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REVIEW

Extracellular Vesicles for Disease Treatment

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Abstract: Traditional drug therapies suffer from problems such as easy drug degradation, side effects, and treatment resistance. Traditional disease diagnosis also suffers from high error rates and late diagnosis. Extracellular vesicles (EVs) are nanoscale spherical lipid bilayer vesicles secreted by cells that carry various biologically active components and are integral to intercellular communication. EVs can be found in different body fluids and may reflect the state of the parental cells, making them ideal noninvasive biomarkers for disease-specific diagnosis. The multifaceted characteristics of EVs render them optimal candidates for drug delivery vehicles, with evidence suggesting their efficacy in the treatment of various ailments. However, poor stability and easy degradation of natural EVs have affected their applications. To solve the problems of poor stability and easy degradation of natural EVs, they can be engineered and modified to obtain more stable and multifunctional EVs. In this study, we review the shortcomings of traditional drug delivery methods and describe how to modify EVs to form engineered EVs to improve their utilization. An innovative stimulus-responsive drug delivery system for EVs has also been proposed. We also summarize the current applications and research status of EVs in the diagnosis and treatment of different systemic diseases, and look forward to future research directions, providing research ideas for scholars.

Keywords: extracellular vesicles, drug delivery, disease diagnosis, disease treatment

Introduction

Over the centuries, numerous diseases have greatly affected human health and wellbeing. In recent decades, various drug and drug delivery systems have been developed. However, chemical drugs can be highly toxic to normal cells, and problems such as treatment resistance still exist, and traditional treatments often cannot achieve the desired results.[1] Traditional methods of disease diagnosis, including direct observation of symptoms, auscultation, palpation, and physical and chemical-based tests also suffer from high error rates and late diagnosis, and improvements are still needed.[2] Consequently, new treatment modalities and diagnostic methods should be urgently developed. Extracellular vesicles (EVs) are spherical lipid bilayer vesicles secreted by cells at the nanoscale, carrying bioactive substances such as nucleic acids, proteins, and lipids.[3] They are present in most living cells, especially dendritic cells, lymphocytes, epithelial cells, and endothelial cells, as well as in various body fluids,[4] thus having a wide range of sources. EVs can be categorized into various subtypes, such as microvesicles, exosomes, and apoptotic vesicles originating from the apoptotic process.[5] The components of which can reflect their cellular origins, and their protein and lipid compositions also affect their pharmacokinetic properties. Their natural components may play a role in enhancing bioavailability and reducing adverse reactions.[6]

Research has demonstrated that drug delivery using a variety of nanocarriers improves the stability and solubility of drugs, and that fewer drugs can be used to reduce the side effects and toxicity that can occur with conventional therapies.[7] Due to the small size of EVs, they effectively avoid phagocytosis by mononuclear macrophages and freely cross the vascular wall and extracellular matrix. The expression of CD55 and CD59 on their surfaces prevents the activation of modulatory and coagulation factors, and thus EVs are widely distributed and stable in biofluids.[8] Due to the presence of membrane proteins. EVs have a strong ability to homing target tissues or cells, penetrate biological barriers

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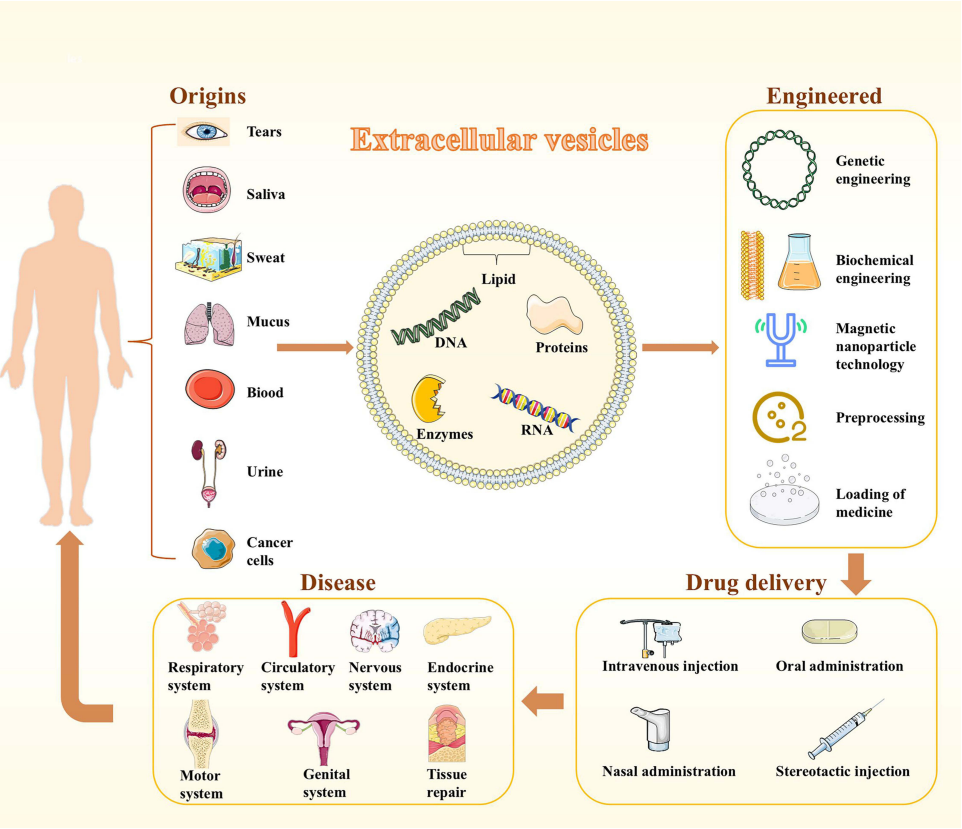
Graphical Abstract



such as the blood-brain barrier (BBB) and the pulmonary hiatus,[9] and have the advantage of natural drug delivery.[10] Furthermore, the endosomal origin of EVs renders them biocompatible and less immunogenic than liposomes and other nano-delivery systems synthesized in vitro.[11] Therefore, EVs are an excellent carrier for drug delivery[12] and have great potential for drug delivery and disease treatment.[13–15] In addition, EVs have the potential to aid in the diagnosis of disease, and the complex cargo of EVs can be easily obtained through liquid biopsy. Multi-component analysis of EVs can be used to determine disease progression and response to treatment.[3] For example, elevated miR-21 levels in circulating EVs have been associated with glioblastoma and pancreatic, colorectal, colon, liver, breast, ovarian, and esophageal cancers, and elevated miR-21 levels in urogenic EVs have been associated with bladder and prostate cancers.[16,17] Liquid biopsy of EVs highlights their potential utility in the diagnosis and prognosis of patients diagnosed with cancer and other diseases.

Despite the considerable advantages of EVs in drug delivery, disease diagnosis, and treatment, limitations remain in the isolation and comprehensive characterization of EVs. The current limitations of EVs research include poor stability and susceptibility to degradation. Over time, EVs lose efficacy and structural integrity due to thermodynamic stress, shear stress, oxidative stress and chemical degradation.[18–20] In terms of thermodynamic stress, there is general agreement among many research groups that short-term storage at 4°C and long-term storage at −20°C to 80°C are acceptable conditions.[3,21,22] However, when bringing EVs products to the clinic or market, there is a reliance on a cold supply chain, which is costly and unsuitable for providing immediate care.[23,24] Furthermore, freeze-thaw cycling has been shown to have various adverse effects on the structure of EVs, but the effects on EV bioactivity and internal cargo are largely unknown.[19,25,26] Various strategies have been explored by researchers to address the poor stability and easy degradation of EVs. Rapid freezing procedures and constant low temperatures (optimally −80°C) as well as minimising the number of

freeze-thaw cycles can be effective in maintaining the number of EVs and cargo integrity.[27] Different sources of EVs (eg, cancer cell source, mesenchymal source, or serum source) require different lyophilisation formulations and conditions. Further investigation of these conditions to assess the overall stability of EVs before and after lyophilisation is an important current direction.[28] In addition, the structure and function of EVs can be effectively protected by the addition of protective agents such as alginate, sucrose, mannitol and amino acids.[29–31] Hydrogels can also provide a microenvironment similar to a physiological environment, protecting EVs from external factors.[27] Based on these shortcomings of natural EVs, modification of EVs to form engineered EVs can function more efficiently.[32] Engineered EVs can effectively carry a diverse array of therapeutic agents. Several techniques can be used to develop engineered EVs, including genetic engineering, biochemical engineering, magnetic nanoparticle technology, and drug loading. Furthermore, advanced preprocessing of EVs can enhance their specific functions. Engineered EVs that have been modified have the potential to better assist in disease treatment breaks and cures.[33–36] Combining EVs with biomaterials can also improve their ability to treat disease.[15,37–39] In subsequent research developments, EVs can be further used in conjunction with biomaterials.

This paper introduces the definition of EVs, their isolation and characterization methods, summarizes the strategies of engineering modifications to obtain multifunctional EVs, and focuses on the application of EVs in drug delivery and disease diagnosis and treatment. Finally, this paper outlines the current research status of EVs for disease diagnosis and treatment, and looks forward to future research directions to provide scholars with research ideas.

Extracellular Vesicles

EVs are biological nanoscale spherical lipid bilayer vesicles secreted by cells that carry nucleic acids, proteins, lipids, and other biologically active substances that play a role in the physiological and pathological processes of the organism.[40,41] EVs are found in most living cells, especially dendritic cells, lymphocytes, epithelial cells, and intracellular cells, and in a variety of body fluids, such as blood, urine, saliva, amniotic fluid, and breast milk.[41–43] EVs can be classified into several subtypes, such as microvesicles (usually 50–1000 nm in diameter), exosomes (usually 40–160 nm in diameter), and apoptotic vesicles originating from the apoptotic process (usually 50–2000 nm in diameter).[42,44] Exosomes are formed by inward budding of the endosomal limiting membrane to form multivesicular bodies (MVBs). Subsequently, exosomes are released into the extracellular space by the fusion of the MVB with the plasma membrane. After being released from the cell surface, exosomes can interact with the extracellular matrix or initiate reactions within the microenvironment or in distant cells.[45,46] Microvesicles are derived from direct outward budding of the plasma membrane, generating heterogeneous populations of EVs of different sizes. Apoptotic vesicles are also produced on the cell surface, although they are only released by dying cells during cytokinesis.[42,45–47] Distinguishing between exosomes and microvesicles has been difficult because of the overlapping size and surface markers of exosomes and microvesicles; therefore, in this review, all types of vesicles are called EVs.

EVs can carry a wide range of bioactive molecules, such as proteins, nucleic acids, and lipids, which are transported from donor cells to target cells and affect their physiological pathways.[40,41] For example, in the communication between tumor cells and immune cells, tumor cell-derived EVs can deliver specific microRNAs (miRNAs) to immune cells and modulate their function of immune cells, thus affecting tumor immune escape.[48,49] EVs can also affect tumor growth and metastasis; for example, tumor cell-derived EVs can induce the activation of tumor-associated fibroblasts, which secrete tumor-growth-promoting factors. EVs can also promote tumor angiogenesis through interactions with vascular endothelial cells, providing nutrients and oxygen for tumor growth. In addition, EVs can carry the genetic information and signaling molecules of tumor cells, which can help tumor cells colonize and grow in distant organs and promote tumor metastasis.[48,50,51] EVs can serve as a bridge for communication between antigen-presenting cells and immune cells, such as T cells. EVs released by antigen-presenting cells carry antigenic information, and after being taken up by T cells, they can promote the activation and proliferation of T cells, enhancing the body's immune response. For example, during viral infection, EVs released by dendritic cells can deliver viral antigens to T cells, activate the immune response of T cells, and help the body clear the virus.[48,52] In addition, EVs released by regulatory T cells are involved in the maintenance of immune tolerance, preventing excessive immune responses by delivering inhibitory signaling molecules.[52] In tissue injury or disease states, EVs can act as mediators of intercellular communication and transmit signals that promote cell

proliferation and differentiation. For example, MSC-derived EVs contain a variety of growth factors and cytokines that can promote cell proliferation and differentiation in damaged tissues and accelerate tissue repair. In nerve injury repair, EVs released from neural stem cells can promote the regeneration of neurons and the proliferation of glial cells, contributing to the recovery of nerve function.[53,54]

Isolation of EVs

The most common method for EVs isolation is differential ultracentrifugation. However, this approach is inherently time-consuming and susceptible to EVs damage. Other techniques currently under development have certain disadvantages. The study of EVs continues to progress; however, their isolation presents significant challenges. Most techniques used cannot completely separate EVs from substances such as lipoproteins.[8] Therefore, future research on EVs must adopt a multi-method approach, combining different techniques to isolate EVs with high efficiency for various purposes and applications.

Ultracentrifugation techniques include differential ultracentrifugation and density-gradient centrifugation. Ultracentrifugation is the most commonly used technique for the isolation of EVs based on differences in density and particles and is suitable for the separation of exosomes with significant differences in sedimentation coefficients. There are two main types of density-gradient centrifugation, one of which uses sucrose as the medium, but EVs and retroviruses are extremely similar in size and density, and a sucrose density gradient cannot effectively separate the two, and Cantin et al found that there was a significant difference in their settling speeds in an iodixanol gradient, which made the successful isolation of EVs and the harvesting of high-purity EVs from HIV-1-infected cells possible.[55]

Size exclusion chromatography and ultrafiltration are size-based separation techniques capable of separating biomolecules based on their size. Ultrafiltration is one of the most commonly used techniques for the separation of EVs, which selectively separates samples through the use of ultrafiltration membranes with different molecular weight cutoff (MWCO). During ultrafiltration, particles larger than the MWCO of a given filter are retained, while particles smaller than the MWCO pass through the filter into the filtrate.[56] Size exclusion chromatography exploits differences in the hydrodynamic radii of biomolecules for separation. When passing through a porous stationary phase, particles larger than the pore size are the first to be eluted from the void between the porous gel and the mobile phase, while smaller particles and molecules penetrate the pore space to varying degrees, depending on their size, and their elution time is prolonged as the size of the particles or molecules decreases.[56] This method requires sample pretreatment by ultracentrifugation or ultrafiltration to prepare EVs that are free of lipoproteins and protein magazines and require hours of run time, are not easily scalable, and cannot be used for high-throughput applications.[13]

Immunoaffinity chromatography recognises and separates uniquely labelled proteins on the surface of EVs by means of antibodies immobilised on surfaces such as magnetic beads, multiwall plates, chromatographic column gels and microfluidic devices.[56] This method can isolate EVs from specific sources as compared to other techniques. The main limitation of this method is that, because the antibodies cannot capture the antigens inside the vesicles, the proteins or antigens used to capture the EVs must be expressed on the surface of the EVs. Compared to other methods, this method isolates EVs with lower yield and higher purity. This method is often reused after enrichment of EVs by ultracentrifugation or ultrafiltration.[13]

Polymer precipitation is often used to harvest EVs by reducing their solubility via centrifugation using polyethylene glycol (PEG) as the medium. The modified polymer co-precipitation (ExtraPEG) method was found to be superior to commercially available methods and ultracentrifugation and is relatively simple to perform, has a short analysis time and is suitable for processing large samples.[57] However, the relatively low purity and recovery may result in false positives and produce polymers that are difficult to remove, thereby compromising subsequent functional test analyses.[58]

The commonly used EVs separation methods with their principles and advantages and disadvantages are shown in the table (Table 1).

Characterization of EVs

Given the heterogeneity of EVs and the challenges associated with isolating them, thorough characterization of EVs formulations is crucial. To characterize EVs, one can use transmission electron microscopy for morphological analysis,

Table 1 Commonly Used EVs Isolation Methods

Separation Method	Separation Principle	Advantages	Disadvantages	Sample Types	Reference
Differential ultracentrifugation	Differences in density, size, and shape	Avoids cross contamination; No sample pretreatment required	High cost; Aggregated blocks, Structural damage, Time-consuming, lipoprotein co-separation, unfavorable for downstream analysis.	CCM (cell culture media), urine	[21,59,60]
Size-based separation techniques	Separation of biomolecules according to their size	Ultrafiltration methods: fast size exclusion chromatography: vesicle integrity, high biological activity, high yield	Ultrafiltration: EVs are easily lost, deformed, vascularized, and cleaved. Size-exclusion group chromatography: time-consuming; Difficult to scale up efficiently or effectively; Not suitable for high-throughput applications	CCM, urine	[21,59]
Immunoaffinity chromatography	Isolation of EVs based on protein-antibody interactions on their surface	Separation of source-specific EVs, High purity	Proteins or antigens must be expressed on the surface of EVs; Low yield	CCM	[61]
Polymer precipitation	Altering the solubility or dispersion of EVs by using non-absorbent polymers to precipitate them out of biological fluids	Simple operation; Short time, Suitable for processing large samples	Lower purity and recovery, False positives; Difficult to remove produced polymers; Unfavorable for subsequent functional experimental analyses	CCM, plasma	[21,59–62]

nanoparticle tracking analysis to determine the size of EVs, and Western blotting for surface protein labeling. The International Society for Extracellular Vesicles advocates the assessment of two protein categories as essential:[22] transmembrane or GPI-anchored proteins affiliated with the plasma membrane and endosomes (comprising CD63, CD81, CD82, and CD47) along with cytosolic proteins (such as TSG101, HSPA8, and HSPA1A). In routine studies, isolated exosomes are usually identified in three dimensions: first, the morphology of exosomes is identified using transmission electron microscopy; second, the size of exosomes is determined by nanoparticle tracking analysis (NTA); and finally, the protein markers on the surface of exosomes are detected using Western blotting (WB). The methods for exosome characterization cover two main categories: external characterization, which mainly involves the detection of morphology and particle size, and endosomal characterization, such as the analysis of membrane proteins and lipid rafts. The rationales and merits of the prevalent methodologies are listed in the subsequent table (Table 2).

Engineered Extracellular Vesicles

EVs derived from specific cells have been shown to exert therapeutic effects in various diseases. However, natural EVs usually degrade easily and are difficult to use for long periods of time. Systemically administered EVs are inefficiently targeted and readily absorbed by circulating cells and the spleen, resulting in extremely high clearance rates, which rarely achieve the desired therapeutic effect.[69,70] However, direct injection of EVs into solution causes rapid loss and low retention of the injected solution, leading to a significant increase in the required dose.[71] It needs to be modified to achieve a better therapeutic effect. Therefore, treatments such as genetic engineering, biochemical engineering, magnetic nanoparticle technology, pretreatment, and loading of drugs are commonly used to form engineered EVs for slow release, stable release, and higher use.[7]

Table 2 Commonly Used Characterization Methods for EVs

Method	Purpose	Advantage	Reference
Electron microscopy	EVs morphology assay	Direct observation of morphological structures.	[63]
NTA	Detecting the size and concentration of EVs	Fast detection, real-time observation, High resolution, Lower limit of fluorescent particle measurement up to 30–40 nm.	[64,65]
WB	Detection of the expression of EVs-tagged proteins	Proven technology, Qualitative and quantitative analysis of labeled proteins, Easier to analyze EVs in cell culture media, Cost-effective.	[22]
Dynamic Light Scattering	Detecting the size of EVs	Lower limit of measurement of 10 nm for monodisperse systems, Fast speed.	[66]
Flow cytometry	Detection of EVs biomarkers	Capable of high-throughput, multi-channel analysis, Fast analysis, Low sample concentration required, and Complete particle phenotyping.	[67]
Enzyme-linked immunosorbent assay (ELISA)	Detection of the expression of EVs-tagged proteins	High sensitivity, High specificity, Fast detection, qualitative and quantitative analysis of tagged proteins, suitable for high throughput analysis.	[68]

Genetic Engineering

Genetic engineering manipulates genes at the molecular level. It is performed by inserting foreign genes into host cells via in vitro recombination, enabling gene replication, transcription, and expression.[72] The most common genetic engineering approach is creating receptor cells highly expressive of target proteins or peptides, enabling the secretion of target-specific EVs. LAMP-2B has become a hotspot in the study of surface proteins in EVs due to its ability to demonstrate targeting motifs. The protein is predominantly distributed in lysosomes and endosomes, with a small amount recycled to the cell surface. Studies have shown that LAMP-2B is abundantly expressed in dendritic cell-derived EVs. The N-terminus of LAMP-2B is exposed on the surface of EVs and can link targeting sequences.[73,74] Tian et al found that fusion of EVs expressing the αvβ3/αvβ5 integrin specific iRGD peptide (CRGDKGPDC) to LAMP-2B and then injected intravenously effectively delivered adriamycin in vitro and in vivo to integrin positive breast cancer cells by delivering adriamycin.[75] In addition, iRGD-modified EVs were used for in vivo specific delivery of small interfering RNAs (siRNA) to A549 tumors carrying αvβ3, achieving specific knockdown of the KRAS gene as well as effective inhibition of tumor growth.[75] LAMP-2B is able to be genetically engineered to fuse with target proteins or antibody fragments, which in turn displays the antibody on the surface of EVs. Compared to binding peptides with moderate affinity, antibodies or affinities can achieve higher affinity and low KD values for receptors on target cells on the nanomolar scale. In the treatment of chronic granulocytic leukaemia (CML), researchers have altered the surface of EVs by fusing interleukin-3 (IL-3) to the N-terminus of LAMP-2B. IL-3 is a natural ligand for interleukin 3 receptor alpha and is highly expressed in CML progenitor cells. IL-3-modified EVs containing imatinib and BCR-ABL siRNA were effective in killing CML cells and prolonging the survival of xenograft mice. In vivo tracking of these engineered EVs showed that they rapidly migrated to CML xenograft tumour sites.[76] In another research, Liang et al[34] integrated zHER2, with its HER2 binding capability, into the N-terminus of Lamp2, abundant in EVs membranes. This fusion was cloned into pLVX-GFP-N1 to yield THLG. HEK293T cells were stably transduced with the lentiviral vectors encoding THLG or LG. Subsequently, the EVs were harvested and purified. zHER EVs exhibited remarkable affinity and selectivity toward HCT-116 colon cancer cells, effectively mediating the targeted delivery of chemotherapeutic agents, namely 5-fluorouracil (5-FU) and anti-miRNA-21, specifically to tumors expressing HER2 in vivo, which consequently reversed drug resistance and improved cancer treatment efficiency (Figure 1A). Alvarez-Erviti et al[73] genetically modified a plasmid that codes for Lamp2b, an EVs membrane protein, fused with a neuron-specific rabies virus glycoprotein (RVG) peptide to facilitate the transfection of dendritic cells. The intravenous administration of EVs, designed to target the RVG, led to precise delivery of the vesicles to neurons, oligodendrocytes, and microglia in wild-type mice, accomplishing the intended gene suppression effect (Figure 1B). Yang et al[77] exhibited that genetically engineered EVs, which were fused with both the RVG and the lysosomal membrane protein Lamp2b, were highly potent in transporting miR-124 specifically to the site of infarction. Systemic injection of miR-124-loaded RVG peptide-modified EVs (RVG-EVs)

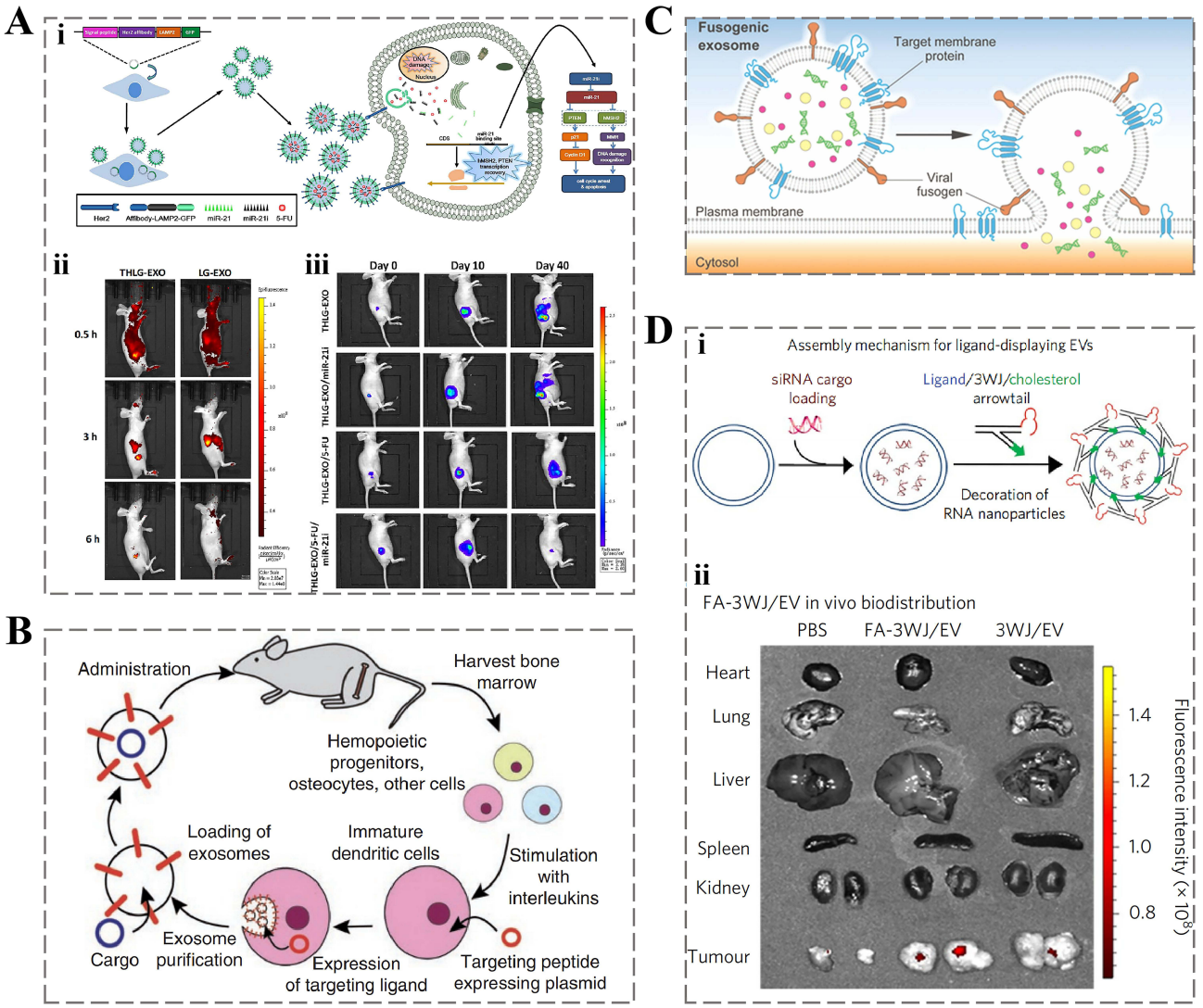


Figure 1 Genetic engineering and biochemical reactions to form engineered EVs. (**A**) i) Schematic diagram of simultaneous delivery of engineered exosome-based nanocarriers for 5-FU and miR-21i to HCT-1165FR human colon cancer cells to enhance chemotherapy efficacy; ii) The in vivo distribution of THLG-EXO; iii) The anti-tumor effect of THLG-EXO/5-FU/miR-21i in vivo. Adapted reprinted with permission from Liang G, Zhu Y, Ali DJ, et al. Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. J Nanobiotechnol. 2020;18(1):10.[34] based on CC BY License, Copyright © 2020, The Author (s). (**B**) Schematic of the production, harvesting, and re-management of targeted self-EVs for gene delivery. Reprinted with permission from Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol. 2011;29(4):341–345. 2011, Springer Nature.[73] (**C**) Schematic diagram of membrane editing by fusing EVs. Reprinted with permission from Yang Y, Hong Y, Nam G-H, Chung JH, Koh E, Kim I-S. Virus-mimetic fusogenic exosomes for direct delivery of integral membrane proteins to target cell membranes. Adv Mater. 2017;29(13):1. © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.[35] (**D**) i) EVs loading and RNA adapter display; ii) In vitro images of healthy organs and tumors collected from mice. Reprinted with permission from Pi F, Binzel DW, Lee TJ, et al. Nanoparticle orientation to control RNA loading and ligand display on extracellular vesicles for cancer regression. Nat Nanotechnol. 2018;13(1):82–89. 2018, Springer Nature.[84]

promotes the differentiation of cortical neural progenitors toward a neuronal fate and mitigates ischemic damage by fostering robust cortical neurogenesis. It has been demonstrated that EVs obtained through genetic engineering can achieve the desired transformation and enhance the targeting capacity in both in vitro and in vivo settings. However, the engineering procedure involved is intricate and expensive, rendering it impractical to use pre-isolated or naturally occurring EVs found in bodily fluids,[72] limiting its practical application.

Biochemical Engineering

Biochemical engineering can also be used to modify EVs and improve their targeting. Compared to genetic engineering, biochemical engineering is simpler, faster, and more effective for modifying EVs without the need to modify cells. Two

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modification strategies are included, direct modification by membrane fusion or hydrophobic insertion and the use of chemical methods to couple functional ligands to the surface of EVs.[72]

Direct Modification by Membrane Fusion

The lipid bilayers of EVs can spontaneously fuse with other membrane structures. Yang et al[35] described a new tool for membrane protein therapy: the direct delivery of membrane proteins to target cells using fused EVs carrying the viral fusion protein, vasculitis stomatitis virus (VSV)-G protein (Figure 1C). Matsuoka et al[78] successfully isolated EVs from cells expressing the tyrosine kinase receptor HER2 and fused these HER2-enriched EVs with phospholipid liposomes using freeze-thaw technology. This technique not only improves the surface properties of EVs effectively reduces their immunogenicity, and enhances their stability, but also significantly extends the half-life of EVs in the blood. Kooijmans et al[79] have proposed an innovative "post-insertion" mechanism designed to enhance the targeting ability of EVs. This approach uses targeting ligands bound to poly-PEG to modify EVs, and researchers first prepared nano-antibody-PEG micelles by combining nano-antibodies specific for the epidermal growth factor receptor with phospholipid (DMPE)-PEG derivatives. When these micelles were mixed with EVs derived from neural 2A cells or platelets, temperature-dependent transfer of nanobody PEG-lipids to the EVs membranes was observed. This process did not alter the morphology, size distribution, or protein composition of EVs. More importantly, by inserting ligand-conjugated PEG-derived phospholipids into the EVs membrane, the cell specificity of EVs was not only improved, but their circulation time in vivo was prolonged, which is expected to increase the accumulation of EVs in the target tissues, and thus enhance cargo transport efficiency. Tareste et al[80] harnessed the electrostatic interactions to enhance the merging of cationic liposomes with EVs. The existence of robust positive charges on liposomes aided the efficient binding and internalization of EVs by the target receptor cells. Incubation of synthesized azide-containing liposomes with donor cells also triggered the cellular secretion of exosomes bearing azide moieties on the lipid membrane. Subsequent biocoupling of these exosomes with a targeting peptide via a controlled click reaction enhances the tumor-targeting ability of EVs.[33] For example, Lee et al[81] loaded EVs coupled with CGKRK peptides and paclitaxel (PTX) for targeted delivery to B16F10 tumors.

Chemical Modification

The amine groups of extracellular vesicle proteins can be readily modified using alkyne groups. Subsequently, the alkyne-labeled EVs proteins are coupled to azide-containing reagent organisms in an orthogonal manner via a copper-catalyzed azide-alkyne cycloaddition (CuAAC) "click" reaction. The glioma-specific RGE peptide established a covalent linkage with EVs through a cycloaddition reaction involving sulfonyl azide. These RGE-modified EVs traverse the BBB upon intravenous delivery and specifically target the tumor site.[33] Most tumors harbor surface CD47s engaging with signal-regulatory protein alpha (SIRPα) found on phagocytic cells. This interaction curtails the phagocytic potential of macrophages toward tumor cells. Koh et al[82] developed an immune checkpoint inhibitor based on EVs that antagonizes the interaction between CD47 and SIRPα. By conjugating a dibenzocyclooctyne-derived SIRPα antibody to azide-modified EVs. Chemically modified EVs targeted to interfere with the CD47-SIRPα checkpoint on the surface of tumor cells improved the phagocytosis of tumor cells by immune cells. Chemical modification of EVs by click chemistry depends on the conversion of the amine moiety of the EVs to an alkyne. However, because of the inability to precisely modify target amino groups or proteins, the reaction is not site specific, which may cause the blocking of some protein-protein interactions and alter the recognition properties of EVs.[33]

An alternative tactic for chemical modification involves embedding amphiphilic molecules within the lipid bilayers of EVs. Previous investigations have revealed that PEG-modified 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG) congregates within EV membranes. Using this approach, DSPE-PEG-RGD nanoparticles were successfully immobilized on EVs. When RGD-functionalized EVs are combined with the tumor-targeting ligand, folic acid, almost all injected RGD EVs are concentrated in the tumor region.[33] Given that sigma receptors are overexpressed in lung cancer, they represent potential receptors for targeted delivery to EVs. Among these, ligands can be selected from anisamides with a high affinity, including aminoethyl anisamide. Aminoethyl anisamide linked to DSPE-PEG has been

successfully coupled to EVs membranes.[83] Compared to unmodified EVs, the lung cell lines exhibited enhanced uptake of aminoethyl anisamide -modified EVs, leading to improved therapeutic efficacy in vivo when used for targeted delivery of PTX. These findings suggest that DSPE-PEG-based EVs hold promise as efficient vehicles for tumor-specific drug delivery. Similarly, Chol can self-assemble into EVs membranes because of its hydrophobic nature. Pi et al[84] modified EVs with Chol coupled to RNA aptamers or folic acid. These targeted EVs delivered siRNAs and miRNAs to the corresponding tumor sites, enhancing the antitumor efficacy (Figure 1D). Other lipids have also been used to bind targeted ligands on the surface of EVs. Zou et al[85] developed a diacyl lipid-DNA aptamer (sgc8) coupler to implement cancer cell-specific therapies.

Magnetic Nanoparticle Technology

The non-toxicity, magnetic targeting, and enhanced retention and permeation effects of magnetic nanoparticles (MNPs) have led to their widespread use in tumor targeting and magnetic resonance imaging (MRI) contrast agents, and they have been studied extensively in biomedical nano-delivery systems.[86] Iron oxide nanoparticles (IONPs) can also be used as an effective drug delivery system to treat BBB and ischemic stroke. By encapsulating the therapeutic agent and iron oxide nanoparticles in magnetic EVs, the magnetic field can achieve precise dynamic regulation and spatial localization, which can promote the aggregation of EVs in the target area and enhance the uptake of the drug by the cells, thus significantly improving the therapeutic efficacy of EVs.[87,88] For example, Kim et al[89] used magnetic nanovesicles (MNVs) synthesized through the integration of IONPs with mesenchymal stem cells (MSCs) in a transient middle cerebral artery occlusion (MCAO)-induced rat model. Following the systemic administration of MNVs, a marked 5.1-fold enhancement in their targeted accumulation was observed compared to the control group. miRNAs are key functional components of EVs and may play an important role in intercellular communication and the regulation of biological functions. Wu et al[90] found that miR-1260a expression was significantly elevated in EVs released from bone marrow mesenchymal stem cells (BMSCs) pretreated with Fe_3O_4 nanoparticles with static magnetic fields. In addition, the expression of miR-21-5p was upregulated in EVs derived from BMSCs treated with magnetic fields, which could effectively promote wound healing[91] and the repair process of tendon and bone[92] by activating the miR-21-5p/SMAD7 signaling pathway. In addition, IONPs can stimulate the upregulation of therapeutic growth factors within MSCs via the activation of c-Jun phosphorylation, which subsequently triggers the engagement of c-Jun N-terminal kinase signaling cascades. IONPs can be assimilated into ferric ions and ferritin, demonstrating their biocompatibility.[93] Magnetic nanoparticle technology holds promise in augmenting the tumor-targeting precision of EVs. Qi et al[94] crafted magnetic EVs by affixing superparamagnetic transferrin to the exterior of EVs sourced from the transferrin receptor-positive blood components. Subsequently, they positioned an external magnet at the location of the tumor within the organism. This innovative method facilitated the precise navigation of magnetic EVs toward the intended tumor cells, exerting a potent inhibitory effect on tumor proliferation (Figure 2A). Although magnetic EVs lack the capacity to directly target cancer stem cells (CSCs), EVs loaded with potent CSC-targeting drugs, which are highly concentrated near solid tumors, can enhance therapeutic efficacy markedly while minimizing adverse effects by confining the drug to the tumor site.[95]

Preprocessing

Obtaining large quantities of EVs is often challenging owing to the limitations of the existing extraction protocols. However, the limitations of low yields can be addressed through pretreatment methods to enhance EVs activity. EVs obtained from cells pretreated with physicochemical factors, such as hypoxia and iron nanoparticles, have enhanced biological activity.[97] Hypoxia, characterized by reduced oxygen levels, is pivotal in numerous pathological phenomena involving decreased oxygen tension within biological systems. Hypoxia affects the size, quantity, and expression of exosomal cargoes.[98] Numerous studies have shown that hypoxia-treated adipose MSC-derived EVs exhibit enhanced pro-angiogenic capacity owing to elevated expression of vascular endothelial growth factor (VEGF), its receptor VEGF-R, epidermal growth factor, and fibroblast growth factor.[97,99,100] It was further found that the expression levels of miR-31 and let-7 were elevated in adipose MSC-derived EVs treated under hypoxic conditions, which contributed to the activation of the protein kinase A signaling pathway in endothelial cells, which triggered an increase in the expression of endogenous VEGF and its receptor, VEGF-R, and ultimately led to a significant enhancement of angiogenic effects.[97,99,100] Xiong

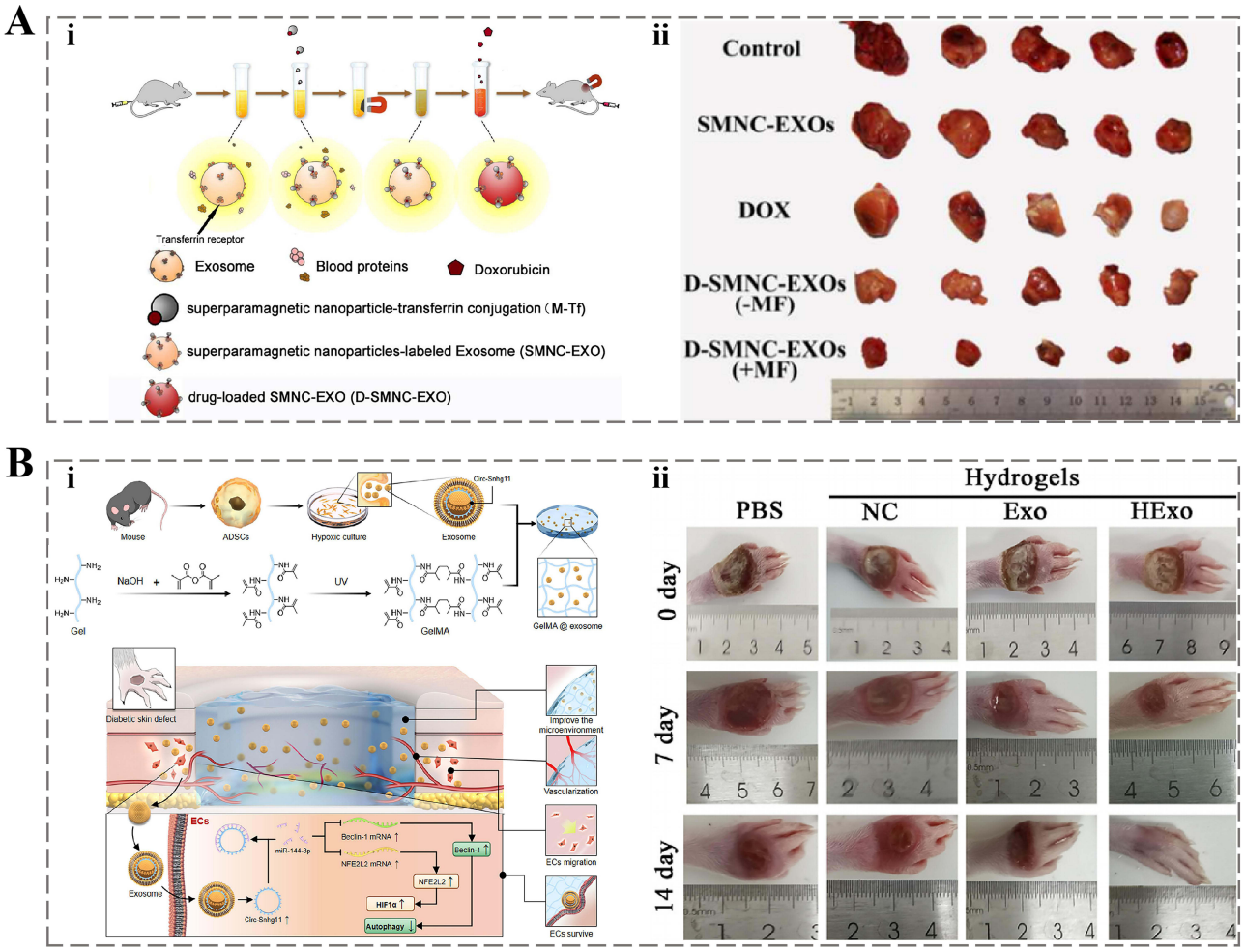

Figure 2 Magnetic nanoparticle technology and anoxic treatment to form engineered EVs, (**A**) i) Schematic representation of the construction and delivery of the drug-loaded SMNC-EVs; ii) Tumor tissue obtained from euthanized mice after administration. Reprinted (adapted) with permission from Qi H, Liu C, Long L, et al. Blood exosomes endowed with magnetic and targeting properties for cancer therapy. ACS Nano. 2016;10(3):3323–3333.[94] based on CC BY License, Copyright 2016 American Chemical Society. (**B**) i) Schematic diagram of the process of encapsulating hydrogels with ADSC-derived EVs preconditioned by hypoxia, and the regulatory mechanism of accelerated healing of foot wounds in diabetic mice; ii) Wound healing progress at different time points between different groups. Reprinted from Acta Biomaterialia, Hu N, Cai Z, Jiang X, et al. Hypoxia-pretreated ADSC-derived exosome-embedded hydrogels promote angiogenesis and accelerate diabetic wound healing. Acta Biomater. 157:175–186. Copyright 2023, with permission from Elsevier.[96]

et al[101] conclusively demonstrated that EVs sourced from bone marrow MSCs that underwent hypoxic preconditioning significantly improved cardiac function, diminished the extent of infarction, and accelerated angiogenesis in the affected heart tissue, promoting recovery. Moreover, miR-125b-5p is upregulated in EVs derived from hypoxic MSCs. Zhu et al[36] treated mice with myocardial infarction (MI) by administering this EVs and observed that the pro-apoptotic genes p53 and BAK 1 expression in cardiomyocytes was inhibited, exerting a significant cardioprotective function following infarction. Hu et al[96] prepared anoxically pretreated ADSC-EVs (ADSC-HEVs)-embedded GelMA hydrogels using non-covalent forces and physical embedding. These materials undergo rapid gelation in the presence of light, thus providing a suitable matrix to treat irregular diabetic wounds. The increased expression of circ-Snhg11 in ADSC-HEVs had a favorable therapeutic effect, as illustrated in Figure 2B. Hypoxic ADSC-EVs have also been shown to influence cartilage regeneration. Xue et al[102] observed that the expression of chondrocyte-related genes was upregulated in hypoxic ADSC-EVs, which induced an increase in the cartilage matrix and proteoglycan production. This indicated a pivotal function of hypoxic ADSC-EVs in cartilage tissue engineering. Hypoxia is a well-established preconditioning strategy. Several alternative approaches have been explored, including the addition of lipopolysaccharide (LPS),[103] hydrogen peroxide,[104] atorvastatin,[105] and pioglitazone.[106] For example, Wu et al[107] reported that EVs secreted by human ADSCs upon stimulation with LPS can

enhance angiogenesis in human umbilical vein endothelial cells (HUVECs). The observed effect stems from the stimulation of CREB, AP-1, and NF-κB signaling cascades, accompanied by the generation of interleukin-8 in recipient HUVECs following treatment with ADSC-LPS-EVs.

Drug Loading

Strategies for incorporating drugs into EVs can be categorized into two approaches: preload integration and subsequent loading. Within the preload integration method, the drug is initially incorporated into progenitor cells, ensuring that, upon isolation or derivation of EVs from these cells, they are inherently loaded with the intended therapeutic agent. Conversely, the subsequent loading technique involves administering the drug to EVs after their extraction, enabling drug incorporation post-isolation. This distinction underscores the flexibility of drug delivery mechanisms tailored to EV-based therapeutic systems.

Preloading Methods

In preloading methods, parental cells receiving drug treatment secrete exosomes or EVs preloaded with the drug. Although this method does not allow precise control over loading efficiency, it has been used in numerous studies owing to its simplicity.

Over the past decade, a growing body of literature has described methods for successfully loading therapeutic drugs into EVs by modulating parental cells that produce these drugs. Primary cell engineering methodologies predominantly involve transfection and activation of progenitor cells.

Transfection is a prevalent and highly effective approach for the introduction of therapeutic proteins and oligonucleotides into EVs. This enables the overexpression of targeted proteins on the EVs' surface membrane and the encapsulation of proteins within their interior lumen. Cell transfection involves techniques involving calcium phosphate and various commercially obtainable lipid transfection agents, among others. Consequently, biomaterials may be encapsulated within EVs to activate or suppress gene expression or to control transcription in the initial cells. Numerous studies have effectively integrated miRNAs into EVs by using miRNA expression vectors.

Although not the best method for loading EVs, cell activation elucidates certain aspects of EVs physiology and function, and has been used to load functional cargo into EVs. Zhang et al[108] demonstrated that upon exposure of the human monocyte cell line THP-1 to diverse inflammatory stimuli, the subsequent generation of EVs exhibited an elevated concentration of miR-150. This upregulation enhanced the migratory capacity of endothelial cells, underscoring the regulatory role of miR-150-laden EVs in cellular migration processes. Moreover, Xin et al[109] co-cultured rat brain MSCs with neurons and astrocytes after middle cerebral artery occlusion and found that miR-133b expression in MSC-derived EVs increased.

Back-Loading Method

A straightforward approach for incorporating cargo into EVs involves co-incubation, in which isolated EVs are directly mixed with the drug. This loading mechanism is fueled by the differential concentration of the drug across the vesicle membrane. Specifically, hydrophobic drugs engage with the lipid bilayer and diffuse into the interior of EVs along a concentration gradient, facilitating efficient cargo encapsulation. Sun et al[110] incubated curcumin with EVs in PBS for 5 min at 22 °C and subsequently purified the EVs. Their findings revealed that curcumin spontaneously organizes into the lipid bilayer of EVs via hygroscopic interactions, enhancing drug stability. Similarly, Zhuang et al[111] used a comparable methodology to isolate drug-laden EVs (designated as EVs-CUR or EVs-JSI124) by integrating curcumin or JSI124 with EVs derived from EL-4 cells in PBS and incubating the mixture for 5 min at 22 °C. Didiot et al[112] devised a technique involving the encapsulation of therapeutic RNA within EVs, which was achieved by co-incubating EVs with hydrophobically tailored small interfering RNAs. The only limitation of this passive loading strategy, which is simple, cost-effective, and does not affect vesicle size or integrity, is its low drug-loading efficiency. Drug-loading efficiency is contingent upon both the hydrophobic nature of the medication and the existence of a concentration gradient.[110]

Electroporation is a well-established method for enhancing drug loading into EVs, which can improve their in vivo stability, blood circulation, and cell targeting efficiency. An electric field creates transient pores in the EVs membrane, enabling the drug to permeate and diffuse, thus restoring the membrane integrity. Such vesicles can be used to load drugs,

such as nucleotides and Adriamycin into EVs.[113] In a study conducted by Wahlgren et al.[114] EVs derived from peripheral blood have been used as carriers for delivering therapeutic siRNAs via electroporation. This study examined the effects of varying EVs concentrations, siRNA dosage, and electroporation settings on the efficacy of the electroporation technique. These findings indicated that variations in siRNA concentration and capacitance factor did not significantly affect electroporation efficiency. The highest efficiency was observed when the EVs concentration was within the 0.25–1 mg/mL range, indicating that EVs concentration is a critical factor influencing electroporation efficiency. Nonetheless, this approach has drawbacks, such as compromising membrane structural integrity and diminishing both efficiency and drug-loading rate. The utilization of high-pressure pulses has the potential to result in the aggregation of EVs. Nevertheless, this approach may compromise protein structure and, consequently, function, which could restrict its broader applicability.

Ultrasonic treatment involves merging EVs obtained from donor cells with a medicinal substance, followed by sonication with a probe sonicator. Remodeling of the EVs membrane under sonication permits the drug to permeate the relatively impermeable lipid bilayer[115] and enter the EVs. Kim et al[116] introduced PTX into macrophage-released EVs using sonication. The technique showed remarkable efficiency in loading and a consistent ability to release drugs without notably affecting protein or lipid levels in EVs. Haney et al[117] used sonication to load catalase into EVs. The procedure involved sonicating a catalase-EV mixture, which was cooled on ice for 2 min and subsequently sonicated again, effectively loading catalase into EVs by sonication. In addition, sonication has been used to load small RNAs into the EVs. However, these loading methods may cause cargo aggregation or degradation, which may limit the potential application of EVs in therapeutic RNA delivery.[118] Lamichhane et al[119] used sonication techniques to introduce functional small RNAs into EVs derived from two cell lines, HEK293T and MCF-7. Nucleic acids were incubated with EVs for 30 min at room temperature in a sonication bath. The frequency, r, was set to 35 kHz. Subsequently, the mixture was transferred to ice for 1 min and sonicated again within the same time frame. Very few detectable aggregates.

The extrusion process involves combining EVs and a medication, placing the blend in a lipid extruder attached to a syringe, and propelling the mixture through a membrane with pore sizes between 100 and 400 nm under controlled temperature conditions. The extrusion procedure disrupts EVs membranes, facilitating vigorous integration with the drug substance, which is then encapsulated within EVs. Haney et al[117] demonstrated the effective extrusion of a catalase-EVs mixture 10 times through a 200-nm pore filter, resulting in the loading of catalase into RAW264.7 macrophage-derived EVs. In another study, Fuhrmann et al[120] introduced various hydrophobic porphyrins into EVs at 42 °C using a portable syringe-operated micro-extruder. They found that extruded EVs may cause cytotoxic reactions, whereas EVs filled with the same porphyrins prepared using other methods showed no significant cytotoxicity.

The freeze-thaw cycle procedure involves mixing a drug with EVs and incubating it at ambient temperature. Subsequently, the solution was subjected to freezing either at −80 °C or liquid nitrogen, followed by thawing at room temperature. This process was repeated at least three times to confirm the effective encapsulation of the drug.[121] Although this method facilitates EVs aggregation, it typically yields a lower efficiency of drug loading compared to sonication or extrusion techniques. Sato et al[122] proposed a novel approach for preparing engineered hybrid EVs, whereby the membranes of EVs are fused with those of liposomes using the freeze-thaw method. EVs sourced from the original 264.7 cells were combined with liposomes featuring fluorescent markers, encompassing phospholipids, polyphospholipids, and phospholipids adorned with fluorescent tags. Afterward, the mixture was subjected to a swift freeze in liquid nitrogen, followed by a 15-min thawing phase at ambient temperature. To further test the applicability of the membrane fusion technology, they investigated the fusion behavior between EVs carrying HER2 and liposomes. HER2 and phosphorylated HER2 were detected in EV-liposome mixtures, suggesting that EV-liposome mixtures carrying specific proteins can be obtained by freeze-thawing methods. Research on cellular internalization using hybridized EVs has shown that altering the composition of liposomes can effectively tailor interactions between engineered EVs and target cells.

Saponin, a surfactant compound, forms membrane pores upon incubation with EVs by interacting with cholesterol and enhancing membrane permeability. Saponin surfactant characteristics and other biological functions, such as hemolysis, are ascribed to their distinctive structural properties and amphiphilic nature, resulting from hydrophilic sugar molecules and hydrophobic genes, such as aglycones.[123] Haney et al[117] used a saponin-based method to load

catalase onto EVs. This involved supplementing a mixture of catalase and EVs (from primitive 264.7 macrophages) with 0.2% saponin and placing them in a shaker for 20 min at room temperature. Although saponins are classified as surfactants, they do not degrade catalase, preserving their enzymatic activity. Hence, this technique demonstrates exceptional loading proficiency, sustained-release properties, and the ability to safeguard against protease degradation through peroxidase activity. Saponin can also help load other hydrophilic molecules into EVs. For saponin-assisted drug loading of hydrophilic porphyrins, the EVs and drug were incubated with 0.1 mg/mL of saponin for 10 min at room temperature. This method has a high drug-loading capacity.[120] Nevertheless, the potential for in vivo hemolytic activity associated with saponins remains a concern. The occurrence of erythrocyte hemolysis seems to stem from the ability of saponins to interact with cholesterol on cellular membranes, triggering modifications in pore architecture, membrane permeability, and negatively charged glycan components of the cell exterior. The precise mechanism by which saponins exert their hemolytic activity remains elusive. When saponin is used as an adjuvant loading method for EVs, maintaining a minimal concentration of saponin and immediately washing the EVs following incubation with saponin is advisable.[124]

Extracellular Vesicles for Drug Delivery

Stimulus-Responsive Drug Delivery Systems

Both natural and engineered EVs have the potential to target-specific tissues and cells, facilitating the delivery of bioactive molecules and drugs. Consequently, they are becoming increasingly important for drug delivery systems. However, EV-based strategies remain constrained by their inability to achieve targeted release in specific environments. It is possible to achieve several stimuli, including internal stimuli such as pH and external stimuli such as light, temperature, focused ultrasound, simple slow drug release, and controlled drug release in complex physiological environments. Therefore, it is possible to develop a more intelligent drug delivery system.

Internal Stimulation

Various techniques can facilitate selective release of EVs at different pH values. Wang et al[38] formulated an injectable, self-healing peptide hydrogel termed FHE@EVs, integrating Pluronic F-127 (F127), oxidized hyaluronic acid (OHA), and poly-ε-L-lysine (EPL). The extrusion procedure disrupted the EVs membrane, facilitating vigorous integration with the drug substance, which was then encapsulated within EVs (Figure 3A). Adipose mesenchymal stem cell (AMSC)-derived EVs display a characteristic negative charge, enabling their loading into hydrogels via electrostatic interactions with EPLs. Because of the high hydrolytic stability of the Schiff base bond at neutral pH and its tendency to break in acidic environments, EVs can be released in weakly acidic environments. Thus, the FHE hydrogel has a slower release rate at pH 7.5 and releases fewer EVs than at pH 5.5. These discoveries suggest that FHE hydrogels are ideally suited for administering drugs to tissues characterized by acidic microenvironments, including diabetic wounds, enhancing therapeutic efficacy and not significantly affecting non-targeted healthy tissue.

Oxidative stress modulates the liberation and molecular cargo of EVs, influencing their functional properties, which modulate the redox state of the target cells. The specific biochemical load of EVs released under oxidative stress can influence signaling pathways in target cells by mediating protective or deleterious signals, depending on the nature of the stress. Consequently, understanding the molecular composition of EVs released in response to oxidative stress can facilitate prediction of their impact on target cells. In a study by Szabó-Taylor et al[126] monocytes were exposed to proinflammatory environments linked to oxidative stress; subsequently, the expression levels of sulfhydryl-reliant oxidoreductase, specifically peroxiredoxin 1, were evaluated both within the cells and in their EVs. Although outer-surface peroxiredoxin 1 is readily detected in secretory cells and EVs, over-oxidized and enzyme-inactivated forms are enriched only in EVs.[126] This suggests that cells respond to oxidative environments by releasing EVs adorned with membrane proteins containing oxidized sulfhydryl groups, preserving a reduced membrane state amidst oxidative stress. Analogously, during the storage of erythrocytes, the thiol segment of the cytosolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) undergoes oxidation, which subsequently leads to the expulsion of the oxidized variant of GAPDH in a manner associated with EVs.[127] This suggests that, as a defensive strategy, proteins within the lumen containing oxidized thiols may also be packaged into EVs for release. The liberation of EVs under conditions of thiol depletion serves a dual purpose: cytoprotection and modulation of inflammation. The oxidized proteins and

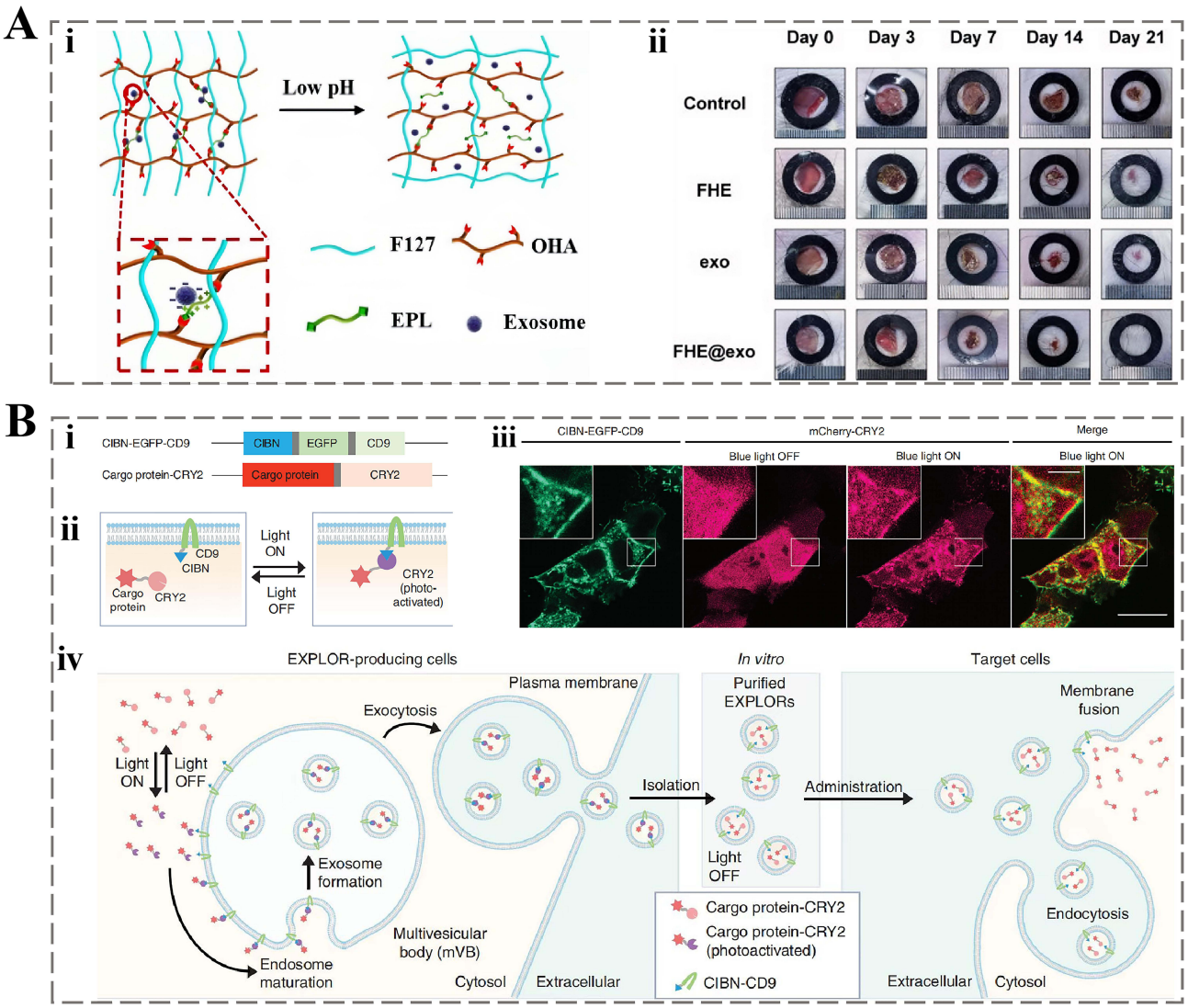

Figure 3 Stimulus-responsive drug delivery systems. (**A**) i) The release scheme of pH-responsive exosomes in FHE hydrogel; ii) Representative images of the wound healing process treated with FHE, EVs, FHE@EVs, and controls. Adapted from Wang C, Wang M, Xu T, et al. Engineering bioactive self-healing antibacterial exosomes hydrogel for promoting chronic diabetic wound healing and complete skin regeneration. Theranostics. 2019;9(1):65–76. https://creativecommons.org/licenses/by/4.0/.[38] based on CC BY-NC License, Copyright © Ivyspring International Publisher. (**B**) i) Schematic representation of the DNA structure used for exosome production; ii) Schematic representation of fusion proteins and their roles; iii) Fluorescence imaging of mCherry before and after 488-nm laser stimulation; iv) Schematic representation of EXPLOR technology. Adapted reprinted with permission from Yim N, Ryu S-W, Choi K, et al. Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. Nat Commun. 2016;7:12277.[125] based on CC BY License, Copyright © 2016, The Author(s).

phospholipids associated with these EVs may serve as inflammatory molecular signatures, contributing to initiating inflammatory responses.[128]

Oxidative sulfhydryl modifications have been observed to facilitate coagulation in an EV-dependent manner. Numerous oxidative and proinflammatory scenarios that deplete sulfhydryl groups have been experimentally validated, leading to the buildup of thrombospondin-harboring EVs, both in vitro and in vivo. The prothrombotic effects of EVs are attributed to phosphatidylserine (PS) and EV-associated tissue factors (TF).[129] PS-rich membranes are negatively charged and efficiently assemble coagulation factors, thereby accelerating coagulation processes.[130] As PS is universally present in the outer leaflet of EVs membranes,[131] augmentation of the secretion of EVs is likely to sufficiently elevate PS availability, fostering PS-mediated coagulation. TF initiates an exogenous coagulation cascade.[132] TF expression and activity are increased in EVs secreted by cells stimulated by sulfhydryl-responsive compounds, suggesting that redox modification is an important regulator of EV-associated TFs.

External Stimuli

The controlled release of EVs can be achieved by constructing light-responsive EVs with enhanced therapeutic effects. Yim et al[125] engineered a light-sensitive protein complex called CRY-CIBN. In this system, CIBN proteins were tethered to the EV-specific CD9 markers, whereas CRY2 was linked to the cargo proteins, enabling a photo-controlled interaction and subsequent release. Under blue light illumination, the cargo-bearing protein CRY2 undergoes intracellular repositioning of the CD9-CIBN proteins, assembling a CRY2-CIBN protein complex. This complex facilitates the transportation of cargo proteins, along with CD9, into EVs. Upon completion of the EVs collection, the light source was extinguished, leading to the dissociation of the CRY2-CIBN protein complex. The resulting cargo proteins are free in EVs and can be released when vesicles reach their destination. (Figure 3B). Li et al[133] introduced an integrated therapeutic approach leveraging tumor-derived EVs decorated with nanoparticles incorporating a photosensitizing agent (indocyanine green) and a tyrosine kinase inhibitor (gefitinib), designated IG@EVs. This strategy potentiates antitumor efficacy against OSCC by synergistically harnessing phototherapeutic and molecularly targeted therapeutic modalities. IG@EVs demonstrate considerable photothermal and photodynamic effects because of their augmented photothermal conversion efficiency and generation of reactive oxygen species (ROS). In vivo, owing to passive and homologous targeting, IG@EVs accumulated efficiently in the tumor and penetrated deeply into the tumor center. The phototherapeutic effect of IG@EVs not only directly elicits substantial damage to cancer cells, but also promotes the liberation and cytoplasmic migration of gefitinib. This significantly inhibits cell proliferation and tumor angiogenesis. The synergistic integration of phototherapy and molecularly targeted therapy ultimately achieves tumor elimination and inhibits lymphatic metastasis.

The controlled release of EVs can be achieved by constructing thermally responsive EVs with enhanced therapeutic effects. Luo et al[134] demonstrated that M2 macrophage-derived EVs (MEs) facilitate angiogenesis by activating the Wnt signaling cascade. Zeng et al[135] designed a dual-layered microneedle wound dressing system (MEs@PMN) that incorporates microencapsulated entities at the needle tips and polydopamine (PDA) nanoparticles within the supporting layer. The ME@PMN hydrogel system exhibited exceptional biocompatibility and pronounced photothermal response. The liberated MEs elicited a substantial transformation in macrophage polarization, shifting from a proinflammatory M1 state to an anti-inflammatory M2 phenotype, and exhibited stimulatory effects on angiogenesis at the wound interface. In vitro and in vivo investigations involving diabetic rat models illustrated that microneedles fabricated with photosensitive hydrogels carrying EVs expedited diabetic wound repair mechanisms, specifically by suppressing inflammatory responses and augmenting the development of new blood vessels (angiogenesis). In addition, studies have confirmed the advantageous effect of moderate heating (approximately 40 °C) on promoting angiogenesis. In a separate study, Yu et al[136] developed an injectable temperature-sensitive hydrogel composed of a poly(glycolic acid) copolymer and triple-block polymer of poly(ethylene glycol) (PLGA-PEG-PLGA) loaded with miR-138-modified stem cell EVs. Hydrogels were injected into the injury sites of Sprague-Dawley rats with spinal cord injury (SCI). In vitro experimental results demonstrated that umbilical cord mesenchymal stem cell-derived EVs (UCMSC-EVs) modified with miR-138-5p mitigated inflammatory reactions in BV-2 cells by regulating the NLRP3-caspase1 signaling pathway. Furthermore, these modified EVs reduced neuronal apoptosis by suppressing intracellular ROS levels via the Nrf2-Keap1 signaling pathway. The results of in vivo experiments revealed that the P-EVs-138 hydrogel effectively promoted neural regeneration and recovery in rats with SCI. The in vivo experimental findings confirmed that the P-EVs-138 hydrogel contributed to the enhancement of neural regeneration and recovery in rats with SCI.

Focused ultrasound (FUS) is a noninvasive, non-ionizing technology that precisely delivers localized acoustic energy to tissues, achieving submillimeter precision. Altering acoustic parameters allows for the induction of diverse biological effects on cells and tissues, encompassing thermal and mechanical effects. Sheybani et al[137] demonstrated that FUS thermotherapy significantly enhanced the release of glioma-derived EVs (GEVs) (approximately 46%) and altered their proteomic profile. This shift includes an increase in the number of markers associated with common EVs, a decrease in the number of markers associated with cancer progression and drug resistance, and a change in the number of markers related to inflammation. Upon pulsing dendritic cells (DC) with GEVs, the original GEVs were observed to inhibit DC production of IL-12p70 in a dose-dependent manner regarding the GEVs. In contrast, GEVs derived from cells subjected

to FUS hyperthermia demonstrated the capacity to stimulate the production of IL-12p70 by DCs, indicating a proinflammatory stimulus. The use of FUS hyperthermia prompts the expulsion of proteomically distinct GEVs. These GEVs can promote important components of innate immune activation. This phenomenon is a potential modus operandi for the interplay between FUS and the tumor immune microenvironment, underscoring the significance of GEV-linked biomarkers in tracking responsiveness to FUS therapy.

EVs Delivery System Route of Administration

Intravenous Injection

Intravenous administration is the predominant route of administration of EVs.[138] Zhang et al[72] delivered exogenous miR-210 to an affected region via intravenous injection, enabling its function in the targeted area and enhancing VEGF expression and angiogenesis. Cui et al[139] experimentally demonstrated that MSC-derived EVs, when injected intravenously, could be traced within the brain of Alzheimer's disease (AD) mouse models, leading to notable enhancements in learning and memory capabilities. These EVs reduced plaque accumulation and amyloid-beta protein levels, while normalizing the levels of inflammatory cytokines, indicating their therapeutic potential. Furthermore, when administered intravenously, EVs loaded with catalase were efficiently transported to the brains of mice with Parkinson's disease. These catalase-laden EVs effectively accumulate within the neurons and microglia in the brain, exerting a robust neuroprotective effect. Specifically, they safeguarded the SNpc neurons against acute oxidative stress-induced damage in mice with inflammation.[117] Despite the widespread use of intravenous administration, the half-life of systemically administered EVs remains insufficient, with a range of only a few minutes to several hours.[72]

Nasal Administration

Nasal drug delivery represents a promising avenue for circumventing the BBB in the context of the central nervous system (CNS) drug delivery.[140] In the context of brain disease research, nasal administration enables more efficient transport of EVs to the brain than intravenous administration. Nasal delivery of EVs has attracted considerable attention because of its noninvasive nature, the possibility of repeated administration, and the fast infiltration of administered EVs into numerous regions of the forebrain. This could enable a lower dose to elicit functional recovery in neurological disease. The findings of this study suggest that the intranasal route is the most efficacious means of delivering EVs to forebrain regions. This finding reinforces that intranasal administration of EVs could serve as a preventive neuroprotectant, mitigating the chronic low-grade neuroinflammation responsible for mild cognitive decline. The proposed approach could be a preventive intervention for AD in the elderly, while also aiding the alleviation of neurodegeneration.[141] Long et al[142] found that nasal administration of MSC-derived A1-EVs reduced inflammation, prevented memory dysfunction following sustained status epilepticus, maintained normal neurogenesis, and preserved cognitive and memory functions. These findings indicate that intranasal administration of A1-EVs may have therapeutic benefits in other neurological disorders with severe neuroinflammation. Kalani et al[143] used curcumin-encapsulated embryonic stem cell-derived EVs (MESC-EVs^{cur}), administered nasally, to rejuvenate neurovascular units in mice subjected to ischemia-reperfusion (IR) injury. Compared to untreated mice, MESC-EVs^{cur} administration restored vascular endothelial integrity, marked by the restoration of tight junction proteins, such as claudin-5 and occludin, as well as the adhesion protein VE-cadherin, in the brains of mice that had undergone IR injury. However, intranasal delivery poses inherent constraints, notably in terms of the constrained dosage capacity, restricted surface coverage of the olfactory epithelium, brevity of drug residence time for effective absorption impacts on the physiology of nasal secretions.[144]

Oral Administration

Oral administration is a crucial route of administration in clinical practice.[72] Oral administration has the advantages of small fluctuations in drug levels in plasma, high patient compliance and acceptance, low cost, and addressing potential problems such as drug solubility and toxicity.[145] For instance, Nazimek et al[146] demonstrated that a solitary systemic administration of EVs enriched with miRNA-150 sourced from regulatory T and B1a cells effectively elicited prolonged attenuation of delayed-type hypersensitivity (DTH) responses in mice. They conducted a comparative analysis of the therapeutic outcomes achieved by inhibiting OVA-specific T cell-derived EVs through various routes of administration (intraventricular, intraperitoneal, intradermal, and oral) in OVA-sensitized mice exhibiting DTH immunoreactivity. Each

delivery mode notably suppressed DTH-induced ear swelling, and the most pronounced effect was observed following oral administration. Aqil et al[14] used milk-derived EVs loaded with curcumin to obtain EVs-curcumin (EVs-CUR). Administration of oral EVs-CUR resulted in a three- to five-fold increase in drug levels compared to that achieved with free curcumin. In addition, using EVs orally represents a low- and high-impact treatment option that is convenient for patients, including children. Although the oral route of administration offers numerous advantages, significant obstacles to the use of EVs still exist. Most EVs remain in the small intestine, with the unabsorbed portion translocating to the colon and a small portion (<5%) diffusing into the liver, which presents a challenge for their diffusion into the brain. Furthermore, the impact of microbial and enzymatic digestion within the gastrointestinal tract on the structural integrity of EVs must be considered.[147] Current studies focusing on the oral administration of EVs are predominantly constrained to those sourced from dietary origins, with cow's milk being the most prevalent example.[148] After purification, the quantity of EVs acquired from non-dietary sources fell short of fulfilling the demand for oral utilization.

Stereotactic Injection

Stereotactic injection entails dissolution or dispersion of a drug and polymer in a suitable solvent, followed by local injection near the target site. The utilization of stereotactic injection effectively addresses the challenges associated with inadequate stability, unintended toxicity to off-target tissues, and erratic drug-release patterns. In addition, it reduces the duration of drug retention, minimizes dosage requirements, and mitigates both toxicity and adverse reactions, enhancing therapeutic efficacy and safety.[149] Orefice et al[150] used real-time monitoring to observe the in vivo dissemination of EVs after stereotactic drug delivery. They encapsulated the unassociated AAV (std-AAV) encoding green fluorescent protein (GFP) into EVs secreted by HEK293 cells and injected them into the ipsilateral hippocampus of mice. The findings of this study indicate that EVs enhance the dissemination of GFP, facilitating its wider spread from the initial injection site to encompass the contralateral hemisphere, in contrast to GFP administered without EVs, which predominantly remain confined within the ipsilateral hemisphere. Furthermore, after injection, the fluorescence mediated by AAV at the injection site with EVs lasted 95 days and 31 days without EVs. Regarding stroke recovery, Gao et al[151] examined the biological functions and mechanisms of cerebral endothelial cell-derived EVs (ECs-EVs) in relation to brain plasticity. Following intravenous administration, the EVs were cleared rapidly through the hepatic and renal pathways within 6 h and disappeared rapidly from murine circulation. Given these findings, researchers have directionally injected ECs-EVs into rat brain ventricles to enhance their accumulation around the lesions and achieve long-term effects. During the recovery period following stroke, miRNA-126-3p-containing ECs-EVs were observed to upregulate the expression of genes related to plasticity signal transduction, alter neuroplasticity in the motor cortex, and actively modulate synaptic plasticity. Despite the advantages of stereotactic brain injections in terms of directness and accuracy, this method presents certain challenges. Stereotactic injections require sophisticated equipment and expertise from a trained professional, and they require imaging guidance to accurately localize the target. Injection deviation can cause significant brain damage. Furthermore, anesthesia and surgical procedures may lead to significant postoperative pain, infection, surgical complications, and neurological complications.[152]

Extracellular Vesicles for Disease Diagnosis and Treatment

Diagnostics

EVs can be identified in various body fluids, including the blood, saliva, cerebrospinal fluid, and urine. The presence of EVs in these fluids may reflect the state of parental cells, making EVs an ideal noninvasive biomarker for disease diagnosis.[153–157] The miRNAs in EVs are the most used tissue-specific biomarkers. Consequently, EVs in biological fluids can be used to precisely diagnose diseases.[158] Glioblastoma multiforme (GBM) releases a considerable number of EVs with cancer-specific characteristics into the circulation.[159] Skog et al[160] demonstrated that EVs can traverse the BBB and contain mRNAs, miRNAs, and angiogenic proteins. The researchers exposed brain microvascular endothelial cells to GBM-derived EVs, noting their dual function of stimulating endothelial tubulogenesis and transferring functional RNA to target cells. In addition, serum samples from patients with GBM revealed tumor-specific mRNA mutations (EGFRvIII variants) and glioma miRNA profiles, suggesting the potential of EVs to offer cancer-related molecular insights through blood tests. Yoshioka et al[161] developed a highly sensitive and rapid technique for analyzing EVs, designated as ExoScreen, to examine the surface proteins in EVs present in patient blood samples. This technique can identify biomarkers of colorectal cancer, permit the monitoring of

circulating EVs in serum with no purification step, and demonstrate superior performance relative to conventional methods such as immunoblotting and ELISA. Furthermore, the ExoScreen method detected EVs double-positive for CD147 and CD9, which were secreted from colon cancer cells in large quantities in the sera of patients with colorectal cancer. This study proposed the ExoScreen technique as a potential instrument for identifying EVs from minimal volumes of serum (as little as 5 μL) derived from patients with cancer. It aims to detect circulating EVs originating from cancer cells, offering a novel approach for early detection or monitoring. Alterations in insulin signaling pathways have been suggested as a possible hallmark of bipolar disorder (BD), underpinned by the prevalent occurrence of metabolic conditions such as type 2 diabetes and obesity among patients with BD.[162] Alterations in insulin signaling pathways can cause a spectrum of metabolic dysfunction. Consequently, EVs metabolites could serve as biomarkers for bipolar disorders, offering a novel perspective for the diagnosis and understanding of the disease.[162] Du et al[163] used liquid chromatography-tandem mass spectrometry to analyze serum EVs in patients with BD and identified 26 differentially expressed serum EVs metabolites in patients with BD compared to healthy controls. Further investigation revealed that 15 EV-derived metabolites could precisely distinguish between patients with BD and healthy controls. Yu et al[164] developed a nanoliquid biopsy assay for precise identification of target tumor-derived EVs. This methodology uses a dual biomarker antigen recognition and sequestration strategy, enhancing the specificity, sensitivity, and cost efficiency of tumor exosome detection. This is accomplished by affixing two complementary capture antibodies onto magnetic and gold nanoparticles, facilitating the precise identification of targeted tumor-derived EVs. Several commercial organizations have embarked on the development of EV-based cancer diagnostics such as Caris Life Sciences, Exosome Diagnostics, and Humsa Bio Med. Among them, Exosome Diagnostics sponsored an observational clinical trial to evaluate the effectiveness of the "ExoDx Prostate Intelliscore" diagnostic test. The study had 532 participants, all of whom exhibited early symptoms of prostate cancer, in part due to high levels of prostate-specific antigen (2.0–10 ng/mL). Participants were divided into two groups: men who had planned their first prostate biopsy and men who had not planned their prostate biopsy. The aim of this study was to assess the urine test performance of men in the first group and its impact on biopsy decision-making. Currently, ExoDx is the only exosome-based test for prostate cancer in the NCCN guidelines, independent of PSA and other standard-of-care information. EVs Diagnostics also launched the ExoDX Lung (ALK), marking the debut of a groundbreaking cancer diagnostic solution globally. This innovative product harnesses the detection of EVs and circulating tumor DNA as its foundation, empowering real-time identification of EML4-ALK mutations in individuals diagnosed with non-small cell lung cancer.[8]

Treatment

Respiratory Diseases

Owing to the diversity of etiologies, drug resistance, delayed diagnosis, and management approaches for chronic diseases,[165] there are still several obstacles in the treatment of respiratory diseases. The low immunogenicity, high safety, targeting of EVs, and their ability to act as carriers for drug delivery are considerable advantages of EVs for treating respiratory diseases.

Coronavirus disease 2019 is a respiratory disease caused by a cytokine storm mediated by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It culminates in severe tissue deterioration, encompassing apoptosis and necrosis, along with the impairment of alveolar epithelial cells and vascular endothelial tissues, leading to persistent lung invasion by sustained immune cells.[166] Jamalkhah et al[167] proposed an innovative strategy for modifying MSC-EVs by manipulating RNA, hindering viral dissemination, mitigating inflammation, and preventing immune evasion in infected cells. This approach also seeks to barricade viral particles from infiltrating uninfected cells and lung tissues, augmenting antiviral immunity. To deliver potential antiviral drugs to specific tissues, Fu et al[168] constructed a method based on the VSVG viral pseudo typing protein to load the SARS-CoV-2 receptor-binding domain (RBD) of virus-spiking proteins onto EVs membranes. Capable of recognizing the Angiotensin Converting Enzyme 2 (ACE2) receptor, which is abundant in type 2 alveolar epithelial cells, RBD-modified EVs demonstrate specific accumulation in the lungs. The GFP signals observed in the lung region are significantly reduced when compared to the control group, indicating that RBD-EVs, with their inherent targeting ability, possess the potential for targeted antiviral drug delivery in the context of SARS-CoV-2 infections. Recognizing the importance of protein palmitoylation in EV-mediated ACE2 targeting and secretion, Xie et al[169] developed engineered EVs (PM-ACE2-EVs) by incorporating S-palmitoylation-

dependent PM targeting sequences fused to ACE2, which increased ACE2 enrichment in EVs, enabling PM-ACE2-EVs to efficiently mitigate the SARS-CoV-2 viral load and protect against lung inflammation triggered by the virus (Figure 4A). Furthermore, Sengupta et al[170] reported that a single intravenous administration of bone marrow-derived EVs showed a significant reversal of hypoxia and cytokine storm in critically ill patients hospitalized with SARS-CoV-2, with no treatment-related adverse effects.

Allergic asthma (AA) is an inflammatory disease of the airways clinically characterized by bronchial hyper-responsiveness, mucosal edema, and airflow limitation.[172] MSC-EVs augment the immunosuppressive capacity of Tregs in patients with asthma and upregulate IL-10 and TGF-β1 in peripheral blood mononuclear cells.[173] Furthermore, intranasal MSC-EVs administration has been demonstrated to promote the expansion of IL-10-producing mesenchymal macrophages

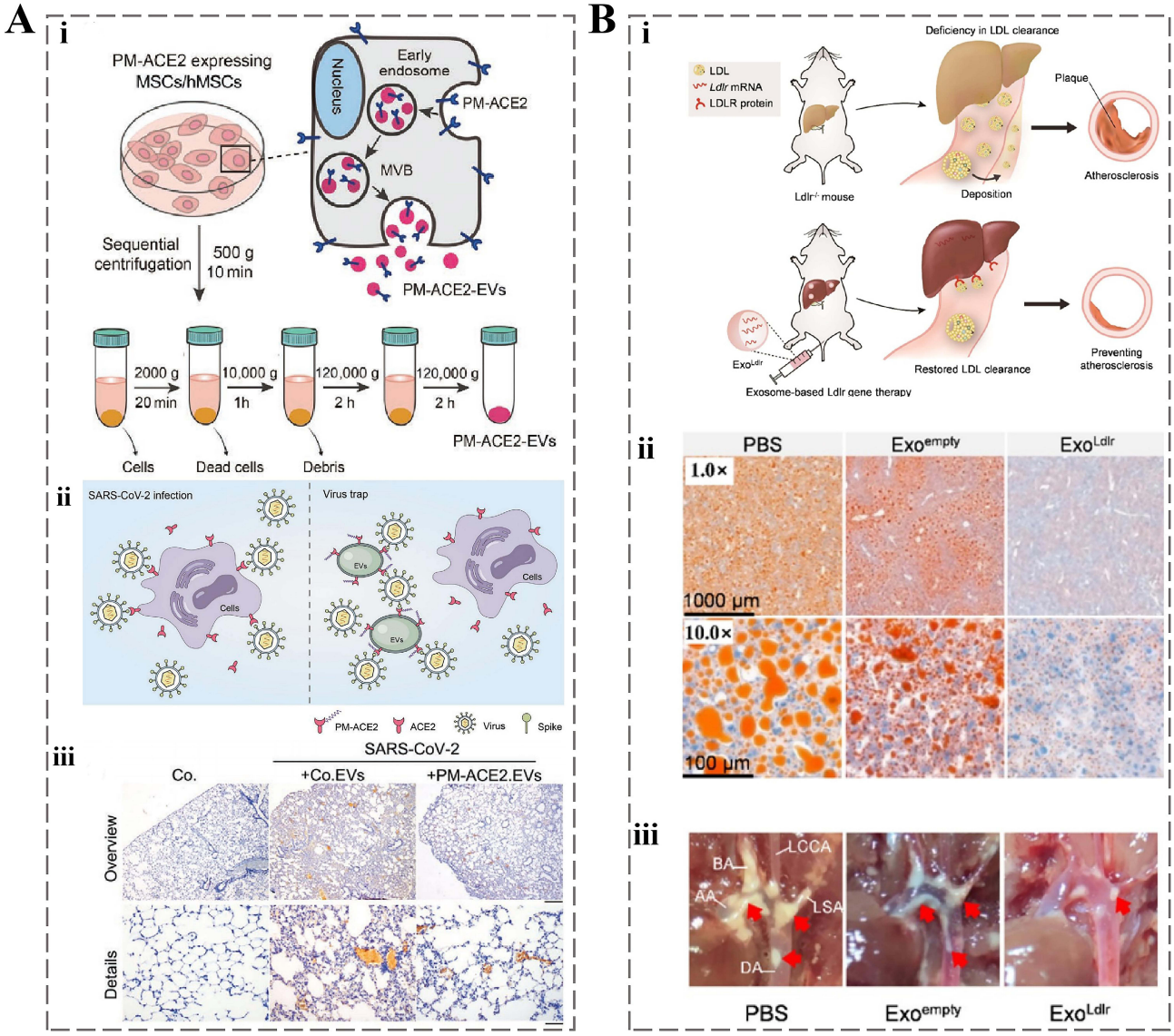

Figure 4 Application of EVs in treatment of respiratory and circulatory diseases. (**A**) i) Schematic diagram of PM-ACE2 EVs biosynthesis and differential ultracentrifugation purification; ii) A proposed model for constructing a SARS coronavirus type 2 trap using EVs enriched with engineering PM-ACE2; iii) Immunohistochemical determination of SARS-CoV-2 S protein antigen in lung slices of ACE2 transgenic mice. Scale bar, 500 μm (top) or 100 μm (bottom). Adapted reprinted with permission from Xie F, Su P, Pan T, et al. Engineering extracellular vesicles enriched with palmitoylated ACE2 as COVID-19 therapy. Adv Mater. 2021;33(49):e2103471. © 2021 The Authors. Advanced Materials published by Wiley-VCH GmbH.[169] . Copyright © 2021 The Authors. (**B**) i) Schematic diagram of the study; ii) Representative images of oil red O-staining of liver samples from a designated group; iii) Representative aortic arch view of atherosclerotic lesions in Ldlr mice treated as directed. Adapted reprinted with permission from Li Z, Zhao P, Zhang Y, et al. Exosome-based Ldlr gene therapy for familial hypercholesterolemia in a mouse model. Theranostics. 2021;11(6):2953–2965. https://creativecommons.org/licenses/by/4.0/.[171] based on CC BY License, Copyright © The author(s).

in the lungs, helping prevent AA.[174] M2 macrophage polarization plays a significant role in the pathogenesis of AA. The long noncoding RNA Dnmt3aos has been shown to influence M2 macrophage polarization. On this basis, Pei et al[175] focused on exploring the combination of polylactic-co-glycolic acid (PLGA)-based nanoparticles with naturally occurring exosome membranes from M2 macrophages to deliver a Dnmt3aos smart silencer to treat AA in mice.

Diseases of the Circulatory System

Owing to the potential adverse effects and limited efficacy of conventional pharmacological interventions, surgical procedures are inherently risky and often require a prolonged recovery period, such as coronary artery disease, for conditions amenable to surgical intervention. Even if treatment succeeds, the risk of recurrence remains high for many circulatory diseases. Although several therapeutic interventions, including cell therapy, have been initiated in the last two decades, the low survival and/or implantation of transplanted cells in ischemic heart tissues has limited their clinical efficacy.[176] Mechanistically, little is known about the principles of cell therapy that improve function. However, some experimental data suggest that cell therapy may have paracrine effects through the release of EVs and other factors.[177,178] Recent research has begun to focus on cell-free therapies, particularly those based on EVs. Compared with conventional pharmacological and surgical treatments, EVs are less immunogenic, targeted, and promote tissue repair. These characteristics make them a promising tool to treat cardiovascular diseases, as they offer a better safety and tolerability profile.

Myocardial infarction (MI), defined as an injury caused by myocardial ischemia, is a significant cause of morbidity and mortality global scale.[179,180] EVs derived from MSCs, induced pluripotent stem cells, and immune cells have been demonstrated to play a key role in cardio protection following MI. This is primarily achieved by promoting cell proliferation and angiogenesis, reducing scarring, inhibiting inflammatory responses, and reducing cell apoptosis. To enhance the cardiac targeting of EVs, several targeting peptides, including cardiac myocyte-specific peptide, cardiac-targeting peptide, and ischemic myocardial-targeting peptide, were fused to the EVs membrane protein LAMP2B. Targeted peptide-modified EVs exhibited higher cardiac retention and enhanced therapeutic efficacy than non-targeted modified EVs.[181] Regeneration of the myocardium following MI is mediated primarily by modified EVs, which are loaded with cargo by manipulating the parent cells. Administration of small EVs (sEVs) following MI has been shown to induce cardiac repair.[182] In a porcine MI model,[183] sEVs derived from cardiospheres exhibited reduced infarct size and promoted neovascularization, demonstrating their therapeutic potential. Furthermore, studies have investigated the potential of sEVs in a human model of Duchenne muscular dystrophy.[183] Cardiosphere-derived sEVs have been shown to reduce arrhythmia and normalize oxygen consumption. Although sEVs therapy is still in its infancy in animal and human models, the findings suggest that sEVs are a viable therapeutic option with potential cardiac benefits comparable to those of direct delivery of parental stem or progenitor cells. Hydrogel patches encapsulating EVs from pluripotent stem cell-derived cardiomyocytes provide sustained, slow release in acute MI rats, enhancing heart recovery.[184] This represents a highly promising avenue for research into MI treatment.

Atherosclerosis, an inflammatory vascular disorder, arises from lipid buildup in the vessel walls, causing plaque formation and lumen constriction. Intercellular communication is crucial for pathogenesis. The vessel wall comprises many cell types, including ECs, vascular smooth muscle cells, fibroblasts, and the extracellular matrix (ECM), and the phenotype of the vascular system undergoes intricate interconnecting changes during atherosclerosis development. EV-mediated cellular interactions are important for atherosclerosis pathogenesis.[185] EVs may promote atherosclerosis or mediate vasoprotection contingent on donor cell status. Atherosclerosis-derived EVs from vascular/inflammatory cells alter the recipient cell phenotype, boosting progression via cell proliferation, migration, or modulation of inflammation. Conversely, EVs containing molecules with anti-atherosclerotic activity can modulate recipient cells to prevent atherosclerosis.[186] Li et al[171] devised a strategy using EVs to deliver Ldlr mRNA, effectively reinstating Ldlr expression and eliciting therapeutic benefits, such as mitigating fatty liver degeneration, inflammation, and atherosclerotic lesions. This innovative approach holds great promise as a therapeutic strategy for the management of cardiovascular disease (Figure 4B).

Neurological Disorders

The intricate structure and function of the nervous system often renders disease mechanisms challenging. A major challenge in this field of neurology is the accurate diagnosis of diseases in their early stages. With the emergence of

symptoms, the disease may progress to a more advanced stage, making an accurate diagnosis more difficult. Numerous neurological disorders remain unresolved. Consequently, treatment largely focuses on alleviating symptoms and enhancing quality of life. Furthermore, the BBB protects the brain from noxious substances while simultaneously impeding the influx of numerous potential therapeutic agents. Due to the difficulty in penetrating the BBB, about 98% of potentially potent drugs, despite promising to improve the treatment of CNS disorders, are difficult to use in the clinic.[187] To break through the limitations of the BBB, researchers have developed a variety of drug nanoparticles.[188] However, the dispersive nature of drug nanoparticles in the bloodstream raises two major issues: the potential toxicity of the nanomaterials and the rapid clearance of these drug nanopreparations by the mononuclear phagocyte system.[189] Furthermore, prolonged use of certain medications may also result in the emergence of adverse effects and development of drug resistance.[190]

Parkinson's disease (PD) is characterized by the degradation of dopaminergic neurons located within the compact area of the substantia nigra, along with the appearance of Lewy bodies, which are intricate accumulations of misfolded α-synuclein proteins (α-syn).[191,192] Accordingly, reducing α-syn expression represents a crucial strategy for treating Parkinson's. Recently, EV-mediated gene delivery systems have attracted considerable attention as potential therapeutic avenues for PD, targeting either the downregulation of α-synuclein expression or mitigation of pathological α-syn aggregates. These systems use electroporation to encapsulate small hairpin RNA (shRNA) microcircuits (shRNA-MCs) within the RVG peptide-modified EVs. Given their nanoscale dimensions, EVs offer advantages over plasmids, while surpassing siRNAs in terms of prolonged gene-silencing efficacy. This innovative strategy ensures the precise delivery of shRNA-MCs to the brain, effectively diminishing α-synuclein aggregations.[53] The encapsulation of DNA aptamers, designed to specifically identify and hinder α-synuclein from recruiting endogenous α-syn into pathological clumps within neuron-targeted RVG-EVs, has been demonstrated to potently diminish the formation of pathological α-synuclein aggregates and ameliorate motor impairments.[193] The approach represents a promising strategy for PD treatment. Besides the use of shRNA-MCs and aptamers, the utilization of antisense oligonucleotides delivered via EVs sourced from BMSCs is a promising alternative pathway for PD therapy. This approach selectively enhances the regulation of α-synuclein expression, offering a novel and targeted strategy for addressing the underlying mechanisms of the disease.[54] Liu et al[194] recently devised a multifaceted nucleoshell delivery system, named the "nanoscavenger", for gene therapy, aiming to elicit a combined therapeutic benefit from genes and chemo drugs. The core component comprised C/ANP/S loaded with siRNA-targeting SNCA (siSNCA) and curcumin. siSNCA attenuates the expression of α-syn proteins, whereas curcumin reduces the presence of α-syn aggregates. RVG-modified imDC-EVs serve as shells that traverse the BBB and target lesion regions, enhance drug bioavailability, and mitigate systemic toxicity. In addition, the presence of imDC-EVs enabled the clearance of immune activation. Improved loading efficiency compared with packing goods directly into EVs (Figure 5A).

Stroke is a leading cause of mortality and disability worldwide.[196] Currently, no pharmaceutical agents are available for its treatment. Increasing evidence suggests that EVs derived from MSCs subjected to distal ischemic preconditioning, endothelial progenitor cells, M2 microglia, and astrocytes can be used to treat stroke, primarily by enhancing angiogenesis and neurogenesis. Moreover, modified EVs from cells transfected with therapeutic miRNAs,[197–199] cyclic RNAs,[195,200] and plasmid cDNAs[201,202] demonstrated enhanced therapeutic efficacy compared to natural EVs. While the encapsulation of bioactive molecules within EVs enhances therapeutic efficacy, a crucial aspect that merits attention is the capacity of these EVs to precisely navigate to damaged brain regions. Yang et al[195] produced NGF-EVs-RV by co-transfecting donor cells with RVG-LAMP2B and pCI-neo-NGF plasmids. NGF-EVs-RV effectively targeted the area of injury and demonstrated anti-inflammatory, pro-survival, and cell proliferation effects (Figure 5B). Tian et al[69] developed a straightforward and potent drug delivery system by conjugating the c(RGDyK) peptide to EVs and encapsulating curcumin, which markedly suppressed inflammation and apoptosis at the injured site.[89] Edaravone (Edv) is a pharmaceutical agent used primarily to treat cerebral infarction. Li et al[203] posited that macrophage-derived EVs encapsulating Edv could facilitate drug release in the ischemic region, enhancing its bioavailability and neuroprotective effects while reducing ischemic cerebral infarction. In the clinical setting, numerous pharmaceutical agents have been evaluated for their efficacy in the treatment

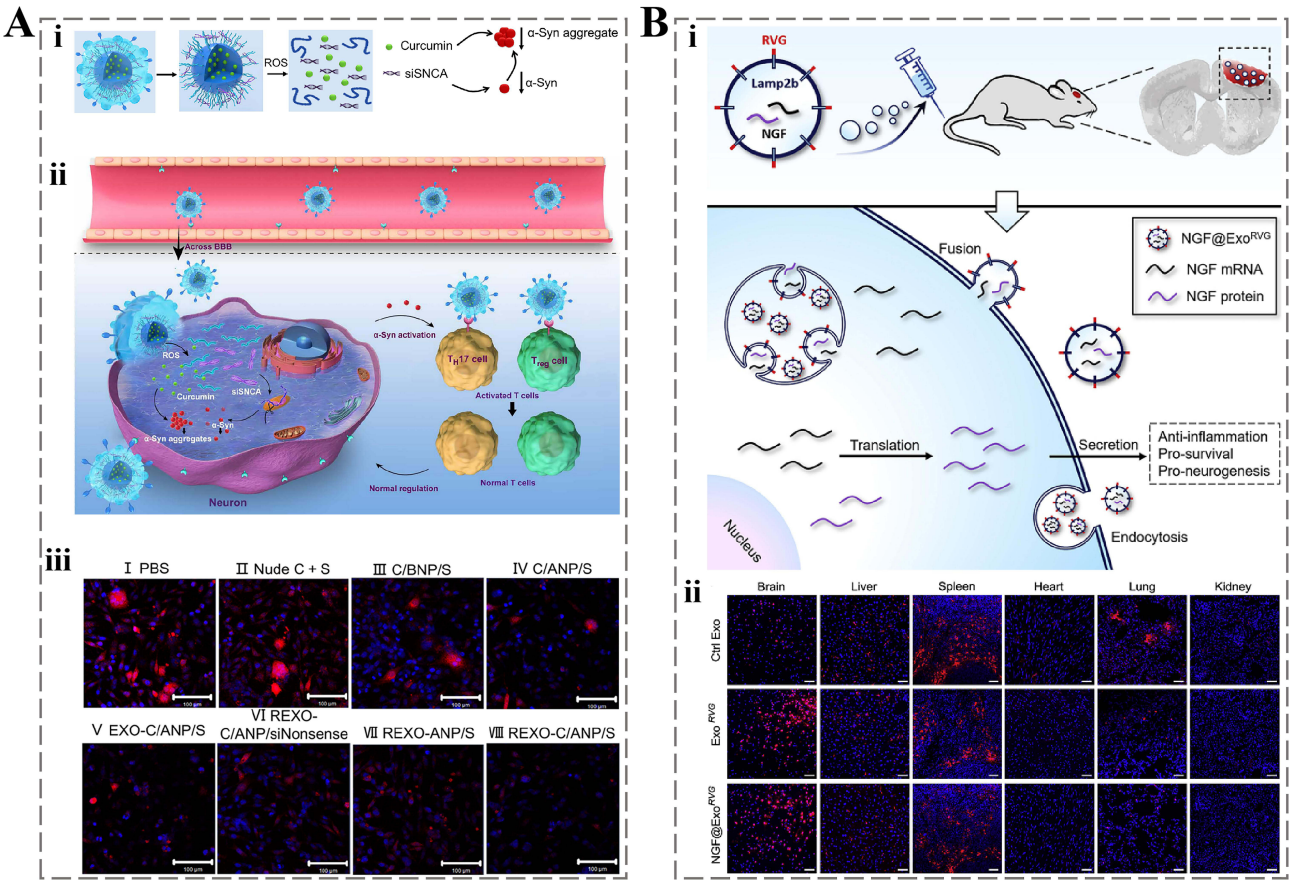

Figure 5 EVs for neurological disease treatment. (**A**) i) Scheme of REXO-C/ANP/S synergistic effect against α-syn; ii) Potential mechanism of nano clearance; iii) The effect of NPs on the reduction of α-syn aggregates. From Liu L, Li Y, Peng H, et al. Targeted exosome coating gene-chem nanocomplex as "nanoscavenger" for clearing α-synuclein and immune activation of Parkinson's disease. Sci Adv. 2020;6(50). Reprinted with permission from AAAS.[194] (**B**) i) Delivering NGF mRNA and protein to ischemic areas and generating significant protective effects through RVG-modified exosomes; ii) NGF @ Exo ^{RVG} effectively delivers NGF into ischemic region. Scale bar, 50 μm. Adapted reprinted with permission from Yang J, Wu S, Hou L, et al. Therapeutic effects of simultaneous delivery of nerve growth factor mRNA and protein via exosomes on cerebral ischemia. Mol Ther Nucleic Acids. 2020;21:512–522. https://creativecommons.org/licenses/by/4.0/.[195] based on CC BY-NC-ND License, Copyright © 2020 The Authors.

of stroke. Loading agents into EVs with brain-damaging targeting capabilities is a promising avenue for enhancing their therapeutic potential.

Endocrine System Diseases

Several endocrine diseases, including diabetes and hypothyroidism, require long-term or lifelong treatment regimens. Furthermore, the long-term use of specific hormonal medications may cause adverse effects, including osteoporosis and immune system suppression.

Diabetes mellitus (DM) is a metabolic disorder. Type 1 DM stems primarily from autoimmune destruction of beta cells, whereas type 2 DM originates from diminished insulin sensitivity. Several enhanced EVs have been developed for diabetes treatment. Xu et al[204] have shown that MSC-EVs can halt the progression of type 1 DM through immunomodulatory mechanisms, promoting β-cell regeneration and enhancing insulin secretion. Furthermore, miR-26a, which is present in β-cells, reverses obesity-induced insulin resistance and hyperinsulinemia via EV-mediated circulation. (Figure 6A). Castaño et al showed that miR-133b-transfected EVs-Fect[205] and miR-690-packed M2-macrophage EVs[206] improved glucose tolerance and insulin sensitivity, offering a novel insulin sensitizer for metabolic disease treatment. Besides EV-based gene therapy, BAY55-9837 peptide has been shown to induce glucose-dependent insulin secretion. However, the peptide exhibits a short half-life, limited targeting efficacy, and suboptimal glycemic response. Zhuang et al[207] loaded SPIONs into EVs to create a BAY-EVs-SPION formulation. This approach overcame the limitations of using the BAY55-9837 peptide alone, resulting in notable enhancement of insulin secretion (Figure 6B).

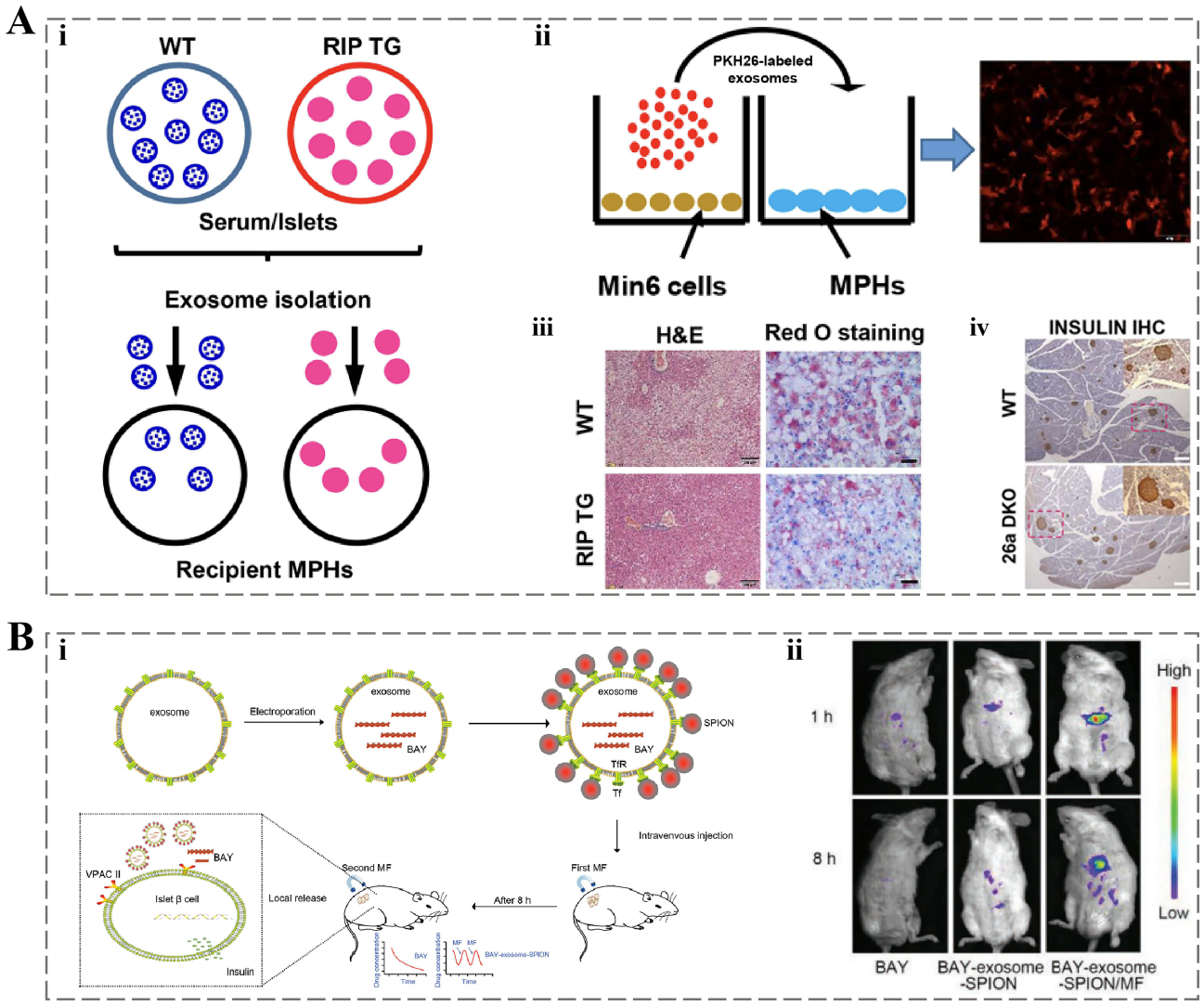

Figure 6 EVs to treat endocrine system disorders. (**A**) i) Schematic of EVs transfer or co-culture experiments; ii) EVs secreted by Min6 cells labeled as PKH26, then added to MPHs; iii) Representative HE-stained and oil red O-stained liver. Scale bar, 100 μm; iv) Representative IHC of pancreatic islets insulin. Scale bar, 200 μm. Adapted reprinted with permission from Xu H, Du X, Xu J, et al. Pancreatic β cell microRNA-26a alleviates type 2 diabetes by improving peripheral insulin sensitivity and preserving β cell function. PLoS Biol. 2020;18(2):e3000603. https://creativecommons.org/licenses/by/4.0/.[204] (**B**) i) Schematic diagram and effect of BAY EVs SPION synthesis; ii) NIRF imaging. Adapted reprinted with permission from Zhuang M, Du D, Pu L, et al. SPION-decorated exosome delivered BAY55-9837 targeting the pancreas through magnetism to improve the blood GLC response. Small. 2019;15(52):e1903135. VCH Verlag GmbH & Co. KGaA, Weinheim.[207]

Autoimmune thyroid diseases (AITD), including Graves' disease (GD) and Hashimoto's thyroiditis (HT), affect approximately 5% of the general population, making them among the most prevalent autoimmune disorders, and are characterized by the production of thyroid autoantibodies and thyroid lymphocytic infiltration. New research suggests that electric cars are linked to AITD. EVs containing undegraded thyroglobulin (Tg) have emerged as potential non-cytotoxic substitutes for conventional Tg-based therapeutic approaches.[208] Patients with GD have elevated blood EVs levels, which are reduced significantly by thiazole antithyroid therapy.[209] The detected rise in circulating EVs signifies the activation of immune and inflammatory cascades alongside the ensuing apoptosis. In the context of AITD, circulating EVs are pivotal in modulating the differentiation of regulatory T cells (Tregs) and helper T cells of the Th17 subtype.[210] Rodríguez-Muñoz et al[210] observed that EVs isolated from patients with AITD hinder the in vitro differentiation of regulatory Tregs while fostering the differentiation of proinflammatory Th17 cells. These AITD-derived EVs exhibit augmented expression of miR-146a and miR-155, which are intricately involved in the differentiation and function of innate and adaptive immune responses. Both miRNAs facilitate the development and functionality of Tregs, whereas

miR-155 specifically enhances the development of inflammatory T cells, particularly Th17 cells. The etiology of AITD has been associated with RNA dysregulation, including miRNAs and long noncoding RNAs.[211] Specifically, four miRNAs—miR-92a-3p, miR-23b-5p, miR-339-5p, and let-7g-3p—have been detected within EVs,[212] suggesting their probable role in augmenting cytokine production in refractory GD. These circulating EVs could function as intermediaries, facilitating communication between immune cells, stimulating cytokine expression in peripheral blood mononuclear cells and contributing to the pathogenesis of GD.[213]

Diseases of the Locomotor System

Orthopedic research has focused on addressing bone defects for decades, with autologous bone grafting being the gold standard therapeutic approach. As bone tissue engineering strategies continue to evolve, tissue-engineered bone stands have emerged as the predominant method for bone repair in the foreseeable future. In treating bone and joint disorders, the spotlight is on MSC-EVs. Owing to their exceptional capacity to differentiate into bone and cartilage tissues, MSC-EVs harbor abundant bioactive molecules that vigorously promote osteogenesis and chondrogenesis. This attribute underscores their immense potential in regenerative medicine applications,[214] particularly in fostering bone regeneration[215–217] and facilitating cartilage repair, showing a profound regenerative capacity.[218,219] Moreover, the design of biomaterials for different bone and cartilage injuries and joint diseases must be closely related to tissue properties. In bone defect regeneration, inorganic materials based on calcium and phosphorus and rigid organic scaffolds based on PLGA have been demonstrated to facilitate bone formation, given that bone tissue primarily comprises inorganic deposits. In contrast, hydrogels such as gelatin and hyaluronic acid are more suitable for cartilage therapy owing to their pressure-bearing and active structures.

Fractures are common trauma, and the bone itself can be repaired through regenerative processes, including inflammation, angiogenesis, stem cell differentiation, osteogenesis, and chondrogenesis. Nevertheless, delayed healing or bone nonunion complicates approximately 5–10% of fractures. A substantial body of evidence from numerous studies has indicated that EVs facilitate fracture recovery by regulating the immune system, promoting bone formation, and enhancing blood vessel growth. In recent years, "osteoimmunology" has emerged as a promising avenue for bone regeneration, underscoring the intricate interplay between immunology and bone biology.[220] The EV-based repair of large segmental bone defects must fulfill several requirements, including promoting osteoblast proliferation, endovascular reconstruction, local delivery, and controlled release of functional EVs at the defect site. In a joint application of modified EVs and biomaterials, Fan et al[221] used an extrusion method to accumulate exosomal mimics (EMs) from suppressed human mesenchymal stem cells (hMSCs), and EMs were further obtained from transgenic hMSCs in which the expression of noggin, a naturally occurring bone morphogenetic protein antagonist, was downregulated to enhance the osteogenic properties of EMs. They used hMSCEMs combined with injectable chitosan hydrogels to treat nonhealing cranial defects in mice and showed robust bone regeneration (Figure 7A). Suitable bone biomaterials should can mediate osteogenesis and manipulate the immune response, thus acting synergistically to achieve satisfactory osseointegration.[222] For example, the implantation of synthetic biomaterials can stimulate M1-polarized macrophages, resulting in the secretion of a diverse array of proinflammatory cytokines. Prolonged exposure to inflammatory cytokines may ultimately fail.[223,224] In contrast, M2 macrophages have been observed to secrete anti-inflammatory cytokines that favor bone regeneration.[223,225] The loading of tannic acid (TA) -sulfonated polyether ether ketone (SPEEK) with EVs results in the polarization of macrophages toward the M2 phenotype via the NF-κB pathway. The doping of TA-modified SPEEK with BMSC EVs endows the material with osteogenic differentiation and immunomodulatory capabilities, ensuring a sustained release of EVs, conducive to improved osseointegration.[225]

Cartilage repair is a significant clinical challenge owing to the lack of self-healing capacity. EVs derived from stem cells reportedly have significant cartilage regeneration potential.[226] The physical properties of biomaterials required for cartilage regeneration differ significantly from those of biomaterials required for bone regeneration. If the biomaterial is extremely rigid, cartilage regeneration is impeded and endochondral heterotopic osteogenesis ensues.[227] The high water content and swelling properties of hydrogels render them an optimal choice as scaffolds for repairing cartilage defects.[228] For example, H. Hu[39] delivered human umbilical cord MSC-EVs (hUC-MSC-EVs) using a combination of GelMA hydrogel and nanoclays. In vitro studies have shown that hUC-MSC-EVs facilitate cartilage regeneration. Furthermore,

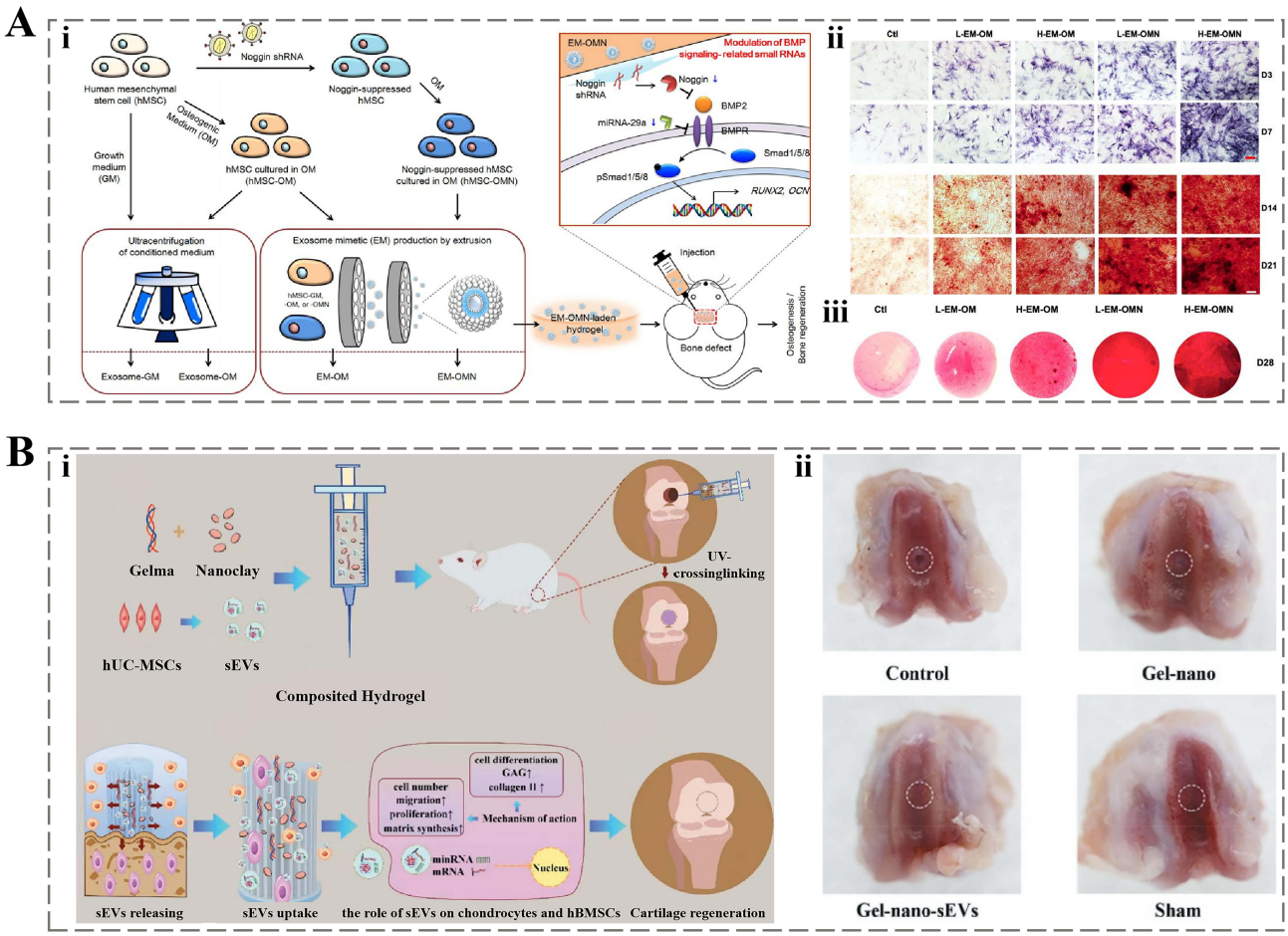

Figure 7 Application of EVs to treat locomotor system diseases. (**A**) i) Schematic diagram of skull regeneration promotion; ii) Detection of ALP expression and mineralization in hMSCs by ALP staining and Alizarin Red staining, respectively. Scale bar, 100 μm; iii) Assessment of mineralization by Alizarin red staining. Adapted reprinted with permission from Fan J, Lee C-S, Kim S, Chen C, Aghaloo T, Lee M. Generation of small RNA-modulated exosome mimetics for bone regeneration. ACS Nano. 2020;14(9):11973–11984.[221] Copyright 2020 American Chemical Society. (**B**) i) Schematic diagram of therapeutic EVs released from Gelma/noclay hydrogel for cartilage regeneration; ii) Visual observation of cartilage defects 12 weeks after surgery. Adapted reprinted with permission from Hu H, Dong L, Bu Z, miR-23a-3p-abundant small extracellular vesicles released from Gelma/nanoclay hydrogel for cartilage regeneration. J Extracell Vesicles. 2020;9(1):1778883. © 2020 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group on behalf of The International Society for Extracellular Vesicles.[39]

in vivo examinations revealed that GelMA-nanoclay hydrogels possess not only favorable biocompatibility but also the capability to enhance cartilage regeneration via the sustained release of EVs. (Figure 7B). In another study, Xing et al devised a porcine-derived decellularized myeloid nucleus.[229] Numerous proteins within decellularized medullary scaffolds were positively charged at the surface, enabling them to adsorb negatively charged ADSC-derived EVs. Concurrently, the injectability and thermal sensitivity of the decellularized medulla hinder EVs leakage during injection, enabling gradual release from a single high-dose injection. Recently, researchers developed a photoinduced imide-crosslinked (PIC) hydrogel containing an o-nitrobenzyl derivative of HA (HA-NB). Light-induced aldehyde formation facilitated in situ hydrogel generation on the tissue surface. This was achieved by the reaction of an aldehyde group with amino groups distributed on other polymers. The resulting hydrogel was seamlessly attached to and integrated into the tissue.[230] The PIC hydrogel displayed excellent maneuverability, biocompatibility, tissue adhesion, and integration, making it ideal for MSC-EV-based cartilage defect repair. The scaffold retained MSC-EVs in vitro and modulated the numbers of chondrocytes and BMSCs. Moreover, the scaffold integrates with native cartilage, fostering cellular deposition and repair at defect sites.[231]

MSC-derived EVs are the preferred choice for bone and cartilage treatments because of their diverse functions and advantageous properties in the locomotor system. Despite the extensive range of applications, most hydrogel-biomaterial

therapeutic systems for osteoarthritic diseases remain limited to simple material scaffolds for loading EVs, and the therapeutic effects depend on EVs. Consequently, the implementation of smart materials may facilitate the introduction of novel avenues for the application of EVs treatment, including 3D-printed scaffolds,[232] gene-polymerized materials,[233,234] composite disease-responsive materials,[234,235] and super lubricating materials for joint cavities,[233] with significant potential for personalized EVs therapy.

Diseases of the Reproductive System

The reproductive system can be affected by several diseases, including those affecting the vagina, endometrium, and erectile tissue. Numerous studies have documented a pivotal role of MSC-EVs in reproductive medicine. An extensive array of studies have harnessed the potential of EVs-integrated hydrogels to fortify the endometrial milieu, alleviate uterine adhesions, accelerate vaginal re-epithelialization processes, and address male erectile dysfunction, offering promising avenues for therapeutic interventions.[236]

Lin et al[236] generated an injectable hydrogel loaded with ADSC-EVs, which, after local injection into the endometrium, increased endometrial angiogenesis and decreased local tissue fibrosis. Using a murine model simulating acute endometrial injury, Xin et al[237] integrated hyaluronic acid (HA) hydrogels enriched with apoptotic bodies derived from umbilical cord MSCs. Their investigation revealed that vesicles induced immunomodulatory effects on macrophages, bolstered cellular proliferation, and augmented angiogenesis in vitro. Subsequent implantation of HA hydrogel-apoptotic vesicle composites significantly augmented endometrial thickness and glandular concentration, attenuated fibrotic processes, and expedited reinstatement of endometrial receptivity and reproductive capacity. Xu et al[238] designed photo triggered imide cross-linked hydrogels containing human urogenic stem cell sEVs. hUSC-sEVs significantly accelerated the healing and epithelialization of vaginal mucosal defects by delivering miR-126-3p to promote the migration and proliferation of VK2 cells.

To address erectile dysfunction, Liang et al[239] formulated thermosensitive hydrogels embedded with PDA nanoparticles, enabling intracavitary delivery of ADSC-EVs through a straightforward in situ polymerization process. The hydrogel enabled sustained EVs release, healing of endothelial cells and neurons, and enhancement of intraluminal pressure in animal studies, restoring erectile function. The exceptional photoacoustic properties exhibited by PDA nanoparticles within thermosensitive gels facilitate precise hydrogel delivery to the leucodermic region, guided by real-time acoustic imaging, enhancing precision and accuracy. Hydrogel-loaded MSC-EVs are anticipated to enhance progress in the development of novel therapeutic options for treating sexual dysfunction and fertility.

Skin/Wound Healing

Wound healing is a crucial and intricate process in living organisms.[240] In most patients, it can proceed without incident. However, in patients afflicted with severe conditions, such as burns, diabetes, or wound infections, the natural healing cascade can be significantly hindered or even halted, leading to delayed or impeded recovery. This can cause a significant medical burden and reduce the quality of life of patients. The use of EVs in wound healing has been widely demonstrated in numerous studies. However, administering free EVs via subcutaneous injection through wounds may cause secondary harm and discomfort. In addition, using EVs directly as wound dressings risks premature degradation, potentially undermining their therapeutic efficacy. Hydrogels are highly comfortable biomaterials that can be used for wound healing because of their breathability, permeability, and malleability. Furthermore, it effectively safeguards and guarantees the activity of EVs throughout the wound-healing process. Consequently, they are frequently used as EVs carriers for wound healing.[241–245] Zhou et al[37] integrated hADSC-EVs with a PF-127 hydrogel, revealing prolonged release of these EVs spanning 72 h. This formulation enhanced cell proliferation, promoted angiogenesis, facilitated collagen remodeling, accelerated epithelial restoration at the wound site, and expedited wound healing. Furthermore, the self-healing nature of the hydrogel preserved the bioactivity of the EVs, protected them against irritation, and significantly contributed to improved wound-healing outcomes. Wang et al[38] crafted a multifaceted hydrogel system designated as (F127/OHA-EPL) @EVs, incorporating PF-127, OHA, and EPL in its formulation. This novel hydrogel boasts self-healing properties attributed to the reversible Schiff base linkages

between OHA and EPL, whereas its temperature-responsive characteristics emanate from the incorporation of PF-127. Despite the significant challenge of achieving scarless healing in wound-healing therapy, Yang et al[15] showcased the capacity of MSC-EVs embedded in shear-thinning HA hydrogels. Their research revealed that the hydrogels enhanced M1 to M2 macrophage transformation, suppressed fibroblast activation, and hindered scar tissue development.

Summary and Outlook

This review focuses on the biomedical applications of EVs and presents a stimulus-responsive drug delivery system for EVs, which is a novel research direction. The ability of EVs to penetrate complex biological barriers and circumvent the challenges associated with traditional therapeutic approaches offers a promising avenue for treating neurological and respiratory diseases. The endosomal origin of EVs makes them biocompatible and low-immunogenic, overcoming their susceptibility to adverse cellular toxicity, which is an undesirable effect of conventional therapeutic modalities. EVs carry a variety of proteins on their surfaces, which enter the cell in a variety of ways upon contact with the cell. It is able to overcome the difficulty of easy degradation of drugs in conventional therapeutic methods. In addition, the stimuli-responsive drug delivery system of EVs enables simple slow release of drugs and controlled release of drugs in complex physiological environments, thus building smarter drug delivery systems for better therapeutic effects. Therefore, EVs can be applied in drug delivery, disease diagnosis, and treatment.

Decades of EVs have made great strides in the biomedical field, but as far as the current technology and results are concerned, there are still several challenges in the application of EVs in drug delivery and disease diagnosis and treatment. First, the precise/effective separation of EVs remains challenging, and the current methods used to separate EVs still have different degrees of limitations. Therefore, different methods should be selected for different purposes and applications, a combination of methods should be used to separate EVs with high efficiency, and better methods should be developed to achieve higher EVs yields. Second, because EV-enriched fluids usually contain multiple impurities and have similar sizes and densities, which lead to difficulties in characterization, better methods need to be developed to better quantify and characterize EVs. The low yield of EVs is also a challenge and can be constructed to build extracellular vesicle mimics (EVMs) by physicochemical means of treating cells so they are segmented into small vesicles of nanometer particle size. Compared with the normal extraction method, the content of EVMs obtained by this method is hundreds of times higher than normally obtained EVs, and they have EVs-like biological activities. Third, the utilization rate of EVs is low, the natural EVs are easy to be degraded and difficult to function for a long period, therefore, it is necessary to use biomaterials loaded with EVs to slow down their therefore, it is necessary to use biomaterials to load EVs to delay their degradation or use genetic engineering, biochemical engineering, etc. to modify them or pre-treat them. The effective translation of EVs into clinical applications is not an easy task, but biomaterials are expected to accelerate this process and have played a key role in the treatment of some diseases. Besides the diseases mentioned in this review, many other diseases require biomaterials for direct treatment with EVs. Digestive and respiratory diseases are typical examples of such diseases. The internal environment of the digestive system is complex and varies, with significant differences in the same area depending on the dietary intake. The combination of high bacterial density, high humidity, and the widespread presence of lipases and proteases in this system makes it difficult for EVs to remain active in the digestive system.[246] In the respiratory system, the use of EVs is also limited due to inhalation delivery and the unique structure of the organs.[247–249] Although research into the use of EVs in these organs and diseases is not lagging behind, the exploration of combining biomaterials with EVs is still in its infancy.[250,251] In the future, combining EVs with biomaterials for other diseases is expected to promote the further development of organ-specific combination therapy strategies.

In conclusion, EVs show great potential for drug delivery, disease diagnosis, and treatment. As isolation and characterization techniques for EVs continue to advance, further studies on the functional modification of EVs and the construction of EVs mimics are anticipated to address the issue of low yields. Furthermore, the construction of engineered EVs and the combination of EVs with biomaterials is expected to solve the problem of low utilization. These are expected to open a new chapter on EVs in drug delivery, disease diagnosis, and treatment. EVs have great potential in the biomedical field, and it is worthwhile for more researchers to focus on and support this emerging research direction.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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