Engineered Extracellular Vesicles

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Contributor: Simona Villata

Engineered Extracellular Vesicles are devices obtained through the surface modification of natural extracellular vesicles, both using direct and indirect methods, i.e. engineering of the parental cells.

The aim of their production is to obtain extracellular vesicles that are more reliable in terms of reproducibility and that present some desired features, that can change depending on the application.

Keywords: extracellular vesicles ; cancer therapy ; drug delivery ; biomedical engineering ; personalized medicine ; direct method ; indirect method

Definition of EVs

Every day, in the human body, cells release in the extracellular space particles delimited by a lipid bilayer that cannot replicate. Such particles are defined as extracellular vesicles (EVs)[1] . This general term encompasses a huge number of structures, referred as exosomes, microvesicles, microparticles, ectosomes, oncosomes, apoptotic bodies, and many other names [1], which differ in biogenesis, release pathways, size, content, and function.

1. Introduction

The nomenclature of these vesicles evolved during the last two decades [2]. The widespread and oldest classification divides the EVs on the base of their biogenetic pathway and, even simplistically, identifies three main classes: the exosomes, the microvesicles, and the apoptotic bodies (Figure 1). The exosomes consist of vesicles with an endocytic origin, ranging in size from around 50 to 150 nm. They originate as intraluminal vesicles (ILVs) of the multivesicular bodies (MVBs) and become exosomes when secreted in the extracellular milieu. The microvesicles originate from the direct outwards budding and fission of the plasma membrane and range in size from 50 nm to 1 µm, and in some case they can reach higher dimensions of up to 10 µm (this is the case with the large vesicles released by cancer cells, named oncosomes). Lastly the apoptotic bodies are vesicles resulting from the disassembly of the apoptotic cells, which are generally defined as 500 nm-5 µm in diameter [3].

In recent years, the International Society for Extracellular Vesicles proposed a new classification based on the size range[1]. In fact, as reported by Thery et al.[1], it is extraordinary difficult to assign an EV to a particular biogenesis pathway due to the lack of specific markers; therefore, a classification on a physical characteristic, such as the size, results as being most appropriate. In the most recent publications, EVs are divided into two main classes, defined as small EVs (< 100 nm or < 200 nm) and medium/large EVs (> 200 nm).

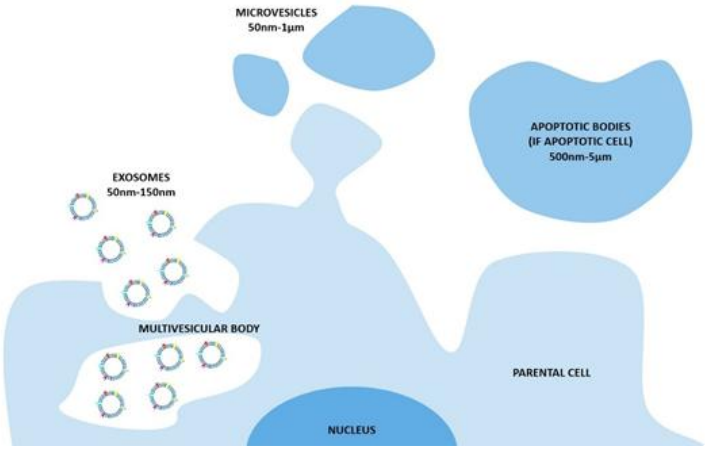

Figure 1. The biogenesis of extracellular vesicles (EVs) and the different pathways according to the current classification. In particular, exosomes consist of vesicles with an endocytic origin, ranging in size from around 50 to 150 nm. They originate as intraluminal vesicles (ILVs) of the multivesicular bodies (MVBs) and become exosomes when secreted in the extracellular milieu. The microvesicles originate from the direct outwards budding and fission of the plasma membrane and range in size from 50 nm to 1 µm. The apoptotic bodies are vesicles resulting from the disassembly of the apoptotic cells, which are generally defined as 500 nm-5 µm in diameter.

After their isolation, EVs can be modified in order to obtain enhanced targeting and biomimetic features[4]. This concept is called engineering of EVs because, starting from naturally-derived EVs, scientists produce a vesicle with the desired behaviour[5]. It is important to highlight that an extracellular vesicle can be modified through both acting on the parental cells (indirect method) and by directly modifying the vesicle once it has been isolated (direct method)[4]. Another important branch of EV engineering is their hybridization after their isolation, where EV membranes are fused with synthetic liposomes[6].

2. Indirect Methods

This method is based on the engineering of parental cells, i.e., the cells that will produce the EVs[6]. First, parental cells can be genetically or metabolically modified to alter the surface expression of the produced EVs and thus enhance their targeting ability and biocompatibility[4]. This can be carried out by inserting the coding sequence of the ligand of interest inframe to the coding sequences between the signal peptide and N-terminus of the mature peptide of a transmembrane protein[5]. Using a retrovirus or a lentivirus as gene transfer vector, this package is transmitted and expressed in parental cells[6]. At this point, these transfected parental cells will produce EVs with the desired peptide expressed on their surface. In Table 1 and Figure 2, some applications of this indirect method are reported [4][6].

Table 1. Applications of membrane functionalization through indirect methods**.**

Parental Cells	Functionalization	Cell Engineering Conditions	Recipient Cells	Treatment Conditions	Application	Reference
HEK293	Tetraspanins (CD63, CD9, CD81)	Transfected at 40~60% confluency using plasmid DNA (1–2 µg/well) for 48h with PureFection Transfection Reagent or FuGENE6 t.r.	HEK293	Cells at confluency of 80% and 50 µg of exosomes	Tracking, imaging and targeting drug delivery	[7]
GM-CSF	Lamp-2b fused to the neuron-specific RVG peptide	Transfected 4 days using 5 µg of pLamp2b and 5 µl of TransIT LT1 t.r.	C2C12 and Neuro2A *IN VIVO*: C57BL/6 mice	Exosomes (12 µg proteins) and 400 nanomoles of siRNA *IN VIVO:* i.v. 150 µg of exosomes	Delivering of siRNA to the brain in mice with a reduced immunogenicity	[8]
Immaturedendritic cells (imDCs)	Lamp2b fused to CRGDKGPDC	Transfected with the vector expressing iRGD-Lamp2b fusion proteins using Lipofectamine 2000 t.r.	MDA-MB-231 *IN VIVO*: BALB/c nude mice	2 mM Dox-loaded exosomes *IN VIVO*: i.v. EVs 3mg/kg Dox loaded exosomes	Targeted tumour therapy	[9]

Neuro2A	GPI	Transfected with pLNCX-DAF-R2 or pLNCX-DAF-EGa1 using TransIT 2020 t.r.	Neuro2A, HeLa, and A431	40,000 cells per well or cells at a confluency of 80–90% and EVs at 5 µg/mL	Promoting tumor cell targeting	[10]
HEK293	GE11 or EGF	Transfected with pDisplay encoding GE11 or EGF using FuGENE HD t.r.	HCC70 HCC1954 MCF-7 *IN VIVO:* RAG2$^{-/-}$ mice	1×10^5 breast cancer cells and 1 µg of exosomes *IN VIVO*: i.v. 1 µg of exosomes, once per week for 4 weeks	Delivering of antitumor microRNA to EGFR-expressing breast cancer cells	[11]
BT474, SKBR3, HER2+, JAWSII DCs, 4T1-HER2, and bmDCs	CEA and HER2 coupled to the C1C2 domain of lactadherin	Transfected with p6mLC1C2 containing either human CEA (nt 1-2025) or human HER2/neu (nt 1-1953)	*IN VIVO*: C57BL/6J and BALB/c mice, hCEA or HER2 transgenic mice	*IN VIVO*: 2.6×10^{10} or 5.2×10^9 or 1.05×10^9 viral particles	Increasing vaccine potency	[12]
HEK293-F, E6, and CT26	PSA and PAP coupled to the C1C2 domain of lactadherin	Transfected with pPSA/Zeo, pPSA-C1C2/Zeo, pPAP/Hygro, or pPAP-C1C2/Hygro using Lipofectamine LTX reagent and PLUS Reagent	*IN VIVO*: Male BALB/c or C57BL/6 mice	*IN VIVO*: 5E7 TCID50 of the MVA-BN-PRO viral vectors once every 2 weeks for a total of three treatments	Targeting of tumor antigens to improve antigen immunogenicity and therapeutic efficacy	[13]

DCs	C1C2 domain of lactadherin	Transfected with modified p6mLC1C2 or pcDNA6-Myc/His using Fugene 6 t.r.	*IN VIVO*: Balb/C mice	*IN VIVO*: six inoculums of YAC exosomes with HLA-A2 or five inoculums of YAC/HLA-A2 exosomes with pMAGE-A3	Usage of antibodies against tumor biomarkers to attach the drug target candidates	[14]
THP-1	RGD- DSPE-PEG and/or DSPE-PEG-SH	Incubated with DSPE-PEG-SH and/or DSPE-PEG-RGD for 2 days	MCF-7 and HeLa *IN VIVO*: tumor-bearing mouse	4×10^5 cells/mL and 100 µL per well of 50 µg/mL exosomes *IN VIVO*: i.v. 200 µL of exosomes at 5 mg/mL	Active targeted chemo-photothermal synergistic tumor therapy	[15]
THP-1	DSPE-PEG-biotin and/or DSPE-PEG-FA	Incubated with DSPE-PEG-biotin and/or DSPE-PEG-folate for 2 days	HeLa *IN VIVO*: C57BL/6 mice	40 µg/mL of EVs *IN VIVO:* i.v. EVs with a total of 1.16 mg iron	Rapid isolation and enhanced tumor targeting	[16]
Cal 27 cells	DSPE-PEG-biotin and DSPE-PEG-folate	Incubated with DSPE-PEG-biotin and DSPE-PEG-folate	MDA-MB-231 *IN VIVO*: BALB/C mice	Series of dose and concentration *IN VIVO*: 18–22 g of EVs via the tail vein	Enhanced target and synergistic therapy for breast cancer	[17]
HUVECs	DSPE-PEG-biotin (to then attach SA-QDs)	Cultured with DSPE-PEG-biotin for several days and then incubated with SA-QDs	EPCs *IN VIVO*: nude mice bearing A2058 xenografts	Short-term incubation *IN VIVO:* injection	Antitumor siRNA delivery	[18]

HUVECs	DSPE-PEG-biotin and SA-FITC	Incubated in modified medium containing 40 μg/mL DSPE-PEG-biotin for several days	HepG2 and 3T3 fibroblast *IN VIVO*: cervical cancer-bearing male BALB/c mice	5×10^3 cells per well and 0, 10, 40, 80, 100, and 200 mg/mL of exosomes *IN VIVO:* exosomes at 5 mg/mL, 200μL per mice	Active targeted drug delivery to tumor cells	[19]
HEK 293T cells	GlucB with sshBirA to conjugate streptavidin–Alexa 680	Transduced with lentivirus vectors, CSCW-Gluc-IRES-GFP or CSCW-GlucB-IRES-GFP, then infection with CSCW-sshBirA-IRES-mCherry lentiviruses	*IN VIVO*: athymic nude mice spiked with EV-GlucB	*IN VIVO*: injected with a bolus of 100 μg EV-GlucB via retro-orbital vein or via tail vein	Multimodal imaging in vivo, as well as monitoring of EV levels in the organs and biofluids	[20]
B16BL6	Streptavidin–lactadherin and biotinylated GALA	4×10^6 cells per dish transfected with the plasmid vector pCMVSAV−LA	MHC class I molecules of DCs	5×10^4 cells per well and exosomes (1 μg of protein) diluted in 0.1 mL of Opti-MEM	Efficient cytosolic delivery of exosomal tumor antigens	[21]

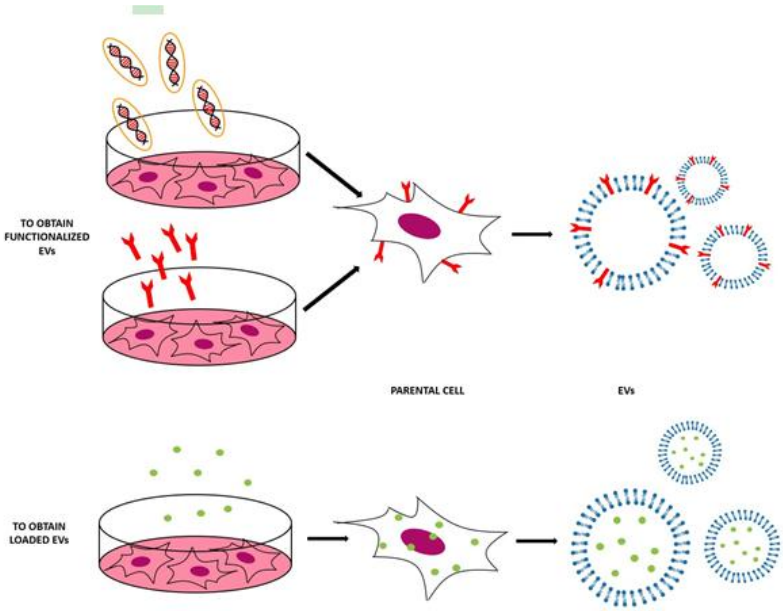

Figure 2. Scheme of the indirect methods used to engineer the EVs, both to functionalize EVs with the molecules of interest (to obtain EVs that expose these molecules on their surface) and to obtain EVs loaded with the desired cargo.

Secondly, parental cells can be incubated with drugs or drug-loaded (or even gene-loaded) nanoparticles (NPs) in a sublethal concentration [4]—after a certain period of time, the therapeutic molecules or NPs will be internalized into the cells and then these cells will produce EVs containing a certain fraction of drug or drug-loaded NPs [4]. In this case, the loading of the cargo is obtained through the engineering of the parental cells[5]. For example, mesenchymal stromal cells (MSCs) can acquire strong anti-tumor activity after priming with paclitaxel (PTX) because MSCs secrete a high amount of membrane microvesicles that will contain the drug[22]. Another study reported how melanoma cells can be loaded with survivin T34A and gemcitabine to produce exosomes that carry the drug to treat pancreatic adenocarcinoma[23]. Doxorubicin and methotrexate have been loaded into tumoral cells and their apoptotic bodies containing the drug have been used to kill tumor cells, with reduced side effects[24]. Cells have been loaded with NPs also— superparamagnetic iron oxide nanoparticles (SPIONs) have been loaded in mesenchymal stem cells to produce charged EVs to treat leukemia[23], while iron oxide NPs and a photosensitizer have been encapsulated in HUVECs and human macrophages to obtain EVs to treat prostate and cervical cancer, respectively [25][26]. Gene therapy can also be carried out with this approach—for example, mesenchymal stem cells have been loaded with different miRNAs to obtain EVs[27]. The purpose of these EVs were varied, i.e., to increase sensitivity of tumor cells to chemotherapeutic drugs (miRNA-122[28]), to inhibit the migration of osteosarcoma cells with miRNA-143[29], and finally to inhibit glioma growth with miRNA-146b[30]. Moreover, chemically modified exogenous mRNA can be loaded in this way into EVs to produce a protein of interest[31].

It is important to focus not only on the technical challenges of producing engineered EVs with indirect methods, but also on the various biological issues that are concerned before, during, and after EV engineering. As a preliminary step before the engineering process, it is important to design the engineered EVs and to make the right choice in terms of parental cells. Many authors decided to use cell lines such as endothelial cell lines (HUVECs) [18][19] or dendritic cells (DCs) [9][12][14], while others worked with more tissue-specific cell lines. From the literature, it is evident that the main challenge in the choice of the parental cells is to become able to work with a patient's derived cells in a controllable way and with introducing scalable protocols. For example, one of the critical issues is to obtain EVs with characteristics compatible to the cells with which they will interact. During the engineering, it is important to choose the proper surface modification to achieve the purpose and also to pay attention to the possible unwanted effects. Another challenge is to identify the most efficient way to obtain the functionalization. One of the most popular choices is to transfect the parental cells with the right plasmid vectors and their building is nowadays an important investigation subject in the biological field[14][8][9][10][11][12][13][14][21]. The other popular approach is to incubate the cells with DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethyleneglycol) to both link and further space the membrane from the targeting molecules. Such functional lipids can be actually bound to targeting ligands such as biotin, folate, thiol groups, or arginylglycylaspartic acid (RGD). Biotin can in turn selectively bind to streptavidin, being used for further functionalization [17][18][19]. Folate is able to target specific cancer cells [16][17], while thiol groups are useful in many binding reactions [32]. RGD is one of the most common sequences of cellular attachment at the extracellular matrix[32].

After the functionalization, the main biological challenge is to choose the most appropriate cell line or animal model to test the engineered EVs. One of the most popular choices is to use immortalized cell lines, for example HeLa [10][32][16], 3T3[17] and Neuro2A [33][8], due to their advantages in terms of cost, ease of use, and ethical concerns. Indeed, even if not specific like the primary cell lines, they allow for the ability to overcome the main biological challenges of EV testing, such as it being time-consuming and having scalability issues, thus allowing movement from in vitro to in vivo testing easily.

Most of the authors that tested their formulation in vivo chose transgenic [11][12] or non-transgenic mice that bear [15][18][19] or do not bear [8][9][13][14][16][17] autologous tumor or xenografts and that could be athymic [20] or not. Unfortunately, these animal models are not complex enough to simulate the human system, and thus more investigation efforts must be pursued to develop more appropriate testing platforms.

3. Direct Methods

Several methods are used to modify the surface of EVs after their isolation. These modifications can be carried out to achieve more specific targeting or mimetic features [6]. Most frequently, the aim is to obtain fluorescent and magnetic labelling to track EVs, their biodistribution, and their pharmacokinetics to investigate their possible diagnostic and therapeutic applications[4]. As EVs are very delicate, it is necessary to pay attention to the reaction conditions to avoid their disruption and aggregation due to inappropriate temperature, pressure, and osmotic stresses[5]. Working in mild conditions can help to obtain the most controlled results[4]. After their isolation, EVs' surfaces can be modified in different ways, as reported in Table 2 and Figure 3.

Table 2. Applications of the direct methods and graphical abstracts from the references.

Parental Cells	Functionalization	Functionalization Step	Recipient Cells	Treatment Conditions	Application	Refer
		Covalent				

PC12 cells	TAMRA-NHS	200 µL of Exos added to 1 mL 0.1 M sodium bicarbonate with 100mg TAMRA-NHS	PC12 cells	1×10^8 cells and 100 µL of exosome solutions	Visualization of cellular uptake and intracellular trafficking of exosomes	[34]
4T1 cells	Alkyne groups conjugated with azide-fluor 545	80 µg of exosomes in PBS, Cu (II) sulfate pentahydrate, 1.44 M l-ascorbic acid, and bathophenanthrolinedisulfonic acid disodium salt trihydrate	4T1 cells	Cells at a confluency of 75% and 5µg of exosomes in 100 µL RPMI	Surface functionalization of exosomes	[35]
Neuro2A and platelets	EGFR conjugated to DMPE-PEG derivatives	Conjugation in a 8.6:1000 molar ratio of nanobody/DMPE-PEG-maleimide micelles and then mixed with EVs	A431 and Neuro2A *IN VIVO*: Crl:NU-Foxn1nu mice with human tumor xenografts	3×10^4 cells per well and 8 µg/mL of EVs *IN VIVO:* i.v. of 2.5 µg of EVs in 100 µL PBS	Enhancing cell specificity and circulation time of EVs	[36]
Bovine serum	DSPE and chemical conjugation by NHS-PEG	Physical: DSPE-PEG-biotin mixed with the EXOs (500 µg in PBS) Chemical: NHS-PEG-biotin reacted with the primary amines (500 nmol) on the EXOs	RAW264.7, DC2.4, and NIH3T3 *IN VIVO*: mice	6×10^5 or 4×10^5 cells per well and EXOs at an ICG concentration of 5.8 µg per well *IN VIVO*: s.i. at a DiI dose of 1.52 µg/kg	Efficient delivery of immune stimulators and antigens to the lymph nodes in vivo	[37]
RAW 264.7 cells and BMM from C57BL/6 mice	DSPE-PEG or DSPE-PEG-AA	Addition of DSPE-PEG or DSPE-PEG-AA at 50 µg/mL	*IN VIVO*: C57BL/6 with induced pulmonary metastases	*IN VIVO:* i.v. injected with the exos at 10^8 particles/100 µl, *n* = 4 per group	Targeted paclitaxel delivery to pulmonary metastases	[38]

HEK293T cells	FA, PSMA RNA aptamer, and EGFR RNA aptamer conjugated to 3WJ	Cholesterol-triethylene glycol was conjugated into the arrow-tail of the pRNA-3WJ to promote the anchorage of the 3WJ onto the EV membrane	MDA-MB-231, KB, LNCaP (PSMA+), PC3 (PSMA–) *IN VIVO*: KB xenograft mice model	Incubation with cells *IN VIVO*: 1 dose of equivalent 0.5 mg siRNA/kg every 3 days for a total of 6 doses	Control RNA loading and ligand display on EVs for cancer regression	[39]
RAW 264.7	NRP-1-targeted peptide RGE	Surface modification with sulfo-NHS that can react with azide-modified RGE peptide, using salts and copper as catalyst	U251 and Bel-7404 *IN VIVO*: orthotopic glioma-bearing BALB/c nude mice	Cells and exos at the equivalent of 15 μg/mL of Cur/SPIONS *IN VIVO*: i.v. of Cur/SPIONS at 800 μg/200 μg Exos/200 μL PBS	Facilitate simultaneous imaging and therapy of glioma in vitro and in vivo	[40]
		Non-Covalent				
HeLa	Cationic lipid formulation, LTX, and GALA	20 μl LTX added to a solution of exosomes and 20 μl GALA and incubated for 20 min at room temperature	HeLa and (CHO)-K1	2 mL with 2 × 10^5 cells and 20 μg/mL of exosomes	Enhancing cytosolic delivery of exosomes	[41]
RTCs	Superparamagnetic magnetite colloidal nanocrystal clusters	1 mL of serum incubated with 200 μL of M-Tfs solution for 4 h at 4°C	H22 cells *IN VIVO*: Kunming mice bearing a subcutaneous H22 cancer	0.1 mg/mL of exos in a simulated blood circulation at 32.85 cm/s (artery), 14.60 cm/s (vein), and 0.05 cm/s (capillary)	Targeted drug delivery vehicle for cancer therapy with magnetic properties	[42]
Human serum and C2C12	Rhodamine-labelled M12-CP05, FITC-labelled NP41-CP05	CP05 (200 μg/mL) incubated with nickel beads, added into the precentrifuged serum (200 μL), and incubated for 30 min at 4°C under rotation	*IN VIVO*: dystrophin-deficient and immunodeficient nude mice and C57BL/6 mice	*IN VIVO*: i.m.1 or 2 μg of EXOs, i.v. EXOs in 100 μL of saline solution	Enabling targeting, cargo loading, and capture of exosomes from diverse origins	[43]

4T1, MCF-7, and PC3	DiR labelling	5µL of DIR, at a concentration of 220 µg/mL in ethanol, was mixed with 220 µg exosomes or liposomes in 100 µL PBS for 1 hour	*IN VIVO*: Balb/c, nude, and NOD.CB17-Prkdcscid/J mice with either 4T1 cells or PC3 cells	*IN VIVO*: i.v. 60 µg DIR-labeled exosomes in 200 µL PBS or i.t. 60 µg of DIR-labeled exosomes in 50 µL PBS	Biodistribution and delivery efficiency of unmodified tumor-derived exosomes	[44]
Glycosylation						
MLP29	Neuraminidase	Surface glycosylation of the EVs was manipulated by treatment with neuraminidase to remove the terminal residues of sialic acid	*IN VIVO*: wild-type mice	*IN VIVO*: i.v. of the EVs	Modification of the glycosylation of EVs to alter their biodistribution in vivo	[45]
U87 and GBM8	Glycosylation and insertion of targeting ligand to DC-SIGN	Treated with a pan-sialic acid hydrolase Neuraminidase for 30 min at 37°C and/or incubated with palmitoyl-LewisY while vortexing for 10 min	MoDCs	500,000 cells incubated with EVs for 45 min on ice to allow receptor binding	Enhancing receptor-mediated targeting of dendritic cells	[46]
HEK293FT	Glycosylation of targeting-peptide-Lamp2b fusion proteins	1.5 mL of 0.971 M sucrose was slowly pipetted underneath the 8.5 ml of exosome solution	HEK293FT and Neuro2A	Cells at 50% confluency and EVs for 2 h at 37 °C	Stabilization of exosome-targeting peptides	[47]
Hybridization						
HEK293FT	CRISPR/CRISPR-associated protein 9 (Cas9) system	Addition of the plasmid–liposome complex to exosomes and incubated at 37 °C for 12 h in a volume ratio of 1:2	MSCs	Incubation with cells at 90% of confluency	Efficiently encapsulate large plasmids and be endocytosed in MSCs	[48]
RAW 264.7, CMS7-wt, and CMS7-HE	DOPC, DOPS, DOTAP, and DOPS/PEG-DSPE	Exosomes (300 µg/mL, protein) mixed with 100 µM liposomes in a volume ratio of 1:1 and then several freeze–thaw cycles	HeLa cells	4.5 µg protein in exosome incubated with 1×10^5 HeLa cells for 4 h at 37 °C	Control and modify the performance of exosomal nanocarriers	[49]

HUVECs and MSCs	Phosphatidylcholine, phosphatidylethanolaminein, and PEG	Liposomes and EVs were mixed at 40 °C in a total volume of 40−200 µL (2×10^{10} or 2×10^{11}objects); liposome/EV ratio of 1:1, 1:9, or 9:1 in PBS. PEG was added at 5−30% (w/v)	THP1-derived macrophages and CT26	100,000 cells per well and hybrid EVs containing 1 mol % of DiR, cells, and 400 µL of mTHPC-loaded hybrid EVs or (3D) 500 cells and mTHPC-loaded hybrid EVs	Design of personalized biogenic drug delivery systems	[50]
J774A.1	L-a-phosphatidylcholine and cholesterol	EVs (200 µg protein) used to hydrate the dry 1000 µg of lipid film in a final volume of 1 mL; then, the solution was extruded through 400 and 200 nm polycarbonate membrane filter	K7M2, 4T1, and NIH/3T3	10,000–20,000 cells and 4 mL of 50 µg/mL of hybrid EVs at 37°C for 3h or 48 h	Tumor targeted drug delivery	[51]

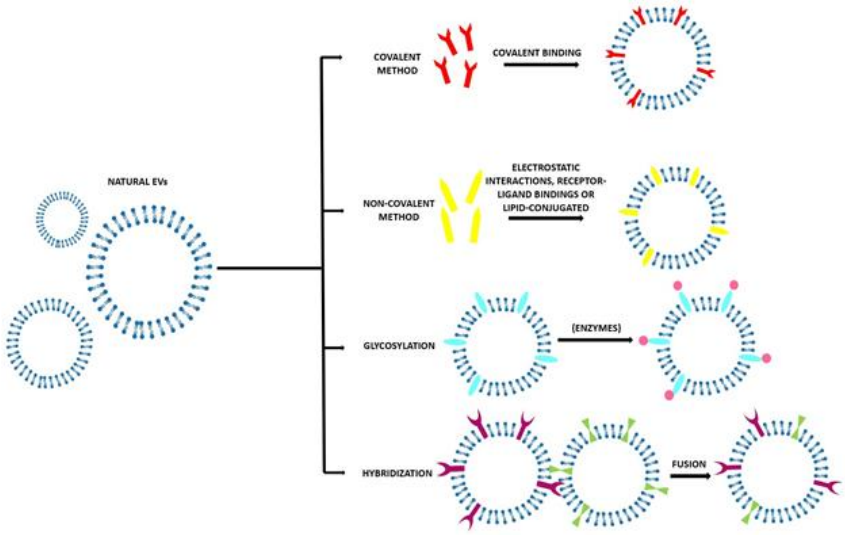

Figure 3. Scheme of the various direct methods to obtain engineered EVs with the desired characteristics and with the molecules of interest on the surface. In particular, covalent method; non-covalent methods such as electrostatic interaction; receptor–ligand binding; or lipid conjugation, glycosylation, or hybridization.

3.1. Covalent Methods

As the classical crosslinking is not enough in terms of specificity and efficiency, the most used covalent method nowadays is the Click Chemistry approach, also known as azide alkyne cycloaddition[52]. With this process, an alkyne moiety reacts with an azide group to form a stable triazole linkage[52]. Some studies also used a copper catalyst to accelerate the reaction[53], but several authors demonstrated that a successful binding can be obtained also without the copper catalyst [54]. One of the strengths of this method is that the experimental conditions are mild and that it can take place in both in organic and aqueous media (water, alcohols, dimethyl sulfoxide (DMSO))[55]. The yield is high, the method is simple, and it does not impact on EV size nor on the target cell uptake[55]. This method does have, however, some drawbacks—the alkyne modification of the EV surface most likely occurs on the amine groups of the proteins instead of those of the phospholipids, introducing the possibility that the EV protein function may be inhibited [52]. By controlling the number of alkyne groups, it is possible to avoid the over modification of EV membrane proteins—with a standard calibration curve it

has been estimated that approximately 1.5 alkyne modifications are made for every 150 kDa of EV protein[35]. A very common approach is PEGylation, the modification of EVs' surfaces with polyethylene glycol to extend the circulation half time of the EVs. The drawback of PEGylation is that the PEG corona also reduces the EV–cell interaction and the cellular uptake of the EVs[36]. This disadvantage can be overcome by functionalizing the distal end of the PEG chain with a targeting ligand[4].

3.2. Non-Covalent Methods

These methods are based on mild reactions, such as electrostatic interactions, receptor–ligand bindings, and lipid-conjugated compounds post-insertion into the EV's lipid bilayer[4]. Electrostatic approaches usually involve highly cationic species adhering on negatively charged functional groups present on the biological membranes [4]. A possible drawback of these methods is that certain cationic nanomaterials can cause cytotoxicity and that they are typically taken up into the cells via endocytosis, leading to lysosomal degradation[5].

3.3. Glycosylation

Glycosylation is at the base of many biological functions of EVs, such as cargo protein recruitment and cellular recognition and uptake [56][57]. Alterations in the glycosylation pattern has been associated with different pathologies, for example, cancer, and these changes are closely correlated with the specific malignant transformation and progression. This evidence has led to make glycan structure a useful target for anti-tumor applications in theranostics [58][59]. The manipulation of glycosylation can be done using either enzymes or not.

3.4. Hybridization

This method implies the fusion of natural EVs with their artificial counterpart, liposomes, to optimize the properties of native EVs [26]. This can be obtained thanks to the lipid composition of the EV membrane. In this way, the colloidal stability of EVs is improved, increasing their half-life in blood and modifying their immunogenicity profile, possibly decreasing it[52]. The lipid composition has been evidenced to impact on the cellular uptake—EVs hybridized with neutral or anionic lipids have a higher possibility to be taken up by cells than those hybridized with cationic lipids[52]. Moreover, hybridization of EVs increases the vesicle size (in a technique-dependent way)—this is a drawback because it decreases the in vivo retention of the vesicles, but also an advantage as it can improve the drug encapsulation efficiency[52]. Native EVs are actually very small in size and thus limited in their ability to encapsulate large molecules, while larger hybridized EVs can carry larger cargos[52].

As for the indirect methods, it is important to remember that the technical challenges to engineer the EVs with the different direct methods are directly correlated with the biological challenges that are fundamental in every step of EV engineering, from the preliminary design to the real environment testing. For what concerns the choice of the parental cells, in some works the authors chose the RAW 264.7 macrophages, an immortalized cancer cell line [38][40][49], while others used immortalized cell lines such as HeLa [41] or Neuro 2A[36], or even extracted the desired cells directly from mice[38] or human serum[43]. As stated previously, the main biological challenge is to find a scalable and controllable way to use the patient's cells as source in order to obtain EVs that are possibly compatible to the patient environment.

Moreover, the best EV engineering method must be carefully evaluated in a specific context, considering advantages and limitations. In particular, for what concerns the functionalization, it is important to find the proper molecule for the desired purpose, and a variety of functionalizations are reported in the literature, as mentioned above. As for the indirect methods, the use of DSPE-PEG [37][38][49] or DMPE-PEG[36], as spacer to expose the functionalization, is a commonly used strategy. Finally, for both in vitro and in vivo testing steps, the biological challenges are the same listed above and analyzed for the indirect methods in terms of choice of the best cell line and/or animal model.

At this point, it is clear that the functionalization of EVs with ligands and other molecules can boost up their stability in blood circulation, have the capability of localizing the target site, and can increase their intracellular delivery efficiency[60]. The main drawback of EV engineering is the introduction of the risks of altering the orientation of membrane proteins, which may compromise their biological functionalities or even induce immunogenicity[6]. Further risks of EV engineering are associated with the hiding of these proteins or to the damage or disruption the EV membrane[50].

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