REVIEW ARTICLE

Emerging chemical engineering of exosomes as "bioscaffolds" in diagnostics and therapeutics

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Abstract All cells release extracellular vesicles (EVs) as part of their normal physiology. As one of the subtypes, exosomes (EXOs) have an average size range of approximately 40 nm —160 nm in diameter. Benefiting from their inherent immunogenicity and biocompatibility, the utility of autologous EXOs has the potential for both disease diagnosis/treatment. EXOs are generally employed as "bioscaffolds" and the whole diagnostic and therapeutic effects are mainly ascribed to exogenous cargos on the EXOs, such as proteins, nucleic acids, and chemotherapeutic agents and fluorophores delivered into specific cells or tissues. Surface engineering of EXOs for cargo loadings is one of the prerequisites for EXO-mediated diagnosis/treatment. After revisiting EXO-mediated diagnosis/treatment, the most popular strategies to directly undertake loadings of exogenous cargos on EXOs include genetic and chemical engineering. Generally, genetically-engineered EXOs can be merely produced by living organisms and intrinsically face some drawbacks. However, chemical methodologies for engineered EXOs diversify cargos and extend the functions of EXOs in the diagnosis/treatment. In this review, we would like to elucidate different chemical advances on the molecular level of EXOs along with the critical design required for diagnosis/treatment. Besides, the prospects of chemical

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engineering on the EXOs were critically addressed. Nevertheless, the superiority of EXO-mediated diagnosis/treatment via chemical engineering remains a challenge in clinical translation and trials. Furthermore, more chemical crosslinking on the EXOs is expected to be explored. Despite substantial claims in the literature, there is currently no review to exclusively summarize the chemical engineering to EXOs for diagnosis/treatment. We envision chemical engineering of EXOs will encourage more scientists to explore more novel technologies for a wider range of biomedical applications and accelerate the successful translation of EXO-based drug "bioscaffolds" from bench to bedside.

Introduction

Exosomes (EXOs), with an average diameter of ∼100 nm, are a subset of extracellular vesicles (EVs) shed from eukaryotic cells. Since the first discovery of EXOs in 1987,[1] they always grasp wide attention in clinics. Their biogenesis involves the origin of endosomes and interactions with other intracellular vesicles and organelles; this process mainly includes four steps: budding, invagination, the formation of intracellular multivesicular bodies (MVBs), and secretion.[2,3] They play an essential role in the "crosstalk" of cell-to-cell/cell-to-tissue communications. EXOs contain different mother cell-type-specific signatures such as membrane proteins, cytosolic and nuclear proteins, phospholipids, and nucleic acids.[4] Since the discovery that EXOs as "crosstalk" tools to transfer their messaging cargos to recipient cells or specific tissues,[5] they have garnered wide interest across areas of theranostics. Although natïve EXOs have been reported in antitumor immunotherapy[6–8] and organ regenerations,[9,10] most of them have been extensively explored as "bioscaffolds" for the delivery of medicines to specific cells or tissues, benefiting from excellent biocompatibility, prominent solubility, metabolic stability, and long circulating half-life. As one of the natural routes for drug transport, innate protein-decorated EXOs contain specific barcodes to possess a unique "homing" effect by receptor-mediated pathway, i.e., the intrinsic ability to target mother cells, and might be expected to avoid the immunological reactions in the human body, such as anaphylaxis, cytokine release syndrome, neutralization of biological activity, etc.[11,12] Meanwhile, they can escape from immunophagocytosis to cross stringent biological barriers, such as vascular endothelium and blood–brain barrier, in distinctly different manners.[12–14] All of these characteristics make EXOs have several distinct advantages over conventional synthetic carriers in theranostics. However, the straightforward utility of natïve EXO-mediated theranostics remains restricted by their inability to track themselves in vivo or low loading capacities. By a variety of orchestrated tailoring on them, EXOs enriched for foreign cargos endow them with better orientation, visual tracking, and excellent performance in biomedical applications, and have already shed light on the next-generation theranostics for various diseases.

With enrichments of exogenous cargos, tailored EXOs as biological entities have captured the imagination of scientists for their clinical performance. If not all, the most commonly used methodologies by membrane rearrangements to enrich exogenous cargos on the EXOs include sonication,[15–17] electroporation,[18–20] freeze-thaw,[21–23] extrusion,[24–26] and passive incubation.[15,27–29] However, the stability of cargos and integrity of membranes by taking these physical approaches across EXOs are still unsatisfactory, extremely restricting their clinical applications in theranostics. To some extent, the surface tailoring of EXOs is essential to determine their possible interactions with conjugates or recipient cells while preserving their natural integrity. The genetic manipulation of mother cells ensures that EXOs shed by such cells are able to express desired cargos of interest and improve their interactions with recipient cells. Besides, genetically engineered-mother cells secrete fluorescent protein-labeled EXOs, which can be directly applied for noninvasive in vivo imaging.[30] What's more, EXOs obtained by genetic engineering can efficiently deliver proteins or siRNA/miRNA as agents for disease therapeutics.[18,31–33] Nevertheless, accumulating evidence indicates that the reproducibility of genetically engineered-EXOs remains controversial, such as inherent biological variability and heterogeneity. Even worse, genetic engineering can only enclose transcriptional proteins or RNAs as theranostic agents in the EXOs and is impotent for other special payloads, e.g., synthetic imaging and therapeutic agents. Nowadays, endowing a diversity of payloads on the EXOs mainly ventures into chemical engineering, which has evolved into a highly sophisticated and versatile methodology. From the discovery of EXOs until 2014, there was a huge blank in chemical engineering on the EXOs. This promising strategy is capable of not only directly tailoring natïve EXOs but also indirectly modifying their mother cells before EXO generation. Firstly, the presence of transmembrane proteins on the EXOs provides plentiful functional moieties, such as carboxylic (-COOH), amine (-NH$_2$), and sulfhydryl (SH) groups, accessible to crosslink reactions by chemical engineering for sorts of payloads[34]; and secondly, an embedding of other foreign groups such as alkyne, azide, amino, maleimide (Mal) and many others into cytomembrane of mother cells by metabolic labeling increases the scalability of cargos on their secreted EXOs, as evidently shown in Figure 1. By employing chemical engineering, EXO-mediated payload transfer could be put into widespread applications in diagnostic readouts (e.g., fluorescent imaging, magnetic resonance
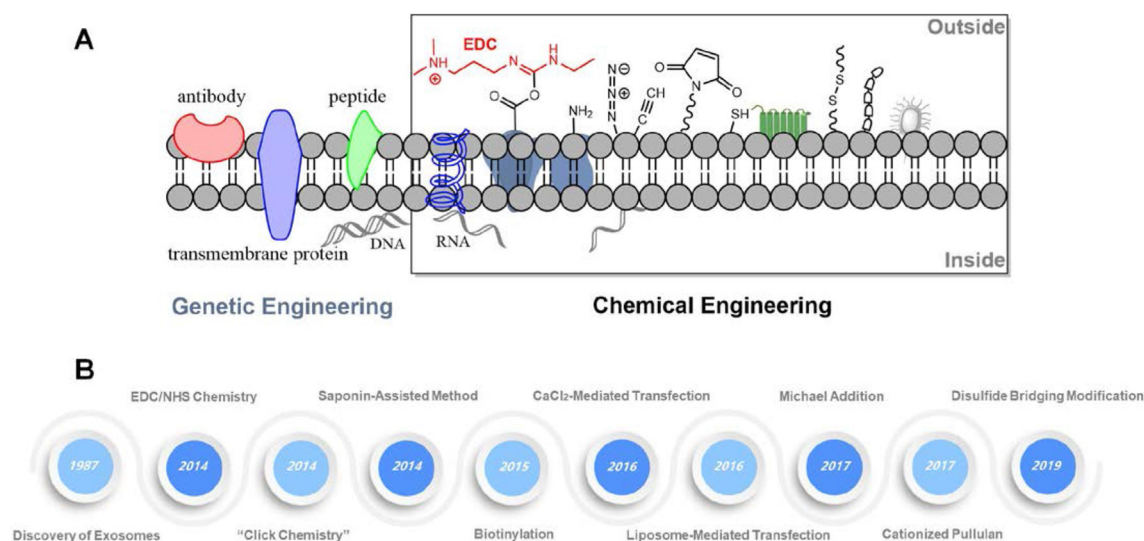

Figure 1 Overview and history of chemical engineering on the EXOs. **(A)** Classification of chemical engineering on the EXOs. Apart from genetic engineering, theranostic agents for diagnosis/treatment can be selectively conjugated on the surface of EXOs by many kinds of chemical molecules or functional groups such as saponin, carboxyl, amino, azide, alkyne, maleimide, thiol, disulfide, biotin, and many others. The chemical engineering of surface modification on the EXOs includes the uses of original groups and foreign moieties. Usually, the foreign moieties are intentionally embedded into membranes by metabolic engineering. **(B)** The history and evolution of chemical engineering on the EXOs. Since the discovery of EXOs in 1987, there was an enormous gap in chemical engineering on the EXOs until 2014.

imaging, and computed tomography) and the transport of medicines for treatment.[35–37]

Over the past years, growing evidence suggests that emerging chemical engineered-EXOs herald a new era of theranostics in diseases. There has been explosive growth in publications describing the wealth of their applications. By chemical engineering, EXOs can piggyback on cargos into regions of interest for early detection, *in vivo* imaging, and targeted drug delivery. A recent review highlights the emerging areas of chemical engineering to EXOs as delivery vehicles for diagnosis/treatment. These chemical strategies of EXOs have been classified into five major categories: (i) chemical transfection, (ii) 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide/N-hydroxy succinimide (EDC/NHS) coupling, (iii) azide–alkyne cycloaddition, (iv) thiol-maleimide addition, and (v) other new-style chemical methodologies; their merits have been also compared with each other, as briefly summarized in Table 1. By laying out different chemical techniques on the EXOs, we seek to provide an overview for gradually knowing more about brief history and recent advances, with perspectives on guidelines for choosing suitable techniques for EXO-based theranostics. However, some critical factors of these chemical approaches on the EXOs remain matters of debate, such as specificity, integrity, morphology and the density of intrinsic cargos, and many others after chemical modifications. Importantly, we have outlined the key challenges and future perspectives of these chemical engineered-EXOs. We also hope that such knowledge will not only make new research insight into the progress of chemical engineering on the EXOs but also spur more chemical protocols applied in EXO-based drug "bio-scaffolds" and their translations for various diseases from bench to clinical reality, including rheumatoid arthritis,[38–40] cardiovascular dysfunction,[41–43] neurode-generative diseases,[44–46] inflammatory,[28,47–49] and even cancer.[50–53]

Loading of theranostic agents on exosomes by chemical transfection

miRNA in EXOs derived from tumor cells extensively acts as a biomarker, commonly providing clinical diagnosis for tumors. Reversely, normal RNA-functionalized EXOs are able to afford reliable tools for gene therapy of diseases by restoring their biological functions to trigger genotypic and phenotypic responses.[54] For the functionalization of EXOs by nucleic acids such as siRNAs and miRNAs, chemical transfection has emerged as a common choice. By traditional chemical transfection, many RNA-based drugs have entered clinical trials. The conventional approach of nucleic acid-functionalized EXOs by chemical engineering is calcium chloride (CaCl$_2$)-mediated transfection. In this fashion, desired RNAs as interference agents are firstly mixed with EXOs in phosphate buffer saline (PBS) containing a low concentration of CaCl$_2$. Then, the mixture is managed by freeze–thaw cycles, thereby ensuring the co-ordinated complexes of RNA and CaCl$_2$ enter into EXOs by membrane rearrangements. After that, RNA-loaded EXOs will be harvested from PBS solution by ultracentrifugation. Ultimately, the EXOs containing desired nucleic acids for diagnosis/treatment of human diseases are delivered to regions of interest. Of note, the EXOs shed from tumor cells are not suggested to be adopted, owing to their roles in tumorigenesis and angiogenesis.[55] So far, a large cohort of studies have developed protocols to load RNAs onto EXOs by CaCl$_2$-mediated transfection for gene therapy of human

Table 1 Emerging chemical engineering of EXOs in diagnostics and therapeutics as mentioned in this review.

Source of EXOs	Chemical strategies	Cargos on EXOs	Applications	Reference	Merits
Serum	CaCl$_2$-mediated transfection	siRNA	Lung inflammation	46	(i) Less time-consuming;
OSCC cells	CaCl$_2$-mediated transfection	miRNA	Therapy of oral cancer	57	(ii) Membrane arrangement; (iii) Destroy of integrity; (iv) Reduction of surface protein density;
MSCs	Lipo-mediated transfection	Photosensitizer	Tracking drug delivery	59	(v) Noncovalent binding;
Fibroblasts	Lipo-mediated transfection	Docetaxel	Metastatic peritoneal carcinoma	61	(vi) Low stability and reproducibility.
Breast cancer cells	Saponin-assisted method	Porphyrins	Photodynamic therapy	64	(i) Less time-consuming;
Macrophage	Saponin-assisted method	Catalase	Parkinson's disease	24	(ii) Membrane arrangement; (iii) Noncovalent binding; (iv) Good stability and reproducibility
HepG2 cells	EDC/NHS	Polyarginine	Anticancer therapy	70	(i) Less time-consuming;
MSCs	EDC/NHS	Aptamers	Central nervous system	71	(ii) Modification on surface protein; (iii) Preservation of integrity; (iv) Nonspecific modification;
Colostrum	EDC/NHS	Folic acids	Therapy of lung cancer	74	(v) Denaturation of surface protein; (vi) Covalent binding;
HEK-293T	NHS	Folic acids	Therapy of breast cancer	75	(vii) Good scalability of cargos; (viii) High stability and low reproducibility.
HEK-293T	NHS	Cy5.5 dye	Bioimaging and immunotherapy	77	
Macrophage	NHS	Cy5.5 dye	Bioimaging and wound healing	79	
Macrophage	EDC/NHS and "click chemistry"	Peptides	Therapy of glioma	80	The same to EDC/NHS chemistry
Dendritic cell	NHS and "click chemistry"	Glycopeptide and Cy5.5	Antitumor vaccine	81	
MSCs	NHS and "click chemistry"	Cy5.5 dye	Imaging and cerebral ischemia therapy	82	
Breast cancer cells	"click chemistry"	Fluorescent tags	Bioimaging and tracking	94	(i) Assists in metabolic engineering;
549 cells	"click chemistry"	Cy5 dye	Bioimaging and tracking	87	(ii) Time-consuming; (iii) Preservation of integrity; (iv) Specific modification;
Breast cancer cells	"click chemistry"	Fluorescent tags	Tracking and organotropic uptakes	97	(v) Covalent binding. (vi) Good scalability of cargos; (vii) Good stability and reproducibility.
Macrophage	"click chemistry"	Antibodies	Cancer immunotherapy	96	
Macrophage	"click chemistry"	Mannose	Intracellular infection	100	
4T1 cells	"click chemistry"	Fluorescent tags	Bioimaging and tracking	69	
MSCs	"click chemistry"	Peptide and miRNA	Cerebral ischemia	102	
Prostate cancer cells	Thiol-Michael addition	Fluorescent tags	Bioimaging and tracking	104	(i) Less time-consuming;
Macrophage	Thiol-Michael addition	Quantum dots	Bioimaging and tracking	106	(ii) Modification on surface protein; (iii) Preservation of integrity; (iv) Specific modification;
HEK293T	Thiol-Michael addition	Aptamer	Targeted therapeutics	107	(v) Denaturation of surface protein; (vi) Covalent binding;
BM-MSCs	Thiol-Michael addition	Oxaliplatin	Cancer therapy	110	(vii) Good scalability of cargos; (viii) Good stability and reproducibility.
Dendritic cells	Thiol-Michael addition	aCTLA antibody	Cancer immunotherapy	111	

Table 1 (continued)

Source of EXOs	Chemical strategies	Cargos on EXOs	Applications	Reference	Merits
Prostate cancer cells	Disulfide bridge	—	Detection of intact EXOs	114	(i) Less time-consuming; (ii) Preservation of integrity;
MSCs	Electrostatic interaction	Pullulan	Therapy of liver injury	115	(iii) Nonspecific modification; (iv) Low stability and reproducibility.
Milk	PEGylation	—	Preventing the degradation of EXOs	116	(i) Less time-consuming; (ii) Preservation of integrity; (iii) Nonspecific modification;
Neuro2A cells	PEGylation	Nanobodies	Targeting tumor cells	117	(iv) Denaturation of surface protein; (v) Noncovalent binding.
Colostrum	Polyethyleneimine	siRNA	Therapy of lung cancer	74	(vi) High stability and low reproducibility.
A549 cells	Polyelectrolytes	DOX	Carrier for DOX loading	118	

disease.[47,56] In 2016, Zhang et al reported a CaCl$_2$-mediated transfection method to directly integrate miRNA mimics onto the isolated EXOs for the first time, instead of transfecting their mother cells, by which a robustly more amount of miRNA could be delivered to recipient cells compared with electroporation.[57] As recently studied, small RNA-loaded EXOs by CaCl$_2$-mediated transfection have been employed to recognize lung macrophages, by which it successfully modulates lipopolysaccharide (LPS)-induced lung inflammation *in vivo*.[47] What's more, EXOs derived from serum were able to successfully circumvent lung immune response. Besides, the feasibility of miRNA-loaded EXOs by CaCl$_2$-mediated transfection has gotten concerns to improve cisplatin resistance during cancer therapy. Sayyed et al have currently reported that miR-155-loaded EXOs by this simple way of transfection reverse cisplatin-resistance for the management of refractory oral cancer to prolong the survival of chemoresistant tumor-bearing mice.[58] Upon administering miR-155-loaded EXOs on the mouse xenograft model, miR-155 was able to directly target forkhead-box protein O3a (FOXO3a), as one of tumorigenesis suggestive when its downregulation.[59] Furthermore, miR-155-laden EXOs simultaneously upregulated the expression of FOXO3a and induced mesenchymal-to-epithelial transition in oral cancer, by which it greatly improved the chemosensitization toward cisplatin during the treatment of oral cancer. Unfortunately, by CaCl$_2$-mediated protocol, the efficiency of RNA loading on the EXOs is still not satisfactory. Even worse, the repeated freeze—thaw cycle can possibly damage the surface proteins of naïve EXOs.

Along with the use of CaCl$_2$ as a medium in EXO engineering, liposome-mediated functionalization of EXOs have expanded their biomedical applications by fusing the membrane of EXOs with liposomes (Fig. 2A).[22] By employing this engineering strategy, the surface of EXOs can be easily modified via liposomes those embedded with exogenous cargos of interest, *e.g.*, peptides or antibodies as well as poly (ethylene glycol) (*i.e.*, PEG), to form Lipo-EXO hybrids, endowing naïve EXOs with exogenous peptides or antibodies.[60–62] Lipo-mediated modification of EXOs substantially relies on PEG-triggered fusion between liposomes and

EXOs to design Lipo-EXO hybrids with tunable composition and properties. The fusion efficiency and critical parameters are generally monitored by fluorescent resonance energy transfer, such as liposome-to-EXOs ratio and bilayer composition.[22] What first intrigued us is that Lipo-EXO hybrids have been directed toward bioimaging agents. A proof-of-concept paradigm has described the point-for-point Lipo-EXO hybrids-based bioimaging for *in vivo* tracking drug delivery. In this paradigm, liposomes were initially labeled by meta (tetrahydroxyphenyl) chlorin (mTHPC), a clinically approved fluorescent anti-tumor photosensitizer; and then, mTHPC-labeled liposomes could fuse with murine mesenchymal stem cells (MSC)-derived EXOs that is under clinical assessment for future use in regenerative medicine. As a result, it is of no surprise that, mTHPC-labeled Lipo-EXO hybrids performed much better than liposomes in the drug delivery to colon cancer cells, and precisely tracked themselves by a fluorescent signal from mTHPC.[60] Except for bioimaging *in vivo*, the versatile Lipo-EXO hybrids have drawn significant attention in cancer therapy. More recently, Lv et al have constructed thermo-sensitive Lipo-EXOs hybrids for the delivery of chemo-immunotherapeutic agents to combat metastatic peritoneal carcinoma (mPC) when receiving hyperthermic intraperitoneal chemotherapy, a deadly disease without effective treatments.[62] In this study, they started with genetic engineering of fibroblasts to produce granulocyte-macrophage colony-stimulating factor (GM-CSF)/CD47-overexpressed EXOs, which were subsequently fused with thermosensitive docetaxel (DTX)-loaded liposomes to form Lipo-EXOs hybrids. By interaction of CD47 and signal regulatory protein alpha receptor (SIRPa) on macrophages, acting as a "don't eat me" signal, Lipo-EXO hybrids escaped from phagocytosis by macrophages to reinforce drug delivery.[63] After administration, Lipo-EXO hybrids efficiently penetrated mPC tumors and released GM-CSF and DTX under hyperthermic conditions, by which they were able to induce repolarization of macrophages towards M1-like phenotypes and apoptosis of tumor cells, respectively, as shown in Figure 2A for clarity. Moreover, they found distribution pattern of Lipo-EXO hybrids on mPC-bearing mice was 2—3 times greater than those of liposomes or EXOs in
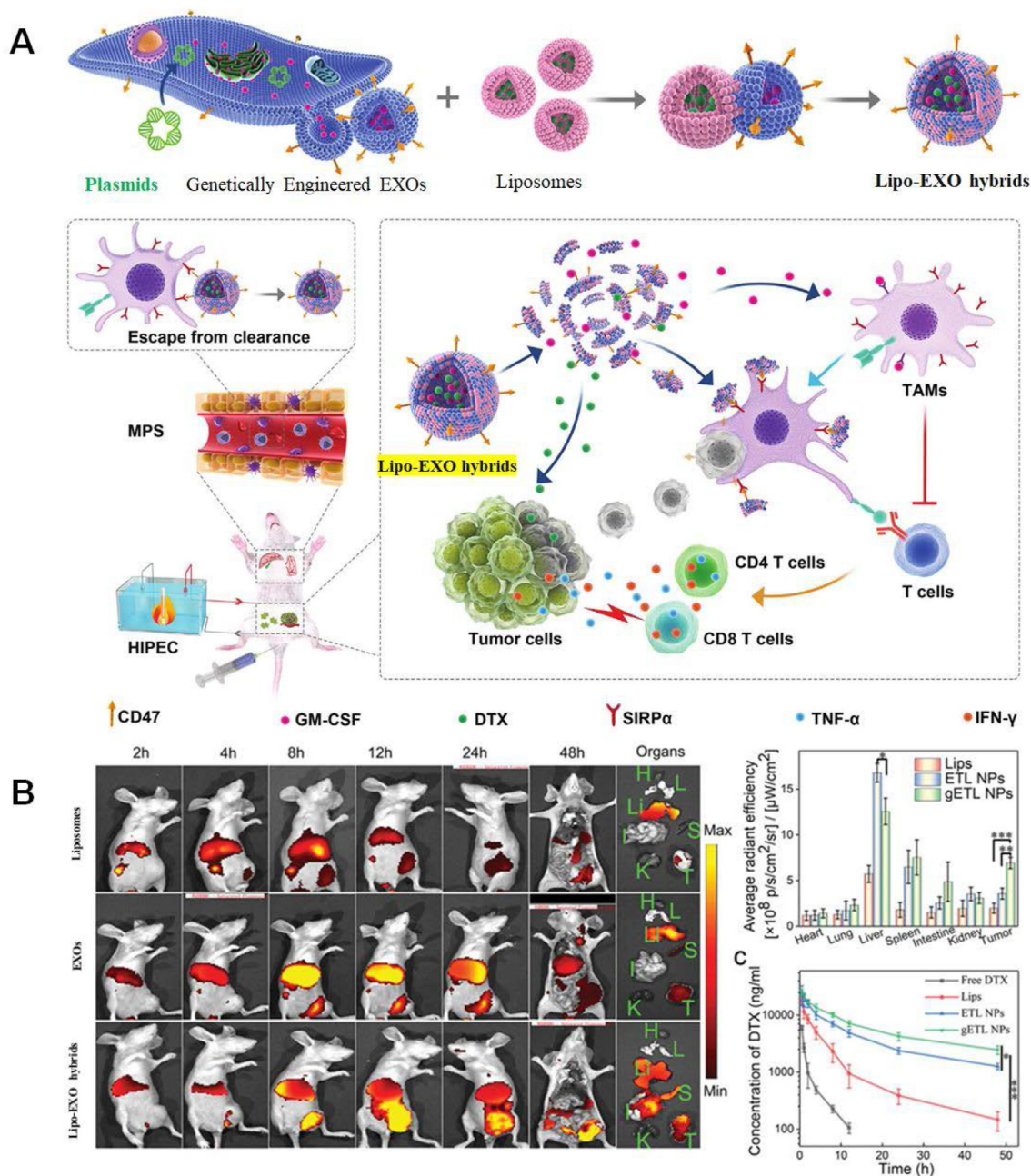
Figure 2 Lipo-mediated EXO functionalization for therapeutics of diseases. **(A)** Schematic diagram of Lipo-mediated EXO functionalization for metastatic peritoneal carcinoma (mPC) treatment. Genetically engineered fibroblasts by transduction with lentiviral vectors produced GM-CSF/CD47-overexpressed EXOs, which then were fused with thermosensitive liposomes encapsulating DTX to form Lipo-EXO hybrids with the loading of GM-CSF and DTX. CD47-overexpressed Lipo-EXO hybrids with the loading of therapeutic agents would escape from phagocytosis of phagocytes, by interaction with SIRPa in phagocytes. Under hypothermia condition of hyperthermic intraperitoneal chemotherapy, GM-CSF and DTX releasing from Lipo-EXO hybrids were able to induce repolarization of macrophages towards M1 and apoptosis of tumor cells, respectively, in the treatment of metastatic mPC. **(B)** Real-time images of liposomes engineered EXOs and Lipo-EXO hybrids with the loading of therapeutic agents and encapsulation of DiR dye in tumor-bearing mice. After 48 h, the DiR in major organs and DTX in plasma were quantitively analyzed. Information is acquired from Wiley Open Access.

the tumors at 48 h post-injection, by monitoring the fluorescent signal of encapsulated DiR dyes, as shown in Figure 2B. The clearance half-time ($t_{1/2b}$) of DTX delivered by liposomes and EXOs were 3.5 h and 6.2 h, respectively, considerably shorter than that (8.1 h) of Lipo-EXO hybrids.

Likewise, it has been reported that chemically transfected-Lipo-EXO hybrids could successfully deliver CRISPR/Cas9 expression vectors in MSCs to potentially cure various genetic diseases such as cancers and inherited disorders by repairing, deleting, or silencing certain genetic
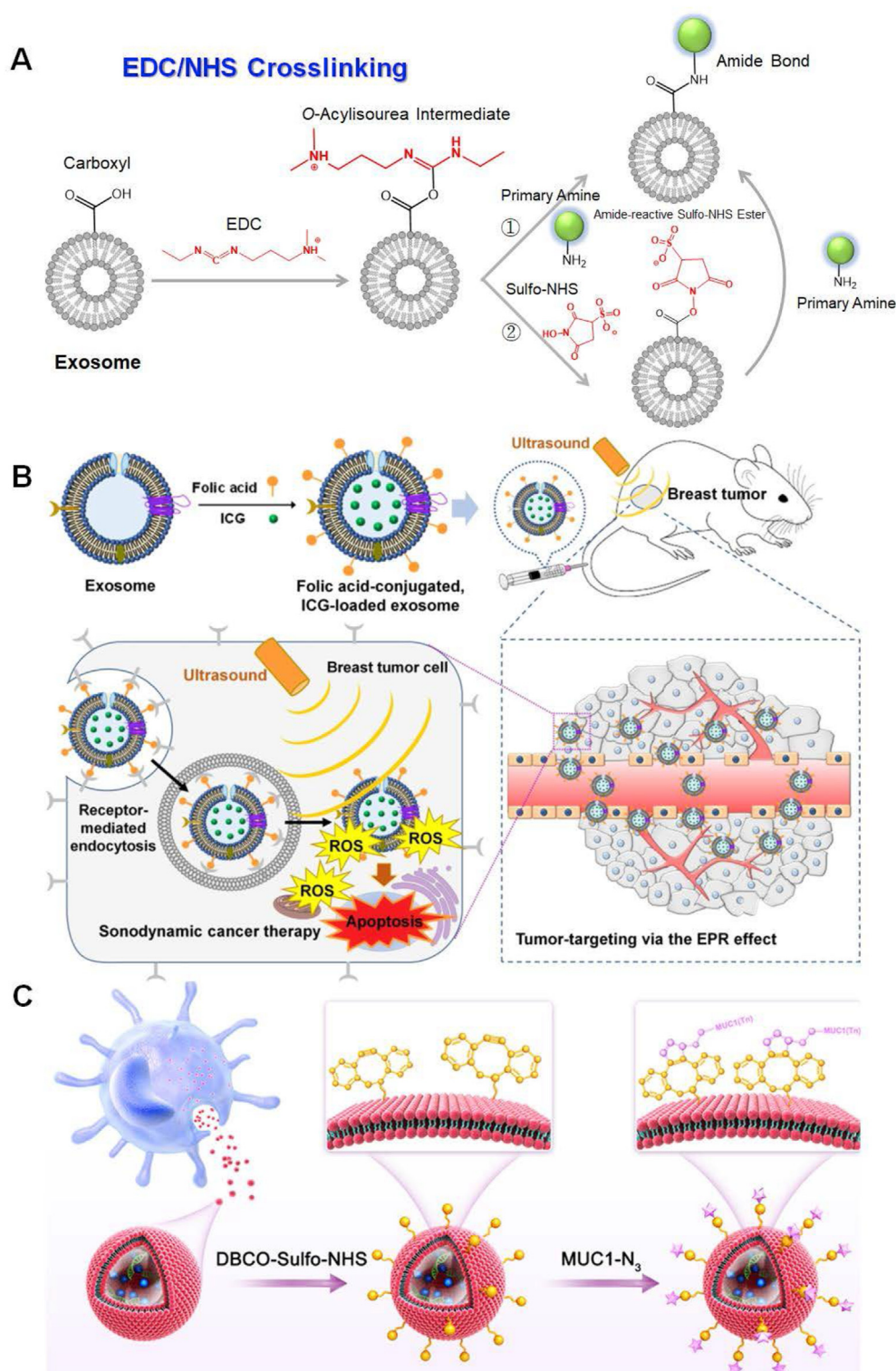
Figure 3 Chemical engineering on the EXOs by EDC/NHS crosslinking. **(A)** Schematic diagram of EDC/NHS crosslinking on the EXOs. (i) EDC reacts with a carboxyl group on the EXOs to form an *O*-acylisourea intermediate that is easily substituted by nucleophilic attack from the primary amino group for the conjugation of theranostic agents. The primary amine forms an amide bond with the carboxyl group on the EXOs, and the EDC-by product is released as a soluble urea derivative. EDC crosslinking is most efficient in acidic (pH = 4.5) conditions. Physiological pH conditions are compatible with this crosslinking, *albeit* with lower efficiency. (ii) NHS or its water-soluble analog (Sulfo-NHS) is commonly adopted in EDC coupling protocols to improve efficiency. EDC makes NHS couple to carboxyl on the EXOs, forming an NHS ester that is more stable than the *O*-acylisourea intermediate. The NHS ester allows for efficient conjugation to primary amine at physiological pH. **(B)** Schematic illustration of sonodynamic therapeutics by FA-conjugated and ICG-loaded EXOs against breast cancer. ICG was successfully loaded on the EXOs by incubating

mutations.[61] By taking this approach to deliver the CRISPR/Cas9 system, it effectively avoided possible cytotoxicity, immunogenic response, long-term expression, and off-target effects. Although the composition of Lipo-EXO hybrids can be tuned by varying liposome-to-EXO ratio, the morphology of naïve EXOs will be transformed after liposome-mediated transfection. Along with that, the size homogeneity of the EXO population will be disrupted. It seems to be worse that a rearrangement and dilution of intrinsic cargos on the surface of naïve EXOs will occur, which results in a distortion of naïve EXO's characteristics and a loss of their recessive functions.

A little more early than chemical transfection, saponin-assisted drug loading on the EXOs has caught attention since it was first reported. Making use of this way, saponins specifically react with cholesterol to form small pores in the bilayer membrane of EXOs, making it convenient for the uptake of exogenous cargos. Dating back to 2015, pioneering work has initiated saponin-assisted methods to incorporate porphyrin into EXOs shed from breast cancer cells. The porphyrin-incorporated EXOs exhibited excellent photodynamic performance in a breast cancer cell model. Their findings rendered a firm basis for the development of EXOs as drug "bioscaffolds".[64] Almost at the same time, Haney et al have fabricated catalase-loaded EXOs by saponin's assistance to provide significant neuroprotective effects in the models of Parkinson's disease.[24] Primarily due to capsulation of catalase by EXOs derived from macrophages, the therapeutic drugs can escape from phagocytosis of macrophages, and their delivery to neuronal cells is greatly increased. As such, chemical transfection or saponin-assisted drug loading on the EXOs can destroy the integrity of EXOs and cause a rearrangement of their membrane, thus affecting the drug delivery function of EXOs. Besides, the scalability and stability of cargos loaded by these methodologies are still open to question.

Loading of theranostic agents on exosomes by EDC/NHS mediated amide reaction

Owing to the presence of proteins and phospholipids, carboxyl (-COOH)/amine (-NH$_2$)-terminated membrane protein and phospholipid moieties of EXOs endows their surface tunability. In this regard, crosslinking primary amine-labeled cargos to terminal carboxylic acids by strong covalent bonds or *vice versa* is a feasible approach for bioconjugations of oligonucleotides, peptides, proteins, and biomolecular probes on the EXOs, which raises the possibility of accurate diagnosis and effective treatment for various diseases. However, to the best of our knowledge, extremely few chemical groups are known to provide specific conjugation to carboxylic acids or amines. The advent of carbodiimide chemistry has led to an influx of

new ideas in EXO-based theranostics, by which the terminal carboxylic acids of proteins or phospholipids are activated for direct conjugation to primary amines via amide bonds (*e.g.*, a crosslinking of two proteins).[65] Among carbodiimide species, water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and water-insoluble dicyclohexylcarbodiimide (DCC) are commonly used reagents for aqueous and non-aqueous cross-linking of carboxylic acids and primary amines, respectively.[66] Considering the physiological restriction, direct crosslink of primary amines to residue-specific carboxylic acids on the EXOs usually adopts EDC-mediated amide reaction rather than DCC. EDC-mediated amide reactions of protein carboxyl groups have the highest efficiency in acidic conditions (pH = 4.5) to form *O*-acylisourea active intermediates, which subsequently tend to react with necleophilic ligands such as primary amine groups.[66] To facilitate this type of reaction at physiological pH (pH = 7.2), the assistance of *N*-hydroxysuccinimide (NHS) is needed to improve the chemical yields of amide bonds,[67] as shown in Figure 3A. Devoid of EDC adducts, direct NHS-mediated conjugations of cargos on the terminal carboxylic acids of membrane proteins or phospholipids for *in vivo* imaging, antitumor, vaccines, and so on, have also been widely verified to be feasible.[68] Despite originating only less than a decade ago, a marriage between EDC/NHS-mediated amine/carboxyl coupling reaction and tunability of EXO's surface, as a milestone in EXO-based drug delivery, has received extensive attention since its first proposal.[69]

Intense efforts have been dedicated to cargo loading on the EXOs by EDC/NHS chemistry. Recently, Xu et al have constructed a novel peptide-equipped EXOs platform, by which polyarginine peptide (R9) was directly conjugated to carboxyls on the surface of the HepG2 cell-derived EXOs via an EDC/NHS-mediated amide reaction.[70] Before the conjugation of peptides, the terminal carboxyl on the HepG2 cell-derived EXOs was initially activated by EDC/NHS chemistry. As one the of cell-penetrating peptides, the presence of R9 peptides could not only enhance the penetrating capacity of EXOs but also correlate EXOs with loading oligonucleotides by static interaction for genetic therapy. Surprisingly, together with these two functionalities, it was noteworthy that R9 peptides on the EXOs by EDC/NHS crosslinking also rendered EXOs with targeting ability to cancer cells. Nowadays, affinity ligand-guided drug delivery presages a new era of targeted therapeutics in diseases. If taking the same strategy, conjugations of affinity ligands, *e.g.*, aptamers and antibodies, to the surface of EXOs may fulfill a specific accumulation of EXOs on the target cells. Inversely, one also can proceed with the conjugation of amine-reactive payloads to terminal amines of membrane proteins or phospholipids on the EXOs by EDC/NHS chemistry. An early report of EDC/NHS chemistry described that carboxylic acid-functionalized aptamers

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HEK-293T-derived EXOs with ICG solution. Under the irradiation of ultrasound, ICG generated ROS to kill tumor cells. FA as a targeting ligand was covalently conjugated to proteins on the surface of EXOs by NHS-mediated amide reaction. Referenced from American Chemical Society (Copyright 2021). **(C)** Dendritic cell-derived EXOs as "bioscaffolds" for the antitumor vaccine. Terminal amine groups on the EXOs were conjugated with DBCO-Sulfo-NHS via amide reaction, subsequently allowing for crosslinking of MUC1 glycopeptides. The MUC1 glycopeptides were able to induce MUC1-specific IgG antibodies, which had strong affinities with MUC1-positive tumor cells. In this way, the EXO-based vaccines increased the killing activity of T cells. Referenced with permission from Elsevier.

were directly conjugated on the primary amines of the MSC-derived EXOs.[48] The goal of particular aptamer decorated on EXOs not only endowed targeting ability of delivery but also suppressed inflammatory response in central nervous system (CNS) disease. Likewise, MSC-derived EXOs cross-linked with aptamers by EDC/NHS chemistry, which has the potential to actively target mucin-1 glycopeptide highly expressed on colorectal tumor cells, were able to safely and specifically deliver Doxorubicin (DOX) to the region of interest for cancer chemotherapy.[71] By far, folate receptor is typically well-known to overexpress on cancer cells.[72,73] Folic acids (FA) have been originally attached to EXOs by EDC/NHS crosslinking, as an effective "platform" for targeted delivery of therapeutic siRNA to treat lung cancer.[74] To this end, FA could guide the endocytosis of whole siRNA-loaded EXOs system by cancer cells to achieve the purpose of genetic therapy. Recently, as one of the emerging treatments, sonodynamic therapy (SDT) that utilizes cytotoxic reactive oxygen species (ROS) generated from indocyanine green (ICG) under ultrasound exposure has received tremendous attention. Chemically FA-modified EXOs through NHS mediation were readily utilized for cancer-targeted delivery of sonosensitizer ICG. An integration of FA on the EXOs greatly improved cellular uptake of ICG, resulting in a significant increase of cytotoxic ROS in breast cancer cells by subjecting them to ultrasound irradiation. By targeted delivery of ICG via FA-modified EXOs, tumor growth within mice was notably suppressed without systemic toxicity,[75] as illustrated in Figure 3B. Current studies of EXO-based delivery not only concentrate on the targeted therapy of diseases but also on bioimaging in living subjects.[37,76–79] Compared with the non-covalent incorporation of lipophilic dyes, the conjugated dyes on the EXOs by EDC/NHS-mediated covalent coupling are difficult to release from "bioscaffolds" in the process of in vivo trafficking and provide precise tracking information. For example, Koh et al have employed NHS-mediated amide reaction to fluorescently label signal regulatory protein a (SIRPa) highly expressed EXOs derived from HEK293T cells. The amine-reactive fluorophore Cy5.5-NHS was successfully conjugated on the EXOs to observe the biodistribution of EXOs under the guide of Cy5.5. Simultaneously, by the interaction between SIRPa of EXOs and CD47 of phagocytes, it was able to restore potent immune responses against tumor cells and mediate tumor phagocytosis by macrophages for cancer immunotherapy.[77] Therewith, a Cy5.5-NHS probe-labeled EXOs derived from anti-inflammatory M2 macrophage was used for in vivo fluorescently tracking delivery of EXOs. Besides, the EXOs from host cells could reprogram proinflammatory M1 to anti-inflammatory M2 macrophage for cutaneous wound healing.[79] Due to the accessibility and flexibility of EDC/NHS chemistry in the membrane proteins on the surface of EXOs, it has paved the way for secondary chemical reactions on the EXOs for loading exogenous cargos.

In the virtue of EDC/NHS chemistry, a previous study originally conjugated 4-pentynoic acid terminated with alkyne (-C≡C) group to murine macrophage cell-derived EXOs, aiming to further crosslink targeted peptide (i.e. neuropilin-1) on the EXOs by emerging "click chemistry", by which EXOs were able to specifically carry superparamagnetic Fe_3O_4 NPs and curcumin to the region of

interest for in vivo imaging and therapy of glioma, respectively.[80] Almost by the corresponding steps, dendritic cell-derived EXOs were firstly functionalized with Dibenzylcyclooocctyne-Sulfo-NHS (DBCO-Sulfo-NHS) by reacting with amine-active EXOs, then allowing for cross-link of azide-modified mucin-1 glycopeptide and Cy5.5 by "click chemistry" to induce mucin-1-specific IgG antibody and track drug delivery, respectively (Fig. 3C). The induced mucin-1-specific IgG antibody by mucin-1 glycopeptide was able to specifically bind to mucin-1-expressing tumor cells, by which it eventually triggered cytotoxic T lymphocyte response and inhibited tumor growth in animal models.[81] This conjugation strategy on the EXOs has been also adopted for cerebral ischemia therapy. After functionalization of DBCO-Sulfo-NHS on the MSC-derived EXOs, the cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide [c(RGDyk)] together with Cy5.5 fluorophore were successfully crosslinked on the EXOs for actively targeting to the ischemic region through specific binding of c(RGDyk) to integrin $a_v b_3$ and bio-imaging, respectively.[82] Taken together, EDC/NHS chemistry can overcome the scalability limitation of chemical transfection. Moreover, amine/carboxyl coupling reaction by EDC/NHS mediation primarily takes place on the surface proteins or phospholipids of EXOs and its simplicity equips EXOs with more tunable surface properties, facilitating the development of "click chemistry" as an effective technique for more cargo loading on the EXOs. Unfortunately, a straightforward modification on the original moieties of EXOs by EDC/NHS chemistry to cause their denaturation, especially their membrane proteins, is also fraught with problems by constraints on functions of proteins that can affect intrinsic properties of EXO, e.g., "homing" propensity. Despite of high stability of cargo loading by EDC/NHS chemistry, the kind of chemical technique on the EXOs does not possess high selectivity or specificity because of the complexity of surface proteins and phospholipids on the EXOs.

Imaging probes and medicines loaded on exosomes by "click chemistry"

In recent years, orthogonal chemistry has emerged as a versatile method of choice within the frontier science of biology and nanotechnology. Azide–alkyne cycloaddition, as one canonical subtype of "click chemistry", is an easy-to-adapt and modular methodology for bioconjugation of theranostic agents to the surface of EXOs by Cu(I)-catalyzed reaction, benefiting from short reaction time, high specificity, and compatibility in aqueous buffers.[69] Owing to a degree of cytotoxicity and DNA damage of Cu(I) ions, Cu(I)-free azide–alkyne cycloaddition by strain promotion will be particularly attractive in the modifications of EXOs, in which the choice of DBCO derivatives is crucial to achieving an efficient reaction.[83,84] Usually, "click chemistry" proceeds under mild conditions and features compatibility with various functional groups, yielding covalent product 1, 5-disubstituted 1, 2, 3-trazole hinge species, as shown in Figure 4A for clarity.[85,86] As one of the efficient and "green" tools, advances of "click chemistry" is able to load not only fluorescent,[87,88] radioactive,[89,90] and MRI agents[80,91] for in vivo tracking injected EXOs, but
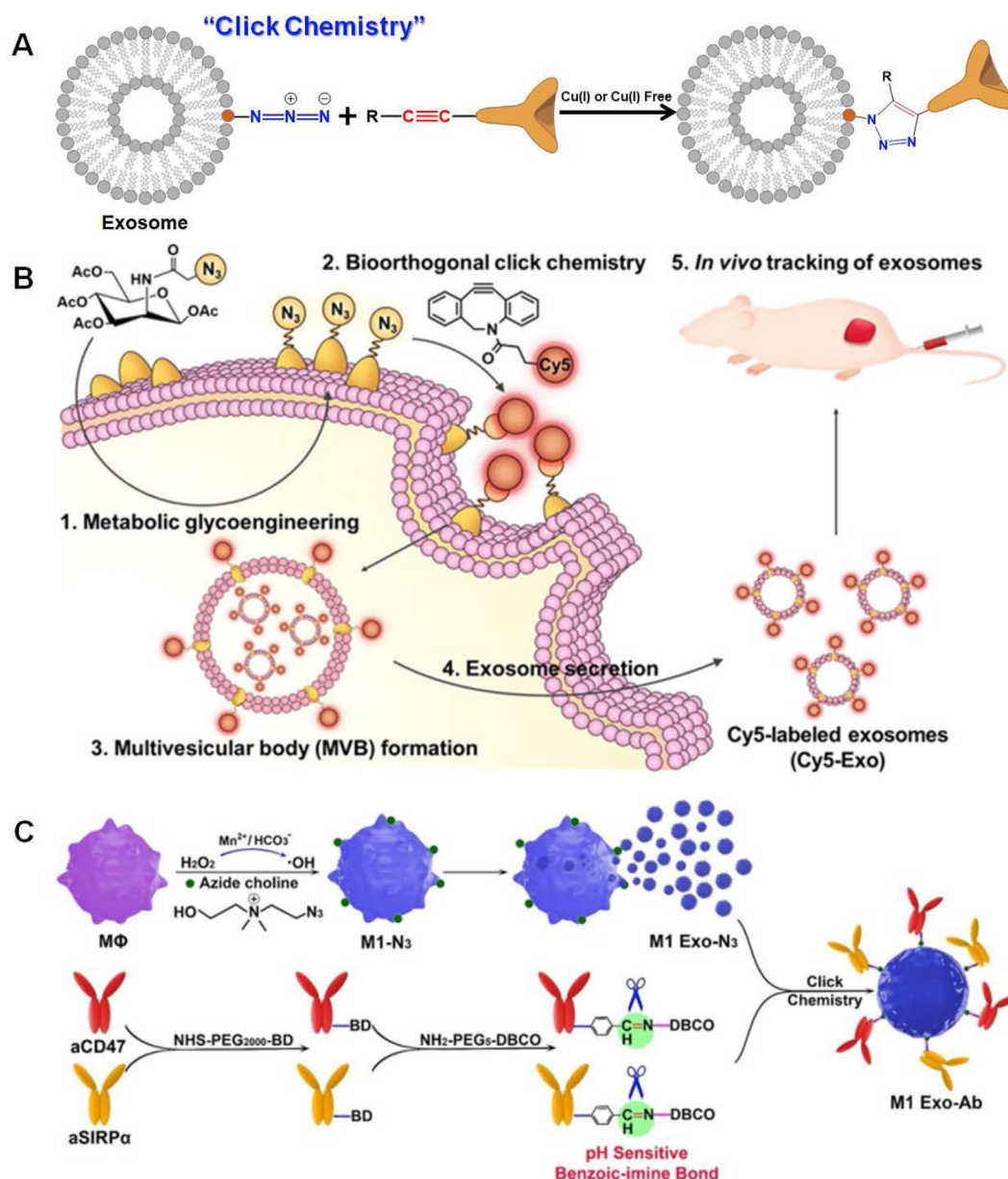
Figure 4 Chemical engineering on the EXOs by "click chemistry". **(A)** Schematic diagram of "click chemistry" on the EXOs. An azide-functionalized EXOs specifically reacted with alkyne-labeled payloads by azide—alkyne cycloaddition to carry probes and biomolecules for the diagnosis/treatment of diseases. The azide—alkyne cycloaddition reaction selectively gives 1,2,3-triazoles linkage. **(B)** Conjugation of fluorescent probes by "click chemistry" onto EXOs for bioimaging. The azide groups were embedded on the surface of A549 cells via glycometabolism. The azide groups were then bioorthogonally labeled with DBCO-Cy5 via "click chemistry". Cy5-labeled EXOs were successfully harvested from parental cells, which were finally used for real-time *in vitro* and *in vivo* imaging and tracking. Referenced from American Chemical Society (Copyright 2020). **(C)** Conjugation of antibodies by "click chemistry" onto EXOs for cancer immunotherapy. The cell membranes secreted from M1 macrophages were modified with azide-functionalized phospholipids by metabolic incorporation. Then, EXOs from M1 macrophages could be conjugated with DBCO-modified anti-CD47 and anti-SIRPa antibodies by "click chemistry". The releases of anti-CD47 and anti-SIRPa in the tumor microenvironment individually blocked interaction with SIRPa on macrophages and CD47 on tumor cells, by which it abolished the "don't eat me" signaling and improved the phagocytosis of tumor cells by macrophages. Meanwhile, the EXOs from M1 macrophages were able to reeducate protumoral M2 toward antitumoral M1 macrophages for cancer therapy. Referenced with permission from Wiley-VCH.

also proteins, nucleic acids, and chemotherapeutic agents for therapeutics. Moreover, this chemical technique is also commonly used for conjugations of the signal transducer,

e.g., metalloenzymes[92] and Raman repoter,[93] to ultra-sensitively detect cancer cell-derived EXOs themselves in the human serum. By taking the approach of "click
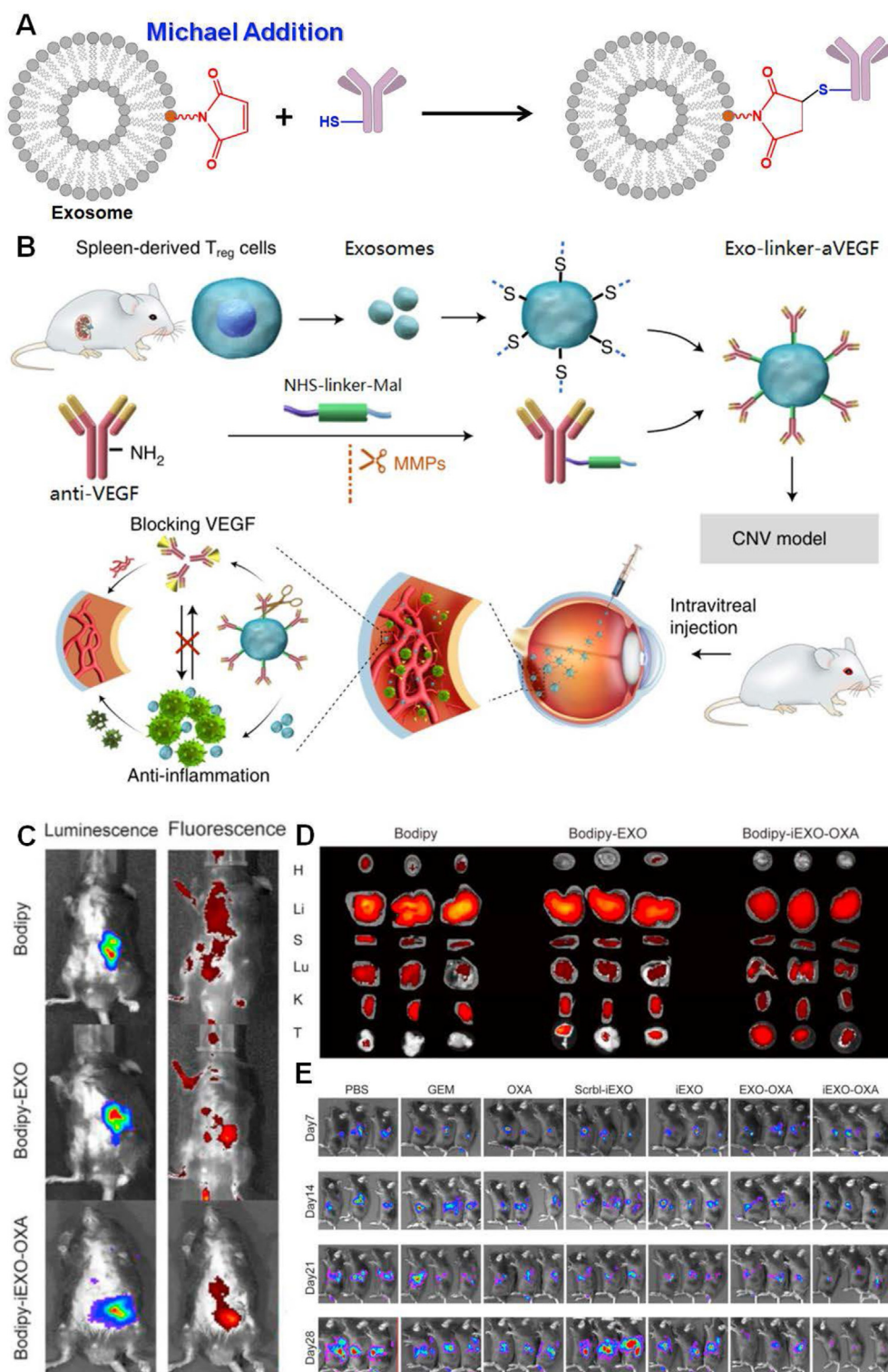
Figure 5   Chemical engineering on the EXOs by Michael addition. **(A)** Schematic diagram of Michael addition on the EXOs. A maleimide-functionalized EXOs specifically reacted with thiol-labeled payloads by the thiol-maleimide reaction to carry probes and biomolecules for diagnosis/treatment of diseases. The thiol-maleimide reaction selectively produces thiosuccinimide linkage. **(B)** Conjugation of anti-VEGFs by Michael addition onto EXOs for suppression of choroidal or retinal neovascularization. The thiol groups were embedded on the surface of EXOs derived from regulatory T cells. Meanwhile, the anti-VEGFs were labeled with NHS-linker-maleimide via NHS-mediated coupling reaction. Finally, anti-VEGFs were successfully integrated onto regulatory T cell-derived EXOs via Michael reaction, which were used for EXOs. Referenced from Springer Nature (Copyright 2021). Oxaliplatin (OXA) and galectin-9-loaded EXOs from bone marrow mesenchymal stem cells (BM-MSC) for treatments of pancreatic cancer: maleimide-functionalized oxaliplatin molecules were successfully conjugated onto EXOs. **(C)** *in vivo* targeting evaluation of OXA-loaded EXOs

chemistry", the surface of EXOs initially needs to label with chemically active alkyne (-C≡C) or azide (-N$_3$) moieties by embedding functional groups to the surface of host cells, e.g., EDC/NHS chemistry,[69,80—82] glycometabolism,[87,94,95] lipometabolism,[96—98] protein labeling[99,100] and many others. Representative examples of azide-crosslinked EXOs by EDC/NHS chemistry have been described previously. As one of the alternative strategies, integration of alkyne-reactive azide moieties onto host cells by azide-labeled saccharide has been strongly recommended, so as to ultimately conjugate fluorescent probes onto EXOs derived from host cells by "click chemistry". Lee et al have provided a proof-of-concept of "click chemistry" to integrate fluorescent tag onto the EXOs and firstly implanted azide groups into cytomembranes of breast cancer cells by incubating these cells with tetraacetylated N-azidoacetyl-D-mannosamine (Ac$_4$ManNAz) solution. Afterward, the EXOs shed from these cells contained azide ingredients on the surface, thereby allowing for the conjugation of DBCO-functionalized fluorescent tags by Cu(I)-free "click chemistry". The fluorescent tag-integrated EXOs were finally applied to infer "homing" propensity, along with their distribution within different organs in breast tumor-bearing mice.[94] Another example of fluorescently labeled EXOs by glycometabolism together with "click chemistry" has been directly operated on their host cells. First of all, EXO-secreted host 549 cells were incubated with Ac$_4$ManNAz to anchor azide moieties on their surface by glycometabolism. Taking the use of "click chemistry" paradigm, the azide groups on the cells were easy to specifically label by DBCO-Cy5. Eventually, Cy5-labelled EXOs shed from 549 cells had the potential to serve as delivery "bioscaffolds" to in vivo track the distributions of therapeutic drugs, as shown in Figure 4B.[87] Apart from glycometabolism, lipometabolism has been also utilized to integrate azide groups into host cells, conferring their secreted EXOs with azide moieties on the surface. Zhang and coworkers have incubated breast cancer cells with newly-synthesized azide chorine, by which it endowed their cytomembranes with azide moieties by metabolic incorporation of azide chorine into phospholipids. Afterward, azide-integrated EXOs from host cells were specifically conjugated with DBCO-functionalized fluorescent dyes for in vivo tracking of their biodistributions and organotropic uptakes.[97] By employing the same way, Nie et al have embedded azide chorine into the membrane of antitumoral M1 macrophages through their intrinsic lipometabolism, by which the EXOs derived from M1 macrophages contained azide functional groups on the surface. Then, both anti-CD47 (aCD47) and anti-SIRPa (aSIRPa) antibodies were simultaneously labeled with DBCO by pH-sensitive benzoic-imine bonds, facilitating their conjugation onto the azide-modified EXOs by "click chemistry" paradigm. Upon accumulation of the aCD47 and aSIRPa-conjugated EXOs in the tumor regions, selective cleavage of

benzoic-imine bonds within the tumor microenvironment resulted in a release of aCD47 and aSIRPa, both of which individually blockade the immune checkpoint CD47 on tumor cells and SIRPa on macrophages, as demonstrated in Figure 4C. The two immune "brake" antibodies were able to abolish the "don't eat me" signaling and restore a potent immune response against tumor cells. On the other hand, the M1 EXOs effectively reeducated the protumoral M2 toward M1.[96] In this study, administration of aCD47 and aSIRPa-conjugated EXOs will be a great way to kill two birds with one stone for tumor immunotherapy. Meanwhile, a pioneering study has proposed L-azidohomoalanine (AHA), an analogue of methionine containing azide moiety, to label proteins by cellular metabolism on the murine melanoma cells. After incubation of murine melanoma cells and AHA solution, these cells would produce azide-integrated EXOs for clicking variable cargos onto EXOs, including theranostic agents and bioimaging probes.[99] Inspired by this work, Yang et al have also adopted almost the same way to integrate mannose onto EXOs shed from macrophages. The mannose-integrated EXOs could be preferentially phagocytized by macrophages to deliver lysostaphin and vancomycin for eradicating intracellular methicillin-resistant Staphylococcus Aureus (MRSA).[100] Notably, chemically-engineered EXOs are also used as a delivery platform of antibiotics to combat the infection occurrence.

Actually, chemically engineered EXOs by robust "click chemistry" firstly track back from the alkyne-modified surface. Starting with the alkyne-modified surface, Smyth et al have carried out 4-pentynoic acid (containing −C≡C group) to chemically modify membrane proteins and phospholipids on the surface of EXOs shed from 4T1 cells for ultimate bioconjugation of azide-fluor 545, which initiates to modify EXOs by "click chemistry".[69] By "click chemistry" for the first time, they successfully labeled EXOs by the fluorescent probe for in vivo bioimaging. The publication of this paper occasioned an explosive growth of "click chemistry" as a versatile technology in the surface modifications of EXOs. Taking use of the same strategy, Xu et al have clicked azide-integrated fluorescence on the pancreatic cancer cell-derived EXOs that were functionalized by DBCO moieties. Unsurprisingly, their findings have confirmed a claim of intrinsic tropism of EXOs for their mother cells, i.e., insight into the "homing" capabilities of EXOs.[101] Besides, another study demonstrated that DBCO moieties were fabricated on the membrane proteins of MSC-derived EXOs by NHS-mediated amide coupling, so as to be further conjugated by azide-linked cyclo(Arg-Gly-Asp-D-Tyr-Lys) peptide [c(RGDyk)], which could actively target the ischemic brain by recognizing integrin a$_v$b$_3$ in reactive cerebral vascular endothelial cells. Next, cholesterol-modified miRNA-210 was loaded onto the c(RGDyk)-conjugated EXOs by incubation, preventing miRNA-210 degradation in the body. When c(RGDyk) targets integrin a$_v$b$_3$ within the

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by a fluorescent signal from Bodipy. **(D)** Accumulation assessments of OXA-loaded EXOs in major organs and tumor tissues by a fluorescent signal from Bodipy. **(E)** Real-time in vivo bioluminescence image at days 7, 14, 21, and 28 after injections with phosphate buffer saline (PBS), gemcitabine (GEM), oxaliplatin (OXA), scrambled siRNA-loaded EXOs (Scrbl-iEXO), gal-9 siRNA-loaded EXOs (iEXO), OXA-modified EXOs (EXO-OXA), and gal-9 siRNA & OXA-coloaded EXOs (iEXO-OXA), respectively. Prior to these performances, luciferase stable-transfected PANC-02 cells were implanted in the pancreas of C57BL/6 mice. Referenced with permission from Elsevier.

lesion region, EXOs carried miRNA-210 cross the blood—brain barrier and were specifically accumulated in ischemia to promote microvascular angiogenesis, by which it ultimately attenuated stroke symptoms.[102] It is conceivable that the inherent advantages of "click chemistry" that have great potentials in EXO-based drug delivery for diagnosis/treatment of diseases are: (i) cargos conjugated by "click chemistry" will not greatly disturb intrinsic properties of themselves and EXOs; (ii) "click chemistry" has excellent selectivity and specificity and extraordinary stoichiometry; (iii) "click chemistry" greatly expands the scalability of cargos loaded on EXOs for diagnosis/treatment. Apart from azide—alkyne cycloaddition, we hope more subtypes that fall within "click chemistry" such as thiol—click reaction, Diels—Alder reaction, nitril oxide cycloaddition, tetrazole cycloaddition, and oxime formation will be utilized in the for bioconjugation of theranostic agents to the surface of EXOs.

Imaging probes and medicines loaded on exosomes by thiol-Michael addition

Except for chemical transfection and saponin-assisted drug loading on the EXOs as previously mentioned, other chemical engineering did not dramatically affect the size and integrity of EXOs. Michael addition, characterized as a reaction of an enolate-type nucleophile to an a, b-unsaturated carbonyl, has a long history of implementation in organic synthesis to afford highly selective products under environment-friendly conditions.[103] This type of chemical reaction has evolved into highly efficient & versatile methods and seemingly arisen in approximate parallel to "click chemistry" in materials science. Exactly, what we are going to next look at is the applications of thiol-Micheal addition in the modifications of EXOs, as typically illustrated in Figure 5A, with particular emphasis on the recent developments in theranostics over the past years. The thiol-Micheal addition has been tailored to progress under mild and solventless conditions to yield a highly efficient reaction. Generally, sulfhydryl is widely distributed in most membrane proteins on the EXOs and can be employed as the binding site for drug loading via interlinkage between sulfhydryl and maleimide. To date, considerable attempts have been devoted to a practical application of thiol-Michael addition on the EXOs. For example, to characterize endocytosis of EXOs by live cells, Roberts-Dalton et al have firstly adopted thiol/maleimide reaction to conjugate Mal-Alexa488 and Mal-Alexa633 onto the prostate cancer cell-derived EXOs. This chemical technique arguably provided flexibility with regard to fluorescent labeling on the EXOs. By the fluorescent signals, they were able to directly observe their interactions with cells and endocytic traffic.[104] In the same way, Luther et al have carried out Mal-Alexa488 to label MSC-derived EXOs, by which they could real-time monitor the uptake of cardioprotective miRNA-21a-5p by recipient cardiac cells to realize cardiac repair.[105] Apart from fluorophores, quantum dots (QDs) anchored onto the EXOs by thiol-Michael addition also enables a moderate and biocompatible labeling strategy. In this study, a DNA hinge was first functionalized with maleimide or biotin on each terminal. Then, the DNA ligament

could be conjugated onto the M1 macrophage-derived EXOs by the chemical reaction between build-in maleimides of DNA and thiols on the EXOs. Afterward, the streptavidin-labeled QDs facilely realized the terminal biotin of DNA hinge to integrate onto EXOs for tumor imaging.[106] Apart from in vivo imaging, another aspect of thiol-maleimide chemistry will focus on the role it plays in the therapeutics of diseases.

Considering the accessibility of thiol-maleimide chemistry, the conjugation of a new class of affinity ligands onto EXO in this way is receiving significant attention in the targeted therapy. Han et al have conjugated aptamers onto the HEK293T cell-derived EXOs by thiol-Michael addition to recognizing prostatic cancer cells. Under the guidance of aptamers, EXOs carried a high dose of siRNA into tumor cells to silence sirtuin 6 (SIRT6) expression, positively correlating with prostate cancer progression.[107] As the report goes, the most effective treatment for ocular neovascular disease focuses on vascular endothelial growth factor (VEGF), which significantly provokes the aberrant growth of blood vessels.[108] Intravitreal injection of anti-VEGF antibodies can block the activity of VEGF and suppress pathogenic angiogenesis in the eyes. Taking the use of thiol-Michael addition, anti-VEGF antibodies were successfully integrated onto the regulatory T cell-derived EXOs to suppress ocular neovascularization. First of all, anti-VEGF antibodies were functionalized with maleimide groups using a peptide linker, which would be subject to cleavage by matrix metalloproteinases in inflammatory lesions. Then, the anti-VEGF antibodies could be crosslinked onto the regulatory T cell-derived EXOs by thiol/maleimide reaction in mild conditions. After intravitreal injection, the engineered EXOs selectively accumulated within neovascularization lesions and the anti-VEGFs on them were released to suppress ocular and choroidal neovascularization, as explicitly shown in Figure 5B.[109] Besides, immunotherapy has raised concern in treating pancreatic cancer since conventional therapies such as chemotherapy could not provide satisfactory survival outcome. A paradigm has described siRNA/oxaliplatin (OXA)-loaded EXOs for therapeutics of pancreatic cancer. In this study, galectin-9 siRNA and OXA were successively loaded on the bone marrow mesenchymal stem cell (BM-MSC)-derived EXOs by electroporation and thiol-Michael addition, respectively. OXA, as an important antitumor agent, was modified with N-(2-Aminoethyl) maleimide to synthesize OXA-maleimide. Then, OXA-maleimide was integrated onto the galectin-9 siRNA-loaded EXOs by thiol/maleimide reaction.[110] Obviously, the use of BM-MSC-derived EXOs could significantly improve tumor targeting efficacy and effectively diffuse into the entire pancreatic tumor mass in contrast with the control group, as dramatically shown in Figure 5C and D. With time, the antitumor effects of siRNA & OXA-loaded EXOs preceded over than other groups such as PBS, gemcitabine, oxaliplatin, scrambled siRNA-loaded EXOs, galectin-9 siRNA-loaded EXOs, and OXA-modified EXOs, as clearly demonstrated in Figure 5E. Except for immune recovery of macrophages by galectin-9 siRNA, the OXA integrated onto EXOs by thiol-Michael addition was able to trigger immunogenic cell death within pancreatic tumors. More recently, anticytotoxic T lymphocyte-associated protein 4 antibody (aCTLA-4) was conjugated onto the dendritic cell-derived

EXOs using thiol-maleimide chemistry to activate tumor-specific cytotoxic T lymphocytes to potentiate tumor therapeutic efficacy.[111] Not only maleimide moiety is successfully conjugated onto the sulfhydryl on the EXOs, but citraconic anhydride can be integrated onto the amine of EXOs by thiol-Michael addition to modifying the surface charge of EXOs. Upon crosslinking of citraconic anhydride to the amine of EXOs, the reduced surface charge made for their superior intracellular uptake by macrophages.[112] Of note, thiol-Michael addition is one of the popular chemical technologies to modify EXOs by specifically aiming at sulfhydryl of their surface proteins, which sufficiently enrich the cargos of interest for diagnosis/treatment. Nevertheless, traditional thiol-Michael addition tremendously depends on susceptible factors such as the basicity of the catalyst, thiol pK_a, types of the thiol, and many others.[103] Even so, we still hope that other types of Michael addition

paradigms such as carbon-Michael reaction, oxa-Michael reaction, and aza-Michael reaction will be adopted in the EXO modifications for diagnosis/treatment in the future.

Imaging probes and medicines loaded on exosomes by other chemical engineering

Many studies also have shown other chemical engineering emerges to reprogram EXOs as "bioscaffolds" for diagnosis/treatment. Serving as attractive surrogates for specific tumor cell-associated counterparts, intact EXOs' detection is of great significance for early-stage diagnosis of cancer.[113] A pioneering work has recently developed molecular imprinting for creating EXO-binding cavities and introducing antibodies and fluorescent reporters inside the binding cavities to detect intact EXOs, as shown in Figure 6A, in
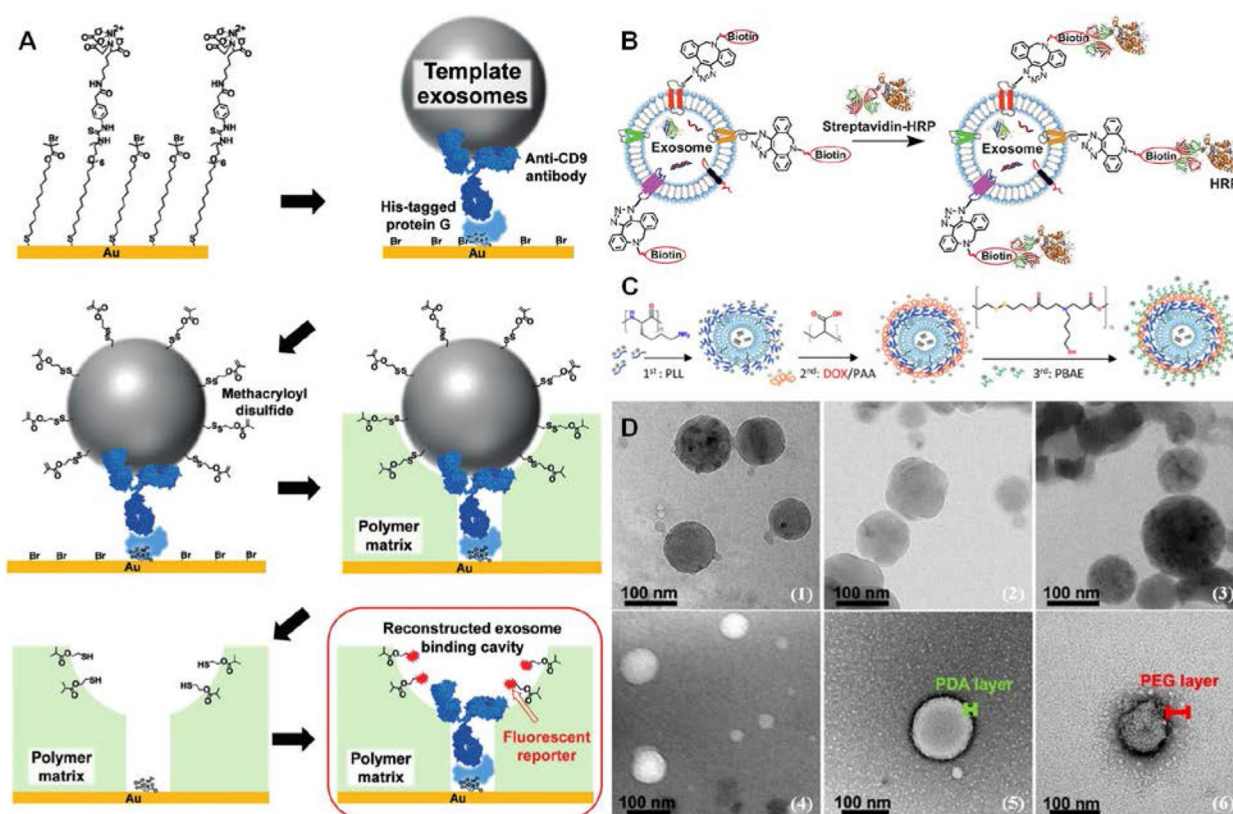

Figure 6 Chemical engineering on the EXOs by different methodologies. (A) Disulfide bridging-modified template EXOs for detecting intact EXOs. Firstly, His-tagged protein G was anchored through Ni^{II} complex with nitrilotriacetic acid (NTA) groups on the gold substrate modified with a mixed self-assembled monolayer, by which anti-CD9 antibodies were immobilized by interaction with His-tagged protein G. The template EXOs were then specifically captured on the substrate through antigen/antibody binding and subsequently, their surface was modified with methacryloyl disulfide. The captured EXOs were imprinted by a polymer matrix on the substrate. After removal of the template EXOs, the disulfide linker would be transformed into a free thiol inside EXO-binding cavities, allowing the fluorescent reporter to be conjugated on the thiol groups. By monitoring the fluorescent signal inside cavities, intact EXOs could be sufficiently detected with high sensitivity. Referenced with permission from Wiley-VCH. (B) Biotinylation of EXOs for intracellular delivery. EXOs were firstly biotinylated with DBCO-PEG$_4$-biotin by "click chemistry". Afterward, the streptavidin-functionalized cargos could be specifically crosslinked onto biotin conjugated on EXOs. (C) Trlayered shell assembly of polyelectrolytes formed on the siRNA-loaded EXOs by layer-by-layer deposition serves as the carrier for DOX loading. 1st layer: cationic poly(L-lysine) (PLL); 2nd layer: anionic poly(acrylic acids) (PAA); 3rd layer: poly(b-amino esters) (PBAE). Referenced from American Chemical Society (Copyright 2020). (D) Morphology characterization of polymer-modified EXOs. Cryo-TEM images of (1) bare EXOs, (2) EXO@PDA, and (3) EXO@PDA@PEG; TEM images of (4) bare EXOs, (5) EXO@PDA, and (6) EXO@PDA@PEG$_{2000}$. Referenced with permission from Wiley-VCH.

which the surface of the template EXOs was modified with a disulfide bridge. In this study, His-tagged protein G was firstly anchored through the Ni[II] complex with nitrilotriacetic acids (NTAs) on the gold substrate. Then, by interaction with His-tagged protein G, anti-CD9 antibodies were immobilized to capture CD9 highly expressed-EXOs. The EXOs were specifically captured through antigen/antibody binding and their surface was subsequently modified with a disulfide bridge using thiolated oleyl poly (ethylene glycol) and 2-(2-pyridyl) dithioethyl methacrylate. Afterward, the captured EXOs were imprinted by a polymer matrix. After removal of the template EXOs, the disulfide bridge would be transformed into a free thiol inside EXO-binding cavities, ensuring the fluorescent tags are site-specifically conjugated on the thiol groups.[114] By monitoring the fluorescent signal inside EXO-binding cavities, this observation signified a successful detection of CD9-specific and intact EXOs. More importantly, such a rapid and highly sensitive "platform" was able to ultimately distinguish cancer-secreted EXOs from normal ones without tedious and time-consuming treatments. Ahead of the disulfide bridge, the surface modification of EXOs with cationized pullulan through electrostatic interaction has been proposed for the development of a new class of therapeutics for liver injury. By a simple mixing of EXOs and cationized pullulan, pullulan-loaded EXOs could be beneficial for targeting ability to hepatocyte asialoglycoprotein receptors. When intravenous administration, more internalization of pullulan-loaded EXOs by hepatocytes resulted in a greater therapeutic effect on liver injury.[115] The regent-free approach was simple to operate while avoiding complex purification steps. Besides, EXOs can be also integrated with biotin on their surface, thereby establishing a specific conjugation of streptavidin-functionalized cargos onto EXOs for diagnosis/treatment of different diseases,[99] as shown in Figure 6B.

Emerging as promising drug delivery vehicles, amphiphilic polymers are good candidates for surface functions of EXOs, facilitating the development of polymer-engineered EXOs for cargo loading. Another study showed, by incubation of siRNA-loaded EXOs with hydrophilic polyethylene glycol (PEG), the surface of EXOs became coated with a PEG corona. When oral drug delivery, the PEG coating could significantly reduce EXOs degradation in the acidic gastric environment and efficiently diffuse across mucosal barriers to enhance their overall permeability.[116] On the other hand, PEGylation allows for decorating EXOs with targeting ligands. To improve EXO characteristics for drug delivery to tumor cells, Kooijmans et al have integrated phospholipid (DMPE)-PEG derivatives onto Neuro2A cell-derived EXOs, by which it facilitated conjugations of nanobodies specific for epidermal growth factor receptor (EGFR) by Michael addition. In this way, the modified EXOs had better specificity to EGFR highly expressed in tumor cells and longer circulation time within the mice body.[117] For different purposes, polyethyleneimine (PEI) coated on the FA-functionalized EXOs could be complex with nucleic acids through ionic interaction. Under the guidance of FA molecules, PEI on the EXOs could effectively deliver a high dose of siRNA to silence gene expression in tumor therapy.[74] Similarly to PEI, one more intricate polymer architecture was successfully constructed on the A549 cell-shed EXOs for selective codelivery of siRNA and chemotherapeutics to cancer cells,

as shown in Figure 6C. The layer-by-layer architecture was designed by sequential deposition of oppositely charged polyelectrolytes. The trilayered shell assembly of polyelectrolytes on the surface of EXOs was composed of poly (L-lysine) (PLL), poly (acrylic acids) (PAA), and poly (b-amino ester) (PBAE) from inside to outside, which served as the carrier for DOX loading.[118] The final layer PBAE was chosen owing to its high gene transfection efficiency and excellent intracellular delivery.[119] Except for this polymer matrix, a recent study employed polydopamine coating to control the surface properties of EXOs, ensuring tailorable conjugations of other cargos.[120] In this study, self-polymerization of dopamine on the EXOs was a simple and mild method, on which the catechol and amine groups conferred conjugations of other cargos by Michael addition or Schiff-base reaction.[121] After PDA coating, the EXO@PDA could be also integrated with PEG-thiol to form EXO@PDA@PEG and fluorescently labeled EXO@PDA@fluorescein isothiocyanate (FITC)-PEG, as shown in Figure 6D. Collectively, modifications by polymers are able to improve the stability of EXOs and facilely incorporate functional molecules, which will expand the potential of EXOs in biomedical applications. Disappointingly, one of the major drawbacks associated with an encapsulation of polymers on the EXOs is a block of the communications of membrane proteins, resulting in a loss of their intrinsic characteristics, e.g., "crosstalk" and tissue-homing capabilities.

Conclusions and perspectives

EXOs secreted naturally by human cells are emerging as a new generation of delivery systems for future clinical development. As one of the natural "bioscaffolds" from the human body, they can carry not only medicines but also bioimaging agents for precise theranostics. It is worth mentioning that a phase II clinical trial of tumor-antigen loaded dendritic cell-derived EXOs as vaccination was successfully carried out in 2018.[122] This review mainly discussed the chemical engineering of surface functionalization on the EXOs. Although each of them differs at the molecular level, their purpose is the same, i.e., to conjugate theranostic agents to EXOs for diagnosis/treatment. Compared with physical and genetic approaches, some of them own better scalability of cargos, more selectivity and specificity at binding sites on the EXOs, and higher stability and reproducibility. Regardless of the unique properties of chemically-engineered EXOs to develop smart drug delivery vehicles that circumvent phagocytosis, immunogenicity, and short circulation half-life, some key points are major hurdles to clinical translation of chemically-engineered EXOs, such as selection of proper donors, preservation of intrinsic characteristics, and standardization of quality control: (i) Firstly, prudent selection of mother cells is one of the critical upstream process steps for chemically-engineered EXOs. The properties of EXOs such as tissue-homing, immunogenicity, and oncogenicity should be considered, which are greatly associated with the mother cells. Of note, contamination and genetic drift occasionally occur during mother cell culture. On demand of theranostic goals, EXOs from mother cells should steadily express desired cargos.[12] (ii) More disastrously, some of the chemical

engineering greatly results in rearrangements of EXO membranes and destruction of their integrity, for example, by chemical transfection and saponin. Besides, the EDC/NHS chemistry and thiol-Michael addition are mainly operated on the functional groups (*e.g.*, carboxyl, amine, and thiol) in membrane proteins or phospholipids of EXOs. If possible, their membrane proteins will be capped when modified by polymers. Collectively, chemical engineering partly alters the original distribution and density of membrane proteins on the EXOs and even denatures them, both of which will disturb their intrinsic functionality such as "crosstalk" and "homing" propensities to affect therapeutic efficacy. Thus, what kind of chemical engineering to choose to modify EXOs should be taken seriously. (iii) The stability and reproducibility of chemically-engineered EXll remain controversial when loading a variety of cargos on them. These important parameters of chemically-engineered EXOs require control of quality and clinical investigation. To further enhance rigor and reproducibility, the International Society for Extracellular Vesicles (ISEV) should initiate the standards of chemically-engineered EXOs and characterization, *e.g.*, the amounts of functional groups and cargos, by which they can achieve equivalent or superior efficacies. Thus, these limitations may render their clinic translation cumbersome and risky. Despite these challenges, the chemical engineering of EXOs is still a rising star as drug delivery vehicles. This technique is still in its infancy and will offer a new route for precise diagnosis/treatment of human devastating diseases, including cancer and neurodegenerative disorders. In this regard, chemically engineered EXOs will be expected to carry other cargos, such as paramagnetic metals for MRI, contrast agents for CT imaging, or microbubbles for sonography. To this end, more ways of chemical cross-linking on the EXOs are expected to be exploited in the future. With concerted efforts from organic chemists, molecular biologists, and clinicians, the chemical engineered-EXOs will become a new theranostic frontier for human devastating diseases.

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Conflict of interests

The authors declare no competing financial interest.

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