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Engineered Extracellular Vesicles for Cancer Therapy

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Extracellular vesicles (EVs) have emerged as a novel cell-free strategy for the treatment of many diseases including cancer. As a result of their natural properties to mediate cell-to-cell communication and high physiochemical stability and biocompatibility, EVs have been considered as excellent delivery vehicles for a variety of therapeutic agents such as nucleic acids and proteins, drugs, and nanomaterials. Increasing studies have shown that EVs can be modified, engineered, or designed to improve their efficiency, specificity, and safety for cancer therapy. Herein, a comprehensive overview on the recent advances in the strategies and methodologies of engineering EVs for scalable production and improved cargo-loading and tumor-targeting is provided. Additionally, the potential applications of engineered EVs in cancer therapy are discussed by presenting prominent examples and the opportunities and challenges for translating engineered EVs into clinical practice are evaluated.

1. Introduction

Extracellular vesicles (EVs) are nano-sized vesicles secreted by all types of cells.[1] EVs carry a variety of bioactive molecules such as nucleic acids and proteins and transfer them from donor cells to recipient cells through multiple mechanisms such as direct membrane fusion, receptor–ligand interaction, and endocytosis or phagocytosis.[2] Increasing studies reveal that EVs mediate signal transduction and play important roles in intercellular communication, thus participating in many physiological and pathological processes.[3] The discovery that EVs transfer bioactive molecules between cells promotes the idea of developing them as potential therapeutic agents and drug delivery vehicles.[4,5] The unique physiochemical characteristics of EVs have endowed them with several advantages to be used as therapeutic nanomaterials. For instance, the lipid bi-layer membrane of EVs protects their cargos from degradation in the circulation. Compared with some traditional nanomaterials, EVs are intrinsically biocompatible, biodegradable, low toxic, and non-immunogenic, which makes them more suitable to be used as nanovesicles (NVs) for drug delivery. In addition, EVs have the potential to escape from clearance by host immune system and to pass through physiological barriers due to specific membrane-bound protein expression pattern and small size.[6,7] Moreover, EVs inherit targeting properties from their producing cells, which is beneficial for the accumulation of therapeutic cargos at local diseased sites after systemic infusion.[8] Together, these unique biological features make EVs one of the promising candidates for nanomedicine.[9]

Previous studies have shown that natural EVs from certain sources, such as tumor cells and immune cells, could elicit antitumor activities and have the potential to be used as cancer vaccines. Further studies have employed EVs to deliver various therapeutic molecules and chemotherapeutic drugs and achieved promising effects.[10,11] However, the use of natural EVs for cancer therapy has several problems. For instance, EVs are prone to be trapped in nonspecific tissues, especially in the liver and lung, leading to insufficient targeting in vivo.[12]

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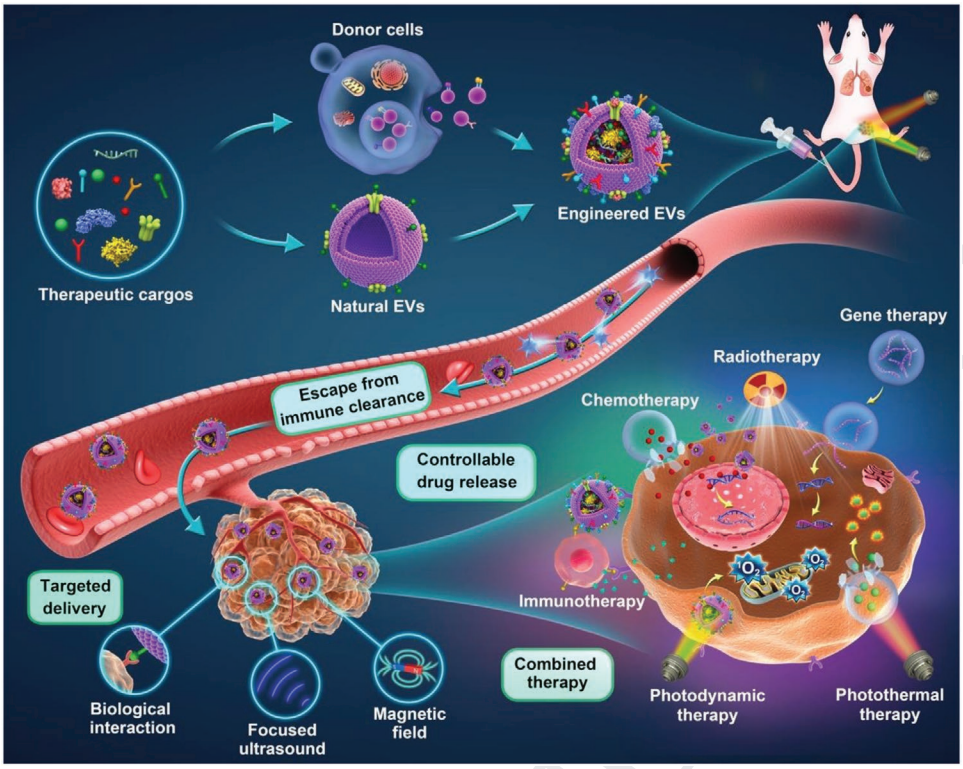

Figure 1. Engineered EVs as advanced nanomaterials for cancer therapy. Traditional and advanced bio-techniques have been used to manipulate donor cells or their derived EVs to generate engineered EVs that deliver a variety of therapeutic molecules, drugs, and nanomaterials. Engineered EVs have superior characteristics to their natural counterparts, including long circulation and high stability, tumor-targeting ability, deep penetration and enhanced accumulation, efficient intracellular delivery, and controllable drug release, which remarkably improve the specificity, efficacy, and safety of EV-based cancer therapeutics.

In addition, the heterogeneity and complicated components of EVs reduce therapeutic efficacy and bring safety concerns when they are used to deliver therapeutic cargos. Moreover, the lack of efficient isolation and scale-up production of EVs and accurate monitoring of the dosage of therapeutic cargos in EVs are also potential problems when used in the clinical settings. Thus, engineered EVs have recently emerged as a new strategy and hold great promise to be used as an alternative approach for EV-based therapy (**Figure 1**).[13] Accumulating evidence suggest that the engineering of EVs enhances their loading efficiency, targeting ability, and therapeutic effect.[14] At present, there are two main strategies for loading a desired cargo into EVs. One strategy is incorporating a cargo into the producer cells and obtaining the cargo-loaded EVs through natural biogenesis process. The other one is harvesting EVs from distinct sources (e.g., cultured cells, human blood, and milk) and introducing a cargo into EVs through traditional and advanced bio-techniques.[15] There is also growing interest in modifying EV membranes to make them target specific tissues and combining EVs with other nanomaterials to achieve improved or synergistic therapy effects.[16] The fabrication of bio-inspired or bio-mimetic EVs with higher production yield and loading efficiency has also been extensively explored.[17]

In this review, we summarized the recent advances in the modification, engineering, and design of EVs as nanovehicles for delivering therapeutic agents with an emphasis on their applications in cancer therapy, which will help better understand the current progress and future research directions of this field.

2. EV Characteristics, Biogenesis, and Contents

EVs are a heterogeneous group of membrane-structured vesicles actively released by all types of cells and are found in many human body fluids such as blood, urine, and ascites.[18] Three main populations of EVs have been proposed according to their size and origin: exosomes, microvesicles (MVs) or microparticles (MPs), and apoptotic bodies (**Figure 2**). Exosomes, with a diameter of 30–150 nm, are vesicles derived from the fusion of multivesicular bodies (MVBs) and plasma membranes, while MVs, with a size ranging from 50 to 1000 nm, are formed as a result of direct outward budding of plasma membranes. Apoptotic bodies are released by dying cells after apoptosis. Most studies focus on the potential of exosomes and MVs/MPs in nanomedicine. There are few studies on the use of apoptotic bodies as therapeutic NVs, which may be associated with their large and uneven size. EVs are enriched in nucleic acids, proteins, lipids, metabolites, and even organelles from donor cells. Initially thought as a way for the disposal of cell waste, EVs have recently been recognized as a key player in regulating intercellular communications. The biological roles of EVs in human

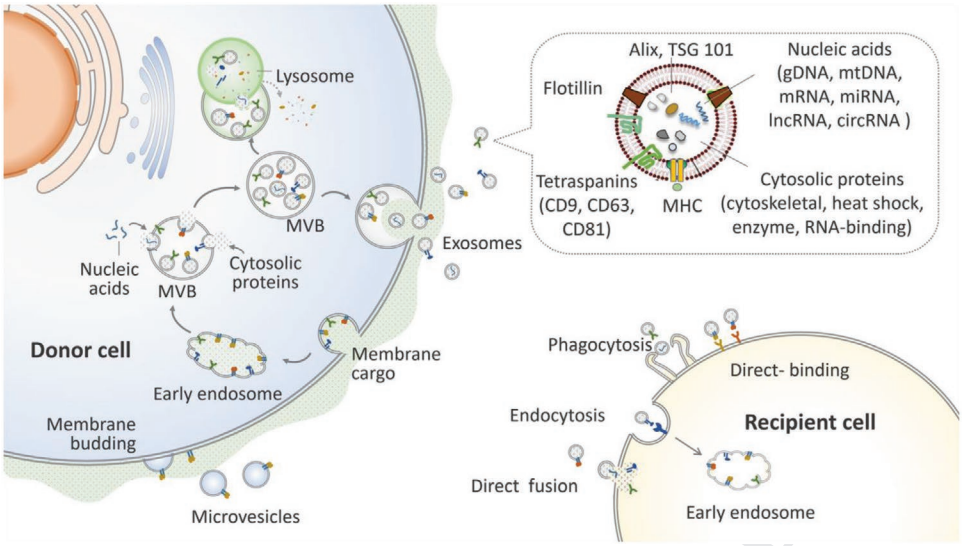

Figure 2. The biogenesis, contents, and internalization of EVs. Exosomes are derived from the fusion of MVBs and plasma membranes. MVs or MPs are formed after direct outward budding of plasma membranes. EVs contain membrane proteins and cytosolic components (nucleic acids and proteins) of donor cells and can transport their cargos to recipient cells though multiple mechanisms such as direct fusion, direct binding (receptor–ligand interaction), endocytosis or phagocytosis. Abbreviations: gDNA, genomic DNA; lncRNA, long non-coding RNA; MHC, major histocompatibility complex; mtDNA, mitochondrial DNA; MVBs, multivesicular bodies.

physiological and pathological conditions including cancer have been widely reported in the past few decades.[1]

Although the mechanisms responsible for specific cargo sorting in EVs are still unclear, previous studies have proposed several possibilities. For example, protein molecules are sorted into MVBs in a ubiquitin-dependent manner with the help of endosomal sorting complex required for transport. In addition, tetraspanin-enriched microdomains also contribute to the sorting of proteins into EVs.[18] For specific loading of RNA (mainly microRNA) into EVs, several key factors such as heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1),[19] adenylation and urylation at the 3′ end of microRNA (miRNA),[20] argonaute 2 (Ago2),[21] and ubiquitinated form of human antigen R (HuR)[22] have been reported to be critically involved. Clancy et al. suggest that the interaction between ADP-ribosylation factor 6 (ARF6)-GTP and Exportin-5 promotes pre-miRNA cargo sorting into tumor MVs.[23] Intriguingly, the specific cargos in EVs could reflect the pathophysiological status of their parental cells, which makes them useful biomarkers for monitoring disease progression.[5] The lipid bi-layer membrane structure of EVs not only protects internal proteins and nucleic acids form degradation, but also maintains the inherent targeting abilities from their parental cells, which endows them the potential to serve as an effective carrier for delivering cargos into recipient cells. Moreover, the specific lipidomic and proteomic profiles of EVs may help them escape from endosomal traps and allow for a direct cytosolic delivery of therapeutic cargos.[9,17] These natural and unique properties make EVs ideal NVs for drug delivery.

However, there are some hurdles when translating EVs from bench to bedside. The low isolation yield and complicated purification protocols make massive production of EVs a challenging task. Thus, it is urgently needed to develop standard, scalable, and cost-effective approaches for EV production. In addition, due to the considerable heterogeneity of isolated EVs and their complex composition and structure, it is difficult to characterize EVs as synthetic nanoparticles (NPs) that are currently used in the clinic (e.g., liposomes). Moreover, tedious cargo loading procedures, relatively low delivery efficiency, and unsatisfactory targeting ability also hinder the therapeutic applications of EVs. If these main problems are properly solved, the applicability of EVs as NVs will be greatly advanced.

3. Natural EVs in Cancer Therapy

The intrinsic ability of EVs to shuttle bioactive molecules has led to extensive exploitation of their function in physiology and pathology. The findings that EVs from immune cells contain bioactive molecules such as major histocompatibility complexes (MHC) suggest an immunomodulatory effect of these small vesicles. In 1998, Zitvogel et al. demonstrated that tumor antigen-pulsed, dendritic cell-derived EVs (DEX) can activate T cells to produce anti-tumor effect in established mouse tumor models.[24] Since then, increasing studies have started to explore the potential of natural EVs in cancer therapy (**Figure 3**).

3.1. DEX and TEX in Cancer Therapy

Early studies of EVs mainly focus on the potential of dendritic cell- and tumor cell-derived EVs (TEX) as cancer vaccines.[25,26] In analogous to their parental cells, DEX present peptide-MHC complexes and a variety of costimulatory molecules on their membrane, which endows them with antigen-presenting ability.[25,27] DEX can also activate natural killer (NK) cells via TNF superfamily ligands.[28] In clinical trials, the administration of DEX into patients with melanoma and non-small cell lung cancer (NSCLC) shows modest T cell activation. In particular, DEX from

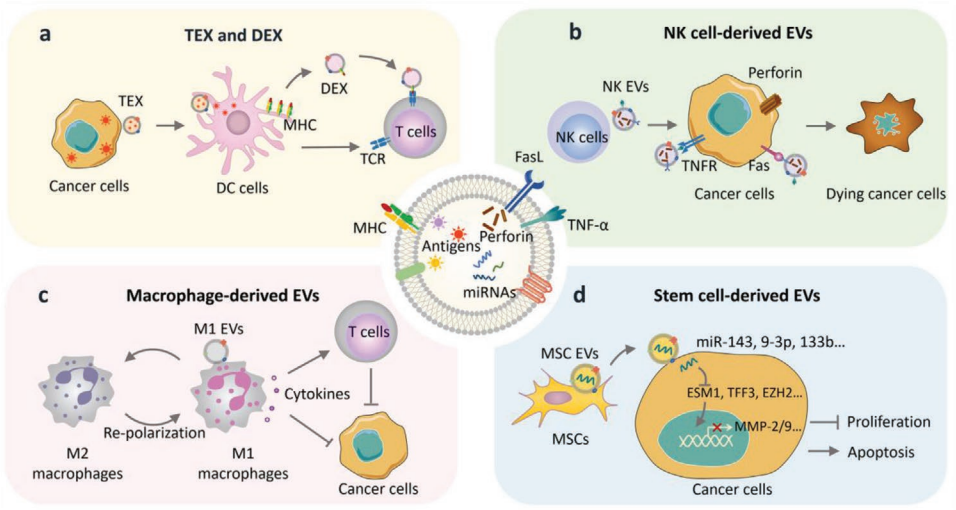

Figure 3. Natural EVs in cancer therapy. a) TEX stimulate immune activation by boosting T-cell expansion and function via APCs especially DC cells. DEX activate T cells by mimicking the role of APCs. b) NK cell-derived EVs induce tumor cell death through FasL, perforin, and TNF-α. c) EVs from M1 macrophages can re-educate tumor associated macrophages from M2 to M1 phenotype, which further activates anti-tumor immunity. d) Stem cell-derived EVs inhibit the growth of tumor cells through miRNA-mediated mechanisms. Abbreviations: DC cell, dendritic cell; DEX, dendritic cell-derived EVs; FasL, Fas ligand; M1 EVs, M1 macrophage-derived EVs; MHC, major histocompatibility complex; MSCs, mesenchymal stem cells; NK EVs, NK cell-derived EVs; TCR, T cell receptor; TEX, tumor cell-derived EVs; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor.

interferon (IFN)-γ maturated DCs boost anti-tumor response of NK cells and achieve better progression-free survival in advanced unresectable NSCLC patients.[29] Although DEX seem to work well in pre-clinical studies, they are difficult to induce highly efficient anti-tumor effects in cancer patients, probably due to the complicated tumor microenvironment. Therefore, in recent years, researchers have further tested the possibility of engineering DEX to improve their therapeutic efficacy.

TEX are used in cancer therapy due to the presence of tumor antigens on their membranes. A common strategy that has been used is to pulse dendritic cells with TEX in vitro, which are then used to stimulate host immune system to boost T-cell expansion and function in vivo.[30] Recently, researchers have collected EVs from the ascites of colon cancer patients and combined them with granulocyte-macrophage colony stimulating factor (GM-CSF) for immunotherapy. The combination treatment regimen induces an enhanced specific antitumor T cell immunity, suggesting the feasibility, efficacy, and safety of this strategy.[31] However, the direct use of TEX for cancer therapy is challenged by their participation in almost all aspects of tumor progression, which hinders them to become safe cell-free cancer vaccines.[32]

3.2. NK Cell-Derived EVs in Cancer Therapy

The ability of NK cells to directly lyse tumor cells in an antigen-independent manner makes them an attractive candidate for cancer therapy. In the past decade, the concept of using EVs from NK cells for cancer therapy has emerged. Lugini et al. demonstrated that EVs from NK cells of healthy donors contain killer proteins such as Fas ligand (FasL) and perforin molecules and can induce remarkable cytolytic activity against leukemia cells.[33] In another study by Zhu et al., they demonstrated that NK cell-derived EVs not only express FasL and perforin, but also produce tumor necrosis factor-α (TNF-α). NK cell-derived EVs show anti-tumor effect on melanoma cells but exhibit no significant effect on normal cells.[34] Shoae et al. suggest that EVs from NK cells that have been previously exposed to neuroblastoma (NB) cells educate naive NK cells to exert greater cytotoxicity against NB tumors, which helps to overcome immune resistance of tumor cells.[35] Moreover, NK cell-derived EVs contain tumor suppressive miRNAs (such as miR-186 and miR-3607-3p) that can induce tumor cell apoptosis and inhibit tumor cell proliferation.[36] More importantly, the anti-tumor potential of NK cell-derived EVs can be further enhanced by interleukin (IL)-15 priming.[37] These findings suggest that NK cell-derived EVs have potent anti-tumor activities, which represents a novel approach for cancer therapy.

3.3. Macrophage-Derived EVs in Cancer Therapy

Macrophages display diverse phenotypes in response to microenvironment and are categorized into anti-tumor M1 and pro-tumor M2 subtypes. M1 macrophage-derived EVs have been used as an immune potentiator in cancer therapy due to their pro-inflammatory effects. Cheng et al. demonstrated that subcutaneously injected M1 macrophage-derived EVs (M1 EVs) can home to lymph node and induce the expression of pro-inflammatory T-helper cell type 1 (Th1) cytokines such as IL-6 and IL-12, which elicits a strong antigen-specific cytotoxic T cell response and enhances the anti-tumor effect of nanoparticulate peptide vaccine tyrosinase-related protein-2 (TRP-2), indicating that M1 EVs can be used as a vaccine adjuvant.[38] In addition, Wang and colleagues suggest that M1 EVs can stimulate macrophages in tumor tissues to release cytokines, which establishes a local inflammatory environment that benefits the anti-tumor effects of chemotherapeutic drugs.[39] Moreover, Choo et al. show that EV-like NVs derived from M1 macrophages (M1NVs) can accumulate at tumor sites and

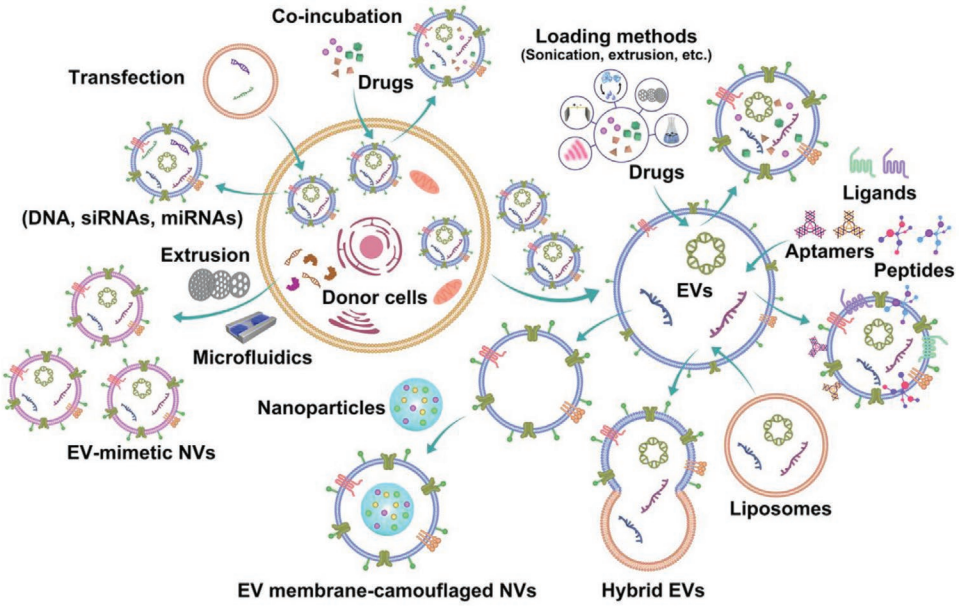

Figure 4. Main strategies for EV engineering. Left: Strategies for engineering donor cells. Co-incubation and gene transfection approaches are used to introduce cargos into donor cells. Extrusion and microfluidic approaches are used to fabricate EV-mimetic NVs and introduce cargos into them. Right: Strategies for EV engineering. Sonication, electroporation, freeze-thaw, extrusion, and saponin permeabilization approaches are used to introduce cargos into EVs. Ligand–displaying strategy is used to anchor targeting ligands, peptides, and aptamers on EV membranes. Nanomaterials including liposomes and micelles can be mixed with EV membranes to fabricate hybrid EVs. Synthetic bio-inspiration strategy is used to develop bio-mimetic EVs. Abbreviations: NVs, nanovesicles.

re-polarize M2 macrophages to M1 macrophages. The combined use of M1NVs improves the anti-tumor efficacy of anti-programmed death ligand 1 (PD-L1) antibody.[40] Similarly, Fan et al. have generated M1 macrophage-derived artificial vesicles (M1mv) and shown that M1mv exhibit anti-tumor effects by inducing cell apoptosis.[41] These results indicate that M1 macrophage-derived EVs provide a novel anti-tumor therapeutic agent.

3.4. Stem Cell-Derived EVs in Cancer Therapy

Several studies have shown that stem cell-derived EVs can suppress cancer progression. For example, EVs from bone marrow mesenchymal stem cells inhibit the growth and metastasis of bladder cancer by miR-9-3p-meiated down-regulation of ESM1 (endothelial cell specific molecule 1) gene expression.[42] In addition, miR-133b in MSC EVs is able to inhibit EZH2 (enhancer of zeste homolog 2) gene expression and block Wnt (wingless)/β-catenin signaling pathway, thus suppressing the development of glioma.[43] In prostate cancer, miR-143 from MSC EVs inhibits cancer progression by targeting TFF3 (trefoil factor 3).[44] Intriguingly, the recent studies have shown that combining stem cell-derived EVs with chemotherapy can achieve better therapeutic effects. For instance, EVs from human umbilical cord MSCs enhance the sensitivity of K562 cells to imatinib by activating caspase signaling pathway.[45] Moreover, EVs from irradiated MSCs are able to stimulate tumor cell death and increase their sensitivity to radiation, which may be associated with the enrichment of tumor suppressor genes such as ANXA1 (annexin A1) in these EVs.[46] However, MSC-derived EVs have been described as a double-edged sword in cancer therapy because they also can promote cancer progression.[47] Therefore, the use of natural EVs from stem cells in cancer therapy still needs further investigation.

4. Strategies of EV Engineering

Proper modification of EVs can increase their delivery efficiency, targeting ability, and therapeutic efficacy.[48,49] EVs from different sources, including tumor cells,[50] stem cells,[51,52] immune cells,[39,53] human blood and urine,[54,55] and milk,[56] have been tested for this purpose. Strategies and methodologies that are commonly used for EV engineering have been well documented in previous reviews and are briefly summarized here.[7,11,15,57] (**Figure 4** and **Table 1**).

4.1. Approaches for Loading Exogenous Cargos into EVs

4.1.1. Active Loading into Donor Cells

Co-Incubation: Chemical compounds, especially small molecule drugs, can be introduced into EVs by co-incubating them with donor cells at different conditions. For instance, paclitaxel (PTX) is loaded into MSC-derived EVs by incubating MSCs with PTX at 37 °C for 1 h with shaking.[58] The similar procedure has also been used for loading doxorubicin (DOX) into cancer cell-derived EVs. Although this method is simple and has no major effects on the structure and contents of EVs, the loading efficiency is influenced by drug properties, incubation periods, and other protocol details.[49]

Table 1. Strategies for cargo loading into EVs.

Strategies[a)]	Methods	Advantages	Disadvantages	Prominent examples	Ref.
Cargo loading into donor cells	Co-incubation	Simple and feasible; No damage to membrane integrity	Cytotoxicity; Poor specificity; Low loading efficiency	Delivery of DOX and PTX	[49,58,109]
	Transfection	Simple and feasible; No damage to membrane integrity	Induce donor cell apoptosis; Impair biological responses; Inefficient packaging	Delivery of Cre recombinase mRNA, let-7c, and HGF siRNA	[59]
Direct loading into EVs	Electroporation	Simple and quick; Higher loading efficiency than transfection	EV aggregation; siRNA precipitation; Not suitable for some RNAs with special structures	Delivery of Cy5-labeled miR-26a, HAL, and BACE1 siRNA	[62,63]
	Extrusion	Efficient packaging	May change the membrane properties	Delivery of hydrophilic porphyrins	[61]
	Saponin permeabilization	Increase drug loading efficiency	Toxicity	Delivery of hydrophilic porphyrins	[60]
	Freeze and thaw cycles	Higher loading efficiency	EV aggregation; Lower drug loading capacity than sonication/extrusion	Delivery of catalase	[65]
	Sonication	Higher loading efficiency	EV aggregation	Delivery of DOX and PTX	[66,111]
General modification of EV membrane	Click chemistry	Rapid and efficient; No damage to membrane integrity	May alter the activity of membrane proteins	Targeted delivery of curcumin/SPIONs and curcumin/cRGD peptide	[68,69]
	Fusion with membrane proteins	Specific targeting	May affect the functions of cargos in the recipient cells	Targeted delivery of CP05 peptide and IMTP peptide	[72]
	Ligand-displaying	Specific targeting; Efficient packaging	Synthetic challenge; Cost of presenting functional ligands	Display folate, PSMA RNA aptamer, and EGFR RNA aptamer	[70]
	Chimeric EVs	Cell membrane and nucleus dual-targeting ability; Low immunogenicity and systemic toxicity	Cost of presenting chimeric peptides	Chimeric peptide engineered EV- mediated delivery of photosensitizer	[139]
New engineered EVs-based platforms	Artificially synthesized EV-like NPs	Simple fabrication procedure; Controllable preparation process; Clean identity, high purity and quantity	Contain some components of EV membrane but may lose their biological function; Hard to incorporation multiple components	Targeted delivery of therapeutic oligonucleotides	[75]
	EV-mimetic NVs	Maintain membrane structures; High production yield	Low homogeneity and purity; Require additional purification steps; Less controllable preparation process	Targeted delivery of chemotherapeutic drugs	[74,77]
	Hybrid EVs	Easy preparation and scalability; Controllable production processes; Adjustable physical parameters; Efficient drug loading	May lose biological functions of integral EVs; Increase the difficulty of fabrication; Low homogeneity	Targeted delivery of CRISPR-Cas9 plasmid and chemotherapeutic drugs	[79,80]
	EV membrane-camouflaged NVs	Maintain complex EV membrane structure; Specific targeting ability; High therapy efficacy	Low scalability; Increase the difficulty of fabrication; Time-consuming	Targeted delivery of proteins, therapeutic RNAs, and imaging agents	[81–83]

[a)]Abbreviations: BACE1, beta-site APP cleavage enzyme 1; DOX, doxorubicin; EGFR, epidermal growth factor receptor; HAL, hexyl 5-aminolevulinate hydrochloride; HGF, hepatocyte growth factor; IMTP, ischemic myocardium-targeting peptide CSTSMLKAC; NVs, nanovesicles; PSMA, prostate specific membrane antigen; PTX, paclitaxel; RGD, arginyl-glycyl-aspartic acid; SPIONs, superparamagnetic iron oxide nanoparticles.

Gene Transfection: Gene transfection is a common strategy for loading cargos into donor cells. Specific cargo-loaded EVs can be obtained from transfected cells by rapid isolation and purification. Exogenous nucleic acids, such as DNA plasmid vector and noncoding RNAs (siRNAs, miRNAs, etc.), are easily to be packaged within EVs through natural biogenesis process.[59] In general, this approach is simple and feasible but has limitations such as poor specificity and low loading efficiency. Further studies are still needed to find ideal donor cells and to improve encapsulation efficiency.

4.1.2. Passive Loading into EVs

Passive Mixing: EVs collected from different sources are mixed with various drugs at different conditions to encapsulate drugs. The loading efficiency can be further improved by saponin permeabilization.[60] This method seems to be more frequently used for hydrophobic drugs, because hydrophobic chemicals can interact with and cross hydrophobic EV membrane, thereby increasing drug bioavailability. The loading efficiency appears to be dependent on the hydrophobic nature of the drug while the chemical lipid composition of EVs is also important.[61]

Electroporation: EVs and exogenous cargos (such as siRNAs and miRNAs) are mixed in conductive solution under an electrical field to create transit pores on EV membrane, allowing the entry of exogenous cargos into EVs.[62,63] This method may induce EV or siRNA aggregation, thereby affecting the integrity of EVs or therapeutic efficacy of siRNAs.[64]

Mechanical Methods: Several mechanical methods are used for cargo loading into EVs, including freeze and thaw cycles, sonication, and extrusion. For freeze and thaw cycles, cargos are incubated with EVs for 30 min at room temperature and then quickly frozen at −80 °C and thawed at room temperature with a repeated procedure for three times.[65] For sonication, EVs and drugs are sonicated on sonic dismembrator (20% amplitude, 6 cycles of 30 s on/off) with a two-min cooling down procedure between each cycle. For extrusion, EVs and cargos are packaged into a syringe-based hand-held mini-extruder with polycarbonate membranes of 400 nm pore size at 42 °C and extruded for 30 times.[61] Studies from Kim et al. and Haney et al. indicate that the loading efficiencies of sonication and extrusion are remarkably higher than that of passive mixing and electroporation.[65,66] However, mechanical force on EVs may compromise EV membrane integrity, which will affect their therapeutic activity and bring safety risks for applications.

4.2. Approaches for EV Modification

4.2.1. Modification of EV Membrane

Proper modification of EV membrane endows them with improved tumor targeting and intracellular delivery capabilities (Figure 4). Chemical modification and gene engineering are two widely used approaches for EV membrane modification. Click chemistry is a copper-catalyzed azide alkyne cyclo-addition reaction that attaches specific molecules into EV membrane.[67] For instance, Jia et al. have conjugated neuropilin-1-targeted peptide (RGERPPR, RGE) to EVs by click chemistry to get glioma-targeting EVs.[68] Tian et al. have conjugated c(RGDyK) peptide to the surface of DBCO-modified EVs for delivering curcumin to ischemic brain.[69] Moreover, other targeting ligands such as RNA aptamers are displayed on EV membrane by similar procedures.[70]

The fusion of target protein with EV membrane protein improves their specificity to be loaded into EVs. For instance, Kooijmans et al. have displayed anti-EGFR ligand on EV membrane via glycosyl-phosphatidylinositol anchor for tumor cell targeting.[71] In addition, the fusion of targeting peptide with EV marker proteins such as CD63 and Lamp2b, have been commonly used to acquire targeting EVs.[72] Furthermore, Yim et al. have described a novel targeting delivery tool called exosomes for protein loading through optically reversible protein-protein interactions, which improved the efficiency of loading cargo proteins into EVs under the control of blue light.[73]

The integration of EV modification and isolation helps rapidly obtain functionalized EVs. By using a 3D nanostructured microfluidic chip, Wang et al. have prepared EVs chemically labeled with dual ligands (biotin and avidin) and packed drugs into the cytosol. This chemical editing approach facilitated the preparation of EVs with specific targeting ability.[74]

4.2.2. Bio-Inspired /Bio-Mimetic EV Generation and Modification

Bioinspired or bio-mimetic EVs, including artificially synthesized EV-like NPs, EV-mimetic NVs, hybrid EVs, EV membrane-camouflaged NPs, have recently been developed for scalable production, efficient cargo loading, and tumor-targeting.[17] Artificially synthesized EV-like NPs refer to those NVs synthesized by using individual bio-mimetic molecules such as EV membrane lipids and proteins to resemble the characteristics of EVs. These NPs share similar physiochemical characteristics to natural EVs but display high tumor-targeting ability, providing an alternative platform to natural EVs for drug delivery.[75]

EV-mimetic NVs are primarily produced by serially extruding donor cells though membrane filters with different sizes of pores or forcing donor cells to move though microchannels of microfluidic devices.[76] Jang et al. have reported the generation of EV-mimetic NVs from monocytes/macrophages with a 100-fold higher production yield. These NVs serve as delivery vehicles for chemotherapeutic drugs and maintain the functions of plasma membrane proteins to achieve targeting ability.[77] To facilitate rapid production, Yoon et al. have developed a microfluidic cell-slicing system based on silicon nitride (Si_xN_y) blades, which can generate NVs via slicing living cells.[78]

Hybrid EVs are constructed by fusing EVs with common biomaterials such as liposomes. Lin et al. have mixed plasmid-liposome complex with EVs to obtain hybrid EVs for delivering CRISPR/Cas9 system.[79] Intriguingly, Zhang et al. have constructed hybrid EVs by integrating red blood cell (RBC) and cancer cell membranes into synthetic phospholipid bilayers, which enables them to inhibit phagocytosis and target homologous cancer cells.[80]

EV membrane-camouflaged NPs are formed by coating synthesized inner core NPs with EV membrane. These NPs protect loaded cargos from immune clearance and promote intracellular drug release.[81] Bose et al. have coated gold-iron oxide NPs with

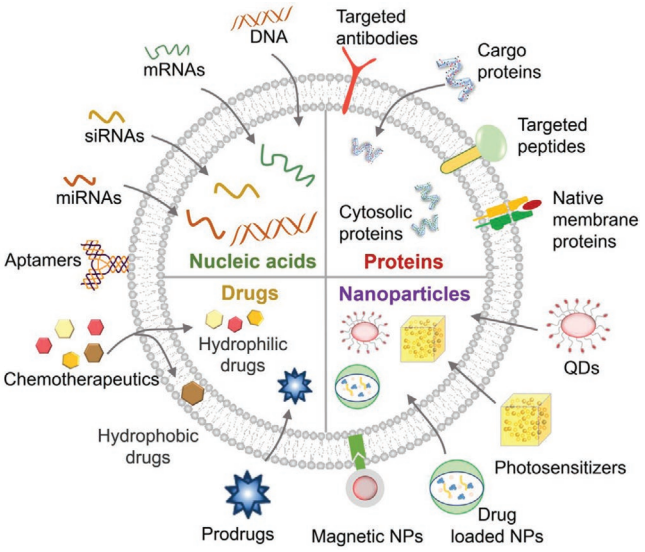

Figure 5. EVs as drug delivery nanovectors. Therapeutic nucleic acids including DNAs and RNAs (mRNAs, miRNAs, and siRNAs) and chemotherapeutic drugs are loaded into EVs. Proteins with targeting abilities or antitumor effects can be incorporated into EVs or anchored on EV membrane. Nanomaterials can be encapsulated by EVs or attached to EV membranes to increase their targeting ability or achieve controllable release. Abbreviations: NPs, nanoparticles; QDs, quantum dots; siRNAs, small interfering RNAs.

the membrane of tumor cell-derived EVs where anti-miR-21 and indocyanine green have been pre-loaded. The multifunctional NVs show significant tumor-specific accumulation and good potential in imaging, drug delivery, and phototherapy.[82] Liu et al. have developed a microfluidic sonication approach to produce EV membrane-coated, poly (lactic-co-glycolic acid) (PLGA)-based NPs for better biocompatibility and targeting efficacy. These EV-biomimetic NPs showed superior homotypic targeting ability and reduced uptake of by monocytes/macrophages.[83] Overall, bio-inspired or bio-mimetic EVs are characterized by massive production yield, convenient preparation protocol, and controllable fabrication process, representing a potential alternative to natural EVs for clinical applications.

5. EVs as Drug Delivery Nanovectors

Since EVs possess high biocompatibility as well as low systemic toxicity when administrated in vivo, they have been widely used as nanovectors to deliver therapeutic molecules (such as nucleic acids and proteins), drugs, and nanomaterials for cancer therapy (**Figure 5** and **Table 2**).

5.1. Nucleic Acids

5.1.1. DNA

Targeted delivery of DNA plasmid vector (e.g., CRISPR/Cas9 system) to recipient cells has a great potential for therapeutic genome editing. For instance, CRISPR/Cas9 expression vectors delivered by hybrid EVs achieve efficient in vivo gene manipulation in MSCs.[79] Gee et al. have developed an all-in-one CRISPR/Cas9 ribonucleoprotein delivery platform (NanoMEDIC) by using EVs, which achieves efficient genome editing in various hard-to-transfect cells, including human induced pluripotent stem cells.[84] Following this strategy, Kim et al. have used cancer cell-derived EVs to efficiently deliver CRISPR/Cas9 plasmids for targeted inhibition of poly (ADP-ribose) polymerase-1 (PARP-1),[50] which induces apoptosis in ovarian cancer cells and enhances their sensitivity to cisplatin.

5.1.2. RNA

mRNAs: In vitro and in vivo delivery of interest RNAs hold promise for gene therapy. EVs have been used as delivery vehicles for therapeutic RNAs such as mRNAs, siRNAs, and miRNAs.[10] Mizrak et al. have engineered HEK-293T cells to express high levels of suicide gene and protein-cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT).[85] The CD-UPRT mRNA/protein complex are loaded into EVs and have tumor cell-killing activity,[86] suggesting a great significance of suicide gene-carrying EVs in cancer therapy. A similar strategy has been used by Kanada et al.[87] and Altanerova et al.[88] to develop EV-based prodrug suicide gene therapy systems, which also achieve promising results.

Cellular nanoporation is a newly developed method to produce large quantities of EVs loaded with therapeutic mRNAs. Yang et al. have used this strategy to incorporate phosphatase and tensin homologue (PTEN) mRNA into EVs with a more than 1000-fold higher loading efficiency than regular transfection.[89] Moreover, to improve the efficiency of message transfer, Kojima et al. have developed a EXOsomal transfer into cells device (EXOtic) to consistently deliver therapeutic mRNA into target cells, indicating the potential of this device for RNA delivery-based therapies.[90]

siRNAs: The delivery of siRNAs into specific cells is important for RNA-based therapeutics. Previously, EVs from human blood have been used to transport exogenous siRNAs into monocytes and lymphocytes.[91] Recently, Kamerkar et al. have utilized EVs for delivering siRNAs or shRNAs specific to oncogenic $KRAS^{G12D}$, with a potent efficacy comparable to liposomes.[92] To improve the efficiency of targeted siRNA delivery, Pi et al. have modified EVs with folate and RNA aptamers to serve as a targeting ligand for binding to specific receptors overexpressed on cancer cells. The engineered EVs are found to successfully deliver survivin siRNAs to prostate and breast cancer cells with less endosome trapping and increased delivery efficiency.[70,93]

To prepare high yield of EVs for RNA-based therapeutics, Lunavat et al. have generated EV-mimetic NVs to load c-Myc shRNA.[94] In addition, to achieve improved drug delivery to lung pre-metastatic niche, Zhao et al. have developed bio-mimetic NPs that contain EV membrane-coated NPs and cationic bovine serum albumin (CBSA)-conjugated S100A4 siRNA. The self-assembled NPs protect siRNA from degradation, show excellent biocompatibility and high affinity toward lung, and exhibit outstanding gene silencing effect.[95] Furthermore, to improve siRNA loading efficiency, Reshke et al. have integrated siRNA sequences into the dicer-independent RNA stem-loop (based on pre-miR-451) to improve their sorting

Table 2. EVs as nanovectors for therapeutic agents.

Cargo types[a)]	Specific substances	Mechanisms	Effects	Cancer types	Ref.
DNA	PARP-1 CRISPR/Cas9 plasmid	Suppress PARP-1 expression	Induce cancer cell apoptosis and enhance chemosensitivity	Ovarian cancer	[50]
	Minicircle DNA that encodes a TK-NTR fusion protein	Enable effective prodrug conversion and tumor cell death	Inhibit tumor growth	Breast cancer	[87]
mRNA	CD-UPRT suicide mRNA/protein	Convert prodrug 5-FC to 5-FU	Inhibit tumor growth	Schwannomas and glioblastoma	[85,86]
	5-FC and yCD::UPRT mRNA	Induce tumor cell death by converting prodrug 5-FC to 5-FU	Inhibit tumor growth	Glioma	[88]
	PTEN mRNA	Restore PTEN expression	Inhibit tumor growth	PTEN-deficient glioblastoma	[89]
siRNA	$Kras^{G12D}$ siRNA or shRNA	Suppress $KRAS^{G12D}$ expression and inactivate KRAS signaling	Inhibit tumor growth	Pancreatic cancer	[92]
	Survivin siRNA	Inhibit Survivin gene expression	Inhibit tumor growth and progression	Prostate, breast, and colorectal cancers	[70]
	CBSA/siS100A4	Inhibit S100A4 expression	Inhibit lung metastasis	Triple negative breast cancer	[95]
miRNA	anti-miR-214	Inhibit miR-214 expression	Inhibit tumor growth and reverse chemoresistance	Gastric cancer	[104]
	miR-122	Alter miR-122 target gene expression	Sensitize cancer cells to sorafenib	HCC	[99]
	miR-206	Regulate TRA2B gene and induce cell apoptosis	Inhibit tumor growth	Osteosarcoma	[98]
	miR-26a	Inhibit cell cycle gene expression	Inhibit tumor growth	HCC	[62]
	miR-126	Interrupt the PTEN/PI3K/AKT signaling pathway	Inhibit cancer metastasis	NSCLC	[100]
	miR-146b	Inhibit EGFR gene expression	Inhibit tumor growth	Glioma	[102]
	miR-124a	Inhibit forkhead box A2 gene expression	Inhibit tumor growth	Glioma	[103]
	anti-miR-9	Inhibit MDR1 gene expression	Reverse TMZ resistance	Glioblastoma	[52]
	let-7a miRNA	Induce cell death	Inhibit tumor growth	Breast cancer	[101]
Proteins	Gelonin	Trigger cell apoptosis	Inhibit tumor growth	Breast cancer	[81]
	Survivin-T34A	Induce cell apoptosis	Inhibit tumor growth	Pancreatic adenocarcinoma	[108]
	Targeting peptides (iRGD, etc.)	Enhance targeting ability to specific tissues	Enhance therapeutic effect and reduce systemic cytotoxicity	Breast cancer and glioblastoma	[109,128]
	CD3 and EGFR (HER2) antibodies	Recruit and activate cytotoxic T cells	Inhibit tumor growth	Breast cancer	[147,148]
Drugs	DOX	Induce cell apoptosis	Inhibit tumor growth	Breast cancer	[109]
	DOX	Anti-angiogenesis	Inhibit tumor growth	Colorectal cancer	[77]
	DOX, t-PA and photosensitizers	Enhance cancer cell death by magnetic targeting	Inhibit tumor growth	Ovarian and prostate cancers	[116]
	miR-21 responded hairpin DNA loaded with DOX	Target-triggered drug delivery and induce cancer cell apoptosis	Inhibit tumor growth	Breast cancer	[41]
	PTX	Inhibit cell proliferation	Inhibit tumor growth	Pancreatic cancer and lung cancer	[56,58]
	PTX and AA-PEG	Target the sigma receptor and inhibit cell proliferation	Suppress tumor growth and pulmonary metastasis	Lung cancer	[111]
	PTX, biotin, and avidin	Increase targeting ability and induce cell apoptosis	Inhibit tumor growth	HCC	[74]
	Pd catalysts and prodrug	Inhibit cell proliferation	Inhibit tumor growth	NSCLC	[112]
	Ganciclovir, CB1954, and TK-NTR	Induce cell apoptosis	Inhibit tumor growth	Breast cancer	[87]
NPs	Magnetic NPs and photosensitizer	Target delivery and enable PDT	Inhibit tumor growth	Ovarian cancer	[115,116]
	SPIONs, CPP, and CTNF-α	Target delivery and induce cell apoptosis	Inhibit tumor growth	Melanoma	[134]
	SPIONs, curcumin, and NRP-1 targeted peptide	Target delivery and inhibit cell proliferation	Inhibit tumor growth	Glioma	[68]

Table 2. Continued.

Cargo types[a)]	Specific substances	Mechanisms	Effects	Cancer types	Ref.
	DOX and SPMNs	Enhance targeting ability and induce cell apoptosis	Inhibit tumor growth	Hepatoma	[54]
	Pt(lau) NPs, HSA, and lecithin	Induce cell apoptosis and suppress cell proliferation	Inhibit tumor growth and metastasis	Breast cancer	[125]
	Nanosensitizer DVDMS	Produce singlet oxygen	Inhibit tumor growth and metastasis	Breast cancer	[117]

[a)]Abbreviations: 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouraci; AA-PEG, aminoethylanisamide-polyethylene glycol; CBSA, cationic bovine serum albumin; CD, cytosine deaminase; CPP, cell-penetrating peptide; CTNF-α, fusion proteins of cell-penetrating peptides and TNF-α; DOX, doxorubicin; DVDMS, sinoporphyrin sodium; HSA, human serum albumin; MDR1, multidrug resistance gene 1; NPs, nanoparticles; PARP-1, poly (ADP-ribose) polymerase-1; Pd, palladium; Pt, platinum; Pt(lau), laurate-functionalized Pt(IV) prodrug; PTX, paclitaxel; QDs, quantum dots; RGD, arginyl-glycyl-aspartic acid; SPIONs, superparamagnetic iron oxide nanoparticles; TK-NTR, thymidine kinase-nitroreductase fusion protein; TMZ, temozolomide; t-PA, tissue-plasminogen activator; UPRT, uracil phosphoribosyltransferase.

into EVs. Compared to liposome delivery, this method apparently reduces the therapeutic dose of siRNAs needed to silence target gene expression.[96]

miRNAs: MiRNAs are packaged in EVs and transmitted between cells to perform biological functions.[97] Researchers have explored the values of EVs in delivering miRNAs for cancer therapy. MSCs and HEK293T cells are two commonly used cell types for producing miRNA-loaded EVs. The delivery of miRNAs (e.g., miR-206, 26a, 122, 126, 146b, 124a, and let-7a) by MSC-derived EVs has been described in osteosarcoma,[98] hepatocellular carcinoma (HCC),[62,99] NSCLC,[100] breast cancer,[101] and glioma and shown promising anti-tumor effects.[102,103] The transfer of miRNA inhibitors (anti-miR-9,[52] anti-miR-214,[104] anti-miR-374[105]) by EVs to cancer cells has also been reported. To improve loading efficiency of specific miRNAs into EVs, Li et al. have fused EV marker protein CD9 with HuR, a RNA binding protein that has high affinity with miR-155. Upon cell transfection and EV production, the fused CD9-HuR successfully enriches miR-155 into EVs.[106] Engineered CD9-HuR EVs can also be used to deliver functional miRNA inhibitor, which provides a novel strategy for improved encapsulation of RNA cargo into EVs.

5.2. Proteins

Delivery of biofunctional enzymes or therapeutic proteins through systemic administration is hindered by protein degradation and poor cellular uptake. EVs have been suggested as ideal delivery vehicles for functional proteins.[107] For example, Aspe et al. have transfected melanoma cells with dominant-negative mutant (Survivin-T34A) to produce Survivin-T34A-contained EVs.[108] Several studies have reported the modification of EVs through anchoring targeting ligands on their membranes. For instance, EVs engineered to express targeting ligands such as iRGD show highly efficient targeting to αV integrin-positive breast cancer cells.[109] In similar, Zhao et al. have utilized plasma membrane vesicles that have high affinity with breast cancer cells that express high level of EGFR.[110] Recently, researchers have developed an EV-based, bio-mimetic NP platform to deliver proteins with high efficiency. Therapeutic proteins are caged in the matrix of metal-organic frameworks by self-assembly and the NPs are camouflaged with EV membrane. This bio-mimetic nanosystem protects cargo proteins from degradation and preferentially delivers proteins to tumor sites.[81]

5.3. Drugs

EV-mediated delivery improves the stability of drugs in the circulation and results in drug accumulation in recipient cells.[54,58,74] Pascucci et al. demonstrated that MSC-derived EVs are able to package and deliver PTX and PTX-containing EVs have a strong anti-proliferative activity on human pancreatic cancer cells. In addition, other cell-derived EVs have also been used for drug delivery.[58] Kim et al. demonstrated that PTX-loaded, macrophage-derived EVs result in more cytotoxicity in P-gp-positive drug resistant MDCK cells than free drug alone.[66] The same group has developed aminoethylanisamide-polyethylene glycol (AA-PEG) modified, PTX-containing EVs, which show high loading capacity and better anti-cancer effect in a mouse model of pulmonary metastatic lung cancer.[111] To find a more suitable source of EVs for drug delivery, Agrawal et al. have used milk-derived EVs to encapsulate PTX and they show that oral injection of PTX-loaded EVs efficiently inhibits tumor growth.[56] To deliver a catalytic cargo into cancer cells, researchers have loaded palladium (Pd) catalysts into cancer cell-derived EVs. Pd-loaded EVs display a preferential tropism for their progenitor cells and perform catalyst prodrug therapy,[112] suggesting that EV-mediated delivery of catalysts into designated cancer cells may offer a new opportunity for targeted therapy. More recently, Belhadj et al. have developed a combined "eat me/don't eat me" strategy to achieve mononuclear phagocyte system (MPS) escape and efficient drug delivery.[113]

5.4. Nanomaterials

The combination of EVs with nanomaterials improves targeting ability and therapeutic efficacy.[114] Qi et al. have developed a dual-functional EV-based superparamagnetic nanoparticle cluster (SPMNs) by anchoring multiple superparamagnetic NPs onto blood-derived EVs through transferrin (Tf)-Tf receptor interaction. This strategy shows enhanced cancer targeting and tumor growth inhibition under the external magnetic field in a murine hepatoma cancer model.[54] To generate vesicles with magnetic and optical responsiveness allowing therapeutic and imaging functions, Silva et al. have developed a macrophage-derived nanovector loaded with citrate-coated magnetic NPs and m-THPC photosensitizer, named theranosomes. This nanovector can be monitored by dual-mode imaging, which

Table 3. Engineered EVs for cancer therapy.

Methods[a)]	EV Sources	EV types	Drugs	Tumour model	Therapeutic outcomes	Ref.
Chemotherapy	Mouse HCC cells	MPs	MTX	HCC mouse model	Inhibit peritoneal tumor growth and prolong survival time without typical side effects	[118]
	Human ovarian cancer cells	MPs	Cisplatin	Ovarian cancer mouse model	Inhibit tumor growth and prolong survival time without typical side effects	[118]
	Human and mouse HCC cells	Exosomes	Porous silicon NPs	HCC mouse model	Inhibit tumor growth by targeting CSCs	[122]
	Human breast cancer cells	EVs	PTX prodrug and CuB loaded nanomicelles	Breast cancer mouse model	Inhibit tumor growth and capture CTCs to suppress cancer metastasis	[123]
	Mouse macrophages and liposomes	Exosome-mimetic NVs	DOX	Osteosarcoma and breast cancer cells	Release drug in acidic condition and enhance toxicity against cancer cells	[124]
	Human fibrosarcoma cells	Exosomes	DOX	Fibrosarcoma mouse model	Target tumor effectively, enhance therapeutic retention, and inhibit tumor growth	[119]
	Mouse macrophages	Exosome-mimetic NVs	DOX	Colon cancer mouse model	Reduce tumor growth to the same extent as 20-fold higher doses of free DOX but without typical side effects	[77]
	RBCs	Exosome-mimetic NVs	DOX	Breast cancer mouse model	Increase tumor accumulation, decrease systematic clearance, and inhibit tumor growth	[80]
	Blood	Exosomes	DOX	Orthotopic glioma mouse model	Inhibit tumor growth and prolong survival time without typical side effects	[121]
	Mouse macrophages	Exosomes	PTX	Lung metastases mouse tumor model	Increase cytotoxicity and inhibit lung metastases	[66]
	Mouse macrophages	Exosomes	NPs composed of a Pt prodrug Pt (lau)	Orthotopic breast cancer with lung metastasis mouse model	Inhibit orthotopic and metastatic tumor growth	[125]
Targeted chemotherapy	Mouse macrophages	AA-PEG modified exosomes	PTX	Lung metastases mouse tumor model	Inhibit lung metastases and prolong survival time	[111]
	Mouse imDCss	iRGD modified exosomes	DOX	Breast cancer mouse model	Inhibit tumor growth without overt toxicity	[109]
	Macrophages	c-Met binding peptide modified exosomes	DOX-preloaded PLGA NPs	Breast cancer mouse model	Possess immune evading ability, target tumor effectively, increase tumor accumulation, and inhibit tumor growth	[129]
	HEK293T cells	lipHA-modified EVs	DOX	MDR breast cancer mouse model	Increase tumor accumulation, drug sensitivity, and inhibit tumor growth	[120]
	Human embryonic stem cells	c(RGDyK) modified exosomes	PTX	Glioblastoma mouse model	Penetrate the BBB, inhibit tumor growth, and prolong survival time	[128]
	HEK293T cells	Anti-HER2 affibody modified liposome-like NVs	DOX	HER2-overexpressing breast cancer mouse model	Inhibit tumor growth	[130]
	HEK293T cells	hEGF affibody modified liposome-like NVs	ICG	Breast cancer mouse model	Increase PTT effect and inhibit tumor growth	[130]
	Plasma	CC8 modified EV-like vesicles	Imperialine	NSCLC	Increase tumor accumulation and inhibit tumor growth with reduced systemic toxicity	[131]
	HUVECs	Biotin and avidin modified exosomes	PTX	HCC mouse model	Inhibit tumor growth	[74]
	Mouse macrophages	Exosome-like NVs	DM4	Lung metastatic breast cancer mouse model	Inhibit lung metastasis	[132]
	Human colorectal cancer cells	A33 antibody modified exosomes	DOX	Colorectal cancer mouse model	Inhibit tumor growth and prolong survival time with reduced cardiotoxicity	[133]
	Blood	SMNC-modified exosomes	DOX	HCC mouse model	Inhibit tumor growth	[54]

Table 3. Continued.

Methods[a)]	EV Sources	EV types	Drugs	Tumour model	Therapeutic outcomes	Ref.
Targeted/ combined gene therapy	HEK293T cells	PSMA or EGFR aptamer, folate modified EVs	Survivin siRNA	Prostate, breast cancer mouse model, and colorectal cancer model	Inhibit tumor growth	[70]
	RBCs	EVs	miR-125b antagonized ASOs	Breast cancer and acute myelocytic leukemia mouse model	Inhibit tumor growth without observable cytotoxicity	[127]
	Human ovarian cancer cells	Exosomes	PARP-1 CRISPR/Cas9 plasmids	Ovarian cancer model	Induce apoptosis, enhance chemosensitivity, and inhibit tumor growth	[50]
	Lipid nanogel	Hybrid NVs coated with exosome membrane	PTX and MDR1 siRNA	Highly drug-resistant ovarian cancer mouse model	Inhibit tumor growth	[135]
	HEK293T cells	Exosomes	anti-miR-214	Cisplatin-resistant gastric cancer mouse model	Enhance chemosensitivity and inhibit tumor growth	[104]
	HEK293T cells	HER2-binding affibody, LAMP2, and GFP modified exosomes	5-FU and miR-21 inhibitor oligonucleotide	5-FU resistant colon cancer mouse model	Enhance chemosensitivity and inhibit tumor growth	[136]
	Mouse M1 macrophages	QDs modified exosomes	DOX and miR21-responded hairpin DNA	Breast cancer mouse model	Inhibit tumor growth	[41]
	Blood	Exosomes	Photosensitizer, nuclear translocation peptide	Breast cancer mouse model	Inhibit tumor growth with minimized systemic toxicity	[139]
	HEK293T cells	IL3 modified exosomes	Imatinib and BCR-ABL siRNA	Chronic myeloid leukemia mouse model	Enhance drug sensitivity and inhibit tumor growth	[161]
Targeted/ combined phototherapy	Urine from gastric cancer patients	Exosomes	PMA/Au-BSA@Ce6 nanovehicles	Gastric cancer mouse model	Enhance penetration and retention, inhibit tumor growth	[55]
	Human macrophages	Nanovectors	Citrate-coated iron oxide NPs and the m-THPC photosensitizer	Ovarian cancer mouse model	Inhibit tumor growth	[115]
	Human breast cancer cells	RGD modified exosomes	TAT peptide modified V_2C QDs	Breast cancer mouse model	Enter into the nucleus to achieve low-temperature PTT with efficient tumor destruction	[140]
	Human macrophages	RGD modified exosomes	DOX and FA-AuNR	Cervical cancer mouse model	Inhibit tumor growth	[138]
	Mouse macrophages	NRP-1 targeted peptide modified exosomes	Curcumin and SPIONs	Orthotopic glioma mouse model	Provide good results for targeted imaging and therapy, penetrate the BBB and inhibit tumor growth	[68]
	Mouse HCC cells	MPs	Bi_2Se_3 nanodots and DOX	HCC mouse model	Inhibit tumor growth	[141]
Targeted/ combined immunotherapy	K562 cells	Exosomes	HLA-A2, CD80, CD83 and CD137L	Not determined	Activate $CD8^+$ T cells	[143]
	Embryonic stem cells	Exosomes	GM-CSF	Lewis lung carcinoma mouse model	Inhibit tumor growth	[51]
	Tumor cells	Exosomes	Functional N-terminus of HMGN1	HCC, pancreatic cancer, and breast cancer mouse models	Elicit long-lasting antitumor immunity and inhibit tumor growth	[145]
	NK cells	Exosomes	miRNA loaded NPs	Breast cancer and NB mouse models	Target tumor effectively and inhibit tumor growth	[146]
	Expi293F cells	Exosomes	CD3 and EGFR antibodies	Triple negative breast cancer mouse model	Induce cross-linking of T cells and $EGFR^+$ cancer cells	[147]
	Expi293F cells	Exosomes	CD3 and HER2 antibodies	HCC mouse model	Redirect cytotoxic T cells toward attacking $HER2^+$ cancer cells	[148]
	Tumor cells	Irradiated tumor cell-released MPs	Not determined	Malignant pleural effusion mouse model	Repolarize tumor-associated macrophages and induce immunogenic death	[149]

Table 3. Continued.

Methods[a)]	EV Sources	EV types	Drugs	Tumour model	Therapeutic outcomes	Ref.
	Tumor cells	Radiation-induced small EVs	Tumor antigens and heat-shock proteins	Hepatoma and breast cancer mouse models	Trigger antitumor immunity and inhibit primary tumor and lung metastasis	[150]
	Human blood-derived leukocytes	Exosomes	Melanoma tumor peptides	Hepatoma and breast cancer mouse models	Enhance antigen presentation ability of exosomes and activate T cells	[144]
	Mouse melanoma cells	CpG DNA-modified exosomes	SAV-LA fusion protein	Melanoma mouse model	Inhibit tumor growth	[162]

[a)]Abbreviations: 5-FU, 5-fluorouraci; AA-PEG, aminoethylanisamide-polyethylene glycol; AuNR, gold nanorods; BBB, blood-brain barrier; CC8, integrin $\alpha 3\beta 1$-binding peptide cNGQGEQc; Ce6, chlorine6; CuB, cucurbitacin B; DM4, cytotoxic soravtansine; FA, folic acid; hEGF, human epidermal growth factor; HLA, human leukocyte antigen; HMGN1, high mobility group nucleosome binding domain protein 1; HUVECs, human umbilical vein endothelial cells; lipHA, lipid mimetic chains-grafted HA; ICG, indocyanine green; MDR1, multidrug resistance gene 1; m-THPC, m-tetra hydroxyphenyl chlorin; MTX, methotrexate; NPs, nanoparticles; PLGA, poly(lactic-co-glycolic acid); PMA, amphiphilic polymer; PSMA, prostate specific membrane antigen; Pt(lau), laurate-functionalized Pt(IV) prodrug; Pt, platinum; QDs, quantum dots; RGD, arginyl-glycyl-aspartic acid; SMNC, superparamagnetic nanoparticle cluster; SPIONs, superparamagnetic iron oxide nanoparticles; V_2C-TAT, vanadium carbide quantum dots modified with TAT peptides.

is valuable in both cancer diagnosis and treatment.[115] They have further enclosed iron oxide NPs and different therapeutic agents into macrophage-derived EVs, which can be manipulated by magnetic force for targeted delivery of drugs.[116] Liu et al. have designed a tumor cell-derived EV-based nanosonosensitizer delivery system, in which high sono-activatable sinoporphyrin sodium (DVDMS) is loaded onto EVs. This system shows targeted accumulation and enhanced DVDMS release under ultrasound exposure, suggesting that EVs are promising delivery vehicles for nanosensitizers.[117]

6. Engineered EVs for Cancer Therapy

EV-based drug delivery has been assessed in many pre-clinical studies and achieved encouraging results. Compared with their free counterparts, therapeutic molecules and chemotherapeutic drugs encapsulated in EVs are more stable in the circulation, ease to cross physiological barriers, possess superior bioactivity, and show low systemic toxicity. Several ongoing clinical trials are investigating the ability of EVs as delivery vehicles for therapeutic cargos. Some commercial companies such as Codiak BioSciences, Therapeutics Solutions International, and Carmine Therapeutics are developing EV-based cancer therapeutics, suggesting a promising clinical perspective of EVs. To further improve the applicability of EVs in precision cancer therapy, researchers have developed novel strategies to produce EVs with high purity and yield and engineered EVs as drug delivery platforms with high loading efficiency, tumor-targeting ability, and controllable drug release capability (**Table 3**).

6.1. EVs as Delivery Vehicles for Chemotherapeutic Drugs

6.1.1. Engineered EVs for Delivery of Chemotherapeutic Drugs

EV-mediated delivery improves the accumulation of chemotherapeutic drugs at tumor sites and reduces the risk of systemic toxicity.[118,119] As a result of the unique integrin expression pattern, EVs from cancer cells preferentially target their parental cancer cells in vitro and home to their original tumor tissues after systemical injection in vivo.[119] EVs-mediated drug delivery also shows high efficacy in the treatment of cancer cells with multi-drug resistance (MDR). Liu et al. demonstrated that HA-functionalized, lipid mimetic chains-grafted HA (lipHA)-modified EVs from HEK293T cells (lipHA-hEVs) enhance the accumulation of DOX in drug resistant breast cancer cells by CD44-mediated cancer-specific targeting and P-gp inhibition.[120] LipHA-hEVs show deep penetration into tumor tissues and effective delivery of DOX into local tumor sites, suggesting that lipHA-hEVs are promising drug carriers for overcoming cancer MDR.

EV-mediated drug delivery can cross blood-brain barrier but the capacity of natural EVs is limited. Bai et al. have developed an EV-based transportation system by using focused ultrasound (FUS), which promotes the BBB-crossing ability of EVs and enhances drug accumulation in glioma cells. The combination of DOX-loaded EVs with FUS treatment results in a remarkable inhibition of tumor growth and an extended survival time with no observable side effects in mouse models, suggesting that it is a potent strategy for brain cancer therapeutics.[121]

6.1.2. Bio-mimetic EVs for the delivery of Chemotherapeutic Drugs

Cell-derived NVs have similar physiochemical properties, such as morphology, size, proteomic and lipidomic profiles, and in vivo biodistribution, to that of natural EVs. The most attractive feature of NVs is that they could be produced with high yield, homogeneity, and purity by a simple preparation procedure. To overcome the relatively low quantities and inconvenient purification of EVs released by mammalian cells, Jang et al. have generated DOX-loaded EV-mimetic NVs from monocytes/macrophages by serial extrusion. DOX-loaded NVs traffic to tumor tissues after in vivo administration and reduce tumor growth without observable adverse effects.[77] Similarly, Yong et al. have developed EV-mimetic porous silicon nanoparticles (PSiNPs) exocytosed from tumor cells as drug carriers. DOX-loaded PSiNPs are of high tumor accumulation, extravasation,

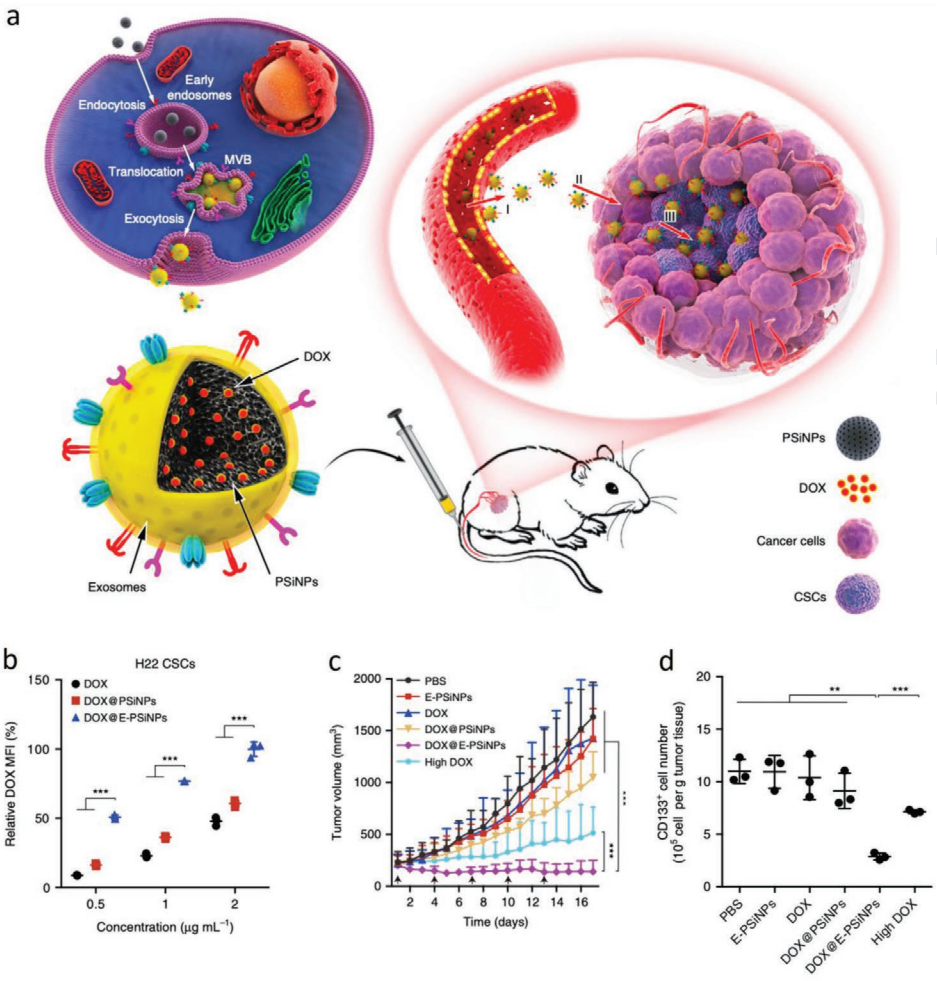

Figure 6. EV-biomimetic porous silicon NPs as efficient drug carriers for targeted chemotherapy. a) Schematic illustration of the preparation and the antitumor effect of DOX@exosome-PSiNPs. DOX@PSiNPs are endocytosed into cancer cells after incubation, then exocytosed into extracellular space. DOX@E-PSiNPs show efficient cancer cells and CSCs killing activities after intravenous injection into tumor-bearing mice due to high tumor accumulation, extravasation, and penetration abilities. b) Relative DOX mean fluorescence intensity of H22 CSCs tumor spheroids treated with free DOX, DOX@PSiNPs or DOX@exosome-PSiNPs at different DOX concentrations. c) Time-dependent tumor growth curves of H22 tumor-bearing mice after different treatments. d) Number of CD133-postive cells in tumor tissues at the end of tumor growth inhibition experiments. Abbreviations: MVB, multivesicular bodies; DOX, doxorubicin; PSiNPs, porous silicon nanoparticles; CSCs, cancer stem cells. Reproduced under terms of the CC-BY license.[122] Copyright 2019, Springer Nature.

and penetration abilities and exhibit enhanced antitumor and cancer stem cell (CSC)-killing activities in multiple cancer models (**Figure 6**).[122] More recently, Wang et al. have co-encapsulated PTX prodrug PTX-S-LA and cucurbitacin B (CuB) into nanomicelles and then coated them with EV membrane via extrusion. This EV-mimetic prodrug nanoplatform targets primary tumor and capture circulating tumor cells (CTCs) to suppress cancer metastasis, which provides a novel platform for intercellular controlled release of therapeutic agents.[123]

Hybrid EVs combine the benefits of synthetic NPs with the intrinsic advantages of EVs, providing a safe and efficient system for drug delivery. Rayamajhi et al. have engineered macrophage-derived EVs with synthetic liposomes to become a refined bio-mimetic hybrid EVs for the delivery of DOX. They demonstrated that drug-loaded hybrid EVs show enhanced toxicity against cancer cells and pH-sensitive drug release in acidic condition, which benefits drug delivery to acidic cancer environment for targeted therapy.[124] By using a similar strategy, Zhang et al. have developed bio-mimetic artificial chimeric EVs (ACEs) as drug delivery nanovehicles by integrating cell membrane proteins from RBCs and cancer cells into synthetic phospholipid bilayers. DOX-loaded ACEs obtain increased tumor accumulation, reduced liver retention, and improved antitumor effects compared to DOX-loaded liposomes (**Figure 7**).[80] Moreover, Xiong et al. have designed bio-inspired EVs that could encapsulate Pt anti-cancer drugs for the therapy of orthotopic breast cancer with lung metastasis.[125] Taken together, these findings suggest that the use of bio-inspired or bio-mimetic EVs to deliver chemotherapeutic drugs further expands the applicability of EVs in cancer therapy and achieves promising therapeutic effects in primary, metastatic, and drug-resistant cancers.

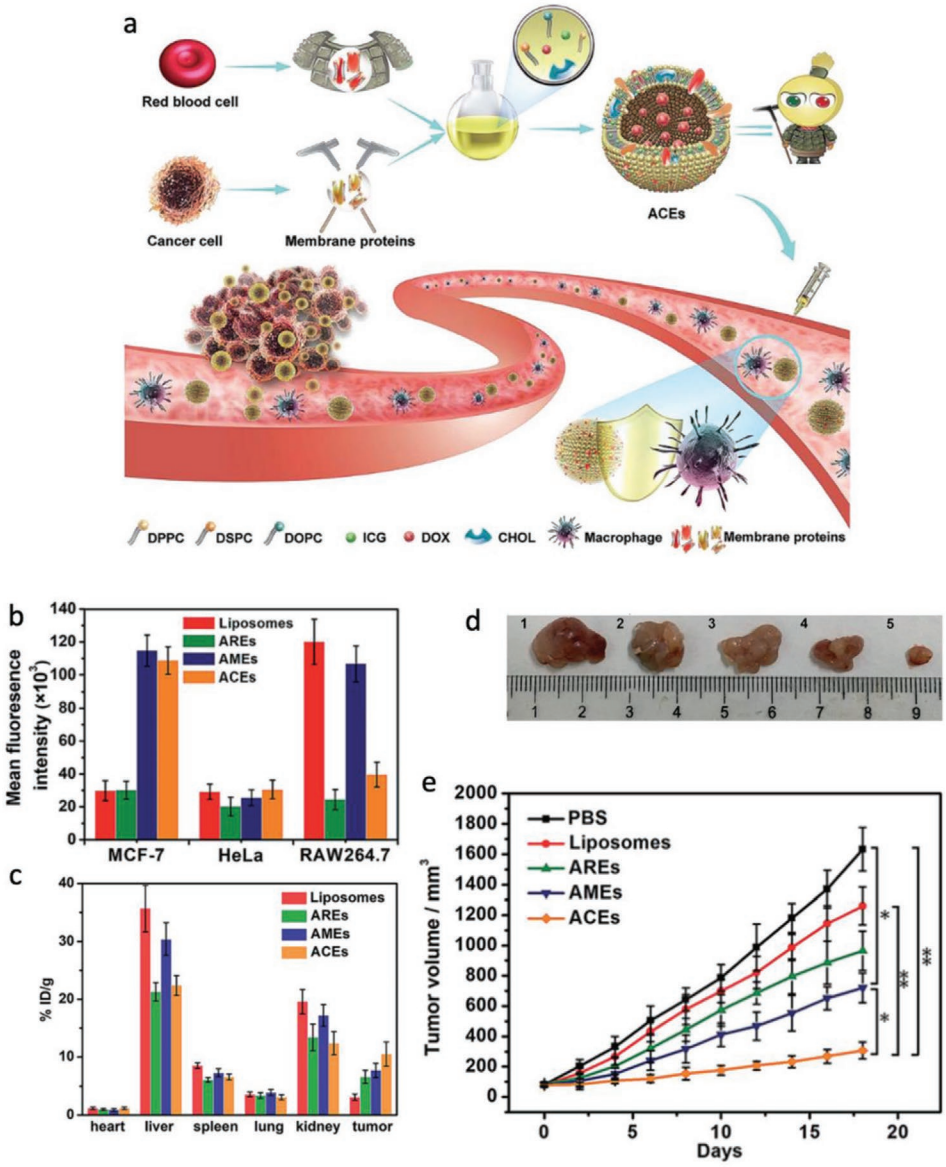

Figure 7. Bio-mimetic ACEs as drug delivery nanovehicles for targeted cancer therapy. a) Schematic illustration of the design of biomimetic ACEs for anti-phagocytosis and targeted cancer therapy. b) Averaged DOX fluorescence intensity of MCF-7 cells, HeLa cells, and RAW264.7 cells after incubation of ACEs for 2 h. c) Biodistribution of DOX at 24 h after intravenous administration of ACEs to breast tumor-bearing nude mice. d) Tumor tissues obtained from tumor-bearing mice after treatment with PBS, liposomes, AREs, AMEs, and ACEs. e) Tumor growth curves of different groups after treatments. Abbreviations: ACEs, artificial chimeric EVs; AMEs, artificial MCF-7 cell EVs; AREs, artificial RBC EVs; CHOL, cholesterol; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOX, doxorubicin; DPPC, 1,2-dihexadecanoyl-rac-glycero-3-phosphocholine; DSPC, distearoyl phosphatidylcholine; ICG, indocyanine green. Reproduced with permission.[80] Copyright 2019, The Royal Society of Chemistry.

6.2. EVs as Delivery Vehicles for Therapeutic Nucleic Acids

Gene therapy targets the diseased genome with high specificity and great flexibility. There is still a lack of safe and effective strategies for the delivery of therapeutic RNAs to most primary tumor tissues. A pioneer study from Kamerkar et al. has shown that iExosomes (EVs engineered to carry $Kras^{G12D}$ siRNAs or shRNAs) escape from phagocytosis by monocytes/macrophages through CD47-mediated "don't eat me" signal. Treatment with iExosomes inhibits pancreatic cancer growth and metastasis in mouse models and significantly increases overall survival, which suggests a new approach for pancreatic cancer therapy.[92] Subsequently, the same group has established a bioreactor-based, large-scale production of clinical-grade iExosomes employing good manufacturing practice (GMP) standards,[126] which has been recently approved by FDA to enter into phase I clinical trial.

Human RBCs represent an ideal source for large-scale EV production since they are readily available in blood banks and devoid of DNA. Usman et al. have developed an interesting strategy to generate large-scale amounts of RBC-derived EVs (RBCEVs) for the delivery of RNA drugs such as antisense

oligonucleotides (ASOs) that antagonize oncogenic miR-125b, which significantly inhibits cancer growth with no observable cytotoxicity.[127] RBCEV platform is also useful for CRISPR/Cas9 system-mediated genome editing. Treatment of leukemia cells with RBCEVs loaded with Cas9 mRNA and 125b-gRNA results in almost complete reduction of miR-125b expression. These findings suggest that RBCEVs is an efficient and versatile delivery system for therapeutic RNAs.

6.3. EVs as Nanocarriers for Targeted and Combined Therapy

6.3.1. Engineered EVs as Delivery Vehicles for Targeted Chemotherapy

Targeted delivery of chemotherapeutic drugs to tumors remains a major challenge for precision medicine. The intrinsic targeting capacity of natural EVs is still unsatisfactory. To this end, increasing studies have explored the potential use of EVs for targeted therapy by specific cargo loading and surface modification. The decoration of EVs with functional ligands is a commonly used strategy to endow them with increased stability in the bloodstream, tropism toward tumor sites, and intracellular delivery of drugs. For example, Tian et al. have modified mouse immature dendritic cells (imDCs) with iRGD peptide and used their derived EVs as drug carriers to target αV integrin-positive breast cancer cells.[109] In a recent study by Zhu et al., they demonstrated that c(RGDyK)-modified, PTX-loaded embryonic stem cell-derived EVs penetrate blood-brain barrier to decrease glioblastoma growth more effectively than free PTX and their unmodified counterparts.[128] Furthermore, Li et al. demonstrated that macrophage-derived EVs loaded with DOX-preloaded PLGA nanoparticles and decorated with a c-Met binding peptide possess excellent immune evading ability and exhibit enhanced tumor-targeting capability.[129] These findings suggest that proper modification of EVs can improve their targeting ability and therapeutic efficacy.

Using bio-mimetic synthetic strategy, Zhang et al. have prepared bio-functionalized liposome-like NVs (BLNs) that can display targeting ligands (such as EGF and anti-HER2 affibody) and encapsulate anti-cancer drugs (such as DOX). Treatment with anti-HER2 affibody-displayed, DOX-loaded BLNs exhibits much better anti-tumor effects than clinically approved liposomal DOX in HER2-positive mouse breast cancer models.[130] Similarly, Lin et al. have encapsulated imperialine into plasma derived EV-like vesicles (ELVs) followed by attaching integrin $\alpha3\beta1$-binding peptide cNGQGEQc (CC8) to their surface for NSCLC cell-targeting chemotherapy. Imperialine-loaded CC8-ELVs show increased drug concentration in tumors and strong anti-tumor activity with low systemic toxicity.[131]

To specifically deliver drugs to metastatic tumors, Cao et al. have constructed EV-like NVs that are loaded with soravtansine (a prodrug of DM4) and anchored with legumain-specific propeptide of melittin (legM) on their membrane.[132] In response to internalization by metastatic breast cancer cells and activation by legumain protease, the prodrug encapsulated in primary NVs convert to facilitate cell death. Then, the damaged cells generate secondary NVs to further release free drug and destroy neighboring cancer cells. This smart drug delivery system displays improved targeting ability for lung metastatic lesions and remarkably inhibits lung metastasis in vivo, providing a new delivery vehicle with controllable drug release for metastatic lung cancer therapy.

The combination of EVs with superparamagnetic iron oxide nanoparticles (SPIONs) further increases their targeting ability.[133] Using this strategy, Qi et al. have developed a blood EV-based SPMNs as targeted drug delivery vehicle for cancer therapy, which can be rapidly separated from blood and exhibit strong responsiveness to external magnetic field to target cancer cells.[54] Jia et al. have loaded SPIONs and curcumin into EVs and then conjugated EV membrane with neuropilin-1 targeted peptide. SPION-mediated magnetic flow hyperthermia and curcumin-mediated therapy show a potent synergistic antitumor effect, which achieves dual-targeting abilities and potent therapeutic effects.[68] To improve the therapeutic effect of TNF-α, Rao et al. have developed cell-penetrating peptides (CPP) and TNF-α (CTNF-α)-anchored EVs coupled with SPIONs, which enhances cancer targeting and efficiently inhibit melanoma growth (**Figure 8**).[134]

6.3.2. Engineered EVs as Delivery Systems for Gene/Drug Synergistic Therapy

Drug resistance is recognized as the main cause of chemotherapy failure. The efficient inhibition of highly drug-resistant tumors still remains a big challenge. The strategy of co-delivering functional small RNAs and anti-cancer drugs by EVs suggests a potential approach to reverse drug resistance. Wang et al. have designed a bio-mimetic lipid/dextran hybrid nanocarrier loaded with MDR1-siRNA and PTX. The knockdown of MDR1 by siRNA promotes the accumulation of PTX in cells, thus achieving an efficient inhibition of highly resistant cancer cells.[135]

To improve the targeting ability of EV-based gene manipulation, Fan et al. have constructed a target-triggered drug delivery system by engineering EV-like vesicles of M1 macrophages with miR-21-responded hairpin DNA and loading them with DOX. The engineered M1mv showed reinforced specificity of drug release and stronger anti-tumor effects.[41] Using the similar strategy, Liang et al. have used anti-HER2 affibody-displayed EVs which encapsulate miR-21 inhibitor and 5-FU to reverse drug resistance in colon cancer.[136]

To co-deliver drugs and nucleic acids to tumor cells precisely, Zhan et al. have recently constructed a nanoplatform where DOX and cholesterol-modified miR-21 inhibitor are loaded into blood EVs, while SPIONs and endosomolytic peptides L17E are attached to EV membrane to improve endosome escape ability and tumor accumulation. This system exhibits efficient inhibition of tumor growth in vivo, demonstrating the potential of the "multi-in-one" nanoplatform in cancer therapy (**Figure 9**).[137]

6.3.3. Engineered EVs as Delivery Systems for Cancer-Targeted Photothermal Therapy

Photothermal therapy (PTT) and photodynamic therapy (PDT) are two hyperthermia therapeutic methods that usually

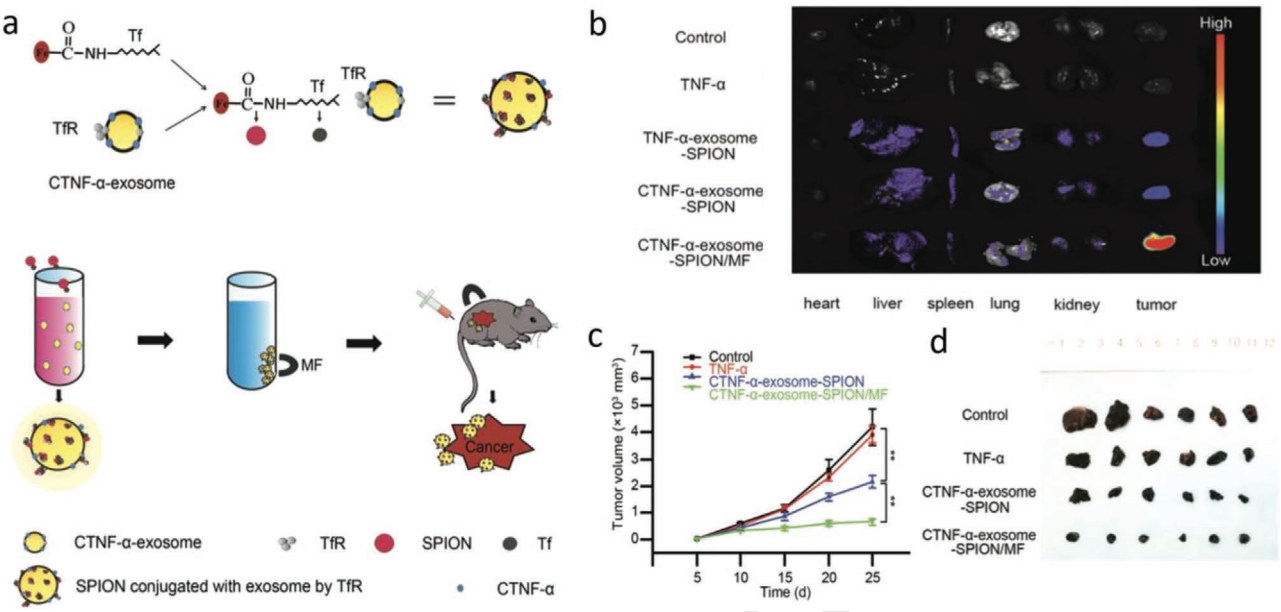

Figure 8. SPIONs-decorated EVs targetedly deliver TNF-α to cancer cell membrane and inhibit tumor growth. a) Schematic illustration for the preparation and targeting ability of the CTNF-α-EV-SPION. b) NIRF optical images of tumors and major organs after intravenous injection in the absence or presence of external magnetic field. c) Tumor growth monitored at different time-points and volume calculation. d) Tumor tissues obtained from tumor-bearing mice after different treatments. Abbreviations: CTNF-α, fusion proteins of cell-penetrating peptides and TNF-α; MF, magnetic field; NIRF, near-infrared fluorescence; SPION, superparamagnetic iron oxide nanoparticle; Tf, transferrin; TfR, transferrin receptor; TNF-α, tumor necrosis factor-α. Reproduced with permission.[134] Copyright 2019, The Royal Society of Chemistry.

employ light-absorbing agents to kill cancer cells under laser irradiation. To achieve effective chemo-photothermal anti-tumor treatment, Wang et al. have designed a photoresponsive EV system that can effectively accumulate at tumor sites via dual ligand (FA and RGD)-mediated endocytosis (**Figure 10**).[138] The localized hyperthermia induced by the conjugated gold nanorods (AuNR) under near-infrared irradiation (NIR) impacts the permeability of EV membrane to enhance drug release, thus inhibiting tumor relapse in a programmable manner.

Following a dual-stage light PDT strategy, Cheng et al. have engineered EVs with chimeric peptide (ChiP-Exo) to exert their activities by sequentially destroying the plasma membrane and nucleus of tumor cells. This strategy exhibits an elevated tumor targeting delivery and a greater tumor growth inhibition with minimized systemic toxicity, providing a new tool for precise tumor therapy.[139] To achieve effective tumor killing, Cao et al. have applied a combined strategy that engineers EV with vanadium carbide quantum dots (V_2C QDs) and photothermal agents for low-temperature nucleus-targeted PTT in NIR-II region (**Figure 11**).[140] The V_2C QDs are modified with TAT (transactivator of transcription) peptides and packaged into EVs followed by RGD modification. The resulting NPs exhibit good biocompatibility, long circulation time, and endosomal escape ability, and thus enter into the nucleus to perform low-temperature PTT with improved anti-tumor effect.

To solve the problems of EVs for clinical application such as unsatisfied yield, complicated labeling procedure, and low drug loading efficiency, Pan et al. have obtained high-purity urinary EVs from gastric cancer patients and efficiently loaded them with multi-functionalized PMA/Au-BSA@Ce6 NPs via electroporation. The engineered nanovehicles are efficiently internalized into cancer cells due to reduced endocytosis of macrophages and prolonged blood retention time. In response to laser irradiation and acidic condition, the engineered nanovehicles are broken and tremendous NPs are released inside, producing considerable singlet oxygen and thus inhibiting tumor cell growth.[55] To achieve comprehensive therapy, Wang et al. have pre-loaded Bi_2Se_3 nanodots and DOX into tumor cells via electroporation and obtained MPs (Bi_2Se_3/DOX@MPs) through irradiation-induced budding. Bi_2Se_3/DOX@MPs show synergistic antitumor efficacy by combining PTT with low-dose chemotherapy.[141] These findings suggest that engineered EVs provide an efficient and safe delivery system for targeted PTT of cancers.

6.4. EVs as Nanocarriers for Immunotherapy

Engineered EVs have been used for improved cancer immunotherapy.[142] For instance, Sueon et al. have constructed human leukemia K562 cells that stably express human leukocyte antigen and various costimulatory molecules to act as artificial antigen presenting cell. EVs from modified K562 cells activate $CD8^+$ T cells more strongly than their unmodified counterparts.[143] Zhao et al. have prepared EVs from murine APCs that have been decorated with tumor peptides on the surface and developed a microfluidic platform for automated and rapid purification. Engineered EVs induce significant higher antigen-specific $CD8^+$ T cell proliferation than native, non-engineered counterparts.[144]

In addition, Kavitha et al. suggest that EVs from GM-CSF-expressing embryonic stem cells have immunogenicity similar to tumor EVs and GM-CSF enhances their immune response

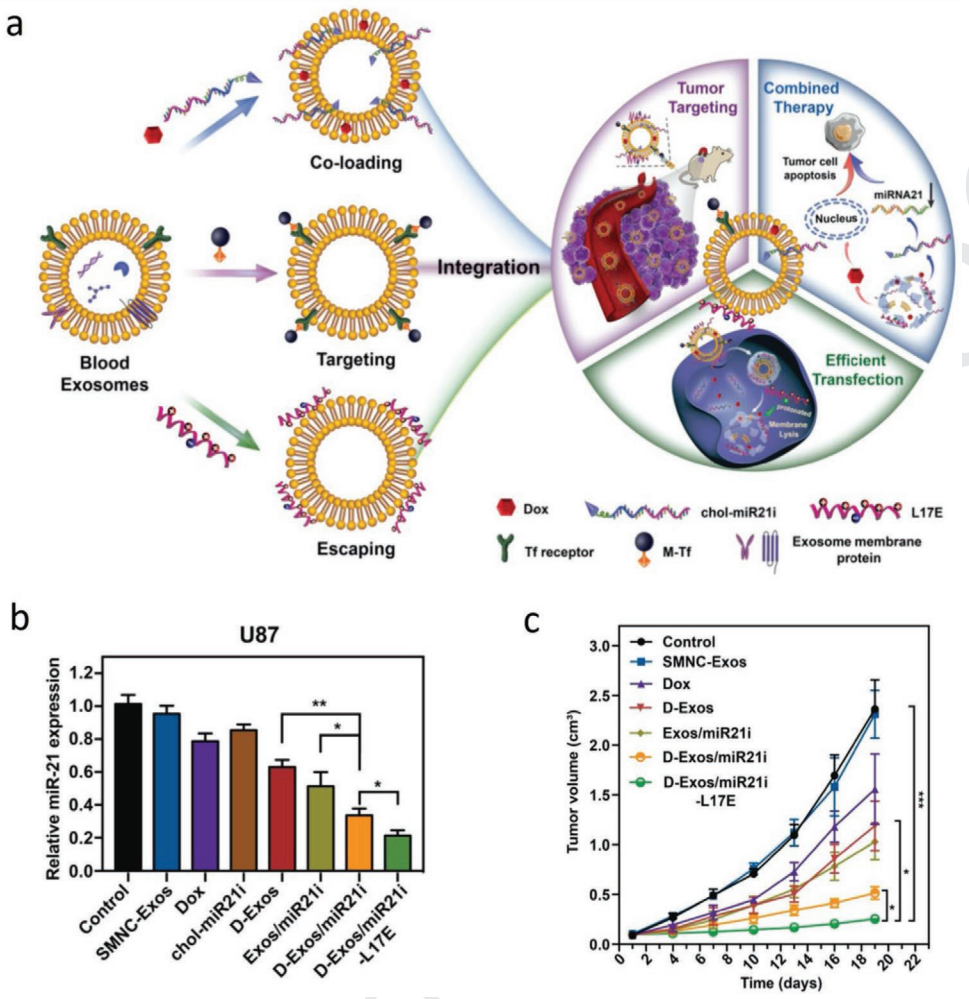

Figure 9. Engineering blood EVs for targeted gene/drug synergistic therapy. a) Schematic illustration of the design and combined antitumor effects of blood EV-based "multi-in-one" nanosystem. b) Relative expression levels of miR-21 in U87 cells after treatment with different samples. c) Tumor growth curves of U87 tumor-bearing mice after different treatments. Abbreviations: Chol-miR21i, cholesterol-modified miRNA21 inhibitor; DOX, doxorubicin; Tf, transferrin. Reproduced under terms of the CC-BY license.[137] Copyright 2020, Ivyspring International Publisher.

ability, which shows considerable anti-tumor effects in mice and thus can be developed as tumor preventative vaccines.[51] Chimeric antigen receptor T-cell immunotherapy (CAR-T therapy) is a promising novel cancer therapy but show unique toxicities. Fu et al. suggest that CAR-T cell-derived EVs that carry CAR on their surface can directly attack tumor cells in a relatively safe way compared to CAR-T therapy. Moreover, CAR EVs do not express immune checkpoint molecule programmed cell death protein 1 (PD1), which maintains their anti-tumor effect not to be compromised by PD-L1 in tumor cells.[53]

To potentiate DC immunogenicity and improve vaccine efficiency, Zuo et al. have constructed TEXs that are decorated with the functional domain of high-mobility group nucleosome binding domain 1 (HMGN1) via an anchor peptide.[145] DCs pulsed by the engineered TEXs show increased homing ability to lymphoid tissues and enhance memory T cell response, resulting in long-term anti-tumor immunity and tumor inhibition effect in tumor-bearing mouse models. Moreover, Wang et al. suggest a cocktail strategy based on NK cell-derived EVs. Bio-mimetic core-shell NPs are self-assembled with a dendrimer core loading therapeutic miRNA such as let-7a and a hydrophilic shell of NK cell-derived EVs. The resulting NN cocktail shows highly efficient targeting and therapeutic miRNA delivery to NB cells in vivo, leading to dual tumor growth inhibition effects.[146]

To reinforce the immunogenicity of EVs, Cheng et al. have developed synthetic antibodies-targeted EVs (SMART-EVs) by genetically displaying two distinct types of antibodies on EV membrane, including monoclonal antibodies specific for T cell CD3 and cancer cell-associated EGFR. SMART-EVs act as an artificial cellular immunity controller to redirect immune effector cells and show potent anti-cancer immunity against EGFR-positive breast cancer cells.[147] The same group has also generated SMART-EVs that express anti-human CD3 and anti-human HER2 antibodies.[148] The resulting SMART-EVs recruit human T cells to kill HER2-positive breast cancer cells, indicating that this strategy is useful for targeted cancer immunotherapy.

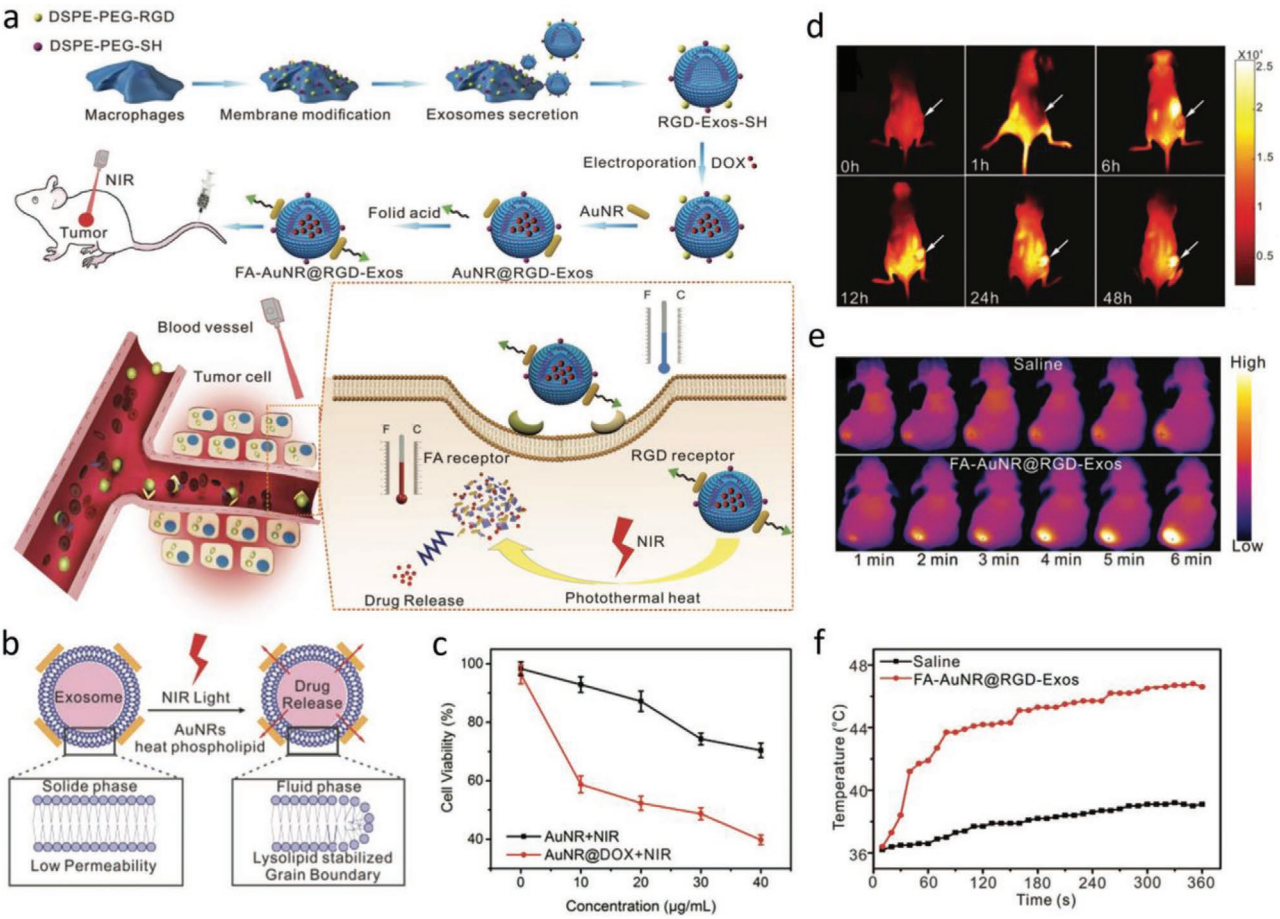

Figure 10. Photoresponsive FA-AuNR@RGD-Exos for targeted PTT. a) Schematic illustration of the design of FA-AuNR@RGD-DOX-Exos and their antitumor effect under NIR irradiation. b) Schematic illustration of drug release from the AuNR@Exos under NIR irradiation. c) Cytotoxic effects of the AuNRs and AuNR@DOX-Exos with NIR laser irradiation on HeLa cells. d) Overall fluorescence imaging of HeLa xenograft nude mice after the injection of FA-AuNR@RGD-DOX-Exos. In vivo NIR fluorescence images were taken before injection and at 1, 6, 12, 24, and 48 h post-injection. e) Thermal imaging and f) photothermal heating curves of FA-AuNR@ RGD-DOX-Exos in mouse tumors under NIR irradiation. Abbreviations: AuNR, gold nanorods; DOX, doxorubicin; DSPE, 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine; Exos, exosomes; FA, folic acid; NIR, near infrared; PEG, polyethylene glycol; RGD, arginyl-glycyl-aspartic acid. Reproduced with permission.[138] Copyright 2018, Wiley-VCH.

In addition to EVs from genetically engineered cells, researchers have used EVs secreted by tumor cells after irradiation for cancer immunotherapy. A recent study shows that irradiated tumor cell-derived MPs (RT-MPs) induce immunogenic death through ferroptosis. RT-MPs re-polarize M2 to M1 TAMs to exert antitumor response.[149] Another study also shows that irradiated tumor cells have enhanced immunogenicity by upregulating the expression of tumor-associated antigen and damage associated molecular patterns in EVs.[150] These results suggest that EVs from artificially manipulated tumor cells may elicit stronger immune response and better immunotherapy effects.

7. Conclusions

EVs have shown great value in drug delivery due to their high biocompatibility and strong bioactivity. In the past decade, great efforts have been made to the studies of EV biology, isolation and detection, therapeutic use, modification, and engineering.[17,151] Compared with traditional nanovectors, EVs are natural nanomaterials that can be used as efficient and safe delivery vehicles. As a result of the unique membrane-enclosed structure and surface protein expression pattern, EVs are able to protect their cargos from degradation and escape from the clearance by host immune system. In addition, the inherent targeting ability from their parental cells endows EVs with the potential of targeted therapy. Compared with free formulation of drugs, EV-mediated delivery shows enhanced capacity to penetrate through tumor blood vessels and across biological barriers to accumulate at tumor sites, which greatly improve their therapeutic efficacy.[14,39] Moreover, the biocompatible properties of EVs also reduce the risk of systemic toxicity that is commonly observed in other nanomaterials.

As a result of these advantages, therapeutic applications of EVs as drug delivery NVs have been explored in numerous pre-clinical studies and several clinical trials. The delivery of therapeutic RNAs and proteins, drugs, and NPs by EVs has been widely reported and shown promising results in various cancers.[152] More importantly, researchers have used many advanced nanotechnologies to modify, engineer, and design

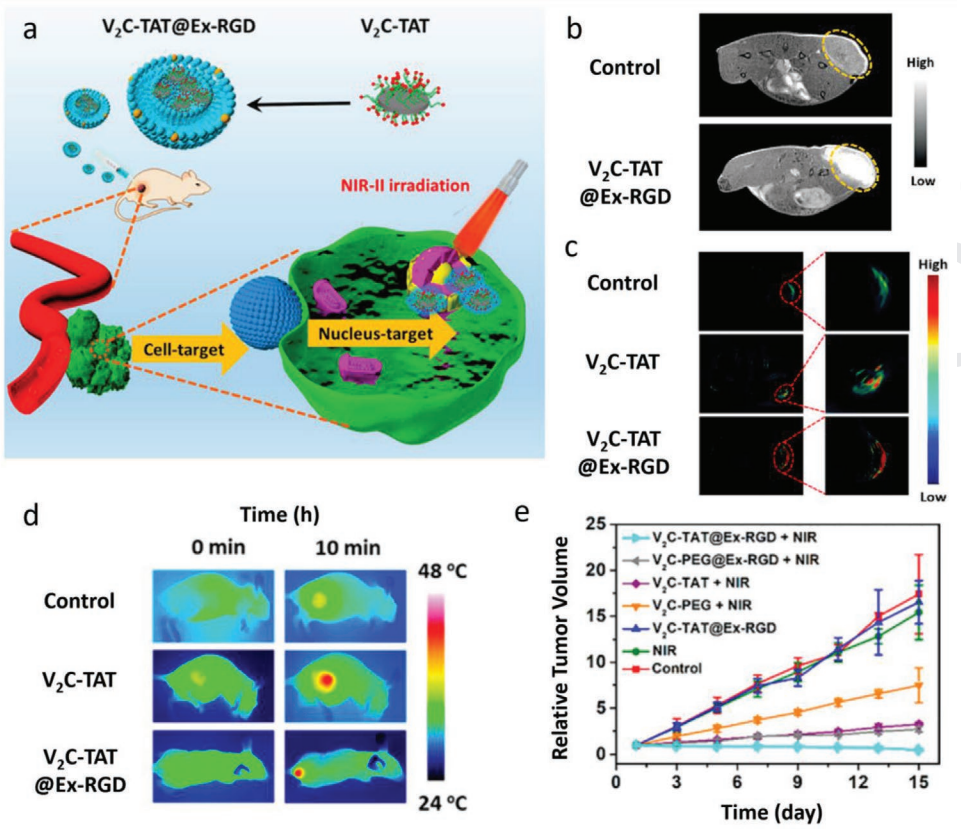

Figure 11. V_2C-TAT@Ex-RGD for low-temperature nucleus-targeted PTT in the near-infrared-II region. a) Schematic illustration of the design and dual-targeting ability of V_2C-TAT@Ex-RGD. b) T1-weighted magnetic resonance images of mice 24 h after intravenous injection of PBS and V_2C-TAT@Ex-RGD. c) Photoacoustic images of mice 12 h after intravenous injection of PBS, V_2C-TAT, and V_2C-TAT@Ex-RGD. d) Infrared thermal images at the tumor sites of the MCF-7 tumor-bearing mice under laser irradiation after different treatments. e) Time-dependent relative tumor growth curves of the MCF-7 tumor-bearing mice after different treatments. Abbreviations: Ex, exosomes; NIR, near infrared; PEG, polyethylene glycol; RGD, arginyl-glycyl-aspartic acid; V_2C-TAT, vanadium carbide quantum dots modified with TAT peptides. Reproduced with permission.[140] Copyright 2019, American Chemical Society.

EVs to improve their loading efficiency, targeting ability, and therapeutic efficacy.[153] In addition, many research groups have developed new methodologies to increase the yield of EVs and to fabricate large scale of EVs for clinical use. The procedure for generating clinical-grade and GMP standard EVs has also been reported. Moreover, bio-inspired and bio-mimetic EV-like NVs have been constructed and used as an alternative to natural EVs for improved drug delivery efficiency and therapeutic effect. Therefore, modified, engineered, and designer EVs represent a new development trend of this field, which is of great clinical value if optimized and integrated properly (**Figure 12**).

Although great progress has been made, there are also several challenges that may hinder therapeutic applications of engineered EVs.[154] The first challenge is massive and stable production of engineered EVs for clinical use. The traditional approaches such as ultracentrifugation have limitations such as poor reproducibility, time-consuming procedures, and low production yield. Large-scale manufacturing of therapeutic engineered EVs may be achieved by increasing the production of EVs using bioreactor and developing streamlined purification protocol via microfluidic devices. For example, Waston et al. have developed a hollow-fiber bioreactor for efficient production of bioactive EVs with more than 40-fold yields compared to conventional cell culture.[155] Alternatively, the generation of cell-derived NVs may also provide a scalable, efficient, and simple production of EVs.[144,156] In addition, the standardization of engineered EV preparations to ensure quality control is also important for their use in therapy. Furthermore, the question of which type of cells is mostly suitable for the generation of engineered EVs still warrants further investigation.

The second challenge needs to be addressed is to improve the efficiency of cargo loading when engineering EVs, which may determine the application potential of these novel nanovehicles in cancer therapy.[15,153] To solve this problem, the optimized methods for drug encapsulation into EVs should be carefully developed to achieve maximum efficiency and reduce the need of using large amounts of EVs. For example, hybrid EVs and EV membrane-camouflaged NVs could combine the advantages of natural EVs with that of synthesized NPs, thus enhancing drug loading efficiency and therapeutic efficacy. In addition, elucidating the factors that critically determine drug loading efficiency is of fundamental importance in therapeutic applications, including the kinetic of release, biodistribution, clearance, the events following the contact with target cells, and the intracellular fate after internalization.[157] Further understanding of EV biology is required to support optimal utility.

Third, considering that EVs harbor a discrete set of proteins and functional immune molecules, the application of engineered

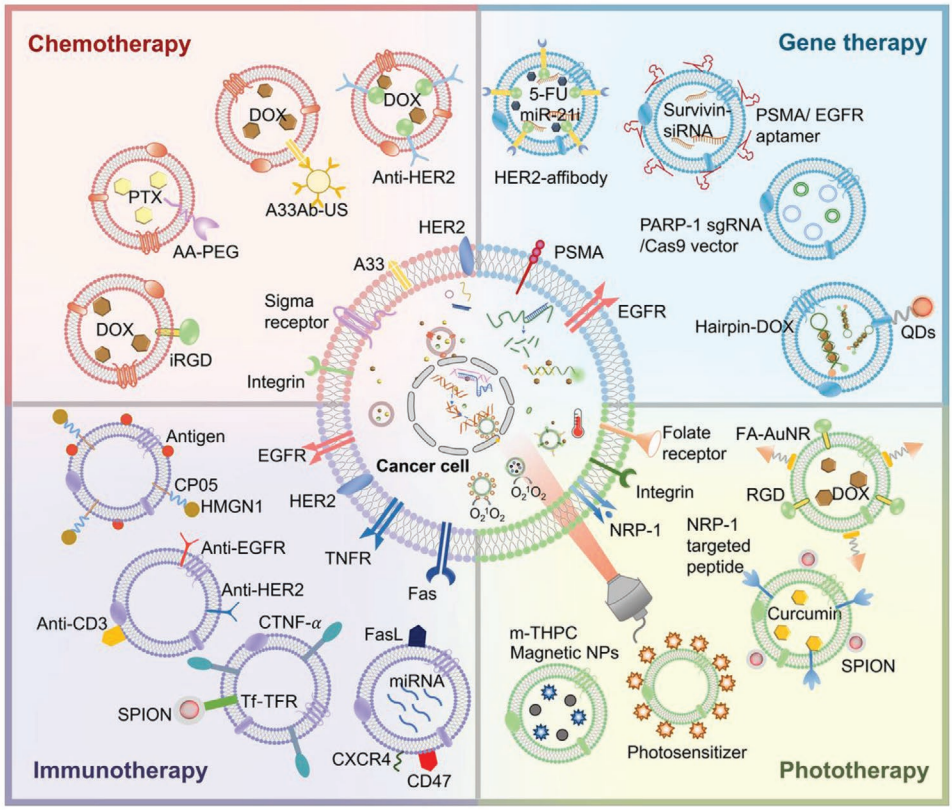

Figure 12. Engineered EVs for improved cancer therapy. Engineered EVs can serve as drug delivery systems for targeted chemotherapy, gene therapy, phototherapy, PDT, and immunotherapy. The modified EVs have better targeting ability, improved therapeutic effect, and higher safety compared to unmodified ones. Abbreviations: 5-FU, fluorouracil; A33Ab, A33 antibody; AA-PEG, aminoethylanisamide-polyethylene glycol; AuNR, gold nanorods; CTNF-α, fusion proteins of cell-penetrating peptides and TNF-α; CXCR4, C-X-C chemokine receptor type 4; FA, folic acid; FasL: Fas ligand; HMGN1, high mobility group nucleosome binding domain protein 1; NPs, nanoparticles; NRP-1, Neuropilin-l; PARP, poly (ADP-ribose) polymerase; PSMA, prostate specific membrane antigen; PTX: paclitaxel; QDs, quantum dots; RGD, arginyl-glycyl-aspartic acid; sgRNA, small guide RNA; SPION, superparamagnetic iron oxide nanoparticle; Tf, transferrin; TFR, transferrin receptor; THPC, m-tetra hydroxyphenyl chlorin.

EVs may trigger potent reactions by host immune systems to a certain extent, resulting in rapid elimination of EV-based drug delivery systems.[158] Therefore, the comprehensive preclinical examinations, including pharmacokinetics, pharmacodynamics, and toxicity profiles, should be addressed in order to prevent potential side effects. Developing therapeutic approaches by using bio-inspired and bio-mimetic EVs may represent a new direction.[17] The discoveries of efficient homing ligands or peptides for different tissues is also helpful to increase the targeting ability of EVs.[159] Moreover, the processes of modification and engineering may change the contents and compositions of EVs, which may compromise their biological functionality and induce immunogenicity. Therefore, it is necessary to develop new methods that engineer EVs with no adverse impact on their biological properties and broaden their therapeutic applications. Finally, previous studies have shown that EVs from distinct sources and distinct EV subtypes have different organ biodistribution patterns and biological functions.[160] For example, CD47-high EVs that deliver $Kras^{G12D}$ siRNA or shRNA efficiently suppress orthotopic human pancreatic cancer growth, while this effect is compromised by pre-incubation of EVs with anti-CD47 neutralizing antibodies.[92] CD54 protein (also known as ICAM1) is involved in the enhanced tumor accumulation of tumor EVs and blockade of CD54 by antibodies significantly reducing drug accumulation in tumor tissues.[14] These findings indicate that distinct EV subtypes may have different delivery efficiency, targeting ability, and therapeutic outcome. The selection of EV subtypes that display favorable targeting properties may provide new insights into therapeutic applications of EVs.

Overall, the recent studies of using engineered EVs to treat different cancers in pre-clinical studies and clinical trials, either alone or in combination with other therapeutics, are summarized in this review. In general, the concept of utilizing engineered EVs as new regimens for cancer therapy is attractive and promising. We expect that the resolution of these key issues would lead to engineered EVs as a novel strategy for cancer therapy in the near future. Q5

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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