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Extracellular Vesicle Nanoarchitectonics for Novel Drug Delivery Applications

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Extracellular vesicles (EVs) can transfer intercellular messages in various (patho) physiological processes and transport biomolecules to recipient cells. EVs possess the capacity to evade the immune system and remain stable over long periods, identifying them as natural carriers for drugs and biologics. However, the challenges associated with EVs isolation, heterogeneity, coexistence with homologous biomolecules, and lack of site-specific delivery, have impeded their potential. In recent years, the amalgamation of EVs with rationally engineered nanostructures has been proposed for achieving effective drug loading and site-specific delivery. With the advancement of nanotechnology and nanoarchitectonics, different nanostructures with tunable size, shapes, and surface properties can be integrated with EVs for drug loading, target binding, efficient delivery, and therapeutics. Such integration may enable improved cellular targeting and the protection of encapsulated drugs for enhanced and specific delivery to target cells. This review summarizes the recent development of nanostructure amalgamated EVs for drug delivery, therapeutics, and real-time monitoring of disease progression. With a specific focus on the exosomal cargo, diverse drug delivery system, and biomimetic nanostructures based on EVs for selective drug delivery, this review also chronicles the needs and challenges of EV-based biomimetic nanostructures and provides a future outlook on the strategies posed.

1. Introduction

Extracellular vesicles (EVs) have received significant attention in recent literature, due to their ability to encapsulate, and transport vital information, in the form of lipids, proteins and nucleic acids (e.g., miRNAs). Furthermore, they have been established as key mediators of cell-cell communication, both in health and disease, including angiogenesis, coagulation, cell survival, and cancer metastasis.[1] EVs is a term used for a heterogeneous population of vesicles, ranging in size, from approximately 40–1000 nm.[2,3] They are lipid bilayer, membrane-enclosed vesicles, and are released by a multitude of cell types. Although the generic term, extracellular vesicles, is used to refer to these membranes bound vesicles, there are in fact, multiple sub-types of vesicles. This classification is based on their size, process of biogenesis, and the expression, or lack thereof, of specific proteins.[3] Broadly,

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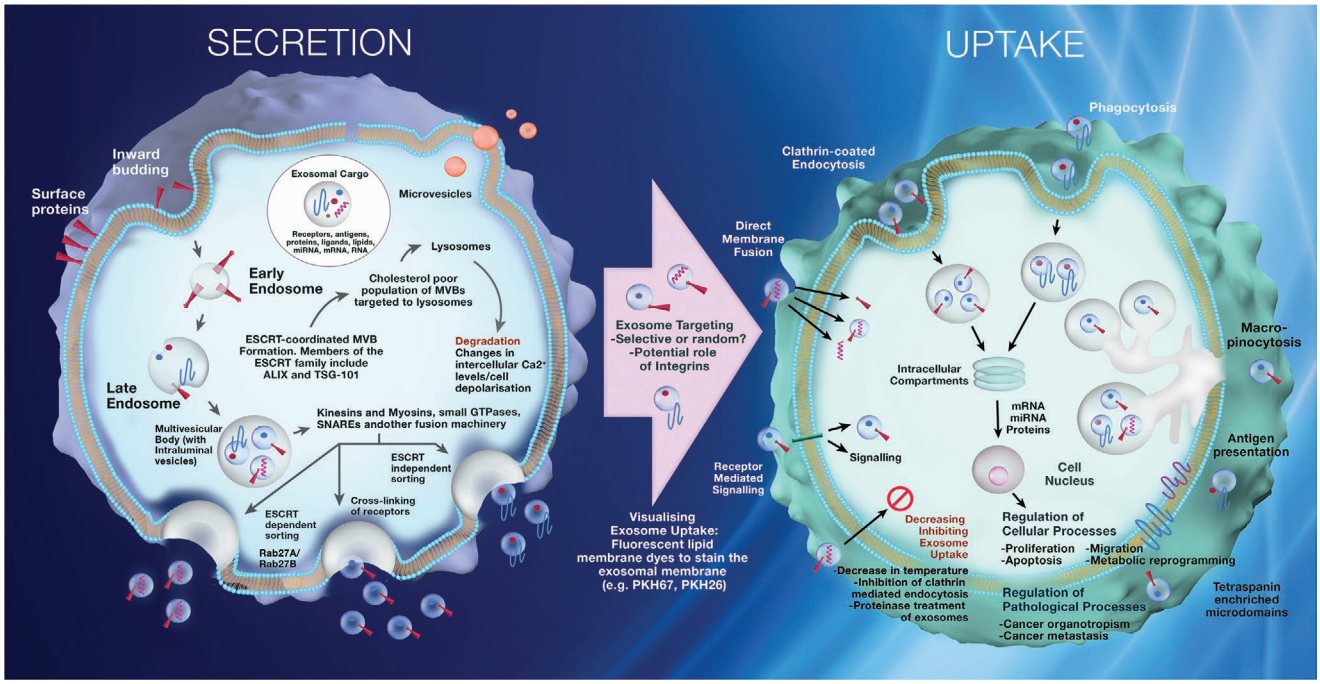

Figure 1. Exosome Biogenesis, Secretion and Uptake. Exosome biogenesis begins with an inward budding of the plasma membrane, leading to the formation of an early endosome. The early endosome than matures to a late endosome stage where inward budding of its membrane leads to the formation of intraluminal vesicles (ILVs). The late endosome is then termed a multivesicular body. The multivesicular body (MVB) can then either fuse with lysosomes, leading to degradation, or fuse with the plasma membrane (through multiple pathways, including: ESCRT-dependent pathways, ESCRT-independent pathways and receptor cross-linking), resulting in the release of the ILVs. Once in circulation, the ILVs are termed exosomes, and they can either interact with neighboring cells or travel to distant target sites, where it can be up-taken through different pathways including Clathrin-coated endocytosis, direct membrane fusion, receptor mediated signalling, macro-pinocytosis and antigen presentation. Once up-taken by the target cell, the exosomal content (proteins, miRNAs, mRNAs, and lipids) can interact with other molecules to regulate cellular processes such as proliferation, migration and apoptosis, among other physiological and pathological processes.

they can be categorized into large vesicles, known as microvesicles, and smaller vesicles, known as exosomes.

Microvesicles further encompass a range of different extracellular vesicles, including microparticles, blebs, apoptotic bodies, and oncosomes.[4] They are generated through an outward budding of the cellular plasma membrane, followed by release into the extracellular space.[5] In contrast, exosomes are smaller extracellular vesicles, of approximately 50–150 nm in size, and their biogenesis begins with an inward budding of the plasma membrane, leading to the capture of membrane molecules, and results in the formation of an early endosome within the cell (**Figure 1**).[6–8] The early endosome then matures to a late endosomal stage where inward invaginations of its membrane form intraluminal vesicles. This allows for the capture of cytosolic molecules. The presence of intraluminal vesicles within the endosome results in the late endosome being termed a multi-vesicular body.[9] The multi-vesicular body can then take one of two routes, it can either fuse with a lysosome leading to degradation or fuse with the plasma membrane, leading to the release of these intraluminal vesicles, which once released into the extracellular space, are termed exosomes.[10] This process of exosome biogenesis, which allows for the capture of both membranous, and cytosolic molecules, is what makes exosomes so intriguing, and a valuable resource to understand the cellular microenvironment. These exosomes can then interact with either neighboring cells, or travel to distant target sites, where they can be up taken through a variety of different methods, such as clathrin-coated endocytosis, direct membrane fusion, and receptor-mediated endocytosis.[2,11] Upon interaction with target cells, these vesicles are able to release their contents to alter the target cell behavior, resulting in physiological changes. Interestingly, exosomal cargo comprises of a range of bioactive molecules, including proteins, lipids, and small RNAs (such as miRNAs).[11] Therefore, these vesicles have gained an immense interest in many research fields, including disease diagnostics as liquid biopsies, regenerative medicine as therapeutics, medicine transport as drug delivery vehicles.[12] While all these molecules have been studied extensively, recent literature has focused on examining the miRNA profile of these extracellular vesicles for multiple applications, ranging from biomarker discovery, and understanding disease progression, to the development of therapeutic interventions.[13,14] Although extracellular vesicles have been identified as having crucial roles in several physiological and pathological processes, there has been a limited advancement in understanding the processes of biogenesis and cargo loading.[15]

The interaction between multivesicular body with lysosomes or plasma membrane can occur through multiple distinct pathways, including ESCRT-dependent, and ESCRT-independent mechanisms. The release of the exosomal content can bring physiological changes in the target cell, and regulate cellular processes, e.g., cell proliferation and migration. They resemble

liposomes in terms of size, shape and structure with complex bilayers and surface-associated molecules.[12,16] The biological composition and ability of exosome to transport biomolecules to recipient cells have made them attractive for drug delivery applications. By exploiting the evolutionary selection of exosome, engineered design, selective drug loading for transport to the disease site, manufacturing challenges in nanomedicine could potentially be circumvented. However, the current challenges associated with exosome sorting, isolation and bulk production have impeded their translation to clinical applications.

With the advancement of nanoarchitectonics, rationally engineered nanostructures hold great promise for improving disease diagnosis and treatment.[17] Nanotechnology is defined as the design, production and application of structures, devices and systems at the nanoscale with higher structural precision. In comparison, the nanoarchitectonics concept combines nanotechnology with various research fields, such as materials science, supramolecular chemistry, and biosciences, to create novel functional materials from nanoscale units.[18,19] Certain features of nanoarchitectonics, including self-organization, mutual effects of multiple components and the unavoidable effects of thermal/statistic fluctuations are also common characteristics of biochemical systems.[20,21] Many functional biological systems possess "soft" flexible architectures based on the self-organization of functional biomolecules and these, in turn, operate effectively even in the presence of (or in some cases, because of) thermal fluctuations. Biosystems can be regarded as the ultimate prototype of nanoarchitectonics. Considering this, nanoarchitectonics is, therefore, likely to provide excellent opportunities for combining artificial nanotechnological systems with biosystems. Hence, nanoarchitectonics is more closely related to biochemical systems than current nanotechnology, which shares more similarities with traditional materials science. Engineered nanoparticles (NPs) with bio-homologous properties (e.g., size, shape, surface environment, and biodistribution and solubility) have extensively been developed for clinical drug delivery. They have also been used to overcome the limitations of conventional drug delivery by improving the spatial and temporal distribution of therapeutic agents, increasing the solubility and stability of encapsulated cargos in the complex biological matrix, promoting transport across membranes and extending circulation times to increase drug efficiency and safety.[22,23] Complex molecular targeting approaches based on NPs, however, have failed in many clinical trials due to complex interactions between NPs and biological groups that mask surface binding sites, ligands and receptor, and causing immunological recognition. Moreover, their clinical efficacy is also limited by their lack of similar (biological moiety) complexity, nonspecific molecular targets, failure to deliver intracellularly, and low bioavailability. Recently, there has been a paradigm shift on EVs and NPs based drug delivery systems, particularly on the amalgamation of EVs with engineered nanostructures for achieving effective drug loading, and site-specific delivery. EVs (exosome) amalgamated NPs can potentially overcome the above issues by incorporating the intrinsic properties of both EVs and NPs.[24,25] The combination of their properties allows for higher drug loading, improved host immune response, excellent tumor targeting ability, enhanced guided delivery to the disease site, and controlled circulation and ejection from the body. These attractive properties render EVs-NPs as promising alternatives to purely synthetic drug delivery vectors. The NPs vehicle can be inorganic or organic with different sizes and shapes, depending on the drug conjugation or encapsulation ability. To date, several reviews and progress reports have discussed potential and challenges of EVs for drug delivery.[12,26–29] Currently, there is extensive literature on NPs based drug delivery system and its comparison with EVs based delivery.[29–32] However, no reviews or reports have yet focused on the amalgamation of EVs with engineered nanostructures for achieving effective drug loading, and site-specific delivery- a growing and exciting research field for advancing nanomedicine and therapeutics for clinics. Therefore, herein we propose a marriage between EVs and NPs as a promising multifunctional drug carrier for personalized medicine.

In this review, we have extensively focused on the recent development of nanostructure amalgamated EVs specifically, exosomes in drug delivery, therapeutics, and real-time monitoring of disease progression. Following the particular emphasis on exosomal cargo, diverse drug delivery systems, and progress of synthesizing vesicles with both exosomal and synthetic NPs, this review also details the recent advancement on engineered nanostructured exosomal cargo design and preparation, sorting, isolation, drug loading and site-specific delivery. With a comprehensive discussion in each section, this review also chronicles the needs and challenges involved in the design and fabrication of EVs-amalgamated nanostructures for efficient drug delivery.

2. Specific Packaging and Sorting of Exosomal Cargo

While exosome biogenesis can be briefly described as being regulated by the endosomal pathway, there are in fact, several key pathways and factors that play a significant role in exosome production, as well as the sorting of cargo into these vesicles. Perhaps the most well-known pathway that has been examined in exosome biogenesis and cargo sorting is the Endosomal Sorting Complex Required for Transport (ESCRT) Pathway.[33] This pathway involves the sequential and coordinated activity of four ESCRT machinery complexes (composed of approximately 20 proteins), known as ESCRT-0, -I, -II, and –III. They are involved in sorting ubiquitylated membrane proteins to membrane domains, thus enabling inward invaginations, leading to the formation of intraluminal vesicles. ESCRT-0 forms domains of clustered cargo, followed by ESCRT-I, and –II, which warp the membrane, to allow the capture of cargo.[34,35] Localization of ESCRT-I, and –II at the neck of the budding vesicle attracts ESCRT-0 ubiquitin domains, followed by eventual localization of ESCRT-III at the neck, which then effectively cleaves the budding vesicles, to form the intraluminal vesicles.[34] Where there is inclusion of the ESCRT proteins, it is known as the ESCRT-dependent pathway, however, exosome biogenesis and cargo sorting can also be independent of the ESCRT pathway (termed intuitively the ESCRT-independent pathway). ESCRT-independent pathways show an active dependence on tetraspanins, such as CD9, CD81, and CD63, as well as a fundamental role for ceramide, and sphingomyelinases.[36] While the process of exosome biogenesis, and cargo sorting into exosomes

are often discussed as independent of each other, they are intimately linked, sharing common pathways and molecules to facilitate both processes.

Efficient distribution of materials internalized through the process of endocytosis is critical for maintaining cellular function and physiology, representing one of the major trafficking pathways within cells. The two key areas at which cellular materials are sorted include the early endosome and the late endosome.[37] The early endosome receives components via several endocytosis pathways, and acts as a sorting station, determining whether molecules are to be recycled to the plasma membrane or targeted for degradation through the lyososomal pathway. Receptors that need to be recycled to the plasma membrane are detached from their ligands, through a process known as acidification, where a low pH environment leads to uncoupling of the ligand from the receptor. The ligands are destined for destruction by targeting them to lysosomes, whereas the receptors are sent to the plasma membrane, through a pathway regulated by small GTPases of the Rab family of proteins.

2.1. Specific Packaging of Proteins

A recent study by Larios et al. showed that the sorting of tetraspanins, such as CD9 into exosomes was dependent on an intact ubiquitination site, suggesting the similarity of this process with the ESCRT-dependent protein sorting, which is also regulated through ubiquitination.[38] Furthermore, they proposed that ALIX, a protein commonly used as an exosomal marker, acts as an alternative recruiter for ESCRT-III to the endosomal membranes, which, in turn, controls tetraspanin packaging. ALIX has also previously been shown to be involved in cargo sorting and exosome biogenesis. The interaction of ALIX with syndecans, through syntenin, has also been established as critical to the process of exosome budding and production.[39] Furthermore, this allows for the segregation of the syndecan cargo to endosomal membranes, and exosomes.[40] Syndecans have been noted as key regulators of receptor trafficking, particularly due to the presence of heparin sulfate chains, which may be involved in receptor signaling and trafficking. Roucourt and coworkers showed that heparanse, which cleaved heparan sulfate, could stimulate the production of Syntenin-1 containing exosomes, through interaction with Syntenin-1 and ALIX.[41] This is of particular interest, as the expression of heparanse has been correlated with tumor progression, with an up-regulation in expression correlated with enhanced angiogenesis, metastasis, and tumor growth. Furthermore, exposure of tumor cells to exogenous heparin has been shown to promote a significant increase in exosome secretion.[42]

The exosomal protein profile varies depending on the cell of origin, with exosomes released by tumor cells showing a profile distinct to that of exosomes released by healthy cells, either in terms of the protein itself or through the levels of protein enrichment. This, in addition to the fact that exosomal content does not mirror that of the releasing cell, reiterates the idea that proteins are specifically packaged into exosomes. Recent literature has shown accumulating evidence to suggest that exosomal cargo is actively selected, based on several factors, including, the cell of origin and its metabolic status, external stimuli, as well as the extracellular environment of the parent cell.[43] Additionally, post-translational modifications on proteins, such as the formation of disulfide bonds, addition or removal of functional groups, as well as cleavage of precursors, have also been implicated in specific protein packaging,[44] Ageta and colleagues showed that ubiquitin-like 3 (UBL3)/membrane-anchored Ub-fold protein (MUB) acted as a post-translational modification, and was fundamental for protein sorting in exosomes.[45] They also reported a 60% decrease in total protein levels in exosomes obtained from UBL3-knockout mice. In addition to proteins, RNAs, specifically, miRNAs, have garnered significant attention as exosomal cargo. Specific packaging of RNAs has also been explored as it provides a lucrative avenue for potential therapeutic options. Although recent literature has focused on miRNAs, exosomes have been shown to contain a variety of small noncoding RNAs, including, tRNA, vRNA, and yRNA.[46]

2.2. Specific Packaging of RNAs

Several studies have reported the utility of exosomal RNAs, suggesting their potential as diagnostic and prognostic biomarkers, in several diseases, including cancers, pregnancy complications, inflammation, and cardiovascular disease.[47–53] Furthermore, the possibility of utilizing exosomes, incorporated with miRNAs and/or siRNAs, has also been proposed.[54,55] Gibbings et al. sought to determine whether exosomes contain proteins essential for miRNA activity, and to do so, they examined exosomes obtained from monocytes.[56] They determined the expression of GW182, and Argonaut 2 (AGO2), two vital components of the miRNA-containing RNA-induced silencing complex (RISC), and found GW182 to be enriched within the exosomes. RNAse treatment demonstrated that abundant miRNAs and mRNAs were protected through exosome encapsulation. Knockdown of ESCRT-associated components, including ALIX, HRS, and VPS36, compromised miRNA activity, suggesting that interfering with ESCRT integrity limits miRNA functions, potentially by altering sorting of GW182 into multivesicular bodies.[56] Mittelbrunn and colleagues showed that RNA, specifically, miRNAs could be transferred in a unidirectional manner, via exosomes.[57] Furthermore, they showed that exosomes derived from T, B, and dendritic immune cells contained miRNA signatures that were distinct to those of the parent cells, and when they inhibited exosome production by targeting sphingomyelinase-2, miRNA transfer between cells was impaired.[57] Comparison of cellular miRNAs with EVs miRNAs, using deep sequencing, revealed that specific miRNAs are preferentially packaged, and thus enriched in extracellular vesicles.[54]

A study by Villarroya-Beltri and colleagues showed that protein heterogeneous nuclear ribonucloprotein A2B1 (hnRNPA2B1), a ubiquitously expressed RNA binding protein, facilitated specific exosomal loading of miRNAs, by interacting with sequence motifs present in miRNAs.[58] These sequence motifs, termed EXOmotifs, guided the loading of miRNAs into exosomes, and mutagenesis of these motifs could modulate miRNA cargo loading. The role of hnRNPA2B1 in miRNA sorting was identified through mass spectrometry analysis.

Streptavidin beads were coated with either biotinylated exosomal miRNAs or cellular miRNAs and incubated with human primary T-cell exosomal extracts. The pulled down proteins were analyzed, and several hnRNPs were identified, with hnRNPA2B1 and hnRNPA1 being the only proteins that bound exosomal miRNAs but showed no binding with cellular miRNAs. Further analysis using Western blots revealed that the weight of the hnRNPA2B1 and hnRNPA1 proteins from exosomes was greater compared to cells, suggesting post-translational modifications within exosomes. It was revealed that the proteins were attached to small ubiquitin-related modifier (SUMO) and that the changes in the molecular weight were consistent with this, suggesting sumoylation. Further, they showed that when sumoylation was inhibited, there was a decrease in the level of hnRPA2B1 and miRNA binding, highlighting the preferential sorting of sumoylated hnRPA2B1 into exosomes, and its role in miRNA loading into exosomes.

Shurtleff et al. also examined RNA binding proteins and noted that Y-box binding protein I (YBX1) was the most abundant protein, based on peptide count and coverage.[59] YBX1 colocalized with GW182 present within exosomes. Furthermore, it was shown that YBX1 was bound to a biotin-miR-223 complex, however, no detectable level of YBX1 was observed in control incubations without biotin-miR-223. Furthermore, they noted that specific miRNAs were more likely to be packaged into exosomes, compared to other miRNAs. They proposed two explanations for this phenomenon, either that sorting motifs were responsible, or that different cells might contain distinct RNA binding proteins, each with a separate miRNA binding preference, and hence they might package distinct miRNAs into exosomes. Santangelo and colleagues identified the role of the RNA binding protein, Synaptotagmin-binding Cytoplasmic RNA-Interacting Protein (SYNCRIP), in exosomal miRNA sorting in hepatocytes.[60] The knockdown of SYNCRIP compromised the loading of specific exosome-enriched miRNAs, and the specific miRNA motif that SYNCRIP was bound to was also identified. To confirm the roles of this motif and SYNCRIP, the motif was introduced into a miRNA that is normally retained within the cells, and insertion of the motif resulted in induction of exosomal export of the miRNA. This highlighted the significant roles of both the motif and SYNCRIP in small RNA loading into exosomes.

A more recent study examined 30 RNA binding proteins to establish their role in shuttling RNA into exosomes.[61] They concluded that RNA binding proteins are also packaged into exosomes, together with RNAs, by acting as RNA-ribonucleoprotein complexes, and had a significant role in transporting RNAs into exosomes and protecting them once they are packaged into exosomes. RNA-binding proteins have gained significant interest, and over 500 of them has been identified in recent years, with RNA binding proteins accounting for approximately 25% of the protein content of an EVs.[62] Due to miRNAs being specifically packaged into exosomes, and their important role in health and disease, recent literature has focused on targeted approaches for exosomal miRNA delivery. However, there are also limitations associated with the use of extracellular vesicles, or exosomes, as therapeutics carriers.

These include the difficulty in choosing appropriate exosomes as drug delivery systems since it has been noted that tumor exosomes, while providing a targeted approach, may also aggravate the patient's tumor malignancy.[63] Furthermore, there is currently a lack of a standardized technique for exosome isolation and characterization, and this may hamper potential downstream applications, such as modification of the exosomal membrane, and the loading of drugs and therapeutics.[64] In addition, the drug loading efficiency and the capability to produce sufficient extracellular vesicles that are required for clinical applications, remain insufficient.[65] Hence, due to the challenges in engineering exosomes for targeted delivery, there has been a shift in the approach, and exosomes in combination with synthetic NPs, have come to the forefront.

3. Nanoparticle-Based Drug Delivery Systems

The main aim of nanoparticle-based delivery is to achieve effective and safe delivery of a therapeutic moiety to its specific target site, organs, and cells. However, the transport of active macromolecules remains a major challenge for achieving effective drug delivery systems.[66] Nevertheless, traditional drug delivery methods have several limitations, such as low site-specific bioavailability, unfavorable biodistribution, low accumulation at the target site, and off-target effects.[67] Hence, there is an urgent need for a smart, and effective novel drug delivery system for localized and targeted drug administration, particularly for biologics (e.g., peptides, proteins, siRNA, mRNA).[68–70] The progress made in the design of delivery vectors includes the incorporation of various markers, such as cell surface cytokines (e.g., integrins[71]), growth factors,[72] and chemical modifications (e.g., folic acid[73]), which are specifically expressed in certain disease conditions. It helps the functionalized delivery carrier to recognize the target site. These nanocarriers, depending on the nature of chemicals, can be classified into organic and inorganic nanocarriers.[74] Organic nanocarriers are usually comprised of polymer-based systems, e.g., carbon nanotubes, dendrimers, liposomes and micelles, and other polymeric NPs. In comparison, inorganic nanocarriers are metal-based systems, such as metallic NPs and silica NPs.

Despite the immense progress in nanocarrier synthesis, the delivery of many biologics is hindered due to premature release, denaturation of carrier, and poor stability of the final formulation. This is mainly due to liver uptake and low drug deposition at the target site.[26] Thus, methods to circumvent these limitations are of the utmost necessity for current ongoing scientific research. To date, many different methods have been developed for enhancing the drug delivery capability of nanomaterials, especially with respect to their target specificity. Therefore, in the following sections, we review the development and applications of various smart/advanced drug delivery systems with a specific focus on the methods to enhance their drug delivery performance.

3.1. Cellular Hitchhiking-Based Nanodrug Delivery Systems

Cancer is one of the major concerns globally and ongoing efforts have been made by researchers towards the utilization of NPs in cancer therapy.[75] Experts have extended the technology

for targeted drug delivery, through the creation of innovative therapeutics, such as cell membrane-coated NPs drug carriers. The advantages of these carriers include: i) Mimicking of cell adhesion properties, ii) Targeted drug delivery towards cancer cells, iii) Enhanced cellular penetration, and iv) Evasion of immune system.[76]

Cellular hitchhiking is one of the emerging concepts in the field of drug delivery and cell biology. This technique utilizes natural mechanisms of circulatory cells to direct vasculature fluid, circumvent immune system clearance, and improve targeting ability. This technique enables the loading of NPs on the back of blood cells. Various biological entities, including proteins, lipids and polysaccharides are associated with blood cells, which offer a range of surface functional modalities. These biomolecules can assist the irreversible conjugation between blood cells and the drug NPs.[77] On the other hand, NPs offer various advantages compared to standard free drugs. These include drug encapsulation, prevention of drug degradation, control of the release profile, targeting ability, and large-scale production. Thus, the combination of synthetic NPs vectors with the circulatory cells may pave the way towards superior drug delivery systems. Moreover, circulatory cells offer the choice (red blood cells (RBCs), lymphocytes and macrophages) to select specific types of blood cells, depending on the desired biological action.

RBCs are abundant and mobile circulatory cells, which interact with various intravenously administered drugs and assist in altering the pharmacokinetic properties of naked drugs. They deliver oxygen to all organs and circulate for up to 3 months. Specific cell surface markers on RBCs (e.g., CD47, CD71, transferrin, and integrins) act as a travel permit to pass the immune system for achieving site-specific delivery. In one study, RBCs-coated recombinant interleukin-2 was found to retain its biological activity, prevent high-dose cytotoxicity, achieve rapid clearance, and possess tumoricidal properties at a very low concentration of about 10,000 i.u.[78] In another study, it was demonstrated that the noncovalent adsorption of RBCs on spherical polystyrene NPs (200 nm or 500 nm diameter) was increased by ≈threefold in the circulatory system, permitted ≈sevenfold transient accumulation in the lung, with a reduction in liver and spleen uptake. Further, RBCs-coated anti-ICAM-1 antibody attached NPs increased lung targeting and its accumulation.[79] However, the delivery of cargo using RBC-coated nanocarriers has some drawbacks. Therapeutic targets, such as extravascular tissue constituents, solid tumors, and the central nervous system are beyond reach for the RBCs. Moreover, the pharmacokinetics of drug release of RBC-loaded drugs, the stability of these nanocarriers and their effects on different communities of the cells within the body are yet to be characterized and fully understood.[80]

Macrophages are known for their unique properties, including their ability to migrate to the site of inflammation, and cross multiple biological barriers. Thus, they can be the ideal drug vector, especially for tumors. Zhang et al. developed biomimetic macrophage-membrane-coated paclitaxel (PTX)-loaded NPs (cskc-PPiP/PTX@Ma) for breast cancer targeted chemotherapy.[81] They decorated the internal areas of the NPs with amphiphilic polymers and performed dual-end PEGylation on selected side chains for PTX loading. In addition, nanoparticle polymers were functionalized with cationic 2-aminoethyldiisopropyl group (PPiP). This modification helped to exert a buffering effect on the extracellular pH of the tumor region. Next, the nanoparticle surface was modified using synthetic d-form oligopeptide (cskc) as a targeting ligand. The designed cskc-PPiP/PTX@Ma showed enhanced biocompatibility and favorable tumor-homing ability in the systemic circulation. Furthermore, lymphocytes (T-cells and B-cells) can also act as a great carrier to target various disease conditions. Likewise, other types of cells, including dendritic cells, and platelets can help to improve the delivery of targeted drug NPs.[82,83] Despite the promising results from these cellular hitchhiking-based delivery systems, numerous factors limit their translation from laboratories to human use. For instance, the logistics associated with the ex-vivo loading of cargo into cells is costly. Moreover, the cells used in these systems possess a circulatory function, making the task of choosing a specific function to use particularly arduous.[73]

3.2. Neutrophils-Coated Nanodrug Delivery Systems

Circulating tumor cells (CTCs) are a sub-population of cells found in the circulatory system of cancer patients having solid tumors and they act as seeds for metastases. Tumor cells can metastasize through the bloodstream via single or multiple migratory CTC clusters. Since CTCs mimic tumor properties and are responsible for initial metastasis, they can be a valuable therapeutic target. Furthermore, inflammatory neutrophils are associated with CTCs and are actively involved in the progression and metastasis of multiple types of cancer. Thus, neutrophil-mimicking targeted drug delivery systems can be achieved by coating neutrophil membranes onto the surface of NPs. Kang et al. developed neutrophil-coated poly(latic-*co*-glycolic acid)(PLGA) NPs and observed 2.59 and 3.58-fold enhanced cellular association in 4T1 cell models in static and shear flow conditions, respectively.[84] The neutrophil-coated NPs further showed precise CTC-targeting in in vivo female Balb/c nude mice and male ICR mice models.

In addition, a second generation of proteasome inhibitor carfilzomib (CFZ)-loaded neutrophil-coated PLGA NPs were found to selectively deplete circulatory CTCs, metastasis, and inhibited the progression of already-formed lung metastasis. In a tumor-bearing mice xenograft model, Cao et al. showed that neutrophil-coated poly(ethylene glycol) methyl ether-block-poly(lactic-*co*-glycolic acid) (PEG-PLGA) NPs possessed selective accumulations at the tumor site compared to uncoated NPs.[85] Further, celastrol-loaded neutrophil-coated PEG-PLGA NPs exhibited significantly enhanced pancreatic tumor inhibition with minimal liver metastases. In another study, neutrophil-coated polycaprolactone-poly(ethylene glycol) (PCL-PEG) block copolymer NPs loaded with sparfloxacin (SPX) displayed precise targeting, prolonged circulation time, and controlled release. A in vivo experiments in female balb/c mice with infected lungs showed a significant reduction in inflammatory cytokines (tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8)) along with rapid recovery rate.[86] Thus, the overall neutrophil coating might have a greater influence in curing the inflammation arisen

from multiple cancers. Neutrophil-coated nanodelivery platforms suffer from some challenges, including low encapsulation capacity of PLGA NPs (especially for biologics), lack of repeatability within the batches of neutrophils due to their susceptibility to epigenetic mutations; and the lack of in-depth understanding of the specific interactions between neutrophil membranes and solid tumor cells within the tumor microenvironment, thereby limiting their efficient transition to clinical use.[87]

3.3. Cancer and Stem Cell Membrane-Coated Nanodrug Delivery Systems

Cancer cell membranes possess certain unique cell adhesion glycoproteins. Thus, by coating cancer cell membranes on various nanocarriers, the enhancement in tumor specificity can be achieved, compared to RBCs or neutrophils. In a study for an anticancer vaccine drug delivery system, cancer cell membrane-coated PLGA NPs displayed 40-fold more cellular uptake in MDA-MB- 435 cells compared to RBCs-coated PLGA NPs.[88] Li et al. developed a breast cancer cell membrane-coated biomimetic nanoplatform for tumor-targeted photodynamic therapy, and hypoxia-amplified bioreductive therapy.[88] They used the bioreductive drug, tirapazamine (TPZ) loaded in porphyrinic metal organic framework PCN-224 which was coated by homotypic 4T1 cell membrane. To define target specificity, they analyzed the cellular uptake in various cells lines, including 4T1 cells. They observed a significantly enhanced internalization in 4T1 cells. Moreover, the in-vivo intravenous administration of 4T1 cell membrane-coated TPZ-loaded PCN-224 NPs into 4T1 tumor-bearing mice showed significant accumulation and therapeutic efficacy on the tumor tissue.

Tian et al. used bone marrow-derived mesenchymal stem cell membrane vesicles (SCV) to target breast cancer cells. SCV were coated on PTX-loaded PLGA NPs (SCV/PLGA/PTX).[89] The coated NPs showed excellent serum stability with a controlled release of PTX. Furthermore, these coated NPs exhibited higher toxicity in 4T1 cells, compared to PTX alone, and PLGA/PTX. Despite this, the IC_{50} of the coated NPs was very low (0.48 μg mL^{-1}) compared to PLGA/PTX (1.29 μg mL^{-1}) and PTX (1.62 μg mL^{-1}), indicating the improved tumor targeting efficacy of the coated NPs. The in vivo biodistribution of the coated NPs in orthotopic 4T1 tumor-bearing female BALB/c mice showed controlled release until 48 hours, with a much higher tumor inhibition (78.4 ± 10.6%) rate compared to free PTX (32.0 ± 6.7%) and PLGA/PTX (53.6 ± 8.3%). Similarly, Gao et al. developed bone marrow-derived mesenchymal stem cell membrane-coated gelatin nanogels (SCMGs) with efficient tumor-targeted drug delivery.[90] Further, they encapsulated doxorubicin (DOX) on nanogel in the formulation and found that the stem cell membrane-coated nanogel exhibited enhanced tumor-targeting and accumulation in tumor sites. Notwithstanding the promising outcomes, cancer and stem cell membrane-coated NPs suffer from some drawbacks, such as a lack of efficient coating methods with high yield. Extrusion is the most commonly used method for the coating, however, cancer and stem cell membranes are much thicker, limiting their efficient curvature around the NPs, leading to fabrication difficulties, such as pore blocking, or partially coated nanovesicles.[91] Therefore, there is a compelling need to develop and optimize the coating method respective to NPs and EVs type.

4. Extracellular Vesicles-Amalgamated Nanostructures for Drug Delivery

EVs have gained considerable attention as natural carriers for drug delivery systems as well as for the treatment of cancer and many other debilitating conditions.[92] EVs comprising exosomes, microvesicles, and apoptotic bodies, are used in physiological conditions as short- and long-distance mediators of intercellular communications.[93] EVs consist of cellular membranes with multiple adhesive proteins on their surface, allowing efficient cell entry and transport of therapeutic cargo. EV membranes with relatively tight bilayers can provide a controlled and prolonged release of the integrated substance, including drugs. EVs exhibit lower immunogenicity (especially autologous EVs) and low cytotoxicity, thus potentially nontoxic for therapeutic use.[94] In addition, EVs can enhance the basic characteristics of a free drug, such as its stability and solubility, and preserve the drug from degradation in the bloodstream. Among EVs, exosomes are nanosized membrane vesicles that are commonly found in eukaryotic fluids, such as urine, blood, and the media of cultured cells.[95] Exosomes combine the properties of synthetic NPs (e.g., increased permeability and enhanced retention (EPR) effect and passive targeting) as well as their own unique properties, including targeting specificity, low cytotoxicity, and different biological effects, depending on the cellular origin.[96,97] The specific origins of exosomes allow them to be involved in intercellular communication by transferring cargo between cells. In addition, they can be combined with specific drugs and NPs to further enhance their targeting abilities/specificities. The above properties have enabled exosomes to be effectively utilized as delivery nanovesicles to deliver RNA, small interfering RNA and microRNA or chemotherapeutics to enhance the cancer treatment process. However, there are many challenges associated with EVs, including isolation, heterogeneity among EVs, and characterization. These challenges need to be overcome before they can be produced at a mass scale and are clinically ready. Additionally, further research into improving the stability of EVs during modifications, and drug loading is necessary.

Synthetic nanostructures, on the other hand, have been extensively used for clinical drug delivery in the last two decades to improve the spatial and temporal distribution of therapeutic agents in the body.[26] NPs have the potential to increase the stability and solubility of encapsulated cargo, facilitate transport across membranes and prolong circulation times. This results in reduced side effects and/or increased therapeutic efficacy and safety. Engineered and spatially designed NPs can overcome the limitations of traditional delivery, such as biodistribution to smaller barriers including intracellular trafficking, through cell-specific targeting, molecular transport to specific organelles, and other approaches.[31] Specific nanoarchitectures, bioresponsive packages and targeting agents improve protein or drug binding, and effective delivery. However, complex molecular targeting methods have failed in clinical trials

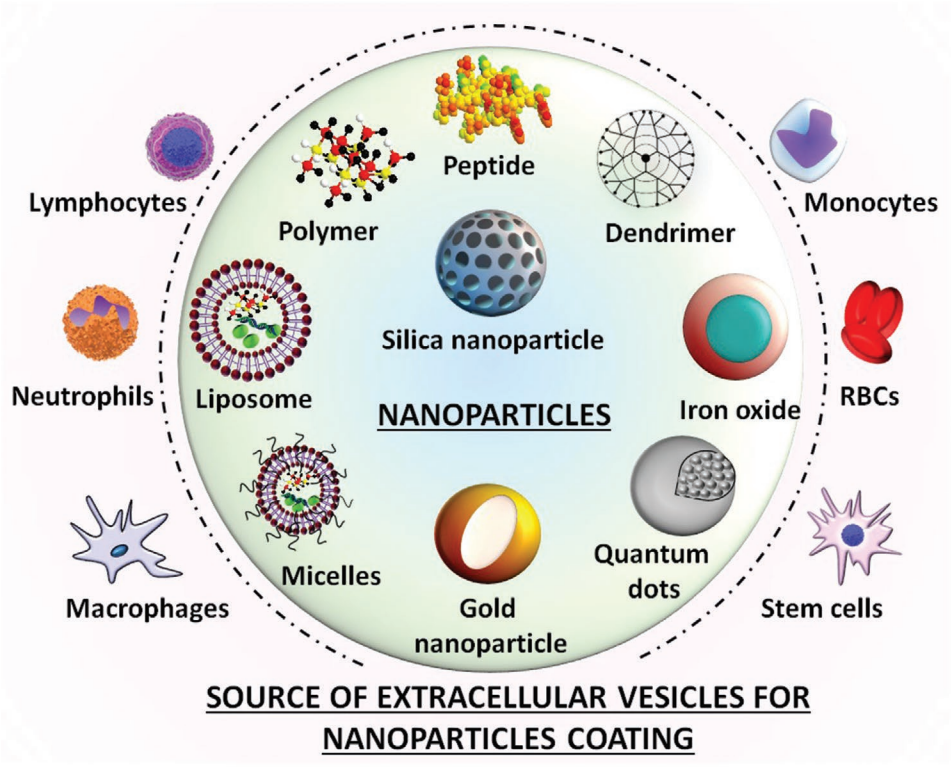

Figure 2. EVs coated engineered nanostructures—A Perfect Marriage. This schematic shows type of nanomaterials which can be coated with EVs and form multifunctional hybrid system to harness the full potential of both EVs and engineered nanostructures.

as complex interactions between NPs and biological groups can mask surface ligands and cause immunological recognition. Likewise, the lack of similar (biological moiety) complexity, nonspecific molecular target, inability to perform intracellular delivery, and poor bioavailability of synthetic NPs have limited their clinical effectiveness.[98] Therefore, it would be premature to conclude who is superior—EVs or synthetic NPs as a better carrier? Herein, we propose a marriage between EVs and NPs as a promising multifunctional carrier for personalized medicine (**Figure 2**).

EVs-coated NPs can bypass the host immune response, possess excellent tumor targeting ability, have better loading into synthetic NPs. Consequently, they serve as a great alternative to all purely synthetic drug delivery vectors. The vehicle can be inorganic or organic in nature with different sizes and shapes, depending on the drug conjugation or encapsulation ability. However, the composition of EVs may vary depending on the source of the donor cells. These EVs possess various protein and lipid markers, which can greatly influence drug targeting. Numerous EVs (especially exosomes) have been implicated as drug/gene carriers, and clinical trials are underway to establish their therapeutic efficacy (**Table 1**).

4.1. EVs—Metal-Organic Frameworks (MOFs)

In recent years, metal-organic frameworks (MOFs) have attracted significant interest as carriers for drugs or therapeutic agents. MOFs are crystalline porous materials formed by the coordination between metal ions and organic linkers.[99–102] They possess controllable structure, composition, and pore size.[103,104] This allows them to be specifically designed to meet the requirements of the targeted application. More importantly, the internal and external surfaces of MOFs can be modified with various functional groups to impart additional properties or to promote specific interactions with biological structures in the body. In drug delivery applications, the large surface area and high porosity of MOFs can lead to high drug loading capacity. The excellent structural tunability of MOF combined with an exosome shell can potentially provide a novel drug delivery platform.

Illes et al. have successfully demonstrated the synergistic combination of MOFs and exosomes for drug delivery by exploiting the advantageous properties of each compound.[105] The iron oxide-based MOF, MIL-88A NPs were first synthesized through a microwave-assisted treatment of an aqueous mixture of ferric chloride hexahydrate (as the iron precursor) and fumaric acid (as the organic linker) at 80 °C. The MIL-88A NPs were successfully coated with exosomes derived from HeLa cell culture via a lipid fusion, thus allowing for the drug loading of exosomes with high efficiency. The lipid fusion process was advantageous compared to the conventional solvent exchange method as it enabled the bilayer of the exosomes to be retained during the coating process without significantly altering the original properties of the exosomes. Using membrane-penetrable calcein as the model cargo, the exosome-coated MIL-88A NPs exhibited effective release of calcein on HeLa cells without showing any sign of early leakage. The therapeutic potential

Table 1. Current clinical trials consisting of EVs for cancer treatment.

Sr. no.	Target disease	Source of EVs	Cargo	Phase	Clinical trials for cancer
1.	Colon cancer	Plant nanovesicles	Curcumin	Phase I	NCT01294072
2.	Nonsmall cell lung cancer	Tumor antigen-loaded dendritic cell-derived exosomes	Peptide	Phase II	NCT01159288
3.	Type I Diabetes	Cell free umbilical cord-blood derived mesenchymal stem cells exosomes	–	Phase II	NCT02138331
4.	Pancreatic cancer	Mesenchymal stromal cells-derived exosomes	KRAS G12D siRNA	Phase I	NCT03608631
5.	Bronchopulmonary dysplasia	Bone marrow mesenchymal stem cell-derived extracellular vesicles (UNEX-42)	–	Phase I	NCT03857841
6.	Oral mucositis associated with chemoradiation treatment of head and neck cancer	Plant (grape) exosomes	Chemotherapeutic drug	Phase I	NCT01668849
7.	Acute ischemic stroke	Mesenchymal stem cell (MSCs)	miR-124	Phase I/II	NCT03384433
8.	Novel coronavirus pneumonia (NCP)	Allogenic adipose mesenchymal stem cells	Aerosol	Phase I	NCT04276987
9.	Novel coronavirus (NCV) pneumonia	COVID-19 specific T-cells (CSTC)	–	Phase I	NCT04389385
10.	COVID-19 SARS-CoV-2 pneumonia	Mesenchymal stem cells exosomes	microRNA	Phase II	NCT04602442

was evaluated by loading these exosome-coated MOFs with the chemotherapeutic drug, SBHA (Suberoyl bis-hydroxamic acid), a histone inhibitor and anticancer drug. Significant HeLa cell death was observed even at very low concentrations. The drug release was promoted by decomposition of the MOF nanocarrier as well as possible endogenous exosomal release mechanisms.

Cheng and coworkers have synthesized EVM-coated protein-loaded MOF NPs with promising potential for tumor targeting and suppression by encapsulating the guest proteins in the matrix of MOFs.[106] This strategy led to superior efficiency (up to ≈94%) and higher loading (by 50-fold) than the surface conjunction method. The protein-encapsulated MOF NPs were further decorated with the EV membrane with an efficiency as high as ≈97%. An in vitro and in vivo experiments revealed that the EV-like NPs could not only protect proteins against protease digestion and evade the immune system clearance but also enhanced the selectivity toward target homotypic tumor sites and promoted tumor cell uptake and autonomous release of the guest protein after internalization. Assisted by biomimetic NPs, intracellular delivery of the bioactive therapeutic protein gelonin significantly inhibited tumor growth in vivo and increased the therapeutic efficacy by 14-fold. This work provides a new concept by looking at the construction of a biomimetic nanoplatform, while also providing a new solution for systemic and intracellular delivery of proteins.

Nanosized MOFs (NMOFs) have been investigated for the encapsulation of DNA or RNA to assess their potential for gene delivery. In early studies, the nucleic acid complexation was often performed on the outer surface of NMOFs. For instance, hexagonal plate-like Zr-based MOF (UiO-66) NPs have been used to enhance the therapeutic efficacy in drug-resistant ovarian cancer cells.[107] The UiO-66 NPs were initially loaded with a cisplatin prodrug through encapsulation and then, multi drug resistance (MDR) gene-silencing siRNAs (Bcl-2, P-glycoprotein [P-gp], and survivin) were incorporated into the NMOFs by means of surface coordination. Here, UiO-66 NPs served multiple roles, including protecting siRNAs from nuclease degradation, boosting the cellular uptake of siRNA, and initiating the escape of siRNA from endosomes to silence MDR genes in cisplatin-resistant ovarian cancer cells. The delivery of cisplatin and siRNAs with NMOFs yielded a significant boost in the in vitro chemotherapeutic efficacy. The siRNA/UiO-Cis could induce significant gene silencing in SKOV-3 cells at 30 nM of siRNA, as determined by ELISA. Moreover, the cytotoxicity assessment by MTS assay revealed that the utilization of siRNA/UiO-Cis led to 11 times higher cytotoxicity relative to the SKOV-3 cells. The high cytotoxicity of siRNA/UiO-Cis to SKOV-3 cells is further supported by the DNA fragmentation observed in SKOV-3 cells treated with siRNA/UiO-Cis compared to those treated with UiO-Cis, and free cisplatin groups, which showed no DNA fragmentation. The siRNA loaded in the NMOFs (red fluorescence) was effectively internalized into the cytoplasm after a 24 h period of incubation to induce MDR-relevant gene silencing. Annexin V conjugate (green fluorescence) was visible in cells treated with siRNA/UiO-Cis but not in cells treated with siRNA/UiO (pooled siRNAs alone) or UiO-Cis (cisplatin alone). These observations indicated that codelivery of cisplatin and pooled siRNAs would induce cell apoptosis in cisplatin-resistant cells by combining the synergistic effects of down-regulating the expressions of MDR-relevant genes and chemotherapeutics.

The iron-based MOF, MIL-101 (Fe) NPs modified with metal NPs (selenium/ruthenium) have been utilized to deliver pooled small interfering RNAs (siRNAs) to enhance cancer therapeutic efficacy by suppressing MDR genes and disrupting the microtubule (MT) dynamics in Taxol-resistance breast cancer (MCF-7/T) cells.[108] Here, selenium NPs were selected specifically due to their good antitumor activity and low toxicity,[109] while ruthenium NPs were employed because of their superior antimetastatic activity in cancer treatment. The presence of coordinatively unsaturated metal sites in MIL-101(Fe) enabled it to strongly interact with the electron-rich functional groups of cysteine, thereby enabling the decoration of the MIL101(Fe) NPs with selenium and ruthenium NPs (termed as Se@MIL-101 and Ru@MIL-10 NPs). The loading of the Se@MIL-101

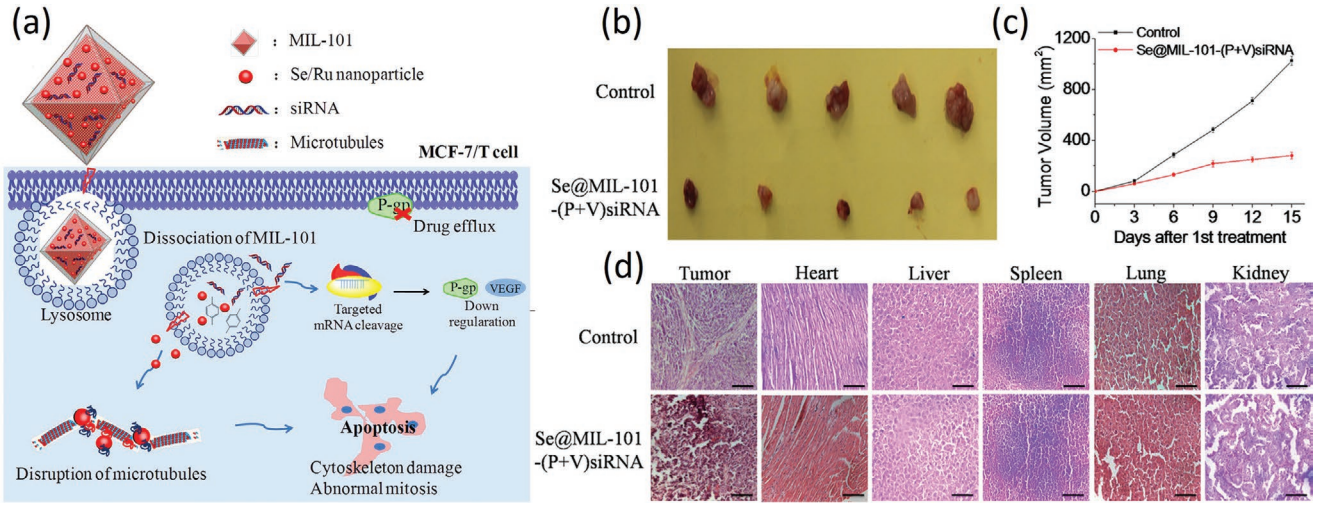

Figure 3. a) Mechanism of the reversal of drug resistance and induced apoptosis by the disruption of microtubule in MCF-7/T (Taxol-resistance) cancer cells. b) Photographs of xenograft tumor from the mice of Se@MIL-101-(P+V)siRNA-treated groups and control groups after 15 days of administration. c) Measurement of tumor volumes at time interval of 3 d. d) Ex vivo analysis of the histological characteristics of tumor in heart, liver, spleen, lung, and kidney tissue by H&E stain. Scale bar is 50 µm. Reproduced with permission.[108] Copyright 2017, American Chemical Society.

and Ru@MIL-101 NPs with MDR gene-silencing siRNAs was achieved through surface coordination. Such loading could effectively protect siRNAs against nuclease degradation, increase the cellular uptake of siRNA, and promote the escape of siRNA from endosomes or lysosome to suppress MDR genes in MCF-7/T cells. These factors led to increased cytotoxicity by inducing apoptosis with the signaling pathways of phosphorylation of p53, MAPK, and PI3K/Akt and the dynamic instability of MTs and limiting the formation of normal mitotic spindle. Moreover, the in vivo study of the Se@MIL-101-(P+V) siRNA NPs on nude mice bearing MCF-7/T cancer xenografts revealed that they could effectively suppress the tumor size and volume (**Figure 3**b,c) while also lowering the systemic toxicity in vivo. Unlike the control group, the H&E stain images of tumor tissues collected from the mice of Se@MIL-101-(P+V) siRNA-treated groups clearly showed significant shrinkage, nuclei fragmentation, and chromosome condensation, thus confirming the high level of induced apoptosis (Figure 3d).

4.2. EVs—Gold Nanostructures

Gold nanostructures, including NPs (Au NPs) are of growing interest in therapeutics owing to their distinctive properties, size tunability, and biocompatibility.[110] Au NPs present themselves as superlative drug carriers with inert moiety and minimal toxicity. Therefore, they can easily pass through the body, thus avoiding any adverse effects or reactions. The surface of Au nanostructures can easily be functionalized through the formation of strong gold-sulfur (Au–S) bonds, which enable the attachment of different targeting molecules, including nucleic acids, proteins, antibodies, peptides, carbohydrates, aptamers, and small molecules for delivering them to selective tissues/disease organs of therapeutic interest.[17,111] Intriguingly, these Au–S bonds are nonlabile, providing high stability to Au NPs-biomolecules to physiologically relevant pH and salt concentrations during different modes of delivery.[112] Moreover, it is relatively easy to achieve Au nanostructures with biologically homologous sizes and shapes. Generally, they can be obtained by control reduction of Au^{3+} to Au, which initiates the nucleation of Au. The selection of the template media, reducing agents and reaction conditions (pH, time, etc.), the size, shape and tunability of Au can be achieved for the desired drug as well as therapeutics.[113,114]

Au nanostructures, including Au NPs, Au nanoshells, Au nanorods (AuNR), and Au nanocages possess surface plasmon resonance (SPR) while absorbing light in the NIR region (700-1000 nm).[115] Therefore, they can efficiently transduce light (optical energy) into heat and destroy malignant cells by hyperthermia, or initiate temperature-driven processes comprising drug delivery and gene expression without harming the healthy tissues.[116,117] Recently, by exploiting their narrow pore size distribution, AuNR have been combined with exosomes for tumor-targeted chemo-photothermal therapy.[118] As shown in **Figure 4**, exosomes were generated from donor cells, cultured in a medium containing arginyl–glycyl–aspartic acid (RGD)-functionalized DSPE-PEG (DSPE-PEG-RGD) and sulfhydryl-functionalized DSPE-PEG (DSPE-PEG-SH), to modify the membrane of the donor cells with RGD and sulfhydryl groups. AuNRs were then attached to exosomes through the formation of Au-S bonds (AuNR@RGD-Exos). The localized laser-induced hyperthermia provided by the AuNRs under near-infrared irradiation enhanced exosome membrane instability, thereby encapsulating the drugs. Additionally, because tumor cells have a lower heat tolerance than normal cells, localized hyperthermia would selectively eliminate the tumor cells without affecting the surrounding normal tissues. In this study, they also improve the tumor cellular uptake efficiency by functionalizing the surface of AuNR@RGD-Exos with folic acid (FA). The synergistic effect of RGD and FA enabled higher loading of exosomes at the tumor sites to provide better therapeutic outcomes.

In another recent report, Au NPs based-passion fruit-like exosome-PMA/Au-BSA@Ce6 nanovehicles were reported for

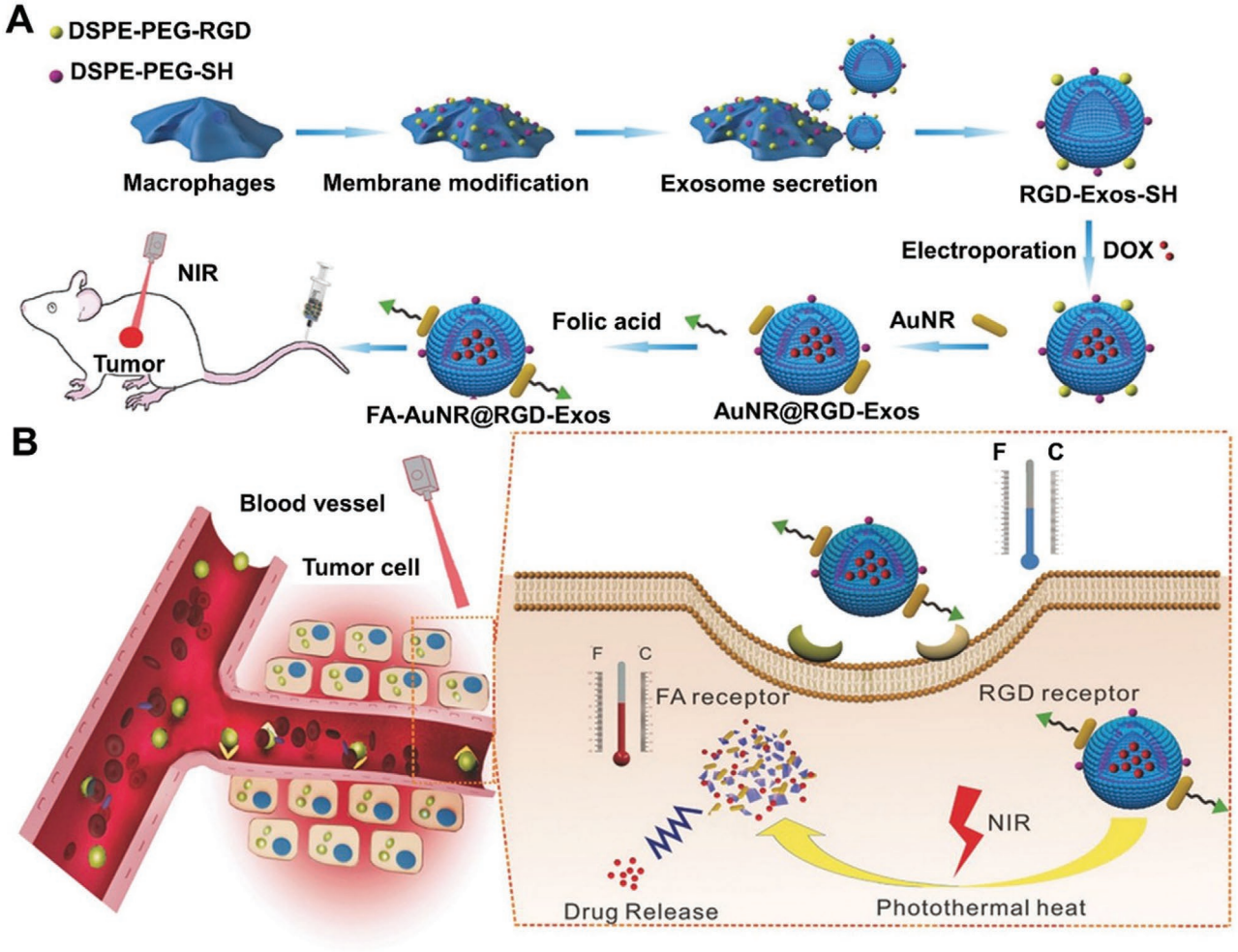

Figure 4. Schematic representation of A) the design of FA-AuNR@RGD-DOX-Exos and their antitumor effect under NIR irradiation in a mouse model and B) development based-targeted delivery and synergistic tumor therapy. Reproduced with permission.[118] Copyright 2018, Wiley-VCH.

real-time fluorescence imaging and enhanced targeted photodynamic therapy.[119] The nanovesicles were obtained by combining urinary exosomes with multi-functionalized PMA/Au-BSA@Ce6 vesicles, through an instant electroporation strategy. A mouse model showed that as-prepared Exo-PMA/Au-BSA@Ce6 nanovehicles could target tumor cells with deep penetration and superior retention performance in tumors. Laser irradiation at 633 nm in acidic conditions led to the breakdown of the structure of nanovehicles, thereby releasing the PMA/Au-BSA@Ce6 inside cancer cells, which produced a substantial amount of singlet oxygen for inhibiting tumor cell growth.

The loading strategy of Au nanostructures onto the desired exosome is a crucial factor to achieve efficient nanocarriers and therapeutics. With the advancement of bioconjugation strategies, several methods have been reported for loading, including incubation, pre- and post-loading, sonication, extrusion, specific antibody modification, etc.[120–122] Among all the methods, extrusion and sonication are most widely used for enveloping exosome membranes on NPs. For instance, exosomes with the RVG and GNSTM peptides were collected and then coated onto the surfaces of Au NPs using the mechanical method or extrusion for enhanced blood-brain barrier (BBB) penetration.[123]

Nevertheless, the mechanical stress and physical interactions involved in these methods (especially sonication and extrusion) could damage the integrity of proteins and exosome membranes.[63,124] Besides, these methods are time-consuming and involve labor-intensive preparation processes. For instance, Sancho-Albero and colleagues reported the incorporation of hollow Au NPs (HGNs) into exosomes secreted by human placental mesenchymal stem cells (MSCs) using the MSC exosome biogenesis pathway.[125] In this strategy, the PEGylated HGNs were incubated with the melanoma B16-F10 cells to achieve exosome membrane-camouflaged HGNs and successfully achieved 50% encapsulation. The PEG coating was highly beneficial, providing high stability and avoiding agglomeration of HGNs. Moreover, the same research group also compared this strategy with other conventional methods, including sonication, electroporation, saponin-facilitated loading, passive loading by diffusion, and thermal shock. These methods generated less than satisfying results, while the internalization of comparatively large NPs into B16-F10-exos was accomplished by nearly all the physicochemical methods studied. However, only about 15% of the exosomes were loaded with NPs and some of these processes had a negative effect on the morphology

and integrity of the loaded exosomes. On the other hand, the integration of PEG-HGNs through the exosomal biogenesis pathway accomplished 50% of NPs internalization without impacting the intrinsic characteristics of exosomes.

4.3. EVs—Iron Oxide Nanostructures

Superparamagnetic iron oxide NPs (SPIONs) have been frequently employed in drug delivery due to their magnetic capability. Using external magnets, SPIONs can be directed to the desired area within the body. However, it is important to stabilize them during the design stage to prevent their agglomeration in biological media. Recent studies have shown a role for SPIONs in cardiovascular diseases, which are currently the leading causes of death.[126] It has been shown that mesenchymal stem cells (MSCs) can be implanted to decrease the infarct size, minimize excessive tissue loss, and improve cardiac function.[127] Unfortunately, there are many limitations associated with implanted MSCs, including low survival rate, limited therapeutic efficacy, and the potential of causing arrhythmias or tumorigenesis. MSC-derived exosomes have shown enhanced therapeutic potential for cardiovascular treatment as they exhibit similar cardiac repair efficacy as that of implanted MSCs and they do not suffer from low cell survival issue, arrhythmia, and risk of tumorigenesis, and can be stored at very low temperatures (e.g., –80 °C) for a prolonged period.

Lee et al. demonstrated the superiority of exosome-mimetic nanovesicles (NVs) derived from iron oxide NPs (IONPs)-incorporated MSCs (IONP-MSCs) compared to normal MSC-derived NVs (N-NVs) for cardiac repair.[128] The enhanced performance was due to the capabilities of IONP-MSCs to enhance the retention of NVs in the infarcted heart, through magnetic guidance, and increase the amount of therapeutic molecules in IONP-NVs via intracellular signaling modifications promoted by the ionization of IONPs in IONP-MSCs. Therefore, the injection of IONP-NVs led to reduction in apoptosis, inflammation, and infarct size, as well as enhanced angiogenesis and recovery of cardiovascular function. These results render IONP-NVs highly attractive for cell-free therapy for myocardial infarction. The Wilhelm's group successfully combined macrophage-derived microvesicles with magnetic NPs for drug loading and targeting of two different types of cancer cells, namely the SKOV-3 human ovarian and PC-3 human prostate cancer cells.[129] These microvesicles could be loaded with various therapeutic agents of different characteristics, including the amphoteric doxorubicin (DOX), amphoteric tissue-plasminogen activator (t-PA), and two photosensitizers (disulfonated tetraphenyl chlorin-TPCS2a, and hydrophobic 5,10,15,20-tetra(m-hydroxyphenyl)chlorin-mTHPC).

In another study, exosomes isolated from A33 positive LIM1215 cells were successfully combined with carboxyl-functionalized superparamagnetic magnetite (Fe_3O_4) NPs (the hybrid termed as A33Ab-US) through a coupling reaction.[72,131] The A33b-US was subsequently loaded with DOX to form the composite A33-Exo-DOX. The in vitro results revealed that A33Ab-US-Exo/DOX could effectively target A33-positive LIM1215 cells. In addition, they exhibited a good antiproliferative effect in LIM1215 cells, as evidenced by the enhanced uptake of the composite. Further, the in vivo study clearly indicated the good tumor targeting ability of A33Ab-US-Exo/Dox as well as its capability to effectively inhibit tumor growth and prolong the life of the mice with decreased cardiotoxicity. This study highlights the potential of targeting ligand-functionalized exosomes as an emerging delivery system for drug delivery towards cancer treatment.

Tumor necrosis factor-alpha (TNF-α) is a cytokine or small protein used by the immune system for cell signaling. It has been used previously in cancer treatment due to its ability to limit or inhibit tumor angiogenesis.[132] However, the use of TNF-α in a clinical setting remains limited due to its high toxicity.[133] When injected directly, TNF-α suffers from poor target specificity which consequently leads to poor distribution and therapeutic effects along with severe side effects. To overcome these limitations, Zhuang and coworkers proposed the coupling of cell-penetrating peptides (CPP) and TNF-α (CTNF-α)-anchored exosomes with SPIONs (CTNF-α-exosome-SPIONs) with cancer-targeting capability.[130] To generate this hybrid, size-controlled SPIONs were initially prepared by carboxylated chitosan (CS). Next, the CS-decorated SPION surface was conjugated to transferrin (Tf) in the presence of carbodiimide (EDC) and N-hydroxysulfosuccinimide sodium salt (NHS). The conjugation process occurred via interaction between the COO^- groups in carboxylated CS on the surface of SPIONs with the NH^{3+} of Tf. As the Tf-modified SPIONs (Tf-SPIONs) contain rich transferrin receptors (TfR), they together with CTNF-α-exosomes could self-assemble to generate CTNF-α-exosome-SPIONs in aqueous solution owing to the high affinity of Tf and TfR. The CNTF-α-exosome-SPIONs could be easily collected by application of an external magnetic field (MF) (**Figure 5**a) due to the presence of SPIONs. The SEM analysis confirmed the uniform coverage of the exosomes by SPIONs, as depicted in Figure 5b,c.

The in vitro cytotoxicity evaluations of the CNTF-α-exosome-SPIONs were carried out using MTT assay against A375, MCF-7, A549, and Colo201 cancer cells. The results revealed that CNTF-α-exosome-SPION/MF (with magnetic field) induced a higher cytotoxic effect on these tumor cells compared to that without MF as indicated by the lower IC_{50} value. Furthermore, the combination of CTNF-α-exosome-SPIONs and MF also led to the largest apoptotic cell death with an approximately 15% increase in G1 population value from 56.4% for TNF-α to 71.5% for CTNF-α-exosome-SPIONs/MF. The assessment of the in vivo activity of the CTNF-α-exosome-SPION using a subcutaneously grafted murine melanoma model revealed that the utilization of CTNF-α-exosome-SPIONs/MF led to a greater decrease in both tumor volume and tumor weight (Figure 5d) compared to TNF-α and CTNF-α-exosome-SPIONs without MF. The immunohistochemistry investigation indicated that in the presence of MF, CTNF-α-exosome-SPIONs successfully induced tumor-targeted melanoma death by means of TNF-α-mediated apoptosis (Figure 5e).

Exosomes also have the ability to cross the BBB, which may remove the limitation of high drug resistance to glioma and the restriction of permeation by BBB during intravenous injection of drugs.[134] Jia et al. reported the loading of exosomes with SPIONs and curcumin (Cur) for combined imaging and

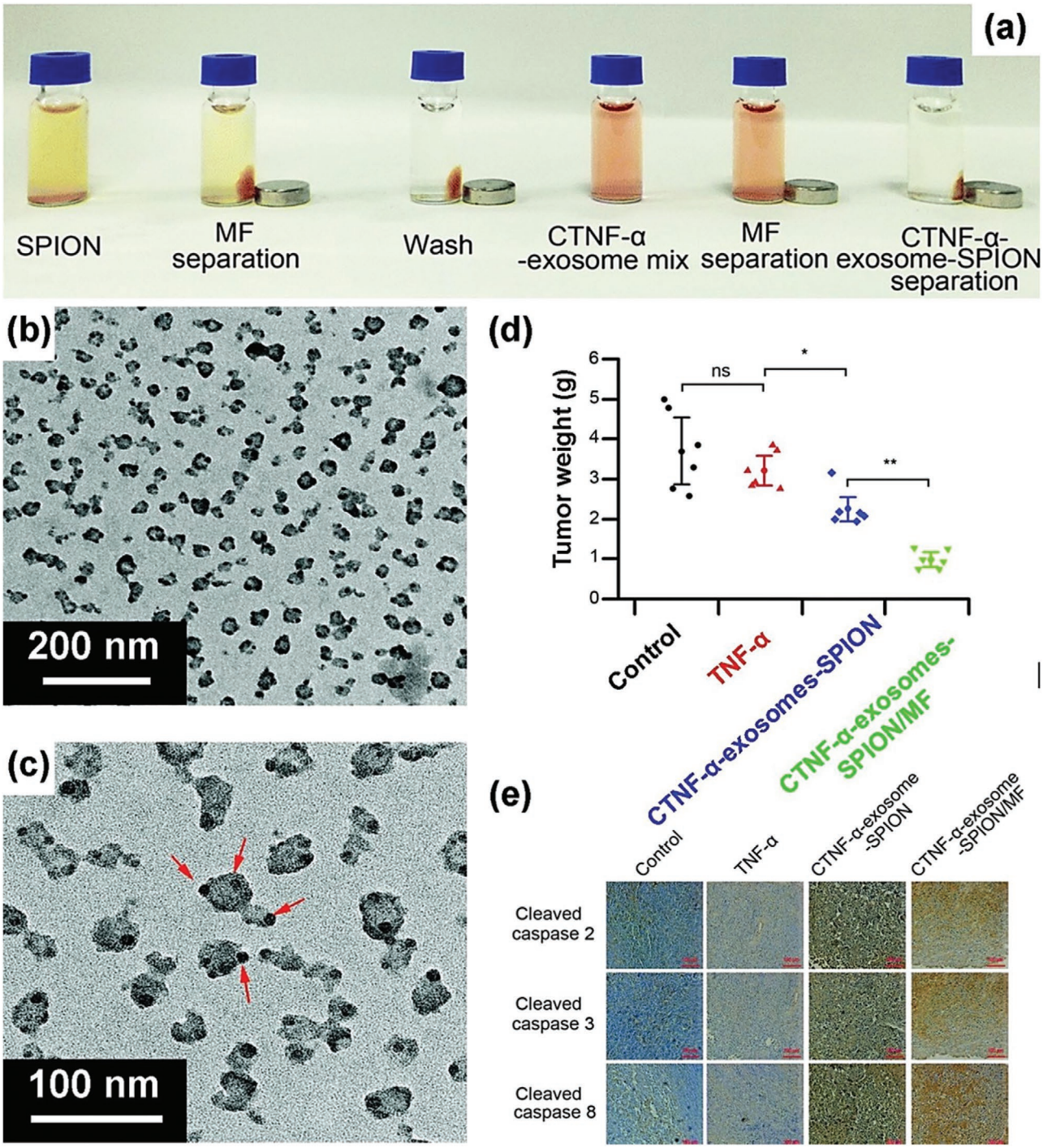

Figure 5. a) Images of the CTNF-α-exosome-SPION construction process. b,c) TEM images of the CTNF-α-exosome-SPION under different magnifications. In vivo antitumor activity and toxicity of the CTNF-α-exosome-SPION. d) Average mass of excised tumors and e) immunohistochemical staining of cleaved caspases 2, 3, and 8 in tumor sections from different groups. Reproduced with permission.[130] Copyright 2020, The Royal Society of Chemistry.

glioma treatment in vitro and in vivo.[135] Herein, the SPIONs and Cur were loaded into exosomes using the "in vitro" procedure followed by the conjugation of the exosome membrane with the neuropilin-1-targeted peptide (RGERPPR, RGE) by using click chemistry. The engineered exosomes could cross the BBB easily, resulting in effective target delivery for glycoma therapy. This type of exosome architecture can serve effectively as imaging and hyperthermia agents as well as therapeutic agent, leading to improved diagnosis and treatment of intracranial tumors.

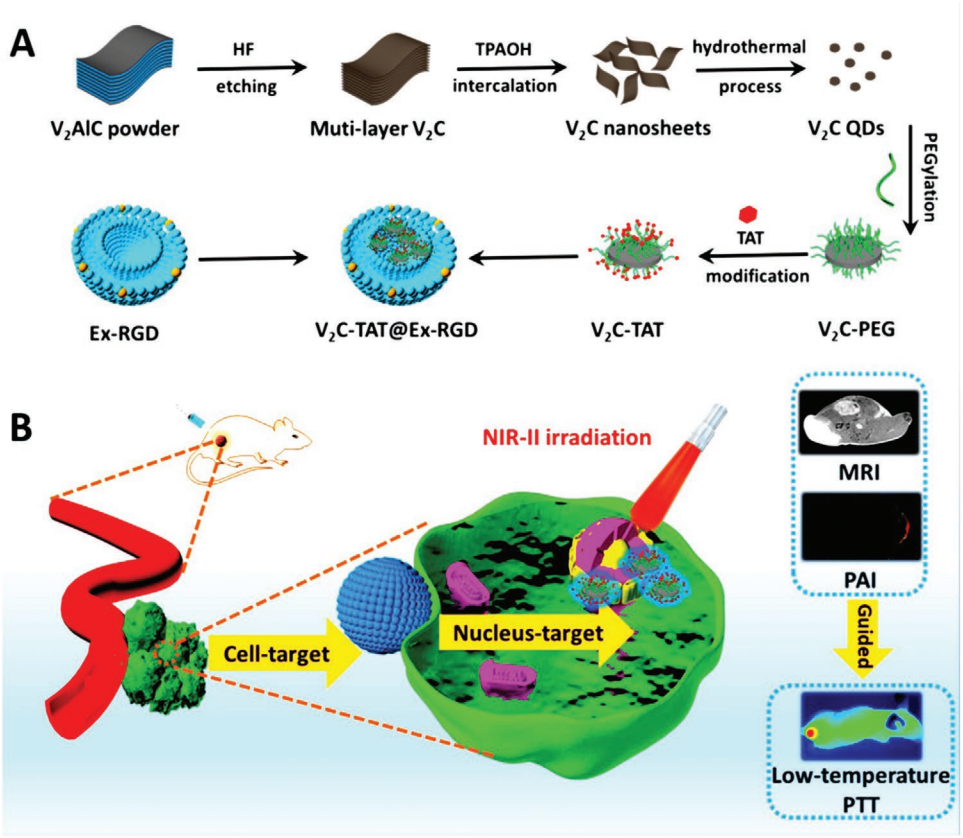

Figure 6. Schematic illustration for A) the preparation of V_2C-TAT@Ex-RGD and B) dual-target V_2C-TAT@Ex-RGD nanoagent for multimodal imaging-guided low-temperature PTT in the NIR-II biowindow. Reproduced with permission.[137] Copyright 2019, American Chemical Society.

4.4. EVs—Quantum Dots

Semiconductor quantum dots (QDs) have garnered considerable attention from biomedical researchers owing to their small size, high water solubility, good fluorescent properties, high stability in physiological environments, and photothermal effect.[131,136] The small size of QDs enables easy excretion from body fluids through the renal system. With the recent development in nano-architectonics, it is feasible to prepare QDs with photothermal effect in the low-energy NIR-II area (1000-1350 nm) and effective tissue penetration relative to the NIR-I window (750–1000 nm). Recently, Cao et al. reported a combination of vanadium carbide (V_2C) QDs photothermal agent (PTA) with an engineered exosome vector for achieving low temperature nucleus-targeted photothermal therapy (PTT) in the NIR-II region.[137] The PEGylated V_2C QDs were modified with cell nucleus-target TAT peptides (V_2C-TAT) and encapsulated into the Arg-Gly-Asp (RGD) peptide endogenous exosome to form V_2C-PEG-TAT@Ex-RGD (**Figure 6**). The small-scale V_2C-TAT QDs (~16 nm) can quickly penetrate the cell nucleus to degrade the genetic substances directly at low temperatures. With laser irradiation at 1064 nm, the prepared V_2C-TAT@Ex-RGD could promote increased cell necrosis in vitro at a low temperature of 45 °C. In addition, the as-prepared V_2C-TAT@Ex-RGD exhibited multimodal imaging-guided cell nucleus-targeted PTT in the NIR-II.

Several other QDs, such as Si QDs (30 nm) and gold-carbon QDs also reported loading onto the outer membrane of exosomes to obtain high-resolution images of live cells and target the metastatic activity of tumor cells with minimal cytotoxicity.[138,139]; However, the comparatively large size of QDs (<10 nm) causes major steric obstruction to the exosome during encapsulation.[140] In order to resolve this limitation, the DNA hinge (functionalized DNA, maleimide-5′-AAAAAA-3′-biotin) as a ligament is built to bind QDs to the surface of the exosomes.[141] In this technique, a moderate and biocompatible exosome labeling approach is used to deliver optimal fluorescent emission benefits from higher quantum yields of QDs. This exosome-DNA-QDs complex labelling strategy can be quickly engulfed by tumor cells and ignited by exosome-DNA-QD fluorescence.

4.5. EVs—Mesoporous Silica NPs

Mesoporous silica NPs (MSNs) are solid materials containing hundreds of hollow mesoporous channels organized in a 2D network of honeycomb-like porous structures.[142] They possess several unique features, including a highly ordered porous network, size homogeneity, high stability and biocompatibility, customizable surface for carrying and controlled releasing of drugs. Besides, both the inner and outer surface of MSNs can be functionalized with a wide variety of hydrophobic and hydrophilic molecules, including polymer, functional groups, small molecule, contrast and imaging agents, and biomolecules.[143–145]

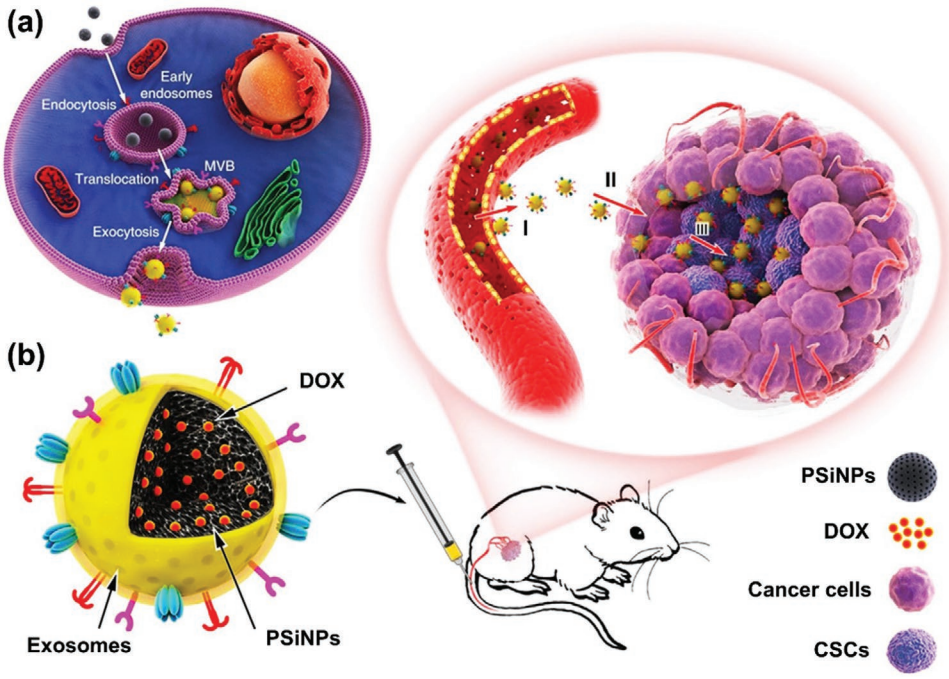

Figure 7. Schematic illustration of E-PSiNPs as drug carriers for targeted cancer chemotherapy. a) The DOX@PSiNPs are endocytosed into cancer cells after incubation, then localized in multivesicular bodies and autophagosomes. b) After intravenous injection of DOX@E-PSiNPs into tumor-bearing mice, the particles are efficiently accumulated in tumor tissues and penetrate deeply into tumor parenchyma to be internalized into bulk cancer cells and CSCs, producing strong anticancer efficacy. Reproduced with permission.[146] Copyright 2019, Springer Nature.

Having these unique advantages, MSNs have become one of the most investigated materials in the areas of nanomedicine and nanobiotechnology. The last few decades have witnessed the tremendous applications of MSNs as highly potential drug delivery systems for a variety of therapeutic agents for treating diseases, however, exosome-sheathed MSNs are very new and highly promising as therapeutics, especially in tissue engineering.

Yong et al. developed a tumor exosome-based NP using luminescent porous silicon NPs (PSiNPs) as efficient drug carriers for chemotherapy.[146] PSiNPs (150 nm) were synthesized by the electrochemical etching of silicon followed by lifting-off PSi film and ultrasonication step. The luminescence properties are then implanted by heating the as-prepared SiNPs in an aqueous solution. To load the exosome into the PSiNPs, a human hepatocarcinoma cell line, Bel7402 cells, were treated with PSiNPs and collected by centrifugation. The field transmission electron microscope (FTEM) analysis revealed that the collected particles are exocytosed exosome-sheathed PSiNPs (E-PSiNPs). The drug-carrying efficiency of E-PSiNPs was also investigated using DOX as a model drug. The DOX drug was loaded into PSiNPs followed by incubation with Bel7402 cells to obtain exosome-sheathed DOX-loaded PSiNPs (DOX@E-PSiNPs) (**Figure 7**a). The DOX@E-PSiNPs exhibited a good cross-reactivity of cell uptake and cytotoxicity against bulk cancer cells and cancer stem cells (CSCs). In addition, they also showed increased tumor accumulation, extravasation from blood vessels, and deep penetration in tumor parenchyma (Figure 7b).

Recently, a biomimetic NP was synthesized using a tumor-cell-derived exosome (E-MSNs) and exosome-camouflaged porous Si NPs for the delivery of drugs ICG and DOX (ID@E-MSNs).[147] When comparing the ID@E-MSNs for realizing the synergistic effects of chemotherapy and photothermal therapy against breast cancer with ID@MSNs (without exosome), the biomimetic NPs ID@E-MSNs were effectively taken up by the tumor cell and enhanced accumulation was achieved with the help of the exosomal membrane. ID@E-MSNs also retained the photothermal effect of ICG and the cytotoxicity of DOX after loading. The total rate of cell apoptosis induced by ID@MSNs was 60.4%, while that of ID@E-MSNs was 78.3%. The in vivo results of 4T1 tumor-bearing BALB/c mice showed that ID@E-MSNs could accumulate in tumor tissue and inhibit the growth and metastasis of the tumor. This type of exosome-camouflaged biomimetic porous silica NPs could contribute to the production of advanced drug carriers based on MSNs for the efficient delivery of anticancer drugs. **Table 2** summarizes different examples of NPs coated with EVs, their coating mechanisms, and the unique features of this strategy for drug delivery applications.[105,106,119,137,146–155]

5. Conclusion and Outlook

The requirements for the effective delivery of drugs in selected tumor tissues have contributed to the emergence and development of a range of drug carriers, such as exosomes, liposomes, dendrimers, etc. The rapid development in exosome science and the incorporation of exosomes with engineered nanostructures (e.g., iron oxides, AuNPs, MSNs, QDs, etc.) have strengthened the translational ability of exosome-nanostructures in clinical practice. In this review, we detail the exosome biogenesis, different packaging module of exosomal cargo, and finally, different nanostructures- integrated exosomes for therapeutics.

The advances in the field of EVs and nanostructures for therapeutic applications reported in recent studies are a sign of flourishing research, however, several issues need to

Table 2. Examples of delivery systems developed by coating EVs onto a variety of nanoparticles.

No.	Source of the EVs	Type of Nanostructure- cargo	Coating mechanism	Target disease	The distinctive feature of the study	Advantage	Ref.
1	Grapefruit	Doxorubicin-loaded heparin -based NPs	EDC-NHS mediated conjugation	Glioma	Unique design of pH-sensitive NP loaded EVs provided unprecedented drug loading capacity	High loading efficiency, prolonged circulation	[148]
2	4T1 cells transfected with Cy5-anti-miR-21	Gold-Iron Oxide NPs (GIONs)	Extrusion	Breast cancer	Combinatorial efficacy of miRNA loaded EV-coated GION platform for the therapeutic and theranostic purpose	Enhanced tumor specific accumulation and tumor targeting	[149]
3	H22, Bel7402, or B16-F10 cells	Doxorubicin-loaded porous silica NPs	Simple incubation of cells with NPs	Breast cancer and hepatocarcinoma	Autophagy-dependent synthesis of EV-coated NPs due to the novel structure of silica NPs	Enhanced cross-reactive cellular uptake, stronger cytotoxicity towards cancer stem cells, targeted drug delivery	[146]
4	A549 human lung carcinoma cell line	Lipid-PLGA NPs	Microfluidic sonication	Lung carcinoma	Fabrication of unique microfluidic system for synthesis of EV-coated NPs, Comparison to cancer cell membrane coated NPs showed the superiority of EV-coated NPs	Enhanced immune system evasion and tumor targeting	[150]
5	MDA-MB-231 human breast carcinoma	ZIF-8 MOF-protein NPs	Ultrasonication and extrusion	Breast carcinoma	Use of hypotonic treatment for the isolation of EV membrane and subsequent reconstitution on NPs	Enhanced protein transduction efficiency due to increased immune evasion, increased blood circulating time	[106]
6	4T1 murine breast carcinoma and J774A1 macrophage cell lines	BPEI-coated gold NPs	Extrusion	Breast carcinoma	Demonstration of EV membrane orientation and composition retention after extrusion	Increased autologous uptake, increased macrophage evasion	[151]
7	MDA-MB-231 human breast carcinoma	Paclitaxel and cucurbitacin-B loaded PEG-PCL NPs	Extrusion	Breast carcinoma	Unique ROS-triggered paclitaxel activation to induce inhibition of tumor metastasis	Enhanced suppression of tumor metastasis, elevation of intracellular ROS for amplified paclitaxel mediated chemotherapy	[152]
8	4T1 murine breast carcinoma cell line	ICG and Doxorubicin loaded mesoporous silica NPs	Extrusion	Breast carcinoma	The utilization of photothermal capacity of ICG for enhanced laser-mediated tumor ablation	Enhanced tumor targeting and cellular retention	[147]
9	B16-F10 murine melanoma cell line	PEG-Hollow gold NPs	Multiple methods used, incubation of cells with NPs found to be most efficient	Melanoma	Comparison of various methods of coating NPs with EVs to demonstrate the most efficient one	Enhanced specific cellular uptake	[153]
10	4T1 murine breast carcinoma cell line	siRNA loaded BSA NPs	Incubation of EVs with NPs followed by extrusion	Breast carcinoma	Highlighted efficiency of siRNA mediated gene silencing to prevent organotropism of breast cancer EVs to lungs	Gene silencing to inhibit breast cancer malignancy, enhanced tumor targeting	[154]
11	HeLa human cervical cancer cell line	MIL-88A MOF NPs	Incubation of EVs with NPs	Cervical cancer	The EV-coated MOF NPs autonomously decompose in lysosomal fluid to release NP cargo demonstrating high drug delivery efficiency	Enhanced targeting and specific cellular uptake	[105]
12	Urine of gastric cancer patients	PMA/Ce6 loaded gold NPs	Electroporation	Gastric cancer	Exploration of the combinatorial therapy effects of acidic environment and laser irradiation for enhanced therapeutic efficiency	Enhanced immune evasion and tumor penetration	[119]
13	MCF-7 breast carcinoma cell line	Antisense oligonucleotide loaded gold NPs	Incubation of EVs with NPs	Breast cancer	Demonstrated the EV-mediated transport of NPs from one cell type to other cell types, thus showing the role of exosomes in malignant transformation of other cell types	Downregulation of metastatic gene expression	[155]
14	MCF-7 breast carcinoma cell line	Vanadium Carbide quantum dots	Electroporation	Breast cancer	Demonstrated nucleus targeted photothermal therapy at very low temperatures	Enhanced cell necrosis and penetration by EV-NPs	[137]

be considered and addressed to transfer EV-NP based drug delivery and therapeutics to clinics, including i) the selection of specific EVs to coat the nanostructures, as EVs released by different cell types show different compositions, ii) the efficient encapsulation of exosomes into nanostructures (Exo-nanostructures) and the loading of selective drugs into the Exo-nanostructures, iii) the stability of Exo-nanostructures in bodily fluids, and iv) engineering of nanostructures so that ultrasmall-sized (biomolecules with homologous shapes and sizes >10 nm) nanostructures can inherit the desired properties required for drug loading, delivery, or action as therapeutics.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomimetic nanostructures, drug delivery system, exosomes, extracellular vesicles (EVs), nanoarchitectonics, nanomedicine

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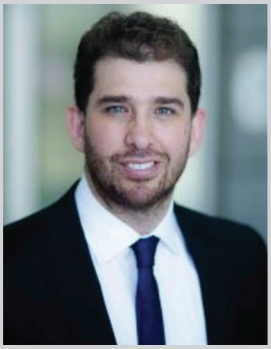

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