a microfluidic chip. This allows the scalable production of liposomes that are able to retain the *in vitro* and *in vivo* activities, compared to the ones produced by conventional extrusion method.[114] The mixing occurs at milliseconds time scale, resulting in the formation of disc-like bilayer micelle intermediates. These micelles grow in size until they start to bend for reducing the surface energy, and finally they produce liposome vesicles at nanoscale dimensions.

It is out of the scope of this review to report on the mathematical details of the model; the interested reader can refer to the paper from Kotouček and coworkers[151] for further information. Researchers have further optimized microfluidic mixing devices based on hydrodynamic focusing in which a central fluid flow of an organic phase containing lipids is interfaced by two streams of aqueous buffer,[152] or also vertical flow focusing in which high aspect ratio mixing channels allow for an enhancement of the size of the lipid/water interface.[153]

Some important examples dealing with AVs production by microfluidics need to be cited in this review. For instance, Petit et al. in 2016 demonstrated the possibility to leverage double-emulsion templated flow-focusing microfluidics for the tunable production of monodisperse liposomes and polymersomes[154] (**Figure 4A**). Importantly, the sizes of both AVs could be easily tuned by varying the flow rates of the two fluids, mainly resulting in microscale systems (Figure 4B-C). However, a further engineering of the AVs production is necessary to reach the nanometer scale, adequate for intracellular delivery. This can be achieved by microfluidic

mixing[151] of an organic water-miscible solvent phase (typically ethanol) containing lipids with an aqueous phase containing molecules to encapsulate (Figure 4D). Such set-up (based on the NanoAssemblr® microfluidic platform) allows for the assembly of liposomes having diameters in the 100 nm scale and can be integrated with gadolinium lipid complexes for applications as MRI contrast agents (Figure 4E). Notably, the high density of gadolinium atoms permits to achieve a sharper visualization of the lipid bilayer by cryo-TEM.

In another report, Albuquerque et al. in 2019 prepared pH-responsive polymersomes using poly([N-(2-hydroxypropyl)]methacrylamide)-b-poly[2-(diisopropylamino)ethyl methacrylate] block copolymers (PHPMAm-b-PDPAn).[155] The authors realized micromixers based on microfluidics chip (Figure 4F). They were able to reduce the sizes of the polymersomes down to about 50 nm by increasing the flow rate of the water phase up to 200 μL min^{-1} (Figure 4G). Furthermore, they successfully loaded them with DOX, permitting a pH-responsive delivery to tumor cells (B16F10 and 4T1) at similar levels compared to free administrated DOX [155] (Figure 4H). As in the case of microfluidic systems, in 2018 the inkjet printing technologies[156] have been employed for emulsions preparations,[157–159] and implemented for vesicle assembly, with high reproducibility and size control.

Indeed, the current inkjet technology has enabled the preparation and loading with cargoes of small unilamellar liposomes, by direct printing of the vesicle-forming amphiphilic molecules into a receiving medium[160,161] (Figure 4 I-J). Notably, the cargoes can be solubilized directly in the receiving medium, where vesicles are assembling. In this way, fragile biomolecules, such as proteins or other biocomplexes, can be quickly encapsulated avoiding any further thermal or mechanical stress, which in some cases might affect their structural integrity.[162–164] In the case of polymersome-based systems, inkjet printing permits to realize biomolecules-rich femtoliter-scale aqueous compartments stabilized within mineral oil drops in which crowding effects are spontaneously generated and lead to interfacial confinement at the water/oil interface.[165]

3. EVs and EV-Inspired Vesicles for Targeted Drug Delivery

Vesicle-based research is crucial for the understanding of fundamental biology functions. Also, translational applications for the development of new diagnostic approaches, together with the use of EVs/AVs as advanced therapeutics, represent important goals of vesicle studies. We have already mentioned how cell culture conditions may be modified/manipulated in order to improve the EV secretion and what are the best and innovative technologies for preparing chemically defined AVs. However, there are additional obstacles to overcome, in order to improve their use in the clinics. Another key aspect in the design of effective vesicle-based therapeutics consists in the selection of the ideal route of administration. Clinical trials evaluating the efficacy and safety of EV/AV-based therapies are currently ongoing, with the aim of finding the best and safest route of delivery vesicles to patients, which often depends on the target site (see **Figure 5**). The vesicle-mediated cargo release needs to be tightly controlled both in space and in time. Importantly, the controlled release of bioactive molecules in a localized fashion may strongly limit the drug side effects, while enhancing its efficacy.[166] The following sections are focused on the best ways to achieve the optimal vesicle delivery, also by using natural or synthetic materials which can ameliorate vesicle release at the target site.

3.1. Delivery and targeting of EVs

The systemic injection is usually the preferred choice since it can be easily performed and potentially allows the distribution of vesicles to all districts. However, once systemically administered, the main problems to face are: (i) the presence of barriers, such as the BBB, and (ii) the clearance operated by the circulating and local immune cells.[167,168] Consequently, many studies have been performed to improve both the biodistribution and the clearance of vesicle carriers.

Subcutaneous injection

In 2005, during the first clinical trial, dendritic cells (DCs)-derived EVs - now known as Dex - have been injected sub-cutaneously or intradermally in patients with metastatic melanoma[57] or non-small cell lung cancer.[58] DCs are the most important antigen-presenting cells (APCs) for the priming of naïve T-cells, and they have been used as vaccines in several cell-mediated approaches against cancer. However, DCs-based vaccinations have encountered many obstacles, including cell-to-donor compatibility, and cell-free approaches have been therefore further explored. Dex have been studied since 1998, when Zitvogel and colleagues demonstrated the presence of functional Major Histocompatibility Complex (MHC) class I and class II in EVs secreted by tumor-peptide pulsed DCs.[169] These vesicles were able to induce an immune response by delaying murine tumor growth or even eradicate the tumor in preclinical models.[169] In 2005, Zitvogel and Lyerly groups separately, conducted two Phase I clinical trials on metastatic cancer patients, who received autologous EVs isolated from culture supernatants of monocyte-derived-DCs loaded with antigenic HLA-presenting melanoma-associated antigen peptides (MAGE). This *ex-vivo* monocyte-derived DCs tumor antigen loading have been pursued following a pre-established protocol from Lamparski et al. in 2002.[170] Once purified from the cellular supernatant, the MAGE-derived EVs were then injected in patients intradermally or sub-cutaneously, once a week, for 4 weeks. The results obtained from these two clinical trials demonstrated the feasibility of Dex production on large scale and the safety of the whole procedure in cancer patients.[57,58]

Intranasal injection

The delivery in the brain, that needs to trespass the BBB, can be improved by intranasal administration. Several studies have been performed where EVs were injected through the nasal cavity. For example, in 2011, Zhuang and colleagues investigated the role of curcumin-loaded EVs in a preclinical model of LPS-induced inflammation. The authors found that EVs injected intranasally reduced the number of activated microglial cells within two hours from the time of

administration. Additionally, the same curcumin-loaded EVs were intranasally administered to mice affected by induced allergic encephalomyelitis, and also in this model the loaded EVs reduced the severity of the disease, compared with the effects of mock vesicles or curcumin only.[171] In the context of neurodegenerative diseases, a 2019 study showed the efficacy of intranasal injection of EVs derived from *ex vivo* cultured stem cells derived from human exfoliated deciduous teeth on 6-OHDA Parkinson's disease (PD) rat models. Such EV treatment significantly ameliorated gait parameters in PD treated rats, with an improved degree of movement coordination and better posture score. Moreover, the authors observed an increase in the tyrosine hydroxylase expression in the Substantia Nigra and in the Striatum regions of the PD brains specifically in EV-treated rats. These data suggest that stem cells-derived EVs may protect dopaminergic neurons from the toxic effects of 6-OHDA, consequently slowing down the progression of the disease.[172] The same year, Gou and colleagues intranasally delivered MSC-derived EVs loaded with PTEN-siRNA (Exo-PTEN) in rats with spinal cord injury.[173] These vesicles efficiently crossed the BBB and migrated to the injured spinal cord sites without any accumulation in the brain, demonstrating that, likewise their original MSC parental cells, MSC-derived EVs are able to sense and target selectively the sites of injury. Also, the authors demonstrated that in injured rats EVs were retained longer in the spinal cord regions compared with healthy control. These Exo-PTEN showed a strong affinity for injured neurons, thus selectively ameliorating motor, sensor and urinary functions. Moreover, Exo-PTEN reduced neuroinflammation and gliosis, favoring also axonal regeneration.[173]

Subcutaneous implantation

Another strategy that is being explored for improving the delivery and the release of EVs in the target site is based on materials, such as hydrogels - biocompatible vehicles used at first for encapsulating cells, and then EVs - into a polymeric matrix. The initial aim of hydrogels was to preserve the integrity and the structure of the implanted cells, especially for brain cell

transplants. For this reason, several types of hydrogels have been produced, since 1980.[174] These hydrogels differ in composition, sustainability and biocompatibility, avoiding the problems due to hydrogel chemistry following their implantation.[175] In fact, biocompatibility is necessary at all steps of polymetric matrix formation, from the precursors to the final products, when the hydrogel should be degraded into biocompatible products.[176] Also, the polymerization process requires attention if, for example, free radicals are formed during the reaction. An appreciable method of polymerization was obtained by Conova et al. in 2012.[177] The authors found that the polymerization process depends on temperature. In fact, the liquid-injected hydrogel may polymerize at physiological temperature, following the injection, thus simplifying the transplantation procedure. For this reason, the hydrogel matrix engineering developed new technologies to have a final product able to mimic the texture and the 3D environment of a natural ECM.[178]

The simplest and easily available gel used to deliver EVs is Matrigel. As previously mentioned, in 2018, Kojima and colleagues tested the Matrigel to improve the EVs delivery in the murine 6-OHDA PD model.[80] Interestingly, most of the studies where the hydrogel matrix was used to encapsulate EVs, have been carried out for promoting tissue repair. Several materials have been developed and tested for this purpose, including Chitosan alone or combined with other materials such as hydroxyapatite,[179–182] but also the FHE (Pluronic F127 (F127), oxidative Hyaluronic acid (OHA) and Poly-ε-L-lysine (EPL)) hydrogel,[183] and the photoinduced imine crosslinking (PIC) hydrogel (EHG).[184]

Another bio-scaffold for MSC-derived EVs has been designed by Chen et al. in 2019 to cure osteoarthritis. The group successfully fabricated a 3D printed cartilage extracellular matrix(ECM)/gelatin methacrylate(GelMA)/vesicle scaffold by using a desktop-stereolithography technology. The 3D printed ECM/GelMA/vesicle scaffold - with radially oriented channels - was able to restore cartilage mitochondrial dysfunction and enhance

chondrocyte migration, thus promoting the osteochondral defect repair in an osteochondral defective preclinical model.[185]

Another line of research takes advantage of the enzyme prodrug therapy (EPT), a set of techniques allowing the enzyme-mediated conversion of an inactive and nontoxic prodrug in an active drug directly in the target site. In 2018 Fuhrmann and colleagues used EVs as carriers for the stabilization of selected enzymatic cargoes within a hydrogel for a local and controlled release and conversion of a benign prodrug, to be transformed locally in an anti-inflammatory drug.[186] The authors loaded the hMSC-derived EVs with β-glucuronidase and encapsulated them within a poly-(vinyl alcohol) (PVA) hydrogel, followed by stabilization with PEG, in order to generate biocompatible hydrogels with a diameter of 8 mm and thickness of 2 mm. In this way they achieved a local and sustained conversion of the curcumin β-D-glucuronide precursor in free curcumin, with a local and acute anti-inflammatory effect. Moreover, for the first time they evaluated the therapeutic potential of MSC-EVs-glucuronidase *vs.* synthetic carriers (liposomes)-glucuronidase within hydrogels in a mouse macrophage cell line challenged with bacterial LPS. The authors demonstrated that EV-hydrogels may have an anti-inflammatory potential greater than liposome-based hydrogels, even in the absence of the substrate.[187] In **Table 1** are listed the hydrogel based approaches for EV delivery and application, with the details about EVs source, hydrogel composition, the physio-pathological context and the specific results.

Other delivery avenues

A completely alternative way for EV delivery has been investigated by Sun and colleagues in 2019. Taking advantage of the technique of ultrasound-targeted microbubble destruction (UTMD) for gene delivery, they applied this method for injecting EVs in refractory tissues, such as heart, adipose tissue and skeletal muscles. Originally this method was applied to inject nucleic acid-based drugs, thanks to the cavitation effect produced by ultrasounds within the

microvasculature of target tissues. However, this approach did not have the desired effects and it has been used only for restricted applications.[188] In their paper, the authors demonstrated the possibility to load EVs in mice refractory tissues by injecting SonoVue (sulphur hexafluoride) microbubbles together with the EVs and inducing UTMD. Microbubble destruction and cavitation effect increased the infiltration of EVs in heart, adipose tissue and skeletal muscles, as well as in liver and the reticuloendothelial system,[188] opening another promising way to increase the selectivity of EV targeting.

3.2. Delivery and Targeting of AVs

As mentioned for the EVs, also in the case of AVs the encapsulation procedure is a promising strategy to physically protect the drugs from the physiological mechanism of clearance, improving the bioavailability. Liposomes constitute since long time the most commonly used class of AVs to deliver selected payloads to specific targets *in vivo*,[189] whereas polymersomes are promising alternatives currently under development.[190] There are many examples on the market of drug formulations loaded in AVs for the enhancement of their therapeutic efficacy. As a matter of facts, liposomes have found application in drug delivery starting from the 1970s, when they have been used for loading and delivery of a broad class of biomolecules, including drugs, toxins, proteins, enzymes, antibodies, and nucleotides.[191] Since then, AVs have been tailored for precise nanomedicine related applications, improving the pharmacokinetics of the encapsulated active molecules.

For instance, a seminal approach used for post-loading doxorubicin into liposomes is the one based on the employ of transmembrane pH gradient,[192] in which the interior of the liposome is at an acid pH. At physiological pH, doxorubicin is uncharged. Upon incubation with the liposomes, it diffuses into them and becomes protonated. The acquired positive charge impedes its leakage from the liposome, so finally remaining trapped inside. This well-established method permits ‘active’ loading to trapping efficiencies as high as 98%, interior drug

concentrations as high as 100 mM, significantly enhancing drug retention within the vesicles. Such extremely high loading is ascribed to the crystallization of doxorubicin in the form of nanorods at the low pH of the vesicles lumen,[193] (see Figure 3B). This unique system enables an optimal drug loading until the liposome reach the tumor site, where the drug release occurs, also in response to the high local concentration of ammonia produced by glutaminolysis, a metabolic process that is typical of tumor cells.[194] Another important active loading approache include the use of phosphate gradients, by employing a transmembrane $(NH_4)_2HPO_4$-gradient,[195] since it allows doxorubicin release at acidic pHs, such as those of tumor tissues.

The enhanced permeability and retention (EPR) effect

The researches in nanomedicine have also been stimulated by the knowledge of the enhanced permeability and retention (EPR) effect, which determines an up-concentration of drug delivery systems (nanoparticles, liposomes and macromolecular drugs) into tumors with respect to healthy tissues/organs.[196] This effect can be explained according to two different factors. Firstly, as a result of their size, nanocarriers can escape the renal clearance, increasing their plasma concentration. The second factor stems from the ability to leak into the tumor tissues, through their abnormally increased inward fluid transport dynamics and the lack of drain by the lymphatic system.[196] In particular, it is well known that liposomes delivery to tumor sites highly benefits from the EPR effect,[197] in particular given the larger sizes of the voids between the endothelial cells in the tumor tissues with respect to the healthy cellular structures, ultimately leading to an efficient drug encapsulated liposome accumulation in the tissue.[198]

Liposomes in the market

The first appearance of AVs-drugs into the market dates back to the 1990s-2000s. The first liposome-based product to be commercialized was the Doxil®, launched in the U.S. market in 1995 for the treatment of ovarian cancer.[199] Since then, liposomes became part of the

formulation of approved products with application in many different therapeutic fields, including viral vaccines (Epaxal® approved in 1993), fungal infections (Ambisome® approved in 1997), photodynamic therapy (Visudyne® approved in 2000) and analgesics (Exparel® approved in 2011). The reported examples represent only the first of many liposome-based medications (>10) currently available in the market, as recently reported by Bulbake et al.[104]. The selected route of administration of drug-loaded liposomes are typically intravenous or intraperitoneal injection.[200] The oral delivery poses some challenges, especially due to the poor stability of the liposome-drug in the gastrointestinal tract – which can be prevented through the use of polymers or polysaccharides. Moreover, research efforts strived to improve the delivery through the BBB, as for brain related diseases. This can be pursued by surface liposomal modification with antibodies or cholesterol pullulan coating, in order to render liposomes more permeable and, thus, able to penetrate into the brain.[201] As mentioned earlier, a convenient delivery strategy to the brain is constituted by intranasal administration, both for hydrophobic and hydrophilic compounds.[202] Researches have looked at the optimization of formulations that can minimize the particle aggregation at nasal pH, ensuring liposomes' stability, and the improvement of the drug loading capacity with the aim to produce injectable microliter-scale formulations.

Hydrogel based formulations

In general, it is fundamental to reduce the burst release of liposome-encapsulated drugs, for preventing potential accumulation and unwanted toxic effects. To this aim, in a similar effort showed for EVs, hydrogel materials have also been investigated for their ability to efficiently load and protect liposomes, permitting a controlled kinetics release by tuning their physicochemical properties (see Table 1). Liposome hydrogel formulations can be obtained by using natural or synthetic polymers. In particular, due to their higher biocompatibility and biodegradability, hydrogels from natural polymers have found more interest[203]. Starting from

the seminal work of Weiner in 1985, on a collagen matrix,[204] many other researchers have investigated the preparation of biocompatible hydrogels based on polysaccharides, given their low cost and the ideal compatibility with tissues[203]. Alginate-based hydrogels were among the first ones to be studied, in the second half of the 90s. In 1995 Rudolph and Monshipouri showed the possibility to encapsulate unilamellar liposomes - loaded with cytochrome-c - within an alginate hydrogel for evaluating their *in vitro* releases[205]. More recently, alginate-based hydrogels have been used for the oral delivery of manganese porphyrin liposomal drug, developed as superoxide dismutase mimic[206]. The alginate hydrogel permitted the drug to resist through the acidic environment of the gastrointestinal tract, and to inhibit the growth of transplanted tumors in mice models. Moreover, chitosan-based hydrogels were firstly investigated in 2002 by the group of Leroux et al.[207]. They obtained an *in vitro* release profile of carboxyfluorescein over at least 2 weeks, which was significantly longer in comparison to the release in absence of the hydrogel (completed within 24 hours). Another remarkable example is constituted by hyaluronic acid, that allowed to improve the liposome-based drug delivery for treating osteoarthritis[208] and ocular diseases, such as human uveitis[209], thanks to the extraordinary compatibility with natural tissues. Conversely, within the field of hydrogels prepared from synthetic polymers, some recent examples deserve to be mentioned. For instance, Suri et al. prepared in 2011 biocompatible polyvinyl alcohol hydrogels produced by freeze-thaw cycles to load unilamellar liposomes.[210] The liposomes were protected from the temperature stresses by trehalose, which permitted to maintain liposomes' hydration during the freeze-thaw cycles. In another interesting example, Gao et al. dispersed cationic liposomes loaded with carboxyl-modified gold nanoparticles within polyacrylamide gels, for treating *S. aureus* bacterial infections onto mouse skin[211]. This preparation sustained nanoparticle release within 24 hours, without producing any toxic effect on the mouse skin within a 7-day treatment. Another recent demonstration of antibacterial applications is provided by the recent example of Liu et al., [212] who loaded octadecylamine grafted liposomes into Ag^{+} loaded thiolated

polyethylene hydrogels, obtaining an interesting system which combines good self-healing and excellent antibacterial properties (tested on *S. aureus* and *E. coli*) thanks to the presence of Ag^{+}. With the aim to further increase the specificity as drug delivery systems (DDS), the surface of liposomes can be functionalized, by coupling ligands on their surface, using either covalent or non-covalent approaches.[213] Usually, the ligand coupling is carried out by means of a hydrophobic phospholipid anchor, which is typically phosphatidylethanolamine (PE), given the presence of a reactive amine in its head group. The covalent approach uses thiol-reactive PE derivatives coupled, through thioether bonds, to the maleimide groups of target proteins, by using a hetero bifunctional cross-linking reagent, like SMPB (H-succinimidyl-4-)p maleimidophenyl-butyrate. The non-covalent route in mainly based on the utilization of the streptavidin-biotin, a high affinity interaction. Firstly, avidin or streptavidin are bound to liposomes containing a biotinylated lipid (usually a derivative of PE) and then bound to the biotinylated ligand. In 2018 Caddeo et al. developed PEG-modified liposomes for the delivery of resveratrol, achieving long-term stability and excellent biocompatibility evaluated by an *ex vivo* model of hemolysis in human erythrocytes.[214]

Polymersomes-based delivery

The low stability, poor flexibility in the synthesis approaches of conventional liposomes, stimulated scientists to produce modified AVs able to efficiently tackle these issues. To this aim, polymersomes vesicles were developed, featured with a higher versatility and the possibility to assembly more complex, stable and chemically versatile particles. Drug loading into polymersomes is typically carried out by a passive or active approach. Whereas in the former, the drug to load is simply mixed in the aqueous or in the organic phase, the active loading considers the employment of a pH or a salt gradient to favor the drug encapsulation whilst polymersome assembling.[215] Once polymersomes are assembled, it is important to block innate immune system driven clearance to increase their potential bioavailability. Such

protection can be pursued using hydrophilic polymers and polysaccharides to coat the polymersomes. Another important aspect is the targeted delivery to favor selective cellular uptake, decreasing the risk of toxicity for the nearby cells. This can be obtained, similarly to liposomes, by chemical functionalization of the polymerosomal surface with antibodies, aptamers or polysaccharides. The antibodies are typically covalently linked on the PEG block, allowing a higher specific binding to target cells.[216] As a possible alternative, small peptides can be designed to selectively bind to and penetrate in recognized target cells thus permitting a precise polymersome delivery.[217] Moreover, polysaccharides have been used to facilitate polymersome selected targeting towards inflammatory tissues.[218]

The studies on polymersomes are more recent (2000s -2010s) compared with the ones on liposomes and show a similar degree of complexity in the range of possible cellular targets. Polymersomes can be accurately designed as stimuli responsive systems for pharmacological purposes.[219] Herein, two classes of responsive polymersomes will be presented: pH and redox responsive. The *pH-responsive polymersomes* are able to release their cargoes in a pH-dependent fashion, allowing to distinguish, for example, between a healthy milieu, with neutral pH, versus a tumoral or inflamed microenvironment, generally presenting a lower pH.[220–222] In this context, Voit and collaborators, starting from 2012, investigated the potentiality of pH-responsive polymersomes based on PEG-g-PDEAMA copolymers.[223–228] In particular, the group demonstrated the effective delivery and pH-triggered release of the drug doxorubicin carried by polymeric particles with different and tunable surface chemistry. The functionalization with folic acid resulted as one of the most promising one for further biomedical applications, since the drug carried by such functionalized particles was effectively delivered to the target cancer cells with selective cytotoxicity *in vitro*.[223]

Another family of stimuli-responsive polymeric particles is composed by the *redox-sensitive polymersomes*, where a disulfide bond is utilized as a linker to covalently bind the polymer blocks. As the polymersomes experience a reductive environment (e.g. in the cytosol after the

uptake process by a target cell) the disulfide bridge is reduced and then cleaved, causing the cargo release.[131] The redox-based strategy has been exploited in 2016 by Y. Zou et al. to develop a polymeric alternative to Doxil® (see above). The doxorubicin-loaded polymersomes have been tested in cell culture and they showed a good chemical stability, as well as glutathione (10 mM)-activated disassembly, with consequent quicker drug release and enhanced activity, compared with the classical liposomal formulations.[229]

Currently, biodegradable polymers, such as PLA and PCL, have been also tested as sensitive component of the polymersome membrane.[230] L. van Oppen et al. in 2018, observed a slower PCL-mediated hydrolysis of polymersomes in the biological media, being a potential novel and efficient biomedical vehicle for enzyme loading and systemic delivery. In particular, the authors demonstrated the efficient delivery of active catalases into living cells, able to improve the protection against the damage of reactive oxygen species (ROS). In addition, they demonstrated that polymersomes conjugated with the transactivator of transcription (TAT) cell-penetrating peptide (derived from HIV virus) may increase their cellular uptake *in vitro*.[231]

In conclusion, polymersomes are highly versatile and biologically stable AVs, in which the active molecule loading, as well as the release capabilities, can be easily manipulated through the use of various block copolymers (e.g. biodegradable and/or stimuli-responsive). Despite the promising results obtained *in vitro* with polymersomes, further preclinical and clinical studies are currently needed to support their use as viable alternative to liposomes.

4. Comparison between EVs and AVs as theragnostics

In general, both EVs and AVs can efficiently protect their payload against degradation, although the active targeting efficiency is currently debated for both systems. By labelling EVs with lipophilic dyes it is possible to follow and verify their targeting ability and biodistribution *in vivo*.[232] Indeed, the identity of the donor cell confers to the secreted EVs targeting properties - or at least a preferential interaction capacity - towards specific target cells. In 2020 Guo and

colleagues demonstrated that EVs derived from pro-inflammatory M1 macrophages and labelled with fluorophore DiD, specifically accumulated within metastatic nodules. On the contrary, labelled liposomes accumulated at a less extent compared with EVs (24-30% lower) and they were equally distributed between the tumor and the liver. They attributed the targeting specificity of EVs to the presence on their surface of the donor cell-inherited chemokine receptor CCR2, which guided the vesicles specifically towards the cancer cells expressing the chemokine CCL2.[233] This is in line with older reports, although the targeting mechanisms of EVs are not fully understood.[234] Moreover, the target specificity may be enhanced by EV functionalization protocols or donor cell manipulation.[235] As an example, EVs may be functionalized with lipid-grafted hyaluronic acid for tumor targeting.[236] On the other hand, surface functionalization is an essential step for AV targeted drug delivery.[122] Liposomes can be functionalized on their surface with tumor-specific ligands, such as folic acid or hyaluronic acid, that are recognized by tumor tissues that overexpress their receptors.[237] Also in the case of polymersomes, a simple chemical strategy based on the electrostatic adsorption of folic acid for targeted delivery to cancer cells has been demonstrated.[238] Although many steps ahead have been done, a more efficient targeted delivery is still needed in the vesicle field. Further research is pivotal to uncover novel strategies for active targeted delivery of carriers transported by both EVs and AVs.

The historical evolution of these systems is remarkably different. EVs have been studied only since the late 1990s/2000s as naturally occurring phospholipid-based systems, offering a plethora of many different applications. For instance, EVs are becoming more and more explored also as potential diagnostic tools, given the possibility to carry endogenously produced molecules related to specific diseased conditions.[125,142,239,240] Differently, AVs have been developed earlier - initially in the form of liposomes - and they represent still the most diffused colloidal DDS in the market. The development of polymersomes is still in progress, being relatively recent (2000s-2010s). The amount of papers published on EVs and AVs in the last

10 years provides another useful picture for understanding the relevance of such colloidal systems in the healthcare field (see **Figure 6**). Liposomes, matter of study since the 70s, remain a very intense topic of on-going research, as evident by the conspicuous number of papers published per year. In comparison, among the AVs, (including polymeric and lipid nanoparticles, as well as emulsions) polymersomes are under intense investigation for to their applications in preclinical and clinical systems. This interest is in line with the growing evidence regarding their biocompatibility and suitability for nanomedicine-related applications. Interestingly, the trend in the field of polymeric/lipid NPs is not positive in the latest years, and in some case a clear decline is visible. Similar situation for the systems based on emulsions, that benefit from the well-established knowledge deriving from pharmaceutic preparation. These systems cannot be considered a truly synthetic analogue for the EVs, but they still possess some tunable properties that make them useful DDSs. On the other hand, as illustrated in **Figure 6**, the interest towards EVs has grown exponentially in the last 10 years, mainly due to the improvement of the technologies of purification and detection, and the extensive range of applications for this class of endogenous vesicles. A positive trend in the EV-related research is envisaged for the years to follow, due the growing interest of the researchers in this field. Apart from the number of publications that provide a quantitative but yet not totally complete picture of the situation, a better confrontation can be ascertained by analyzing the major advantages and disadvantages of EVs *vs.* AVs, under three complementary aspects: (1) production, (2) biocompatibility and (3) suitability as DDS. These parameters are summarized in **Table 2**.

Production

As illustrated above, the production of the EVs is significantly more challenging compared with the synthesis of the AVs. As a result, EVs have been less used as DDS compared with liposomes, since limitations emerged in terms of scalability of the production methods and loading of

exogenous bioactive molecules. In fact, EVs are produced in living cells with relative low yields, requiring efforts to obtain optimal amounts of particles.[241] In this regard, different technologies can be implemented to bridge this gap, including ultracentrifugation/gradient techniques, size-exclusion chromatography, ultrafiltration, microfluidics chips, ultimately improving the quality (i.e., size, density, ζ potential and biochemical composition) and the yield of EVs. In particular, novel technologies based on innovative 1D[242–245] and 2D materials[246–248] may largely facilitate the capture, the detection, the quantification and the quality control of the purified EVs, helping to gain a larger space in the healthcare and nanomedicine market.

In contrast with EVs, AVs are synthesized in laboratory and industry by using reproducible protocols that can be scaled up given its independence from living systems as a starting source. As described above, this is true for liposomal formulations that entered already in the market as DDS.[189,249] The efficient drug loading, adjustable size, as well as tunable surface chemistry and charge contributed to accomplish remarkable and robust preclinical and clinical evidences for liposomes as good carrier for therapeutics. One issue is the higher production cost, which can be solved in future by polymersomes. Indeed, given their higher flexibility in production and easier functionalization, polymersomes can be envisaged as an advantageous and cheaper alternative to liposomes.

Biocompatibility

EVs are characterized by an excellent biocompatibility that has been extensively evaluated both at preclinical and clinical stage, especially under conditions of repetitive administrations.[77,250] As described for example by Kordelas et al. in 2014, EVs from 4 x10^7 allogenic MSCs (arbitrarily defined as 1 Unit) were gradually administered to a patient with graft-versus-host disease (GvDH) starting with the dosage of 0,1 unit per kilo of body weight to reach the dosage of 4 units during the course of the 15 days-therapy. The EV administration was well tolerated and no side effects were observed.[77]

However, the fact that they are naturally occurring vesicles does not preclude their toxicity, as evident for tumor-derived EVs.[251] Therefore, it is important to evaluate carefully which is the best source of EVs, depending on the application of interest.

In the Era of personalized medicine, to improve their therapeutic efficacy and safety, EVs can be isolated, modified and finally re-injected in the patient. For example, EVs purified from biological fluids or from MSCs are used on the same subject in an autologous way.[57,58] This possibility allows to meet specific patient needs, reducing the insurgence of immune responses.[252] As reviewed elsewhere, autologous EVs are currently studied in cancer care, as patient-compatible nanocarriers for targeted delivery of anti-cancer therapeutics.[253] However, regarding the use of autologous MSC-derived EVs, one of the main issues is the time needed for cell extraction and cultivation, as well as for EVs isolation and purification. Considering all the passages, this process takes about 1 month.[254] This time frame may be acceptable for certain therapies or scheduled surgeries but, of course, it is not possible for other approaches requiring prompt actions. For this purpose, many ongoing clinical trials are further evaluating the safety of allogenic EVs administration (NCT03384433, NCT04276987).[255]

On the other hand, liposomes have been used for several years in the clinical practice and are in general biocompatible, given their similar composition with respect to EVs. The researchers have optimized the production of AVs to implement the translation from bench to clinical production settings. In fact, the conventional multi-step liposomal preparations in bench including hydration of the liposomal thin film followed by size reduction such as extrusion is a lengthy and tedious process that significantly hampers its application to the industrial production. An interesting scale-up approach is based on the injection of lipid rich ethanol dispersion in water, followed by ultrasonication. This process permits to obtain liposomes at costs of about this $ 30/liter of 5 mM liposomal suspension. [256] A further evolution is constituted by scalable microfluidic platforms that involve a fine mixing control of an organic phase containing the phospholipids with an aqueous phase, resulting in the spontaneous

assembly of quasi monodisperse unilamellar liposomes with controllable sizes without the need for any post-processing steps to reduce size variance.[114]

Of note, uncharged liposomes can be hardly internalized by cells, resulting in a lower drug delivery efficiency. To improve this aspect, cationic liposomes have been employed, in order to favor the interaction with the negative potential of the cell membrane. However, this can result in higher toxicity following a systemic administration.[257] Importantly, liposomes can be easily recognized by the hosts immune system,[258] and rapidly cleared from the organism.[259] Polymersomes biocompatibility is currently matter of debate, given the possibility of having contamination from organic solvents and other impurities deriving from their synthesis, which can prolong their unwanted and potentially damaging accumulation in the system.[50] Their clearance is generally lower in comparison to liposomes, adding issues of unwanted accumulation in the body that still need to be fully addressed by researchers. Nevertheless, it is envisaged that these issues will be solved in the future, thanks to the physicochemical proprieties, such as pH, temperature and redox potential, also with a more controlled cargoes release, for a sustained systemic delivery.[131]

Cargo loading and delivery

Given their peculiar origin, EVs and AVs show remarkably different features also in terms of cargo loading and delivery. The EVs have the great advantage of loading and delivering different molecules from the cytosol of producing cells (such as enzymes, miRNAs etc.) permitting to achieve a combinatorial effect. On the other hand, while it is easier to deliver cargoes which are naturally synthetized by the donor cell, loading exogenous ones (synthetic molecules for instance) poses more challenges. In fact, the exogenous cargoes may be loaded into the EVs either before their isolation from the donor cells or afterwards, by direct loading into the EVs. The strategies to generate the so-called “modified-EVs” have been thoroughly reviewed by García-Manrique and colleagues in 2018.[260] To summarize, the pre-isolation

approaches use the EV-producer cellular machinery. They are divided into three classes, based on the method used: I) transfection of donor cells with plasmids encoding for proteins fused with vesicle membrane proteins; II) activation of the vesicle biogenesis pathway signaling by using nucleic acid sequences; III) overexpression of the gene of interest, or passive/active loading of the gene product within the donor cells. Instead, the post-isolation approaches are divided in two additional classes: IV) passive insertion of molecules into the external membrane of EVs or V) physical[261]/chemical[262] modification of the EVs to allow the cargo entrance in the vesicles.[260] However, the low permeable nature of the natural occurring phospholipidic bilayer of the EVs limits the applicability of the post-isolation approach.[263] In addition, although several experimental procedures have been conceived to load biomolecules within the EVs, such protocols may strongly reduce the release of the cargo from the EVs, thus reducing the effectiveness of the treatment.[264]

Differently, in the AVs the loading of the cargo can be carried out either during vesicles assembly or afterwards, through the use of the chemical functionalization procedures (see Section 3.2) that add a great flexibility for the nanomedicine purposes. Regarding the AVs loading, whereas in the pre-insertion method the biomolecules are inserted during AVs assembly, in post-insertion, the molecules are coupled to PEG micelles and then transferred from such micelles through the external layer of pre-formed AVs. This versatile method allows different ligands to be inserted into a variety of pre-formed liposomes. Loading biomolecules, including nucleic acids and proteins, into AVs is inherently less efficient in comparison to EVs, where this operation is orchestrated by the cellular machinery itself. The release of cargos from AVs can be finely engineered by introducing responsivity to external stimuli (pH, light, temperature), feature missing in EVs.

In particular, the synthetic nature of AVs enables to leverage temperature stimuli to favor the cargo release by heating them slightly above physiological temperature – a remarkable advantage with respect to EVs. To this aim, the formulation of phospholipids that can be

subjected to a gel-to-liquid phase transition at temperatures of about 40°C can be realized by the combination of Dipalmitoylphosphatidylcholine (DPPC), with other systems such as cholesterol, distearoyl phosphocholine, hydrogenated soy phosphocholine, and/or PEG-conjugated lipids.[195] Another possibility is constituted by the employ of lysolipids (a lipid derivative which has only one acyl chain) and also synthetic temperature-sensitive polymers. As firstly demonstrated in 1999 by Anyarambhatla and Needham,[265] lysolipids tend to form defects into PEGylated DPPC membranes, which result in efficacious drug release upon heating. Such lysolipid-based systems have also been developed as a commercial product to deliver doxorubicin into tumors at high efficacy (ThermoDOX®), being Phase III clinical trials to be completed in July 2020 (NCT02112656). Synthetic polymers have also been leveraged for engineering thermo-responsive systems. Indeed, it is possible to distinguish two classes of thermo-responsive polymeric moieties, considering their miscibility in a suitable solvent as a function of temperature, in particular the lower critical solution temperature (LCST) and the upper critical solution temperature (UCST). More specifically, LCST and UCST are the respective critical temperature values below and above which the polymer and solvent are totally miscible,[266] and can be described as entropically or enthalpically driven effects, respectively. Notably, most polymeric systems are characterized by LCST, such as poly(*N*-substituted acrylamides), poly(*N*-vinylalkylamides) and poly(*N*-vinylethers)[266]. These polymers have been demonstrated as suitable for improving the responsiveness of thermoresponsive liposomes. In fact, they can be anchored to lipid membranes by hydrophobic interactions. At the LCST, they tend to separate from the lipids, producing defects in the liposomes and finally favoring drug release. Polymer LCST can be tuned by copolymerization with a variety of monomers, the choice of which also affects the extent of drug release. In this scenario, it would be interesting for healthcare applications to use polymers with an UCST moieties, with the final aim to mimic thermoresponsive liposomes by using wholly synthetic systems. In this regard, the researchers are still at a very early stage, being of notable importance

to highlight the work from Wolf et al.,[267] who prepared poly(ethylene alkyl phosphonate) copolymers with PEG self-assembling into polymersomes exhibiting adjustable UCST phase separation in water in the temperature range between 43-71 °C.

Comparative studies

A number of research groups carried out comparison studies, highlighting differences between EVs and AVs. In 2016, Stremersch and colleagues directly compared the functionalities of AVs, in particular liposomes, *vs.* EVs. The authors demonstrated that even though EVs showed a good cell-penetrating potential, they possess a lower efficacy in terms of cargo release when compared with fusogenic anionic liposomes.[263] Two years later, as mentioned above, Fuhrmann and colleagues, compared the therapeutic potential of natural EVs *vs.* synthetic liposomal carriers, loaded with β-glucuronidase and encapsulated into PVA hydrogels, in a mouse macrophage cell line treated with bacterial LPS. They demonstrated that EVs, when administered to cells, display greater anti-inflammatory effects compared with the synthetic counterpart, even in the absence of the curcumin β-D-glucuronide substrate.[187] EVs are able to offer a more physiological environment for the delivery of biomolecules, especially for membrane proteins, which can be incorporated in the particle membrane and exposed with the proper orientation and post-translational modification for the interaction with the target cell. On the other hand, liposomes can enable virtually any kind of biomolecular structure delivery, that is not possible with other nanomaterials as metal colloids and with some extent by polymeric particles. Recently, in 2020, Lenzini and colleagues investigated the mechanisms by which EVs diffuse through the meshes of extracellular matrix (ECM).[6] They engineered hydrogels to mimic the natural ECM and evaluated the extent of EVs transport and release. The EV release was seen higher in stress relaxing hydrogels compared to nanoparticles and liposomes and was adjuvated by the presence of aquaporine-1 on the EV surface which allow their deformation thus facilitating their escape from the meshes of hydrogel and decellularized matrix.[6]

Hybrid vesicles

A promising compromise between EVs and liposomes was made by Piffoux and collaborators, in 2018.[268] They fused EVs with liposomes to allow a better cargo loading while preserving the natural vesicular membrane moieties, stability and thus the EV identity. The fusion protocol was optimized by fusing 1,2-dioleoyl-sn-glycero-3-phospho-Lserine-N-(7-nitro-2−1,3-benzoxadiazol-4-yl) (NBD-PS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE) lipids within the membrane of nonfluorescent EVs, resulting in the increased NBD fluorescence, due to the lack of fluorescence resonance energy transfer (FRET). The authors also characterized the effect of PEG-8000 concentration upon the fusion, obtaining 36% NDB fluorescence after 2h treatment with 30% (w/v) PEG-8000 (**Figure 7 A-B**). Subsequently, PEG was used to induce the fusion of EVs with chemically defined liposomes, in a process that produced larger particles, called hybrid EVs, which were able to efficiently load mTHPC, a fluorescent clinically approved antitumor photosensitizer. The authors found that the hybrid EVs had a significantly higher uptake in CT26 colon cancer cells with respect to Foslip (liposomal non-clinically approved form) and mTHPC alone, resulting in morphological changes and cytotoxic activity when internalized (Figure 7 C-D). Moreover, such PEGylated hybrid EVs demonstrated to be less internalized by THP1-derived macrophages compared with control EVs, thus allowing a prolonged bioavailability in the blood circulation and a sustained cargo release following hybrid EVs systemic injection.[268]

Novel and innovative hybrid vesicles have been developed in the last 5 years. In this context, a seminal paper has been published by Sato et al. in 2016. [269] The authors showed a direct membrane fusion strategy based on the freeze–thaw method to prepare hybrid vesicles starting from EVs and phospholipid liposomes. This approach resulted in a new kind of particles characterized by optimized properties, such as lower immunogenicity, higher colloidal stability, and improved circulation time in blood, respect to the unmodified EVs.[269] These optimizations

have been obtained by the surface modification of EVs through properly choosing the chemical composition of the liposomes to fuse with. In general, the fusion of EVs with PEGylated, fluoro-labeled, or chemically modified liposomes, leads to the introduction of the desired chemical moiety on the resulting hybrid vesicles. Certainly, the surface properties of delivery systems are key factors for their internalization in living cells, so tuning such interfacial features is a point of high relevance in advanced colloidal drug formulations. On the same line, Lu and colleagues in 2018, demonstrated that EV-mimicking liposomes gained a superior properties in terms of biocompatibility and systemic stability, as well as higher cellular uptake rate, when compared with standard synthetic liposomes.[258] Specifically, they compared Lipo-2000 and DOTAP liposomes with EV-mimicking liposomes, observing for the latter a strongly ameliorated stability, reduced cytotoxicity *in vitro* and *in vivo*, coupled with a longer shelf-life. Finally, a further interesting approach to obtain hybrid lipid nanocarriers consists in the combination of cell membrane fragments with exogenous phospholipids, to obtain hollow particles sometimes referred as *ghost vesicles*. Ghost vesicles are relevant systems due to their comparable physicochemical features with EVs, and a higher production yields respect to EVs[270]. A recent development in the field of ghost vesicles has been shown for the preparation of colloidal dexamethasone to contrast sepsis.[270] In terms of vesicle preparation, a protocol based on the treatment of cells with an alkaline solution to obtain membrane fragments has been illustrated. The following steps of sonication and ultracentrifugation led to a phospholipid colloid of articles in the 100-200 nm scale, purified from intracellular proteins and nucleic acids, in which dexamethasone can be loaded during the vesicle formation process.[270] A second recent example of ghost vesicles resulted from the studies of the group of M. Machluf, [271] that had already reported about the feasibility of ghost vesicle preparation from the cytoplasmic membrane of human mesenchymal stem cells.[272] In fact, they highlighted that the ghost vesicles retain the composition, orientation and function of the membrane of cells from which they have been obtained by disintegration. Then, such vesicles can target and infiltrate

malignant tissues, while rapidly clearing from other organs.[272] In their recent work, they showed the preparation of ghost vesicles from mesenchymal stem cells by an osmotic pressure based approach, and deeply investigated the effect of PEGylation on the particle morphology and targeting mechanism.[271] The ways of synthesis of the above-described EV-like carriers are broadly depicted in a recent review, and they can be divided into two main types, depending on the protocol used for their production: I) the donor cells are reduced in units and used as starting bricks to generate EV-like carriers *ex-novo*; and II) individual molecules (lipids, proteins, etc.) are assembled to generate complex carriers.[260]

5. Conclusion

Since the 1970s and in particular within the last 20 years, tremendous technological progresses have been made in nanomedicine in order to improve the methods for delivering drugs, to increase their efficacy and, at the same time, to decrease their associated toxicity and detrimental side effects. Colloidal drug carriers, as EVs and AVs, represent a promising tool to achieve such goals. In fact, enclosing a small synthetic molecule, or a biomolecule within an amphiphilic shell, represents the safest approach to preserve its activity. Accordingly, the carried payload is protected from the microenvironment (e.g. degrading enzymes, low pH etc.). Additionally, once administered, such lipidic carriers (when adequately functionalized) may reach selectively the target site and release their cargoes directly where they are meant to act, thus reducing the levels of potentially severe side effects. This is of pivotal importance for example in the case of anti-cancer drug formulations, such as the above-mentioned Doxorubicin encapsulated in liposomes (with longer circulation time and less extravasation safety issues, when compared to free Doxorubicin).

Natural occurring vesicles, such as EVs, have the advantage of being more compatible with cellular membranes, and they show a better systemic biostability. Moreover, being transporters of endogenous signal molecules, EVs represent a valid carrier not only usable as therapeutic,

but also as a diagnostic tool. As disadvantage, EVs are not easy to be synthetized in larger scale. Novel bioreactors and special genetically modified EVs-producing cell lines, with a higher vesicle secretion rate, are currently under study, in order to produce a natural carrier both low-cost and with high production yields. The artificial counterpart, AVs, especially the more characterized liposomes, are already the vesicle-vehicles of choice for improving the formulation of drugs, widely present on the market, and currently used in patients. In particular, AVs have the greater advantage to be easier to be functionalized with antibodies or other functional groups which can control the release of the transported cargoes both in a space- and in a time-dependent fashion.

In order to approve vesicle-delivered therapeutics, the route is even longer than for classical drugs. In general, it is more complicated to test molecules carried by vesicles in clinic, because such studies need to be more controlled (meaning high costs and higher number of enrolled patients). Nevertheless, in the era of personalized medicine, the use of EVs and AVs can impact positively on the healthcare system aiming to a safer delivery of both old and innovative drugs. The future goal of nanomedicine research will be to optimize a novel generation of vesicles potentially merging all the advantages of EVs and AVs, thus overcoming their respective issues.

Conflict of Interest

The authors declare no conflict of interests relevant to this work.

Acknowledgements

This work was supported by the “Brains2South” grant (Fondazione con il Sud - Bando Capitale Umano ad Alta Qualificazione 2015). L. Leggio and G. Arrabito contributed equally to this work.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

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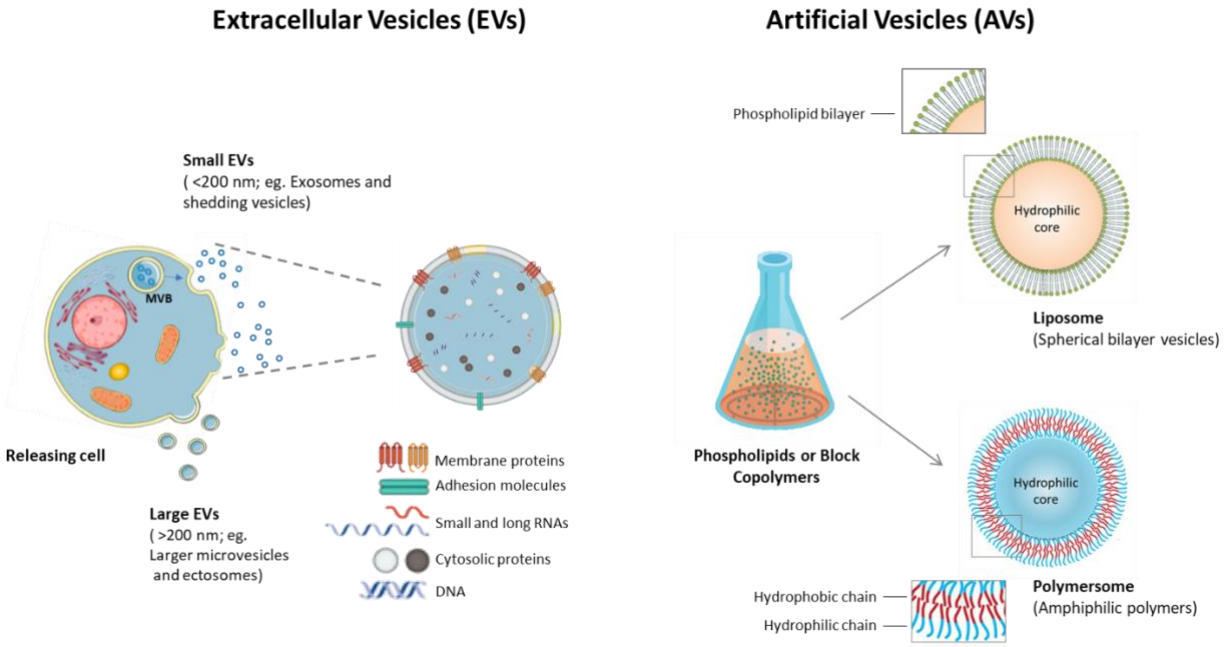

Figure 1. Schematic representation of extracellular vesicles released by a donor cell *vs.* the synthetic production of chemically defined liposomes or polymersomes.

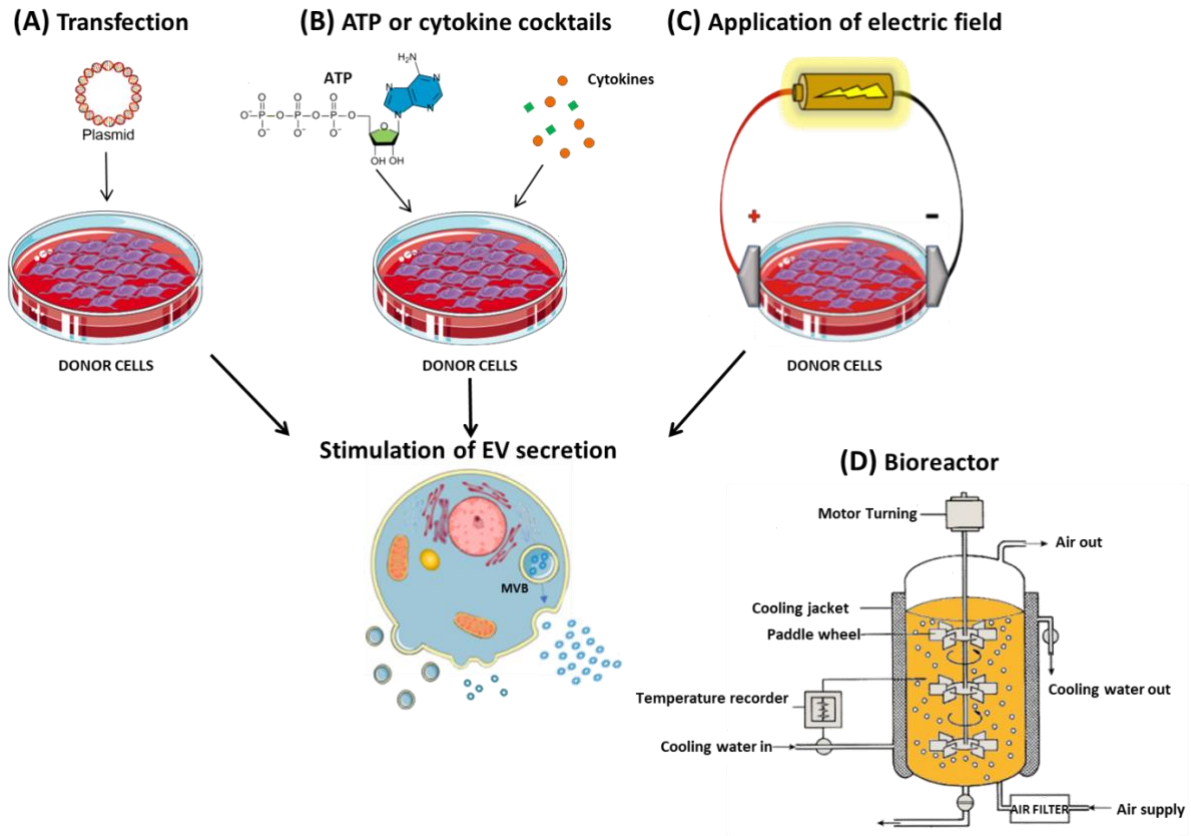

Figure 2. Strategies to increase the production of EVs. **A)** transfection of cells to induce the expression of genes involved in EV production and release; **B)** Stimulation of EV secretion by treating cells with ATP or cytokine cocktails; **C)** EV release stimulated by application of low

level of electricity on donor cells; **D)** High amounts of EVs can be obtained by increasing the number of donor cells that are cultivated into bioreactors.

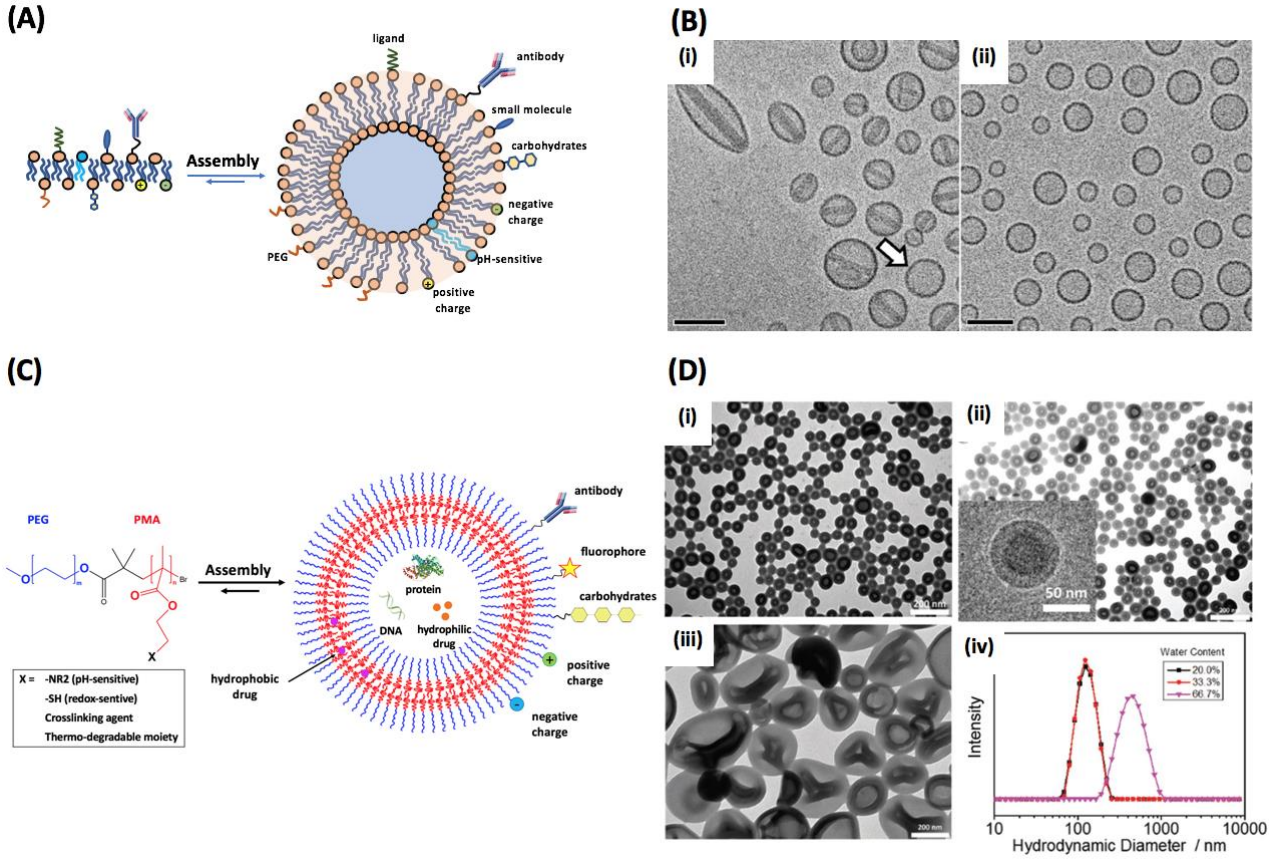

Figure 3. Artificial vesicle assembly and characterization. A) Schematic of liposome self-assembly from lipids. The composition of lipids can be engineered (charge, pH-sensitive) and/or pre-functionalized with different molecules (antibodies, proteins, carbohydrates, PEG and other ones) to confer new properties (steric stabilization, specific targeting, etc). **B)** Cryo-TEM images of (i) single liposomes containing doxorubicin (concentration of 2 mg/mL) and (ii) empty liposomes in a buffer containing 10% sucrose, 10 mM histidine, pH 6.5. The arrow shows a doxorubicin-free liposome. Scale bar = 100 nm. Reproduced with permission.[119] Copyright 2018, Elsevier. **C**) Schematic representation of polymersome self-assembly from a representative PEG-b-PMA copolymer. The polymersomes can be modified both at their surface (biomolecules, fluorophores and charged groups by physi-/chemisorption), at the inner layer of the membrane (cross-linking, pH- and redox-sensitive moieties, indicated as X), and in terms of loading in the aqueous core and in the hydrophobic part of the multilayer. **D**) TEM images (i-iii) and corresponding DLS characterizations (iv) of PEG_{45}-*b*-PS_{206} polymersomes prepared at different water amounts (expressed in % v/v), (i) 20.0%, (ii) 33.3% and (iii) 66.7%. The inset in panel (ii) shows a single polymersome imaged by cryo-TEM. Scale bars are reported in the figure. Reproduced with permission.[273] Copyright 2016, Royal Society of Chemistry.

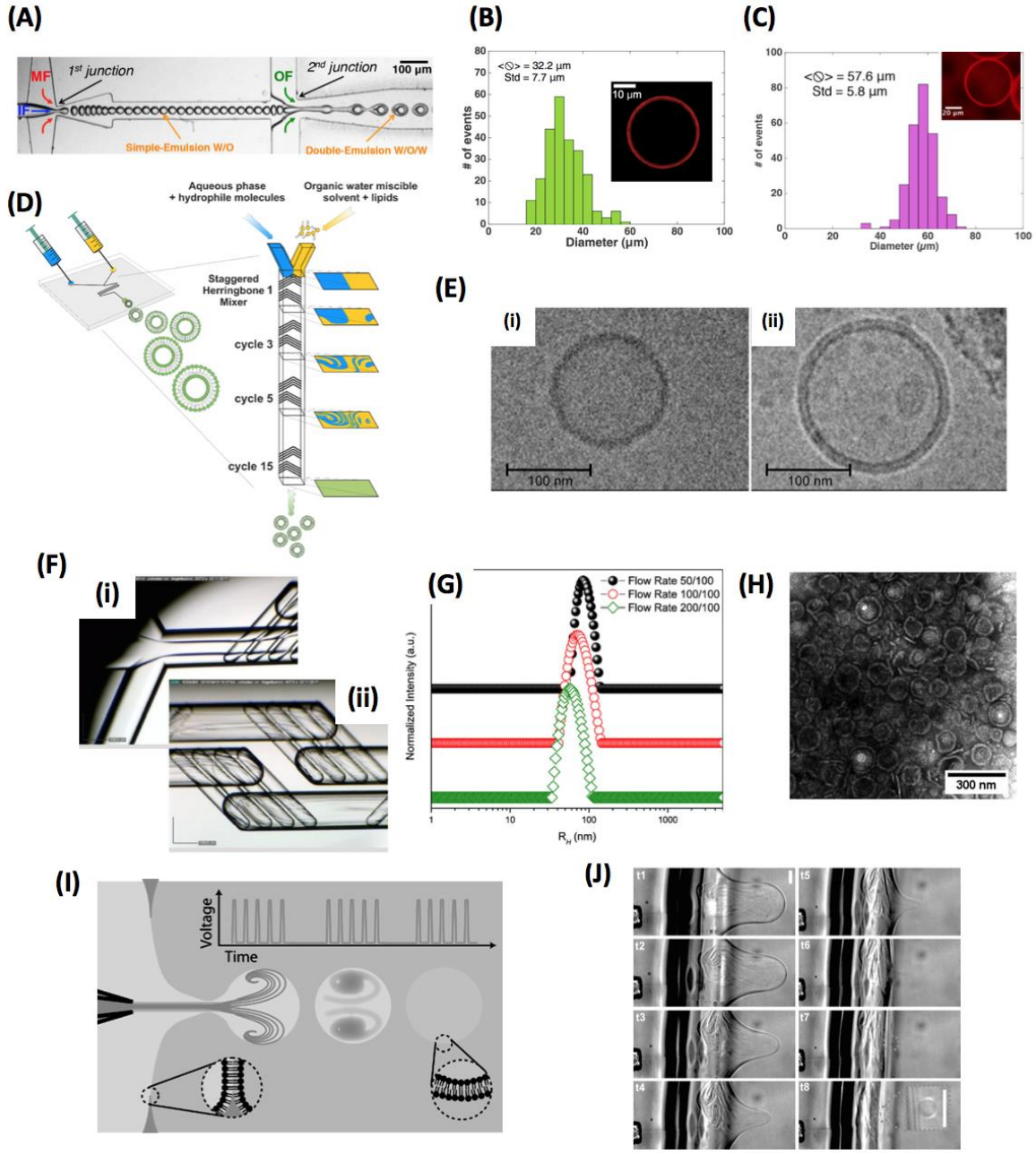

Figure 4. Microfluidics and printing approaches for AVs production. A) Scheme of the W/O/W emulsions for AV production by microfluidics. The inner aqueous phase (IF) is sheared by an oil phase (MF), producing a single-emulsion, which is turn sheared at the second junction by an aqueous outer phase (OF) leading to double-emulsion. **B)** Liposomes diameter distribution (mean value of 32.2 ± 7.7 µm); **C)** Polymersomes diameter distribution (mean value of 57.6 ± 5.8 µm); Reproduced under the terms of the Creative Commons 4.0 license.[154] Copyright 2016, the authors. **D)** Microfluidic mixing (NanoAssemblr® microfluidic platform) of an organic water-miscible solvent phase containing lipids with an aqueous phase containing molecules to encapsulate. **E)** Corresponding cryo-TEM characterization of the (i) as-formed liposomes (ii) liposomes containing 5% of Gd-lipid. Reproduced under the terms of the Creative Commons 4.0 license.[151] Copyright 2020, the authors. **F)** Microfluidic chip for producing PHPMA35-b-PDPA75 polymersomes. The micromixer input orifice is depicted where mixing occurs in (i) focused stream and in (ii) mixing chambers. **G)** Radius distributions for PHPMA35-b-PDPA75 polymersomes at different flow rates (aqueous phase/organic phase) in µL min–1. **H)** TEM images of the polymersomes produced at flow rate 200/100. Reproduced with permission.[155] Copyright 2019, American Chemical Society. **I)** Scheme for the inkjet

printing production of individual liposome vesicles. **J)** Time lapses showing the formation of liposome vesicles. Reproduced with permission.[161] Copyright 2009, The Royal Society of Chemistry.

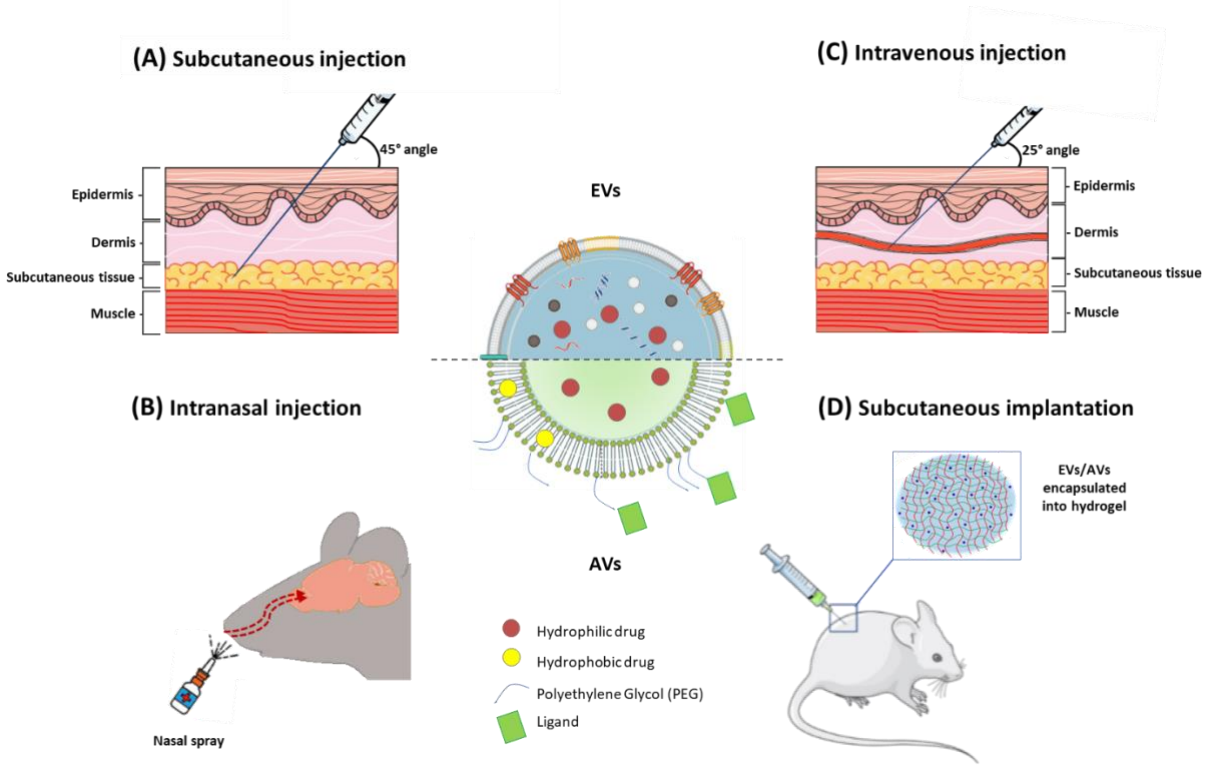

Figure 5. Different routes of administration for EV/AV. A) Subcutaneous injection used in the first clinical trials with dendritic cells derived EVs; **B)** Intranasal injection of vesicles for brain targeting bypassing the BBB (used at the moment only in animal models); **C)** Systemic injection of EVs/AVs, used both in pre-clinical models and some clinical trials, for reaching several body district; **D)** Subcutaneous implantation of hydrogel encapsulated EVs/AVs.

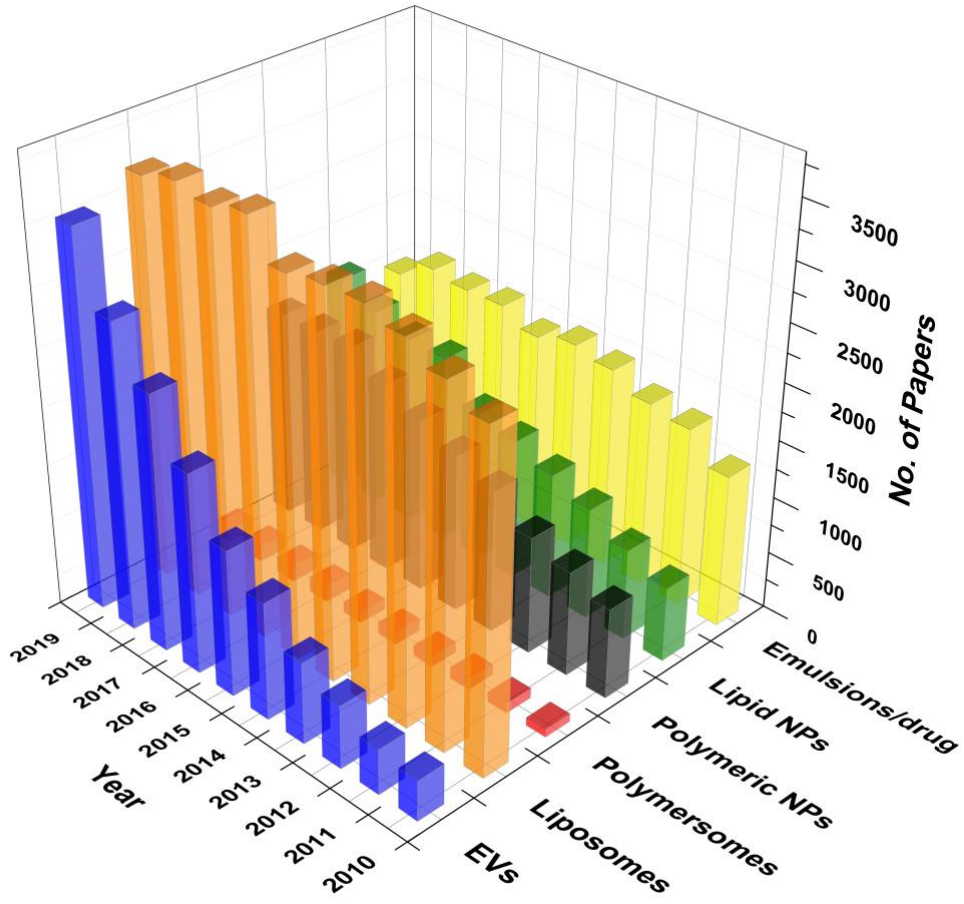

Figure 6. Number of papers published from 2010 to 2019 reporting on the utilization of different classes of EVs and AVs for medical applications (source: Scopus). Concerning the EVs, the data represent the sum of the number of papers containing the different terms used in the literature to indicate this class of vesicles (i.e. extracellular vesicles, exosomes, microvesicles, shedding vesicles and oncosomes), in order to take into account the EV nomenclature evolution. Redundancy in the counting was avoided by the use of the "AND NOT" Scopus function.

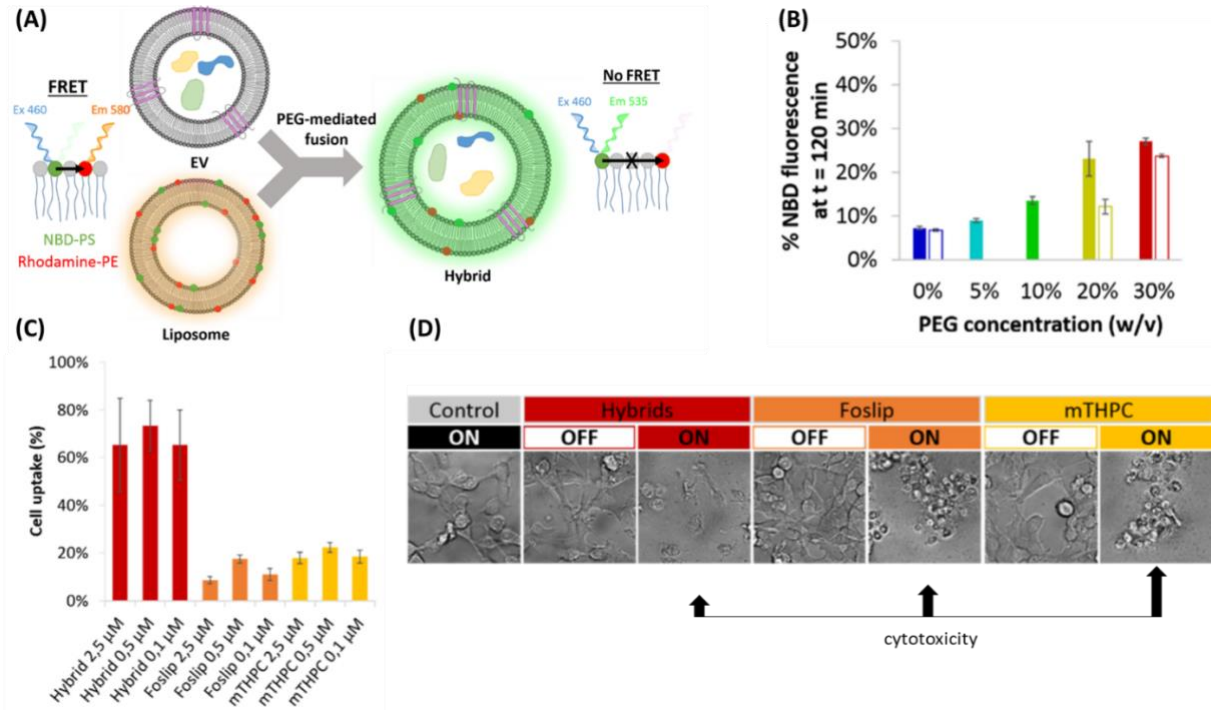

Figure 7. Towards better nanomedicine: EVs/AVs hybrids. A) PEG8000-mediated fusion between HUVEC-derived EVs and fluorescent liposomes produced by extrusion. **B)** PEG concentration dependent fusion after 2 hours of incubation of fluoro-labelled liposomes (empty bars indicate PEGylated liposomes) with MSC-derived EVs and liposomes. **(C)** Hybrid EVs/AVs loaded with mTHPC, a fluorescent antitumor photosensitizer (clinically approved), leads to a higher uptake in CT26 colon cancer cells compared with Foslip (liposomal non-clinically approved form) and mTHPC alone. **(D)** Bright-field imaging of CT26 cells, before (OFF) and after (ON) laser irradiation (650 nm, 10 J/cm2), shows increasing cytotoxicity following treatment with encapsulated or free mTHPC: Hybrids<Foslip<mTHPC. Reproduced with permission.[268] Copyright 2018, American Chemical Society.

Table 1. Hydrogel-based approaches for subcutaneous EV and for AV delivery. Abbreviations: Chol: cholesterol; DMPG: 1,2-dimyristol-sn-glycero-3-phosphoglycerol; DOTAP: Dioleoyl-3-trimethylammonium propane; DPPC: Dipalmitoyl phosphatidylcholine; DSPC: 1,2-distearoyl-sn-glycero-3-phosphatidylcholine; EPC: egg phosphatidylcholine; EPL: Poly-ε-L-lysine; GelMA: gelatin methacrylate; OHA: oxidative hyaluronic acid; PC: Egg phosphatidylcholine; PEG-DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[methoxy-poly- (ethyleneglycol)-2000]; PG: Egg yolk phosphatidylglycerol; PVA: Poly-(vinyl alcohol); PI-b-PEG: poly(isoprene)-block-poly(ethylene glycol); PMOXA-*b*-PDMS-*b*-PMOXA (2-methyl oxazoline-block-polydimethylsiloxane-block poly(2-methyl oxazoline); PNAM-*b*-PChA-*b*-PNAM: poly(N-acryloyl morpholine)-block-poly(cholesteryl acrylate)-block-poly(N-acryloyl morpholine; PS-*b*-PIAT: polystyrene–block-poly(isocyanoalanine-(2-thiophene-3-yl-ethyl amide)); PVA: Poly-(vinyl alcohol).

Source of EVs	Hydrogel composition	Experimental model	Functional outcome	*Ref.*
HEK-293T cells engineered to secrete more EVs and overexpressing the therapeutic catalase Mrna	Matrigel	6-OHDA PD mouse model	Attenuation of neurotoxicity and neuroinflammation	[80]
Human placenta-derived MSCs	Thermosensitive Chitosan Hydrogel	Mouse hindlimb ischemia model	Functional recovery of ischemic tissues	[179]
Synovial MSCs highly expressing miR126-3p	Hydroxyapatite-Chitosan composite hydrogel; Chitosan	Diabete rat model	Increase of blood vessel neoformation and promotion of re-epithelialization	[180,181]
Human gingival MSCs	Chitosan-silk Hydrogel Sponge	Diabete rat model	Extensive deposition of collagen fibers orderly arranged in the wound bed; increase in micro-vessel and nerve density	[182]
Stimuli-responsive adipose-derived MSCs	FHE (Pluronic F127 (F127), oxidative Hyaluronic acid (OHA) and Poly-ε-L-lysine (EPL)) hydrogel	Chronic diabetic wound rat model	Proliferation, migration and angiogenesis of HUVEC cells *in vitro*; neovascularization and cellular proliferation at the level of the wound site *in vivo*	[183]
MSCs derived from human induced pluripotent stem cells (iPSCs)	Photoinduced imine crosslinking (PIC) hydrogel glue (EHG)	Full-thickness articular cartilage defect in rabbit model	Repair and regeneration of articular cartilage	[184]
MSCs	3D printed cartilage extracellular matrix (ECM)/gelatin methacrylate (GelMA)/vesicle scaffold	Osteochondral defective rabbit model	Recovery of condrocyte mitochondrial dysfunction and repair of osteochondral defect repair	[185]
Human MSC-derived EVs loaded with β-glucuronidase	PVA hydrogel stabilized by PEG	Cell model of inflammation: mouse macrophage cells (RAW 264.7) challenged with bacterial lipopolysaccharide (LPS)	Sustained local conversion of the curcumin β-D-glucuronide precursor in free curcumin, with an acute anti-inflammatory effect. The anti-inflammatory effect was higher with EVs compared with liposomes	[186]
Formulation of AVs	**Hydrogel composition**	**Experimental model**	**Functional outcome**	***Ref.***
Plurilamellar vesicles containing egg or DPPC	collagen matrix	untreated diabetic rat	peptide hormones, insulin and growth hormone release	[204]
Large unilamellar liposomes of DPPC	alginate hydrogel	in vitro cytochrome-c release	functional recovery of ischemic tissues	[205]
Large unilamellar liposomes containing EPC, Chol, DMPG and DSPC	chitosan–b-glycerophosphate	/	optimized in-vitro carboxyfluorescein delivery (> 2 weeks)	[207]
Unilamellar liposomes containing PC, PG, Chol, PEG-DSPE	OHA	lipopolysaccharide (LPS)-induced model of anterior uveitis (EIU model)	sustained delivery of vasoactive intestinal peptide (VIP) up to eight days after intravitreal injection	[209]

Cationic liposomes consisting of egg PC and DOTAP	polyacrylamide gels	Staphylococcus aureus bacteria as a model mouse skin infection	Release of carboxyl-modified gold nanoparticles onto mouse skin	[211]
Adhesive cationic liposomes containing lecithin, cholesterol and octadecylamine	Ag^{+} loaded thiolated polyethylene hydrogels	liposomes (A-LIP) loaded with Bone morphogenetic protein 2	local bone remodeling of osteoporotic fractures in rats	[212]
Polymersomes of the triblock copolymer PNAM-*b*-PChA- *b* -PNAM	PVA hydrogel matrix	C2C12 mouse myoblast cell line	release of the small cytotoxic depsipeptide drug thiocoraline	[274]
Polymersomes of the triblock copolymer PMOXA-*b*-PDMS-*b* -PMOXA	PVA hydrogel matrix	blood plasma	calcein release as model fluorescent dye sensitive to encapsulation	[275]
Polymersomes of the diblock copolymer PI- *b*-PEG	hydroxyethyl cellulose hydrogel	in-human olfactory studies	sequestration of trimethylamine (TMA) via polymersome transmembrane pH gradient for trimethylaminuria treatment	[276]
Polymersomes of the diblock copolymer PS-*b*-PIAT	OHA based matrix	proof-of-principle of an enzyme-loaded polymersome continuous-flow batch reactor	encapsulated enzymes Candida antarctica lipase B (CALB) and glucose oxidase (GOx)	[277]

Table 2. Critical comparison between EVs and AVs (liposomes and polymersomes). EVs and AVs are compared as a function of three major targets of interest for nanomedicinal applications: production, biocompatibility and efficacy as DDS. For each target, the respective level of accomplishment is classified as (+) acceptable, (++) good, (+++) optimal, and is motivated in detail by the reported descriptions.

	Production		*Ref.*	Biocompatibility		*Ref.*	Efficacy as DDS		*Ref.*
Extracellular Vesicles	**+**	Difficult massive production and purification; Biomolecules as only building blocks; Limited tunability of the physicochemical properties	[87] [241]	**+++**	Generally safe; Extraction from biological sources with high biocompatibility	[80] [83]	**++**	Excellent loading of biomolecules; Challenging loading of synthetic molecules; Good delivery efficiency; Rapid clearance	[187] [188]
Liposomes	**++**	Easy production and purification; Pre and post-synthetic functionalization; Tunable physicochemical properties	[124] [161]	**+++**	Generally safe, unless modified with charged phospholipids; Similar composition to EVs, but without cell-mediated modifications	[103] [214]	**++**	Good natural and synthetic molecules loading; Pre-assembly and post-assembly loading; Good delivery efficiency; Tunable clearance properties	[199] [200]
Polymersomes	**+++**	Customizable massive production; Pre and post-synthetic functionalization; Tunable physicochemical properties	[136] [155]	**+**	Possible accumulations in the body; Possibility to introduce biodegradable polymers	[230] [231]	**+**	Pre-assembly and post-assembly loading; Cell uptake dependent on the vesicle and cell membrane characteristic; Tunable clearance properties	[223] [225]

Loredana Leggio (LL) received her M.Sc degree in Cellular and Molecular Biology in 2013 and her Ph.D. in Geological, Biological and Environmental Sciences in 2017, both from the University of Catania, Italy. She is postdoctoral fellow in the group of Dr. Iraci at the Department of Biomedical and Biotechnological Sciences (BIOMETEC, University of Catania). Her current research focuses on the identification of new mechanisms of cell-to-cell communication mediated by extracellular vesicles (EVs). **LL** is responsible for the analysis of EV content – nucleic acids and proteins – and the evaluation of their protective roles in the context of neurodegeneration.

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Lipid vesicles are emerging as fundamental tools for the development of novel theragnostic approaches in nanomedicine. This review describes the properties of these natural (i.e. extracellular vesicles) *vs.* artificial (e.g. liposomes, polymersomes) "delivery systems". The most recent progresses for the preparation and the application of both systems, together with the perspectives for the realization of highly-performing hybrid vesicles are provided.

Keyword: Vesicles

L. Leggio, G. Arrabito, V. Ferrara, S. Vivarelli, G. Paternò, B. Marchetti, B. Pignataro,* and N. Iraci*

Title: Mastering the Tools: Natural vs. Artificial Vesicles in Nanomedicine

ToC figure