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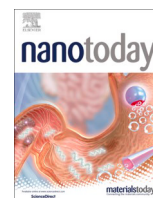Link to publication record in Queen's University Belfast Research Portal

Review

Exosome nanovesicles: A potential carrier for therapeutic delivery

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ABSTRACT

Exosomes are small nanosized biovesicles that form when multivesicular bodies and the plasma membrane fuse and are released into the surrounding body fluids. They are best known for their multifunction in mediating intercellular communication by transferring various biomolecules, including DNA, RNAs, proteins, and lipids, in a short- and long-distance manner and have been identified as health and disease messengers. Importantly, exosomes are necessary for various physiological processes in health and disease. The generation of exosomes depends on the status of the disease, which usually exhibits opposite roles by inducing enhanced cellular stress and damage. Recently, exosome-based nanotechnologies have provided unprecedented opportunities to boost the developments of exosome-related biology, chemistry, pathology, and therapeutics in different diseases based on their unique structural/compositional/morphological characteristics for next-generation nanomedicines. Herein, we provide a comprehensive overview of the recent advances in exosome nanotechnology research, including their classification, isolation and preparation, constitution, biological function, and nanobiomedical applications in disease treatment and diagnosis. Furthermore, future prospects were also concluded. This review will provide more inspiration for promoting the development of exosome-based advanced theranostic nanoplatforms and nanotechnology.
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Introduction

Exosomes are small membrane vesicles of endocytic origin secreted by most cells [1,2], with sizes ranging from 30 nm to 150 nm, different from extracellular vesicles and apoptotic bodies in their origin, size and inclusions[3,4]. Exosomes are extracellular cell-derived phospholipid nanocarriers that function as signalosomes and transmit bioactive molecules to specific recipient cells for intercellular communication over short and long distances [5], where cargoes, including DNA, RNA, protein, lipid, carbohydrate and other small molecules, within exosomes can execute their functions in targeted tissues to regulate the proliferation, differentiation, migration, survival, gene expression, cell metabolism, *etc.*, of recipient cells [6]. To date, exosomes have been identified to be complicated within almost every disease [7–11]; for example, to maintain cellular homeostasis, exosome secretion is beneficial for removing harmful cytoplasmic DNA from cells [12]. At the same time, due to the high heterogeneity of inclusions, exosomes act as a double-edged sword in disease biogenesis, progression, and inhibition[13–15]. Therefore, exosomes have been effectively regarded as diagnostic biomarkers [16–18], therapeutic targets [19,20], delivery platforms[21,22], and therapeutics[23,24] in various diseases. In particular, exosomes that act as platforms for disease-targeted delivery have been developed rapidly and achieved excellent outcomes[25,26] and have been regarded as next-generation theranostic platforms of advanced nanotechnology[27–29]. In addition, there are 287 clinical trials of exosomes in various diseases (ClinicalTrials.gov), highlighting the importance of exosome-based technologies in biomedicine.

Exosomes from various kinds of cells, including mesenchymal stem cells (MSCs), cancer cells, immune cells, and food and plant cells, have been isolated and prepared [30–32]. At present, a standardized exosome isolation method is still not available, and many approaches have been established through exploration of the biochemical and physicochemical features of exosomes, including differential centrifugation, filtration centrifugation, density gradient centrifugation, immunoaffinity capture, size exclusion chromatography, microfluidic technology, *etc.* [28,33,34]. Many methods are adopted for purification, quality control and storage [35–38]. Due to the dynamic homeostasis of the biological body, the results of exosome preparation are also different from each other; therefore, many methods, such as adjustment of physical and chemical factors, increase in intracellular calcium concentration, drug stimulation, and gene overexpression, have been utilized to enhance the generation ratio of exosomes[39,40]. Furthermore, the identification of exosomes is based on their physicochemical properties, including size and protein expression, and exosomes are usually characterized by transmission electron microscopy (TEM), western blotting, nanoparticle tracking analysis (NTA), and protein concentration determination.

Compared with other delivery platforms, exosomes exhibit overwhelming advantages when applied in disease-associated applications, including i) maintaining high stability in the long term; ii) high targeting capability, by passive and active targeting manner, of exosomes improve the efficiency of drug use and reduce the frequency of drug usage; iii) autogenous exosomes promote low immunogenicity that reduces body clearance; iv) high drug loading capability, including the broad species of loading cargoes, including nucleic acid drugs, virus, protein drugs, small molecule drugs and so on, and loading efficiency; v) exosomes benefit from effectively increasing drug solubility and implementing multiple drug release; and vi) therapeutic potential itself to achieve synergistic therapeutic outcomes jointly. All these advantages have boosted their applications in biomedical fields [39,41–43]. Beyond natural exosomes, engineered exosomes defined by various modifications are another branch of the exosome-based scientific community that can enhance targeting specificity and efficacy to improve therapeutic outcomes significantly [44–46], and they have been applied in cancer treatment[47,48], neurodegenerative diseases[24,49], cardiovascular diseases [50,51], mental illness[52], orthopedic diseases[53], metabolic diseases [54] and so on.

Although a plethora of breakthroughs in exosome-based science have been made in recent years, many obstacles still need to be resolved urgently, such as standard preparation and quality control methods and effective quantification methods for their clear, complete and simultaneous inclusion[55–57]. On the other hand, there are a plethora of studies focusing on the summary of exosome-associated science, from production, purification, storage, quality control, modification, and biomedical application[58–63]. However, many advances need to be summarized and explored. Herein, in this review, we make our best efforts to provide a

comprehensive overview of the recent advances in exosome na-notechnology research, including their classification, isolation and preparation, constitution analysis, biological function, and nano-biomedical applications in disease treatment, especially in the fields of delivery of various therapeutic agents.

Exosomes

Exosomes arise from endosomes between 40 and 100 nm in size [7]. They are present in most human fluids, including blood, saliva, and urine, and are released by all cell types. Exosomes are nano-spheres with bilayer membranes that contain several proteins obtained from their parent cell, including transport proteins, heat shock proteins, proteins linked with multivesicular body (MVB) biogenesis, and tetraspanins, to name a few. In addition to proteins, they contain several lipids, including cholesterol, sphingolipids, phosphoglycerides, ceramides, and saturated fatty acid chains [7,8]. Exosomes act as biomarkers and indicate their involvement in bio-logical processes, making their composition crucial.

They play a vital role in signal transduction and physiological response initiation [9]. Exosomes were first implicated in the re-moval of superfluous proteins, even during the development of cells. Based on the origin of the exosomes, their roles vary. By transporting prostaglandins, platelet-secreted exosomes contribute to proinflammatory reactions [10]. In addition to sharing proteins and lipids between recipient cells, exosomes have been demon-strated to transport nucleic acid cargo, with mast cells secreting exosomes carrying mRNA and short RNA, which are subsequently transmitted to selected recipient cells and translated by the re-cipient cell [11].

Given that exosomes can be extracted from almost every kind of cell, are engaged in cell-to-cell contact, and participate in both normal and pathobiological pathways, several studies have focused on their diagnostic and therapeutic applications. For instance, exosomes are capable of inducing tissue regeneration by delivering growth factors, proteins, microRNAs, messenger RNAs, noncoding RNAs, and lipids [12,13]. Furthermore, exosomes produced from stem cells and endothelial progenitor cells could regenerate cardiac tissue and neovascularization in a model of myocardial infarction and kidney damage [14]. Plant exosomes are widely accepted moieties and are mostly incorporated into edible items. It is useful in numerous therapeutic applications, such as inflammation, tu-mors, and infectious diseases. Plant-derived exosomes are a novel approach to natural exosomes. A study concluded that there are different concentrations of exosomes in different edible contents, such as 1.76 mg/g and 2.21 mg/g in grapes and grapefruit, respec-tively [64]. Citrus fruits are the matter of choice regarding the production of plant-based nanovesicles. Citrus fruit-derived exo-somes have shown significant antioxidant activity in various dis-eases[65].

MicroRNAs can improve the condition of cardiac disease, such as myocardial infarction (MI). Delivery of miRNAs is facilitated by using human exosomes derived from human blood. It ensures targeted drug delivery and cellular communication, which increase efficacy and nullify adverse events[66]. Exosomes derived from breast milk elevate the growth and health of neonates. A study on porcine epi-thelial cells showed a positive response, which was concluded by an increase in the proliferation of cells[67].

With the advancement of technology and science, artificial exo-somes are also considered an alternative for drug delivery and therapeutics. A study on rabbits concluded that APO2 L/TRAIL, when conjugated with liposomes, shows a better therapeutic effect in ar-thritis[68]. Although accepted and supported by clinical data, it was found that the mass that reaches the tumor was only 0.7% of the total dose[69]. When compared to plant exosomes, human exosomes

show an alert for the therapeutic index. However, the production scale of human exosomes is limited compared to that of plant exo-somes[70]. Safety concerns are present in artificial molecules, and delivery systems are recommended for vegetable- and plant-derived exosomes[71].

Exosomes are classified as either natural or designed de-pending on whether they have been purposefully altered [15]. Ultimately, natural exosomes are split into those originating from animals and those obtained from plants. Additionally, food-de-rived exosomes offer promising future potential. In recent years, researchers have discovered that plant-derived exosome-like na-noparticles (ELNs) have structural similarities with exosomes from mammals. Ginger-derived particles may prevent the devel-opment of liver disorders, while ELNs generated from grapes, carrots, grapefruit, and ginger have anti-inflammatory properties and can preserve intestinal homeostasis [16–18]. Exosomes have been separated from various plant species and their sections (*i.e.*, fruits, roots, leaves, and seeds) using standardized isolation and purification techniques discussed below. Wang et al. investigated whether nutrient uptake by plant-derived nanovesicles has mul-tiple functions with some health benefits and could be useful for efficiently delivering therapeutics without eliciting inflammatory responses[72]. Using nanovesicles made from vegetables and fruits might provide a novel technique to complement the average human diet. Several recent studies have revealed that plant-de-rived nanovesicles may be taken up by both macrophages and stem cells, with anti-inflammatory and regenerative benefits. The antioxidant impact of organic agriculture-derived nanovesicles was shown to be much greater than that of intensive agriculture-derived vesicles. The ability to acquire antioxidants without the use of chemicals was a significant accomplishment in this field. This delivers antioxidants in vegetables and fruits in their natural and active forms. Furthermore, when antioxidants are en-capsulated inside the lipid membrane of nanovesicles, they are shielded from rapid oxidation [73]. A study by Logozzi M et al. suggested a series of data supporting the use of plant-derived nanovesicles in daily human supplementation to prevent and treat human diseases [74]. Whenever antioxidants are consumed inside nanovesicles, they are protected from rapid oxidation and lysis and enzymatic digestion in gastric and intestinal solutions. A phase I clinical trial is presently being performed to evaluate the delivery potential of plant-derived exosomes conjugated with curcumin in colon cancer (ClinicalTrials.gov Identifier: NCT01294072). In addition, clinical trials have been developed to study the oral administration of grape-derived nanovesicles to abolish oral mucositis related to neck and head cancer treatment (ClinicalTrials.gov Identifier: NCT01668849). Finally, the ability of ginger- and aloe-derived exosomes to alleviate insulin resistance and long-lasting inflammation in polycystic ovary syndrome (PCOS) patients will also be assessed in clinical trials (Clinical-Trials.gov Identifier: NCT03493984).

It is ideal to achieve optimal therapeutic efficacy, avert off-target outcomes and toxicity, and reduce large-scale production costs when creating new therapeutic treatments. Based on this require-ment and the above clinical examples, it is evident that plant-de-rived nanovesicles appear feasible for drug delivery and targeting. Furthermore, due to their natural source, exosomes can be isolated in large volumes in an ecological manner.

At present, exosomes are categorized mostly based on their ori-gins. However, this categorization does not comprehensively analyze the properties and functional applications of the numerous exosome types. Further classifications of organophilic, biological dispersion, and immunogenicity may be proposed in the future [19].
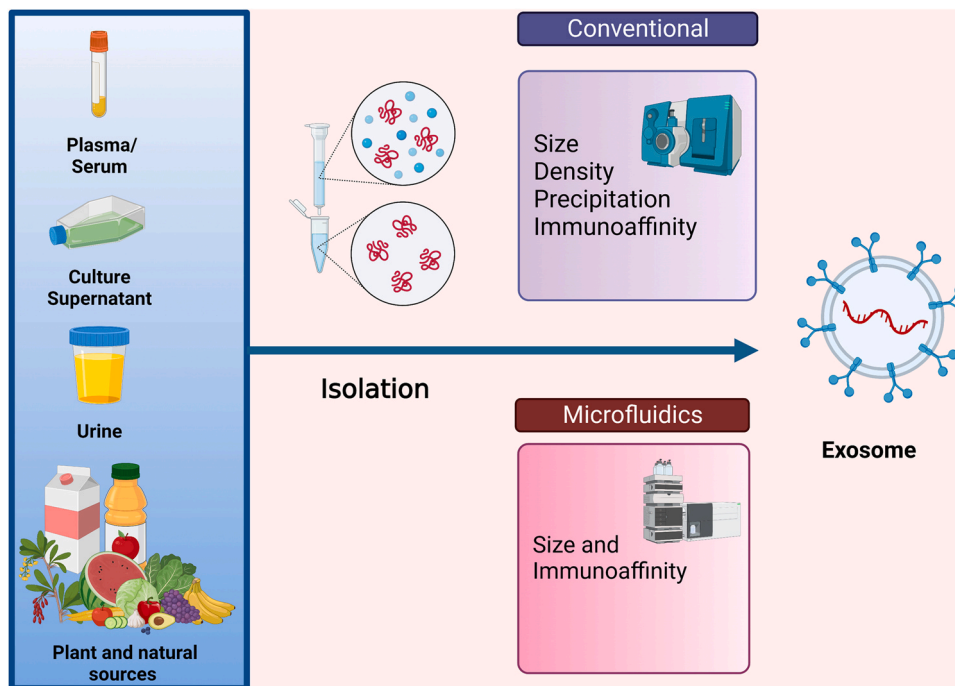
Fig. 1. Methods employed to isolate exosomes from various biological matrices, such as plasma, cell or microorganism cultures, urine and plant/vegetable/fruit sources. The basic principle of these purification techniques is to separate the exosomes from other elements in the sample based on particle size and physical properties. Frequently used exosome isolation techniques are immunoaffinity separation, size exclusion chromatography, differential centrifugation, PEGylation and filtration.

Isolation, extraction and purification of exosomes

Methods adopted for the isolation and purification of exosomes

It is an essential part of exosome research. However, it is challenging to extract exosomes due to their small size and low number and heterogeneity in their content, function and source [75]. Due to the exhibition of great potential in unraveling disease mechanisms, targeted drug delivery, and diagnostic biomarkers, advancements in exosome separation techniques are gaining more attention [21]. Various methods, such as ultracentrifugation, ultrafiltration, precipitation, immunoaffinity separation, and size exclusion chromatography, have been developed based on their purpose and application (Fig. 1). The following is a brief description of different methods.

a) Ultracentrifugation is categorized into two types: differential ultracentrifugation and density gradient ultracentrifugation. Differential ultracentrifugation is the most common method to isolate exosomes from biological fluids. This technique involves initial sets of minimal-speed centrifugation (4 °C) to eliminate cell and apoptotic fragments followed by two cycles of high-speed centrifugation (4 °C) at 100000 x g for a longer duration to separate larger vesicles and precipitate exosomes, respectively, as pellets [22,23]. Despite the effective isolation of exosomes, this method is time-consuming, laborious, and expensive, produces low yields, and compromises purity[24]. On the other hand, the density gradient centrifugation technique involves the addition of a sample into an inert gradient media (linear sucrose or iodixanol gradient) for centrifugal sedimentation or equilibration, ultimately resulting in the settling of distinct sample components to their isodensity region, thereby leading to the separation of exosomes. The advantages of this method are higher separation efficiency and purity of exosomes, maintenance of the architecture of extracted exosomes, and nonmixing of separated

components. However, it is a highly time-consuming method and produces low yields.

b) The ultrafiltration or size-based exclusion technique is a rapid method that employs a membrane filter with specific molecular weights or size exclusion constraints to separate exosomes. Here, a smaller volume of samples can be used. However, the use of force may deform or break larger vesicles, resulting in misleading results [75].

c) The principle involved in size exclusion chromatography (SEC) is that macromolecules are not able to penetrate the gel apertures and will be flushed with a mobile phase along the pores of the gel. Nevertheless, tiny molecules persist in the porous matrix and are eventually eluted with the help of an eluent. It is an easy technique of isolation that is quicker, less costly and has no effect on biological characteristics [75].

d) The precipitation technique involves settling exosomes by altering their solubility or dispersibility. This is achieved by engaging water, excluding polymers such as polyethylene glycol (polymer precipitation), adding salt solution such as sodium acetate (salt precipitation), or adding protamine sulfate (charge precipitation) [25]. The procedure involves incubating the sample and collecting the precipitate containing exosomes by low-speed centrifugation or ultrafiltration.

e) Immunoaffinity chromatography involves the covalent bonding of antibodies to filters or other matrices that bind to the surface protein or antigen present on the surface of exosomes [26]. To add value to this method, Zarovni and team developed an immunoaffinity magnetic capture technique employing submicron-sized magnetic beads coated with a monoclonal antibody that attaches to certain receptors on the surface of exosomes [27]. However, the fundamental drawback of this method is that eluting exosomes from magnetic beads is difficult, and the bound exosomes cannot be used in subsequent studies [5]. Therefore, Huang and colleagues created unique magnetic graphene oxide nanoparticles (MGONs) for efficient exosome capture using Fe_3O_4

@SiO$_2$ magnetic nanoparticles coated with graphene oxide through dopamine. On the surface of MGONs, CD63 aptamers were added, which could detect and bind to CD63 on the exosome membrane [28].

Various commercial kits are currently marketed based on the isolation mentioned earlier, such as EXO-Prep, qEV separation columns, exoEasy Maxi kit, Exo-spin isolation kit, RIBO exosome isolation reagent, and Minute Hi-Efficiency Exosome Precipitation Reagent. After thorough analysis, commercial kits have shown the benefits of saving time, good yield, and excellent structural architecture. However, due to uneven extraction processes, no kit can extract optimal exosomes from a combination of samples. In addition, the kits are expensive, and the purity and yield of exosomes are not particularly good [76].

With the growing clinical potential of exosomes, it is more important than ever to improve and optimize their isolation process for optimum yield, purity, and assay consistency. Because of constant research, development, and innovation, scientists have access to numerous novel approaches for optimizing established technologies from one or more viewpoints, all of which have promising application potential. Microfluidic technology is one of the recent advancements in the isolation of exosomes. The approaches employed in microfluidic techniques are immunoaffinity-based extraction using particular biomarkers such as antibodies, integrating microfluidics with acoustic waves and dielectric electrophoresis to accomplish tag-free isolation of exosomes based on their electrical and physical attributes, and filtration-based extraction. Tayebi and coworkers designed a microfluidic device with grids to effectively capture a multitude of individual microbeads with abundant exosomes at the extent of a single particle based on the principle of passive hydrodynamic trapping [29]. Similar to the immunoaffinity approach, nanoplasmonic enhanced scattering (nPES) employs antibodies against the biological markers CD81, CD63, and CD9, which are abundant on most exosome surfaces, to collect and identify all exosomes present in a sample [77]. On-chip isolation of exosomes employs an exosome total isolation chip (ExoTIC), which is designed to simplify the isolation of exosomes based on a simple filtration method wherein intact exosomes in sizes ranging from 30 to 200 nm are concentrated and purified by filtering through a nanoporous membrane. Few researchers have modified the membrane to porous silicon nanowires, acoustic nanofilters, and so on for better extraction and purification [30,31]. The method can be utilized for point-of-care exosome testing for disease diagnostics and in resource-limited situations because it is simple, rapid, cost-effective, scalable, and produces a high yield of exosomes. Lim and coworkers developed antibody cocktail-conjugated nanowires to isolate exosomes from the plasma of breast and lung cancer patients. This resulted in the rapid extraction of homogenous exosomes with high yield and purity from even smaller amounts of a sample [33].

Although several techniques for isolating and purifying exosomes have already been developed, they need to meet all the needs. An amalgamation of multiple extraction strategies might be more effective than separation with a single method. As a result, many scientific groups have started to fuse multiple approaches to improve separation efficiency and enrichment, thereby acquiring optimal exosomes with high yield and purity [34–36]. Wang et al. used an acoustic fluid platform that coupled acoustics with microfluidic technology to isolate and purify saliva-derived exosomes depending on size and found a 15-fold greater yield than differential centrifugation [33]. Additionally, isolation techniques such as tangential flow filtration [37,78], flow field-flow fractionation (FIFFF) [39], hydrophobic interaction chromatography (HIC) [40], and deterministic lateral displacement array [41] also offer many enrichment opportunities.

However, the change in biological features during separation and the scalability of current technologies for clinical setup are two major challenges in exosome isolation and extraction. Variations in preanalytical stages, such as sample collection, use of anticoagulants, presence of contaminants, and sample processing time involved in the isolation and characterization of exosomes, are among the technical obstacles that affect exosome analysis. The key concerns to be considered are exosome loss and limited yield. Separating disease-associated subpopulations of exosomes from normal exosomes is very difficult, necessitating an integrated strategy. Aside from genetic considerations, physiological and environmental factors linked to sample heterogeneity have a negative impact on exosome separation. As a result, greater research into the genesis, function, and number of exosomes is needed[28]. Aside from technology, adequate throughput and validation are required for upcoming applications.

Identification of exosomes

Exosomes can be identified explicitly by detecting nucleic acid information (aptamer and induced mDNA release), recognizing the lipid bilayer (cholesterol, polyglycerol, phospholipids, *etc.*), recognizing surface proteins based on immunoreaction and aptamer affinity (tetraspanins (CD63, CD9, CD81, CD82), heat shock proteins (Hsp 60, Hsp 70, Hsp 90), biosynthetic proteins (TSG, Alix), surface growth factor (EGFR), *etc.*); surface modification (nucleic acid recognition, radioactive material, nanoprobe); overall recognition (image analysis, flow cytometry, molecular imprinting); morphological features (scanning electron microscopy, transmission electron microscopy, atomic force microscopy, cryogenic electron microscopy) [42] and other methods (microfluidic chip, placental alkaline phosphatase, alkaline charge) [79].

Exosome drug loading techniques

The strategies developed for loading drugs into exosomes include passive drug loading (incubation), transfection, active drug loading, and *in situ* assembly and synthesis. The most straightforward method includes incubating a drug with exosomes at a specific temperature for a particular period, allowing drug diffusion into exosomes based on a concentration gradient. Incubation with donor cells entails administering the drug to the cells, producing exosomes containing the drug [41]. Despite its simplicity and ability to maintain membrane integrity, this strategy has been linked to decreased drug loading efficiency and the inability to manage the amount of drug distributed into exosomes. Transfection is a technique for loading nucleic acids, proteins, and peptides into exosomes in a stable manner. Using transfection reagents, certain plasmids are transduced into cells to ectopically produce desired nucleic acids, proteins, or peptides, which are then packaged into exosomes. This approach has two primary drawbacks: contamination and poor loading efficiency. Active drug loading entails creating micropores in the exosomal membrane or recombining the membrane to allow medications to enter the exosomes. Sonication, electroporation, extrusion, freeze−thaw cycles, surfactant treatment, and dialysis are techniques used for active drug loading. These methods are more efficient at loading exosomes, but they damage the exosomal membrane, make exosomes visible to immune cells, inactivate proteins, and cause exosome aggregation. In situ assembly and synthesis [42] is a noninvasive method for loading nanomaterials onto the surface of exosomes or inside exosomes. Although this approach preserves exosome integrity, it has a complicated operation process and technological limitations. Exosomal drug loading has been approached in various ways, and each method has its benefits and limitations. The incubation technique has been

demonstrated to be an easy strategy for maintaining the integrity of exosomes.

Challenges in exosome isolation and extraction

The change in biological features during separation and the scalability of current technologies for clinical setup are two significant challenges in exosome isolation and extraction. Variations in preanalytical stages, such as sample collection, use of anticoagulants, presence of contaminants, and sample processing time, involved in the isolation and characterization of exosomes are among the technical obstacles that affect exosome analysis. The key concerns to be considered are exosome loss and limited yield. Separating disease-associated subpopulation exosomes from normal exosomes is difficult, necessitating an integrated strategy. Aside from genetic considerations, physiological and environmental factors linked to sample heterogeneity have a negative impact on exosome separation. As a result, detailed research into the genesis, function, and number of exosomes is needed [42]. Aside from technology, adequate throughput and validation are required for upcoming applications.

Mammalian-derived exosomes are typically rich in sphingomyelin and cholesterol, but plant-derived exosomes have abundant phospholipids, including phosphatidic acids, phosphatidylethanolamines, and normal plant lipids [80,81]. In addition to compositional differences, plant-derived exosomes also possess anti-inflammatory properties [82]. A low pH is a defining feature of tumor malignancy, and it may influence exosome release and absorption by cancer cells [83]. Parolini and colleagues demonstrated that I enhanced exosome release and uptake at low pH compared to a buffered state, and (ii) exosome uptake by melanoma cells occurred through fusion using changing pH conditions as a regulator of exosome traffic [80]. Caveolin-1, a protein important in melanoma growth, is significantly supplied by acidic exosomes. The exosomes and tumor pH are critical targets for future anticancer treatments by demonstrating that exosomes may be exploited as a delivery vehicle for paracrine dissemination of tumor malignancy.

Composition and genetic modifications

Composition

Exosomes comprise a diverse combination of proteins (proteins of the plasma/endosome membrane), lipids, and other cytosolic components but no proteins from the nucleus, mitochondria, endoplasmic reticulum, or Golgi apparatus [44]. The lipid families sphingomyelin, phospholipids, ganglioside GM3, and cholesterol are often present in exosomes [45]. Nevertheless, the relative quantity of these lipids in exosomal envelopes may vary based on the kind of producer cell, the physiological stage of the producer cell, and the destination and functionality of the exosome [46]. Importantly, the transport of lipids into exosomes and other EVs is a continuing phenomenon that reacts to several variables. Global interactions in the lipidic structure of reticulocyte-derived exosomes were identified in response to metabolic responses in the cell during development to erythrocytes, indicating that the sorting of lipids for exosome synthesis adjusts to the cell's needs [47]. Exosomes include a large concentration of transport proteins, such as tubulin, actin, and actin-binding molecules, and several proteins linked to certain secretory cell activities [48]. Exosomes have been shown to spontaneously transport RNAs from one cell to another [50,84]. As per Mashouri and colleagues [51], "Adhesion molecules such as CAMs, integrins, tetraspanins, MHC class I, II displayed on B lymphocytes and dendritic cells, as well as transferrin receptors (TfR) on the surface of reticulocytes, are examples of typical forms of exosome proteins. In contrast, a variety of fusion and transferring proteins, such as Rab2, Rab7, flotillin and annexin; heat shock proteins, such as Hsc70 and Hsc90; cytoskeleton proteins, such as actin, myosin, and tubulin; and proteins, such as Alix, that mediate MVB formation are nonspecific protein types of exosomes."

Exosome-identifiable proteins are exclusively localized in the cell cytosol or endosomes, never in the endoplasmic reticulum, Golgi apparatus, mitochondria, or nucleus. Endosomal compartments also include plasma membrane proteins, which are also present in exosomes. These data support the theory that exosomes originate as intracellular vesicles of late multivesicular compartments [85]. Formation of the internal vesicles of multivesicular structures by inward budding from the limiting membrane entails a budding event with a membrane orientation opposite to that of classical intracellular budding events. All occurrences of inverse budding are associated with an inversion of the transmembrane partition of phosphatidylserine [53,85].

Genetic modifications and surface engineering

Natural exosomes may be modified for therapeutic applications, including the integration of pharmaceuticals and other therapeutic agents, as well as the alteration of the surface charge for quicker drug absorption. Exosomes derived from various natural sources, such as mixed fruit and vegetable juices and mammalian biological fluids, have been modified in several studies to demonstrate their medicinal potential. Exosomes may be changed in two distinct ways: inner modification, which alters the structure of the cargo inside the exosome, and surface modification, which modifies the exosome's outer surface [54]. However, genetic engineering has inherent flaws, such as complex modifications and a limited number of relevant proteins. Now, chemical alteration supplies us with some fresh ideas. Chemical conjugation utilizes electrostatic interactions or covalent attachment to affix chemical ligands or bioactive compounds to exosomal surfaces [55]. For instance, a recent approach known as 'postinsertion' decorates EVs with specific moieties coupled to PEG, thereby enhancing cell selectivity, prolonging circulation length, and obviating the need to change EV-secreting cells [86]. However, hydrophobic probes are randomly placed on the surfaces of exosomes and need more accuracy and selectivity. In addition, covalent ligations often require harsh conditions for biochemical processes, which may change the structure and functionality of exosomes and raise safety issues [57]. Sumit et al. recently encapsulated exosomes using a nanofilm supramolecular combination of ferric ions (Fe^{3+}) and tannic acid. They showed the surface modification of a cloaking film to add chemical ligands or active biomolecules [58]. The procedure is straightforward, quick, and substrate-independent. Thus, the chemical alteration of exosomes must still overcome several obstacles, and further *in vivo* tests are required to confirm their safety and effectiveness.

Exosome-based formulation and stability

Exosome-based formulation and structure

The membrane-bound cytosol comprises particles excreted into the extracellular space *via* mostly all living cells. According to Wang and colleagues, "EVs originate in bodily fluids comprising blood, urine, saliva, breast milk, cerebrospinal fluid, sputum, bile, semen, amniotic fluid, bronchoalveolar lavage fluid, and ascites [87]." The characteristics of EVs, such as their content, size, and membrane composition, hinge on their cellular cause and physiological surroundings [59]. Usually, apoptotic bodies are comprised of larger vesicles ∼ 800 nm– 5 μm in diameter released during programmed cell death. In addition, microvesicles are normally smaller in size (50–1 nm diameter) and are generated through the budding of the

plasma membrane and linked to the cell-shedding process. Smaller EVs are exosomes (40–- 150 nm) released through MVB fusion along the plasma membrane [60]. Owing to their different and dynamic nature, EV subcategory distinction is difficult. Cell communication is based on EVs and several physiological and pathological characteristics [61,62]. Furthermore, EVs obtained from cancer cells have been revealed to stimulate angiogenesis and coagulation, support tumor progression, and generate premetastatic niches [63]. "A varied display of quantitative methods is accessible for extracellular vesicle/exosome characterization. The exosome sizes and morphologies can be evaluated *via* transmission electron microscopy and cryogenic electron microscopy[88]. Nanoparticle tracking analysis[89], tunable resistive pulse sensing, dynamic light scattering (DLS) [90], and high-resolution flow cytometry [91] are not only able to determine exosome sizes but also offer evidence concerning the concentrations of exosomes. Conventional methods for the isolation of exosomes include ultracentrifugation, density gradient centrifugation, size exclusion chromatography, ultrafiltration, and gel filtration using the size and buoyant density of exosomes." A polymer-based precipitation method found a comparatively new technique to isolate exosomes *via* changes in exosome solubility utilizing volume-excluding polymers, *e.g.*, polyethylene glycol (PEG), to induce aggregation[92]. Further new isolation approaches include combined microfluidic systems with on-chip immune isolation and lipid-based nanoprobe systems, allowing for extemporaneous exosome labeling with quick consequent magnetic enrichment[93].

CD63 and CD9 are utilized as exosome enrichment markers[94]. Because of their similarities with liposomes, it is anticipated that exosome drug delivery systems will display characteristics similar to liposomes [95]. For this reason, it has been proven that carefully selected exosome sources and their exosomes can be utilized as highly specific targeted drug delivery systems. Over the past several years, numerous instances have represented preclinical achievements employing exosome-based delivery [96].

Exosomes for treatment purposes

Exosomes derived from macrophages and catalase were encapsulated to protect against neuroinflammation in a rodent model of Parkinson's disease [97]. In addition, targeted exosomes have been used to deliver siRNA to mouse brains. In addition, Zhou et al. developed pancreatic cancer-targeted exosomes from bone marrow mesenchymal stem cells/BM-MSCs. Exosomes were loaded with gelactin-9 siRNA by electroporation, and their surface was modified with oxaliplatin (OXA) prodrug as an immunogenic cell death (ICD) trigger. Therefore, surface-modified exosomes increased targeting efficacy toward the tumor *via* the accumulation of drugs at the tumor site. The results of the studies demonstrated that BM-MSC exosomes were able to accumulate in pancreatic cancer sites more significantly in mice owing to their interaction among BM-MSCs and pancreatic tumor tissue, in place of being mostly disseminated to the liver and spleen, as observed in healthy mice (Fig. 2). Furthermore, exosomes combined with immunotherapeutics have great antitumor effects that lead to a significant decrease in tumor size as well as tumor bioluminescence with extended survival compared to standard drug treatment in pancreatic cancer-bearing mice. Furthermore, there was no obvious weight reduction or organ toxicity in any of the experiments presenting the safety of BM-MSC exosomes. Finally, exosomes stimulate antitumor immunity by reversing tumor-suppressive macrophage (M2-like tumor-associated macrophage (M2-TAM) polarization, cytotoxic T lymphocyte recruitment and Treg downregulation and attain substantial therapeutic efficiency in cancer management [98].

Similarly, Jang et al. prepared bioinspired exosomes mimicking nanovesicles from U937 and RAW264.7 cells loaded with chemotherapeutic agents for malignant tumor treatment. The cell-derived exosomes and nanovesicles had similar characteristics and could enhance TNF-alpha-induced endothelial cell death in a dose-dependent manner. The results of *in vivo* studies exhibited a significant reduction in tumor growth with no adverse events compared to the free drug-treated rodent group. Furthermore, doxorubicin-loaded A33 antibody-containing exosomes were developed *by* Master et al. for the targeted treatment of colorectal cancer. However, Dox-loaded LIM1215 exosomes crosslinked with A33 Ab and carboxyl superparamagnetic iron oxide nanoparticles with A33 antibodies (A33Ab-US) showed that A33 Ab can bind to A33 +ve exosomes and form a complex to target A33 +ve colon cancer cells. From the experiments, it was found that A33Ab-US-Exo/Dox had excellent tumor targeting ability and was able to suppress the growth of tumors by almost 194.63 ± 13.75 mm^3. 3.04- and 2.90-fold lower than the Dox-treated group (477.50 ± 93.14 mm^3) as well as the A33-Exo/Dox (553.88 ± 78.06 mm^3) groups on day 16. It also prolonged the survival rate of the mice up to 61 days, with decreased cardiotoxicity (Fig. 3). Therefore, exosomes are modified by targeting ligands by coating with high-density antibodies, which might be evidence of a unique delivery system for targeted drugs against cancer [99].

Almost all drug delivery systems need to be stable for their stated efficacy. The preisolated, isolated, or downstream formulations must also be stable. Exosome stability can be defined as resistance to accumulation, structural damage, and protein degradation. Therefore, to estimate exosome stability, several techniques need to be employed. Surface marker quantification, for instance, CD63/CD81 quantification, was utilized to evaluate protein degradation. Concerning exosomal drug delivery, exosomal colloidal stability is a significant parameter determined *via* surface zeta potential, size distribution, and concentration. Electron microscopy is the only method used to examine physical morphology and accurately represent the whole sample[100].

Exosome stability upon storage and different pH conditions

In addition, storage factors have a significant impact on exosome stability. Storage temperature, the time required for storing the formulation, and freeze−thaw cycles for lyophilized exosomes also play a vital role in exosome stability determination. Osmotic pressure and pH influence stability; however, they have not been explored as stability parameters for exosomes. The size of the exosomes decreased when stored at 37 °C and 4 °C, and a nominal size change was observed after multiple freeze−thaw cycles (−20 °C) [26]. When exosomes were stored in a refrigerator (4 °C) and deep freezer (−80 °C), they increased particle size compared to freshly isolated exosomes. In addition, deep freezer (−80 °C)-stored exosomes displayed a reduction in absolute zeta potential, which caused aggregation of exosomes. Moreover, exosomes stored at 24 °C showed a time-dependent decrease in concentration compared to those stored at − 20 or − 80 °C for a month, as evidenced by flow cytometry [101,102].

Researchers have found that exosomes are impervious to freeze−thaw cycles in liquid nitrogen when augmented with 1 mg/mL albumin. Other cryoprotectants, such as DMSO (1% v/v) and glycerin (5% v/v), cause lysis of exosomes or cannot shelter exosomes from degradation. On the other hand, exosomes stored at − 20 °C were found to be stable compared to those stored at refrigerator temperature. Trehalose is an excellent cryoprotectant for exosome lyophilization. Trehalose-PBS-supplemented lyophilized exosomes were more stable in terms of the polydispersity index. Moreover, the protein concentration and activity remained unaffected in trehalose-PBS-supplemented lyophilized exosomes [101]. While recurrent freeze−thaw cycles of exosomes in the presence of PBS resulted in a nominal growth in particle concentration and the standard deviation of size when measured *via*
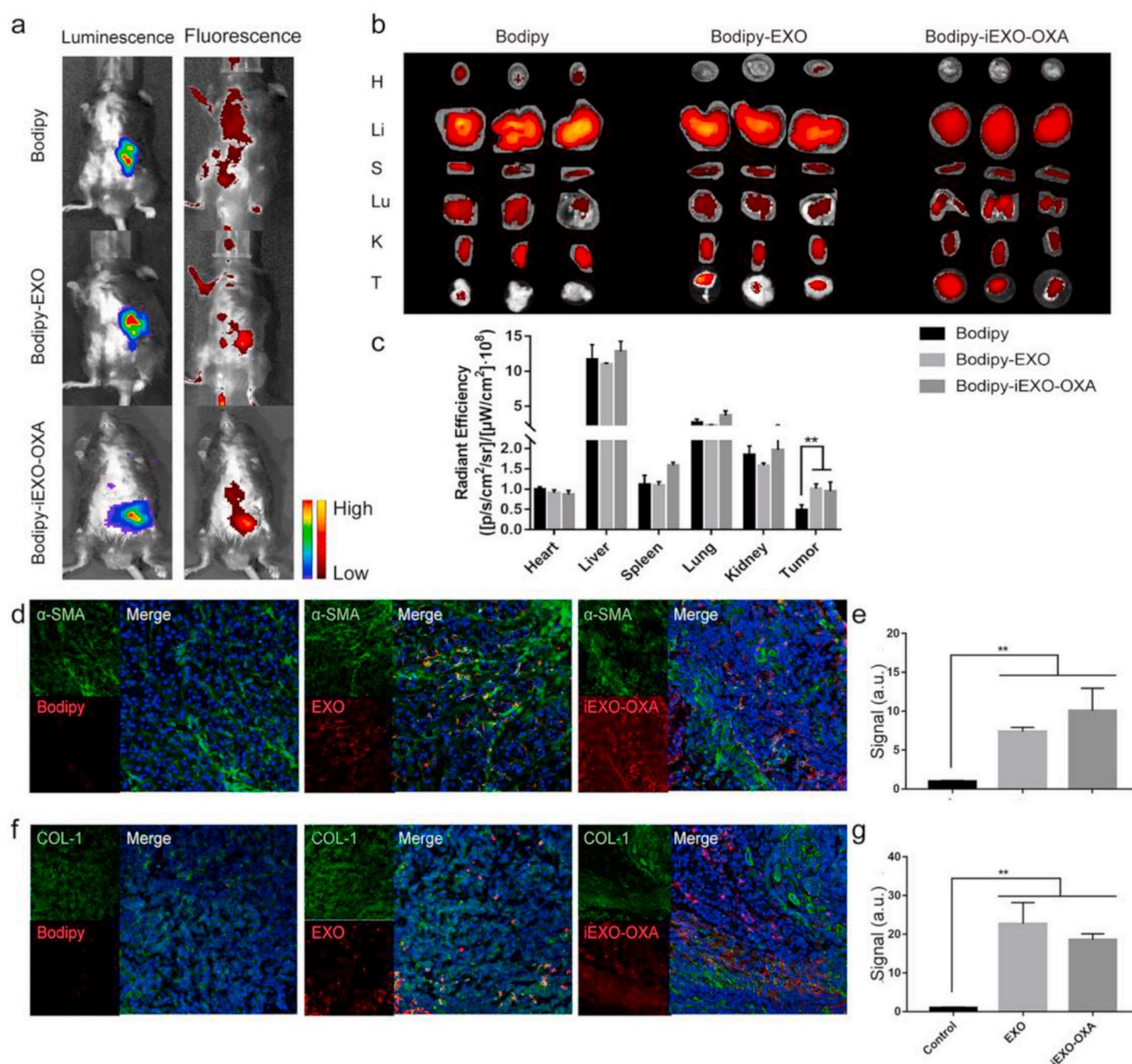
Fig. 2. *In vivo* targeting effect of iEXO-OXA. a) In vivo IVIS imaging of mice 24 h after tail vein injection with BODIPY, BODIPY-EXO or BODIPY-iEXO-OXA (n = 3, 5 mg OXA/kg, ~108 exosomes per mouse). b) *Ex vivo* IVIS imaging of major organs and tumor tissues from PANC-02 mouse models 48 h after treatment with different formulations (n = 3). c) Quantification of the fluorescence intensity by measuring the region of interest (ROI) in (b) (n = 3). d) and f) Tumor tissue immunofluorescence images under LCSM. (blue: DAPI for nucleus; green: Alexa Fluor 488 labeled α-SMA or COL-1; red: BODIPY, BODIPY-EXO or BODIPY-iEXO-OXA; original magnification = 200). e) and g) Statistical results of the BODIPY signals of different groups (n = 3). Data are presented as the mean ± SD, one-way ANOVA, $^{**}p < 0.01$.
Adapted with permission from ref [98].

nanoparticle tracking analysis, it was not observed with trehalose-PBS-containing exosomes. Instead, trehalose-PBS-dispersed exosomes displayed a smaller particle size, reduced standard deviation, and augmented particle concentration. These results demonstrate that trehalose reduced exosome accumulation or aggregation [103,104].

Moreover, exosomes stored at an acidic pH of 4 or 10 showed a higher loss of exosome protein than those stored at physiological pH (pH 7). Captivatingly, storage at 37 °C and 60 °C for 24 h was not as destructive to exosome concentration as freeze−thaw cycles. Similar to other biological samples, exosomes should be frozen (−80 °C) or lyophilized for long-term stability, and they can also be stored at 4 °C for very short times [105].

Diagnostic potential of exosomes and novel biomarkers

Recently, many biomarkers, such as angiogenic factors, cytokines, cell-free DNAs, circulatory miRNAs, and proteins, have been explored for their potential role in diagnosing various diseases. Nonetheless, exosomes are also emergent biomarkers being considered for their vital role in disease diagnosis. Exosomes are bilipid membrane-bound nanovesicles that contain subcellular materials such as miRNAs, mRNAs, cell-free DNAs, and proteins. This signifies the disease state of their cell of origin. These exosomal nanobubbles are released into the extracellular circulation and body fluids [106]. They are released from many types of cells, such as lymphocytes, dendritic cells (DCs), platelet epithelial cells, endothelial cells, mast cells, and
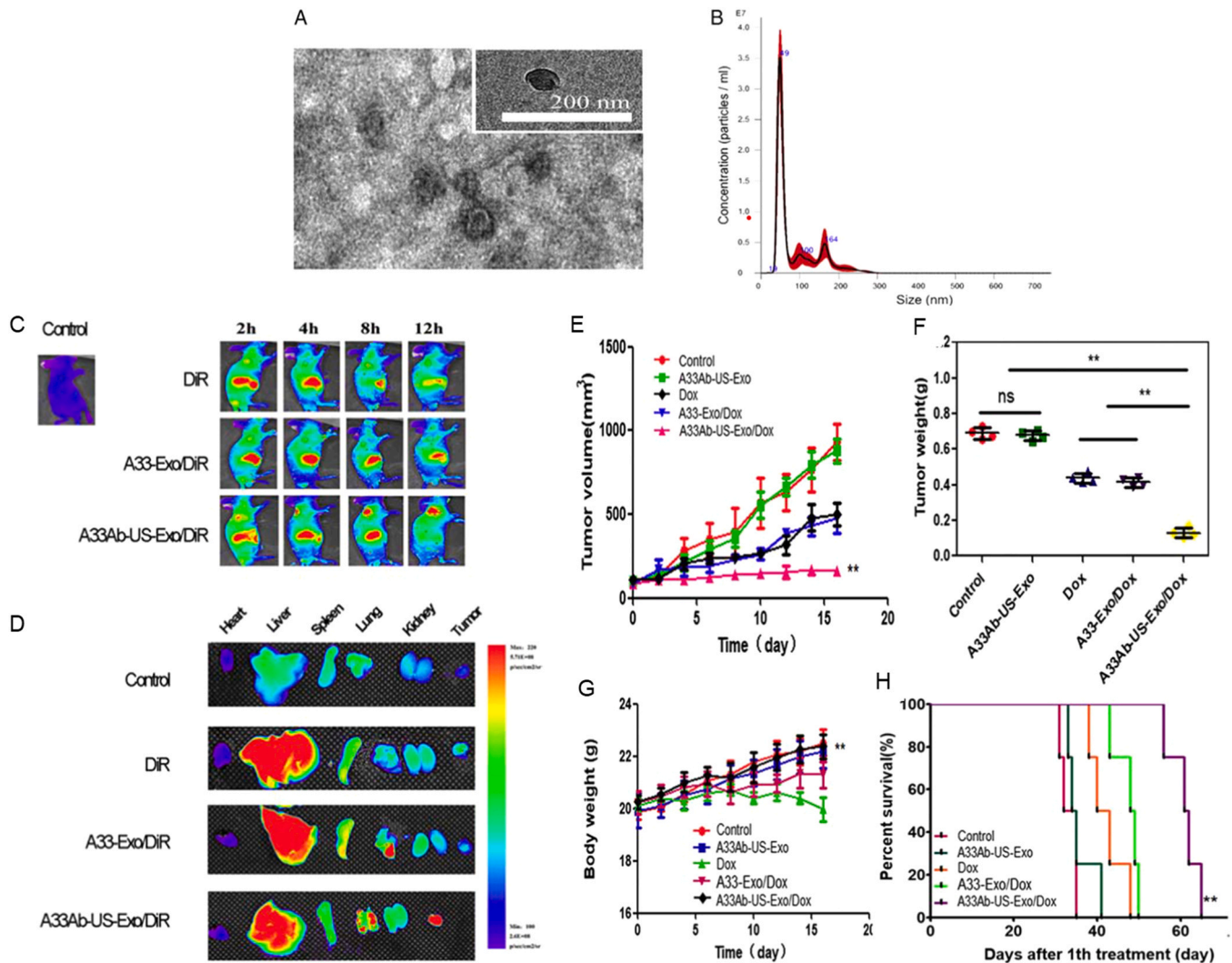
Fig. 3. Characteristics of A33Ab-US-Exo/Dox. (A) TEM image of deflated football-shaped A33 exosomes. (B) Size and concentration distribution of A33-Exos comprising a mean particle diameter of 85.1 ± 1.5 nm and concentration of 9.83 × 108 ± 5.80 × 107 particles/mL. (C) *In vivo* fluorescence signals of mice bearing LIM1215 cell-derived tumors after intravenous injection of saline, DiR, A33-Exo/DiR, and A33Ab- US-Exo/DiR (n = 4). Dose: DiR equivalent 50 µg/kg. The mice were scanned at different times (2, 4, 8, and 12 h) after injection using an imaging system. (D) Fluorescence imaging of excised organs from tumor-bearing mice 12 h after IV injection with DiR, A33-Exo/DiR or A33Ab-US-Exo/DiR (n = 4). (E) *In vivo* efficacy evaluation of A33Ab-US-Exo/Dox through IV injection in LIM1215 xenograft tumor-bearing nude mice. (A) Tumor volume of tumor-bearing mice treated with saline, A33Ab-US-Exo, Dox, A33-Exo/Dox, or A33Ab-US-Exo/Dox every 4 days × 4 *via* the tail vein. Dose: Dox equivalent 5 mg/kg. Mean ± SD, n = 5. * * P < 0.01 *vs.* Dox group. (B) Weight of the excised tumor tissues from all groups. Mean ± SD, n = 5. ns, not significant. * * P < 0.01 compared with the indicated groups. (C) Body weight changes of mice in all groups during treatment. Mean ± SD, n = 5. * * P < 0.01, A33Ab-US-Exo/Dox *vs.* Dox group. (D) Survival of tumor-bearing BALB/c nude mice treated with saline, A33Ab-US-Exo, Dox, A33-Exo/Dox or A33Ab-US-Exo/Dox. * P < 0.01 *vs.* Dox group. Mean ± SD, n = 4.
Printed with permission from ref [99].

neurons. These exosome nanobubbles are released into the extra-cellular circulation and body fluids, including blood, saliva, urine, breast milk, amniotic fluid, hydrothoracic fluid, and ascitic fluid, by exocytosis in response to a range of physiological or pathophysio-logical conditions [107]. In the last two decades, various studies have revealed that exosomes comprise nucleic acids and proteins linked to cancer, neurodegenerative, infectious, metabolic, and many other ailments [108,109]. Additionally, exosomes can be separated from biofluids that can easily be collected for testing, such as blood and urine, for diagnostic application.

Cancer diagnosis

Various cell types release exosomes, including cells from tumors. Exosome vesicles circulate in many biological fluids, including blood and urine, and can be considered possible circulating biomarkers for cancer diagnosis [110].

While exosome functionality seems to be determined by its specific protein content, proteomic characterization has been carried out on derived exosomes (*in vitro* and *in vivo*) in various studies. These analyses have shown that all exosomes contribute to certain general characteristics, such as lipid bilayer structure, density, size, and protein makeup. Exosomes consist of various biological sub-stances (Fig. 4), including 1116 lipids, 9769 proteins, 2838 miRNAs and 3408 mRNAs, according to the exosome database (http://www.exocarta.org). Some proteins, such as "signal transduction proteins (protein kinases, heterotrimeric G-proteins), cytoplasmic proteins (annexins and Rab proteins, tubulin, actin, actin-binding proteins), MHC class I molecules, and heat-shock proteins (Hsp70 and Hsp90), are commonly associated with all exosomes. The tet-raspanin (including CD9, CD63, CD8, and CD82) protein family is the most commonly linked with exosomes as a potential exosome marker for certain cancers [111]. Since cancer-originated exosomes show some commonly shared proteins, they also express a range of tumor antigens that reveal the initiating tumor cells. Although
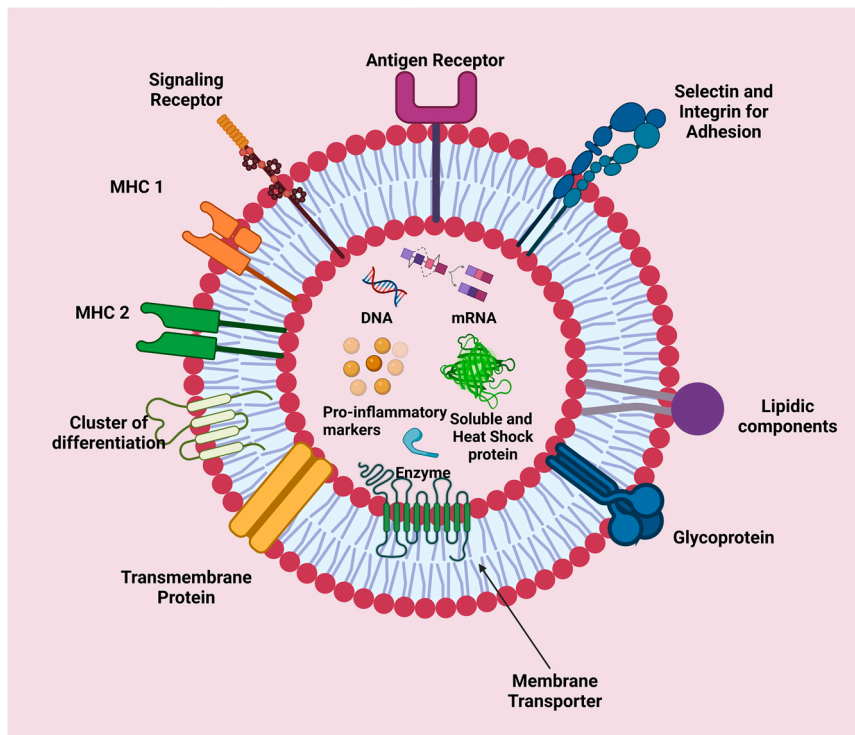
Fig. 4. Exosome biological features include many proteins, nucleic acids, lipidic components, membrane transporters and receptors.

exosome release can be exhibited in many proliferating cell types, exosome release is intensified in tumor cells, as demonstrated by their elevated presence in plasma, ascites and pleural malignant effusions of cancer patients [112]." Exosomes also provide information on diverse functionalities of tumors, such as growth, metastasis, immunomodulation, and therapy. In addition, exosome proteins are highly stable and protected from external enzymes by the lipid bilayer, and phosphorylated proteins can be isolated from frozen exosome samples for up to 5 years [113,114]. This exalted occurrence of exosomes in the numerous body fluids of cancer patients has led researchers to investigate the role of exosomes in cancer diagnosis [115]. Because CA IX is a good diagnostic for prostate cancer and its expression increases in hypoxia, the acidic microenvironment of tumors may alter CA IX expression and activity in human prostatic cancer cell lines and exosomes produced from them. Furthermore, WB analysis revealed that under acidic conditions, the expression of the CA IX 54 kDa band increased at both the cellular and exosome levels [116]. As a diagnostic biomarker, exosomes have been evaluated in several cancers, such as melanoma and ovarian, prostate, and cervical cancers, as described in Table 1. At the same time, clinical trials are listed in Table 2.

Exosomes as a diagnostic marker in pregnancy

EVs participate in many vital biological processes and facilitate fetal-maternal connections in healthy pregnancy development [128]. The placenta, umbilical cord, amniotic fluid, and amniotic membranes (Fig. 5) can produce exosomes. As a result, the amount of EVs is considerably increased in maternal circulation during pregnancy. The amount of placenta-derived EVs also increases in circulation with progressing gestational age. In addition, exosomes isolated from the maternal circulation during normal pregnancy exhibit changes in their bioactivity (described as the ability to induce cell migration) with advancing gestational age [129].

The critical role of EVs in angiogenesis, embryo implantation, and endothelial cell migration during pregnancy is also directly connected to various diseases, such as gestational diabetes mellitus (GDM), gestational hypertension, fetal growth restriction, and preterm birth. For example, exosomes are released due to oxidative stress in the placenta, and the number of placental exosomes in circulation is higher in preeclampsia than in normal pregnancy [130]. In addition, exosomes also assist in the transport of signaling molecules and their effect on sperm function, playing vital roles in male and female infertility (Fig. 5).

EVs have a time- and tissue-specific pattern and source cells during pregnancy and are important as biomarkers for women at risk of pregnancy-related diseases. In addition, early diagnosis and prognosis of disorders during pregnancy may be detected by isolation and analysis of the exosome cargo. Therefore, exosome vesicles could be used to diagnose/prognosis during pregnancy (Table 3).

Diagnosis of neurodegenerative disease

Parkinson's disease is a neurodegenerative disease in which there are many pathological changes in the structure of neurons. A major mutation and proliferation of alpha-synuclein genes lead to alpha-synuclein overexpression [132]. An *in vitro* study concluded that alpha-synuclein is transmitted to healthy cells from preinfected cells. This process is facilitated by membrane exosomes[133]. Therefore, the evaluation of exosomes helps in predicting the presence of disease. The separated exosomes were capable of transferring alpha-synuclein into healthy cells. Furthermore, it was not only limited to the cells, but it was observed that it could be successfully transferred into neurons, which might contribute to degeneration [134]. Another disease that is affected due to the transmitting property of exosomes is a prior disease. A study suggested that the abnormal isoform (PrP^{Sc}) of the main protein (PrP^{C}) is a major culprit for the spread of disease [135]. Exosomes bound to PrP^{C} infect the lymphoreticular system and can significantly transmit it to the brain, which leads to the destruction of crucial parts [136,137]. Exosomes can be used as biomarkers, but researchers are still muddled by their

Table 1

Exosomes as diagnostic biomarkers.

Cancer type	Biofluid	Associated proteins	Analytical technique	Reference
Breast cancer	Plasma samples	Plasma exosome microRNAs (miR-1246 and miR-21)	qRT–PCR	[117]
Lung cancer	Plasma	Higher levels of the tetraspanins CD151 and TSPAN8 and the cell adhesion molecule CD171 cancer.	EV array	[118]
Liver cancer		HepC2-derived Vasorin (VASN) protein	Western blot analysis and qRT–PCR	[119,120]
Prostate cancer	Urine	Novel miRNA-based PCa biomarkers in and used RT–qPCR	miR-196a-5p and miR-501–3p were downregulated in prostate cancer samples.	[121]
Prostate cancer	Plasma	Exosomal survivin	Human Survivin Immunoassay and Western Blot Analysis	[122]
Gastric cancer	Tissue	Gastrokine-1 (GKN1)	qRT–PCR	[123]
Cholangiocarcinoma	Uuman bile	Long noncoding RNAs	Real-time RT–PCR	[124]
Colorectal cancer	Plasma	Exosomal miRNAs (miR-126, miR-1290, miR-23a, and miR-940)	qRT–PCR	[125]
Oral squamous cell carcinoma (OSCC)	Plasma	Exosomal miR-130a	qRT–PCR	[126]

association, which leaves uncertainty regarding their development into potent biomarkers[136,137].

Exosomes for therapeutic delivery

Extracellular vesicles and exosomes, in particular, are highly stable particles enclosed with a protective membrane, making them potential candidates for delivering therapeutics. Furthermore, their biocompatible nature enables them to act as carriers for complex molecules such as RNA, DNA, proteins, peptides, *etc.*, thereby garnering immense attention from both the pharmaceutical and biopharmaceutical scientific communities. A particular property of exosomes, making them unique above other drug delivery carriers, is their capability to cross biological barriers and even invade the immune system, which is of prime importance when catering to a broad range of disorders, including cancer and ever-growing infectious diseases.

Proteins and peptides

Protein- and peptide-based therapeutics are some of the most widely researched and sought-after biomolecules, as evidenced by their wide presence in the pipeline of drugs approved by the FDA over the past decade [138]. Innovative formulation strategies are being devised for delivering these biomolecules owing to their degradation-prone physico-chemical profiles. Exosomes are recognized as potential delivery vehicles because of their important role in intercellular communication. A pseudotyping approach, commonly used for the production of recombinant viruses, was used by Lu et al. to load exosomal membranes with reported protein cargo [139]. They used a vesicular stomatitis virus glycoprotein (VSVG) and demonstrated its utility as a genetically encoded pseudotyping platform for loading and enhancing the intracellular delivery of therapeutic proteins *via* exosomes [139]. An exosomal vaccine approach was explored by Kuate et al. for developing a system against SARS coronavirus wherein they replaced the cytoplasmic and transmembrane domains of the G protein of VSV with the S protein of SARS coronavirus [140]. The approach was tested *in vivo* in mice. It was as effective as the adenoviral vector vaccine in inducing neutralizing antibody titers and in a SARS-S-expressing tumor challenge model [140]. It has been found that in the case of diseases where the classical vaccine approach exhibits limitations, a targeted exosome-based strategy can be utilized to enhance therapeutic efficacy. Exosomal targeting significantly enhanced antitumor effects in a HER2 + transgenic animal model [141].

In addition to carrying therapeutic protein cargo, exosomes have also shown efficacy when decorated with membrane proteins on their surface to achieve targeting. For example, exosomes functionalized with TNF-related apoptosis-inducing ligand (TRAIL) on their membrane were found to induce apoptosis in cancer cells and control tumor progression *in vivo* upon systemic administration [142]. The system was proposed as a platform for delivering genetic material for intratumor therapies. Engineered exosomal vesicles can also be used to deliver membrane proteins into cells. A biocompatible virus-mimetic fusogenic exosome platform consisting of fusogenic exosomes with viral fusogen and VSVG was developed to deliver integral membrane proteins inside cell membranes *in vitro* and *in vivo* using GLUT4 as a model protein [143]. The delivery strategy posed by exosomes for protein and peptide therapeutics looks very promising; however, it still needs to overcome obstacles such as the development of standardized protocols for large-scale production, maintaining the purity of actives, deciding on an ideal human dose, immunogenicity concerns and achieving efficient therapeutic payloads [144].

Table 2
Clinical trials examining exosomes for cancer diagnosis and patient response to therapy.

Clinical Trial No	Tumor Type	Purpose	Status
NCT02977468	Triple Negative Breast Cancer	Assess response and change in tumor to Pembrolizumab	Recruiting
NCT02892734	HER2 Negative Inflammatory Breast Cancer	Monitor patient response to combination of Ipilimumab and Nivolumab	Terminated
NCT02662621	Infiltrating Nonmetastatic Breast Cancer	Quantify exosomes displaying HSP70 membrane protein in blood and urine for solid tumor diagnostic	Completed, no results posted
	Breast Cancer with First Metastasis Ovarian Cancer, Stages III and IV Metastatic NSCLC		
NCT02971761	Androgen Receptor Positive and Triple Negative Breast Cancer	Monitor patient response to combination of Pembrolizumab and Enobosarm	Active, not recruiting
NCT03830619	Lung Cancer	Detection of exosomal long noncoding RNA as potential lung cancer diagnosis	Recruiting
NCT03317080	Lung Cancer	Monitor circulating tumor DNA in surgical patients with lung cancer	Recruiting
NCT02921854	NSCLC	Monitor response to radiotherapy and chemotherapy by analyzing patient serum	Completed, no results posted
NCT02869685	NSCLC	Monitoring plasma exosomal levels of PD-L1 before and after radiotherapy	Unknown
NCT03108677	Primary Osterosarcoma, Lung metastases	Analyze RNA profile of circulating exosomes for early detection of metastases	Recruiting
NCT03228277	T790 M Positive NSCLC	Monitor response to Olmutinib in T790 + NSCLC using DNA extracted from bronchial lavage fluid	Completed, no results posted
NCT03432806	Colon Cancer, Liver Cancer	Guide treatment of colon and liver cancers	Recruiting
NCT02439008	Colon Cancer, Live Cancer, Kidney Neoplasms	Monitor response to radiotherapy before, during, and after administration	Terminated
NCT02393703	Pancreatic Cancer	Use exosomes to monitor patients	Active, not recruiting
NCT03032913	Pancreatic Cancer	Patient disease monitoring[127]	Completed
NCT03791073	Pancreatic Cancer	Analyze interstitial tissue fluid for novel biomarker identification	Recruiting
NCT03250078	Pancreatic Cancer	Development of blood-based screening for pancreatic cancer	Recruiting
NCT03334708	Pancreatic Cancer	Early stage pancreatic cancer diagnosis	Recruiting
NCT03410030	Pancreatic Cancer	Using GPC1-labeled exosomes, and additional biomarkers for monitoring patient response to combination of Ascorbic Acid, nanoparticle Paclitaxel protein bound Cisplatin and Gemcitabine	Recruiting

Genetic materials

Exosomes exhibit an inherent cargo-carrying capacity, especially toward materials of genetic origin, such as DNA and RNA, to their target cells. Therefore, they have been utilized widely to design gene therapy [145]. They also serve to protect these unstable genetic materials from degradation. Therefore, siRNA delivery to target cells using exosomal carriers has been a sought-out strategy. In a study, exosomes derived from embryonic stem (ES) cells were found to induce changes in hematopoietic progenitor cells (HPCs) by stimulation using surface ligands and delivery of ES-derived mRNA, further evidencing the intracellular horizontal transfer of genetic information [146].
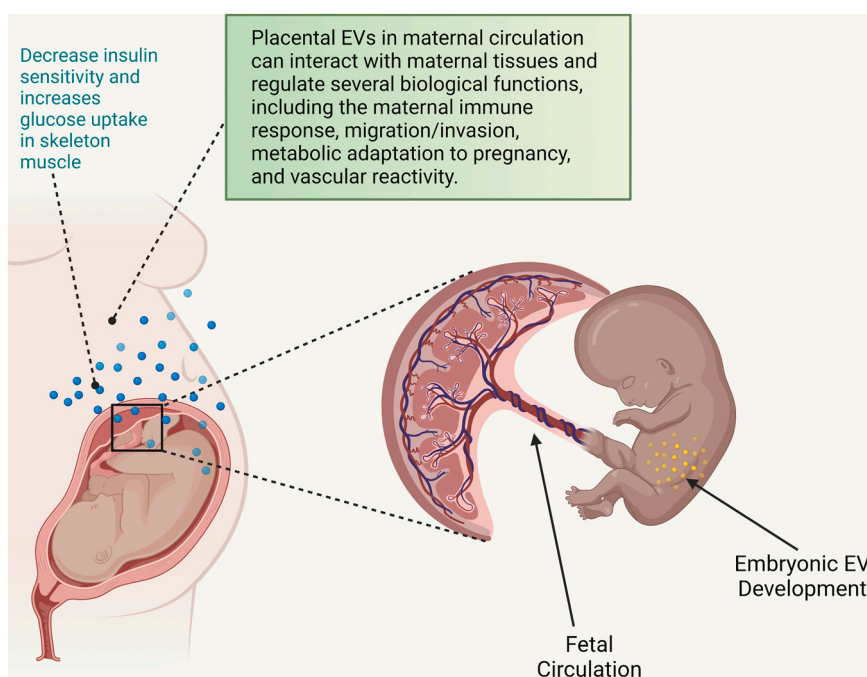
Fig. 5. EV circulation during pregnancy. EVs—including small EVs such as exosomes and large EVs such as microvesicles and apoptotic bodies—are released from the human placenta into maternal and fetal circulation during pregnancy. Placental EVs in maternal circulation can interact with maternal tissues and regulate several biological functions, including the maternal immune response, migration/invasion, metabolic adaptation to pregnancy, and vascular reactivity. In addition, EVs present in fetal circulation are associated with fetal development. GDM, gestational diabetes mellitus; PE, preeclampsia.

Table 3
The clinical value of EVs in pregnancy complications [131].

Pregnancy complications	Source of EVs	Pregnancy stage	Targets	Isolation method	Detection method	Clinical value
Preeclampsia	Plasma	Early	Total exosomes, exosomal PLAP	Ultracentrifugation, differential centrifugation	ELISA	Elevated in PE at early pregnancy (AUC 0.745 and 0.829)
	Plasma	Late	Exosomal PLAP to total exosomes ratio	Ultracentrifugation	ELISA	Reduced in PE; lower in late-onset PE than early-onset PE
	Plasma	Late	miRNAs profile	Commercial kit	Nanostring counter system miRNA assay	Potential markers of PE and subtypes of PE
	Plasma	Mid and late	miR-210	Commercial kit	qRT–PCR	Elevated in PE; higher in severe PE
	Plasma	Early	miR-486-1-5p, miR-486-2-5p	Ultracentrifugation, differential centrifugation	RNA sequencing	Elevated in PE at early pregnancy
	Plasma	Mid and late	miR-136, miR-494, miR-495	Ultracentrifugation	qRT–PCR	6.4-, 3.9- and 2.1-fold higher in PE than normal pregnancy
	Serum	Late	miR-155	Ultracentrifugation, differential centrifugation	qRT–PCR	Elevated in PE
	Serum	Late	miR-548c-5p	Commercial kit	qRT–PCR	Reduced in PE
	Plasma	Early	miR-517-5p, miR-520a-5p, miR-525-5p	Commercial kit	qRT–PCR	Reduced in PE at early pregnancy (AUC 0.719)
	Plasma	Late	PLAP⁺NEP⁺ EVs	Size exclusion chromatography	FCM	Elevated in PE
	Urine	Late	Podocin⁺ EVs-to-nephrin⁺ EVs ratio	Without isolation	FCM	Elevated in PE; correlated with renal injury
	Urine	Late	ENaC, NKCC2	Differential centrifugation	WB	Elevated in PE; correlated with renal injury
Gestational diabetes mellitus	Plasma	Early, mid and late	PLAP⁺EVs	Ultracentrifugation, differential centrifugation	ELISA	2.2-fold higher at early gestation in GDM than normal pregnancy
	Plasma	Early, mid and late	PLAP per exosome	Ultracentrifugation, differential centrifugation	ELISA	63% lower at early gestation in GDM than normal pregnancy
	Oral fluid	Early	Total exosomes	Commercial kit	NTA	Elevated in GDM at early pregnancy (AUC 0.81)
	Plasma	Late	miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p, miR-224-5p	Ultracentrifugation, differential centrifugation	RNA sequencing, qRT–PCR	Elevated in GDM; related to metabolism
	Serum	Early	10 miRNAs	Differential centrifugation	qRT–PCR	Elevated in GDM at early pregnancy
	Plasma	Late	DPPIV⁺PLAP⁺ EVs	Without isolation	FCM	Eightfold higher in GDM than normal pregnancy
Preterm birth	Plasma	Late	78 proteins	Ultracentrifugation	SWATH-MS	Potential markers of GDM
	Plasma	Early, mid and late	173 miRNAs	Ultracentrifugation	RNA sequencing	Potential markers of PTB
	Urine	Late	16S rRNAs derived from Ureaplasma and Veillonellaceae	Differential centrifugation	RNA sequencing	Elevated in PTB
	Plasma	Late	72 proteins	Differential centrifugation, size exclusion chromatography	SWATH-MS	Potential markers of PTB
Fetal growth restriction	Plasma	Early	62 proteins	Size exclusion chromatography	LC–MS	PTB predictor at early pregnancy
	Plasma	Late	PLAP⁺ exosomes to total exosomes ratio	Ultracentrifugation, differential centrifugation	NTA	Reduced in FGR; corrected with birth weight percentile
	Serum	Mid	miR-20b-5p, miR-942-5p, miR-324-3p, miR-223-5p, miR-127-3p	-	RNA sequencing	Elevated in FGR

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; LC–MS, liquid chromatograph-mass spectrometer; NTA, nanoparticle tracking analysis; qRT–PCR, quantitative polymerase chain reaction; SWATH-MS, sequential windowed acquisition of all theoretical mass spectra; WB, Western blotting.

Advanced systems such as exosome-liposome hybrids have also been investigated for gene delivery to expand the application scope of exosomes. The freeze−thaw method was used by Sato et al. for fusing exosomal membranes with liposomes to develop a hybrid exosome system [147]. Cellular uptake studies revealed that this approach could transport exogenous hydrophobic lipids to target cells along with hydrophilic cargoes within exosomes, leading to a versatile drug delivery platform [147]. Another advanced system termed NanoMEDIC was developed based on ribonucleoprotein using a dual mechanism: CRISPR−Cas9 protein insertion using chemically induced dimerization and a combination of viral RNA packaging signal with two self-cleaving riboswitches for releasing sgRNA into the nanovesicles [148]. Genetic materials have been exploited in numerous complex therapies, including chemotherapy, antiviral, and neurological therapies; hence, these have been described individually in the following subsections.

Chemotherapy

Cancer has been hovering over human civilization for an eternity, and efforts to find ways to combat old and newly emerging cancers are persistent globally. Research conducted in this direction over the past decade has revealed numerous limitations of the existing therapeutic strategies involving conventional chemo- and radio-therapies. To overcome these limitations, advanced drug delivery vehicles such as nanoparticles of both polymeric and lipidic origin have been developed to achieve longer circulation times and targeting efficiency in specifically designed systems. However, their fate in the reticuloendothelial system is still a challenge that restricts these vehicles from achieving therapeutic efficacy. Although first reported in 1987, exosomes are now identified as potential carriers for the delivery of complex therapeutics such as anticancer drugs. The inherent nature of exosomes confers them with the ability to enable intercellular communication [149]. Numerous scientists have explored this property; for example, Wang et al. revealed that exosomes derived from lung cancer stem cells promoted the migration and invasion of cancer cells due to the specific binding of exosomal miR-201−3p to FGFRL1 (fibroblast growth factor receptor-like 1), thereby emphasizing the targeting potential of exosomes [150]. In another study, high-yield and high-purity exosomes were isolated from human embryonic kidney cells (HEK-293), and siRNA labeled with Atto655 was loaded into the exosomes using electroporation followed by excess removal using gel filtration [151]. The exosome isolation technique using ultracentrifugation onto a sucrose cushion gave good yield and purity, and the exosomes delivered siRNA into the cancer cells *in vitro* [151]. Apart from their passive targeting ability, exosomes have also been designed to perform active targeting in cancer. For instance, Leonard et al. developed a platform for actively loading engineered RNAs into exosomes using a targeted and modular EV loading (TAMEL) approach using exosome-mediated delivery of mRNA and protein to a prostate cancer cell-based model system [152].

Exosomes isolated from bovine milk and coated with bcl-2 siRNA were found to dramatically inhibit the migration and invasion of cancer cells, making them a promising therapeutic alternative [153]. Mesenchymal stem cells (MSCs) are another important source of exosomes and have been widely studied for their drug-carrying properties. Genetically engineered MSCs have also been developed for deriving target-specific exosomes; for instance, exosomes derived from 5TR1 aptamer-modified MSCs were used to deliver doxorubicin (DOX), and they could specifically bind to the Mucin-1 receptor on the surface of colorectal cancer cells, thereby reducing drug-related cytotoxicity in healthy cells [154]. Dendritic cell (DC)-derived exosomes are also being studied for their role as drug carriers. The protein tyrosine kinase 7 (PTK7)-specific aptamer sgc8 was used as a targeting moiety on the surface of DC-derived exosomes, and it was found to deliver DOX to cancer cells overexpressing PTK7.

These Apt-Exos (aptamer-functionalized exosomes) were proposed as a promising cancer theranostic platform [155]. Scientists have also developed exosomes derived from cancer cells to explore the Trojan horse concept. Indocyanine green and DOX were loaded in tumor cell-derived exosome-camouflaged porous silicon nanoparticles and were used as a synergistic system for chemotherapy and photothermal therapy against breast cancer. The system could accumulate in the tumor tissue in a BALB/c mouse tumor model and inhibit its growth and metastasis, making it a promising drug delivery carrier for combination chemotherapy [156]. Another category studied for exosome synthesis is erythrocyte-derived exosomes. A new strategy included the insertion of phosphatidylcholine into the membrane lipid layer of reticulocyte-derived exosomes. Two model therapeutic agents, namely, DOX and anti-miR21, were loaded into the formed system, and remarkable accumulation into cancer cells was observed along with enhanced *in vitro* antitumor activity, thereby making the system a potential drug delivery carrier [157]. Federici C et al. studied the role of extracellular acidosis and nanovesicle (exosome) release in human tumor cell resistance to cisplatin (CisPt), as well as proton pump inhibitors (PPI) potential to interfere with these tumor cell properties [70]. Low pH settings significantly reduced CisPt absorption by human tumor cells, according to the findings. Compared to untreated cells, PPI pretreatment boosted the cellular absorption of CisPt in an acidic-dependent manner. Further investigation revealed that *in vivo* PPI therapy resulted in a significant decrease in the plasmatic levels of tumor-derived exosomes, which also contained reduced amounts of CisPt. Overall, these data lead to the discovery of a dual mechanism that human malignant melanoma uses to resist a horrible cellular toxin such as cisplatin. This resistance system incorporates both low pH-dependent extracellular sequestration and exosome-mediated clearance. Proton pump inhibition significantly impairs both processes, resulting in enhanced CisPt-dependent cytotoxicity.

Antiviral therapy

Exosomes are being highly exploited for their potential in diagnosing and treating viral invasion because of their ability to carry biological markers and enable a targeted approach toward infections. It has been reported that some specific exosomes, such as macrophage exosomes, possess a cell entry mechanism similar to that of invading hepatitis B virus (HBV) and thereby show the efficiency of delivering interferon alpha (IFN-α), resulting in anti-hepatitis B virus activity [158]. Another study was performed on clinical specimens of exosomes from patients treated with PEGylated IFN-α and supernatants of IFN-α-treated macrophages, and exosomal miRNAs were analyzed [159]. Exosomes can transfer IFN-α-related miRNAs from macrophages to HBV-infected hepatocytes, further possessing anti-HBV activity against the replication and expression of the virus [159]. In addition, interferon-inducible transmembrane proteins (IFITMs) 1, 2, and 3 have been identified as potential effectors of enveloped viral infections. In particular, intracellular and extracellular levels of IFITM3 can be utilized for predicting dengue virus infection, thereby suggesting exosome-mediated IFITM3 delivery as a potential anti-dengue viral therapy [160]. Such findings pave the way for in-depth research toward applications of exosomes in antiviral therapies.

Multiple components of exosomes have been found to play a role in HIV infection, making them effective prediction tools for the disease [161]. Exosome markers from plasma were investigated for their role in HIV pathogenesis in patients receiving anti-retroviral therapy (ART) [162]. The results of this study suggested that the exosomes in these patients were carriers of proteins responsible for immune activation and oxidative stress; they further showed immunomodulatory effects on myeloid cells, and Notch4 was suggested to be a potential biomarker of immune activation in HIV infection [162]. The contribution of exosomes to the pathogenesis of

Table 4

Clinical trial summary of exosomes used in the delivery of therapeutics [168–174.]

Clinical trial No	Conditions	Study Title	Status
NCT05043181	Familial Hypercholesterolemia	Exosome-based nanoplatform for Ldlr mRNA delivery in FH	Not yet recruiting
NCT04879810	Irritable Bowel Disease	Plant exosomes + /- curcumin to abrogate symptoms of inflammatory bowel disease	Recruiting
NCT03608631	KRAS NP_004976.2:p.G12D Metastatic Pancreatic Adenocarcinoma Pancreatic Ductal Adenocarcinoma Stage IV Pancreatic Cancer AJCC v8	Phase I Study of Mesenchymal Stromal Cells-Derived Exosomes With KrasG12D siRNA for Metastatic Pancreas Cancer Patients Harboring KrasG12D Mutation	Recruiting
NCT01294072	Colon Cancer	Phase I Clinical Trial Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Malignant Colon Tissue	Recruiting
NCT01854866	Malignant Pleural Effusion, Malignant Ascites	Phase II Study of Tumor Cell-derived Microparticles Used as Vectors of Chemotherapeutic Drugs to Treat Malignant Ascites and Pleural Effusion	Unknown
NCT02657460	Malignant Pleural Effusion	Clinical Trial of Tumor Cell-derived Microparticles Packaging Chemotherapeutic Drugs to Treat Malignant Pleural Effusion	Unknown
NCT03857841	Bronchopulmonary Dysplasia	A Safety Study of Intravenous Infusion of Bone Marrow Mesenchymal Stem Cell-derived Extracellular Vesicles (UNEX-42) in Preterm Neonates at High Risk for Bronchopulmonary Dysplasia	Terminated (The study was discontinued due to a business decision; no safety concerns were noted).

retroviral infections such as HIV and HTLV-1 (human T-cell leukemia virus), particularly in the multiplication and infection phases, provides a broad avenue for their exploration in the prevention and therapy of retroviral infections [163]. Exosomes are being explored during the ongoing COVID-19 pandemic to develop a therapeutic strategy. MSC-derived exosomes are widely researched for numerous applications. Their impacts on the complications arising from COVID-19, such as cytokine storms, acute respiratory distress syndrome, and acute lung injury, are being studied to further this research [164]. Potential exosome-based therapies highlighted for COVID-19 include using the MSC secretome, incorporating specific miRNAs and mRNAs into exosomes, and using exosomes as drug delivery carriers [165]. MSC-derived exosomes are also a potential tool for treating SARS-CoV-2 pneumonia due to their anti-inflammatory, immunomodulatory, and tissue regeneration abilities [166]. Clinical trials on the study of aerosol (inhalation) and intravenous administration of MSC-derived exosomes for treating SARS-CoV-2 pneumonia have also been undertaken. A clinical study was conducted on 24 PCR-positive COVID-19 patients to evaluate the safety and efficacy of one marrow MSC-derived exosome delivered intravenously. Single-dose therapy showed a significant reduction in cytokine storms with improvement in oxygenation, thereby exhibiting promising outcomes [167]. Table 4 summarizes the clinical trial summary of exosomes used to deliver therapeutics.

Other therapies

Targeted exosomes were developed by engineering dendritic cells to express the exosomal membrane protein Lamp2b fused with the neuron-specific RVG peptide [175]. Electroporation was used to load the GAPDH siRNA into the vesicles to achieve specific gene knockdown in the brain. Sixty percent knockdown of mRNA and 62% knockdown of protein of the Alzheimer's therapeutic target BACE1 were demonstrated in the studies performed, showcasing the therapeutic potential of the developed system [175]. A core-shell hybrid-modified exosomal system comprising RVG peptide-modified exosome curcumin/phenylboronic acid-poly(2-(dimethylamino)ethyl acrylate) nanoparticle/small interfering RNA targeting SNCA was developed for the treatment of Parkinson's disease. The developed nanoscavenger system was proposed as a platform for clearing α-synuclein aggregates to reduce their cytotoxicity to aid in neurodegenerative therapy [176]. Neuron-targeted exosomes were fabricated as coating materials to enable brain-targeted delivery of gold nanoparticles. The exosomes were engineered to express RVG peptide

– Lamp2b fusion protein to allow specificity to the gold nanoparticles and help them cross the blood−brain barrier [177].

MSC-derived exosomes have also been reported to possess unique features that could attenuate their use in managing kidney injuries. Exosomes derived from specific and conditioned media, such as human urine, human placenta, human amnion epithelial cells, and glomeruli, have been shown to play a role in renoprotection [178]. MSC-derived exosomes were found to follow the Keap1-Nrf2 signaling pathway to accumulate in injured renal tissue and into renal proximal tubular epithelial cells, thereby promoting kidney repair [179]. Exosomal miR-146a-5p was explored to target and degrade the 3'UTR of interleukin-1 receptor-associated kinase 1 (IRAK1) mRNA followed by inhibition of nuclear factor (NF)-κB signal activation and restriction in the infiltration of inflammatory cells to achieve renoprotection [180].

Challenges for exosomes as therapeutic carriers

Exosomes have been considered one of the most important EVs with enormous potential to be therapeutic carriers; however, designing and engineering exosomes that can portray drug delivery specificity is an ongoing challenge. The tools and machinery to produce uniform exosomal carriers are most difficult to assemble, along with putting the quality control criteria in place at every step of production. The commercial viability of exosomes as therapeutic cargoes would require close monitoring of the mechanical and purification parameters of the final delivery system. Batch-to-batch consistency would be needed to ensure worldwide approval by leading regulatory bodies. Citing the biological nature of these carriers and the ultimate delivery system, a close check on the stability and allied efficacy of the systems would need to be assured by multiple evaluation studies. Visualization of the exosomes being produced at the molecular level will have to be conducted to allow in-process quality checks, and methods such as next-generation sequencing or NGS would pave the way for such specific analysis during the production and scaling of these advanced drug delivery systems[180].

Theranostics potential of exosomes

Inherent structural and biocompatible features, the ability to traverse biological barriers and accumulate at specific sites, and the feasibility of incorporating various diagnostic or imaging and therapeutic agents enable the development of exosomes as personalized

theranostic platforms. However, it is challenging to simultaneously incorporate contrast agents/biomarkers and therapeutic cargo within exosomes. Most of the attempts at developing theranostic exosomes involved the use of inorganic nanoparticles. In addition to reproducible preparation, inorganic nanoparticles offer the advantages of biocompatibility, multifunctionality, and stability and further facilitate long-term *in vivo* tracking [181]. These nanoparticles may be incorporated into exosomes postisolation by surface conjugation or using techniques including electroporation, extrusion, or sonication. The chemical linkers for conjugation or nanoparticle fusion techniques should be chosen so that the exosomal structural integrity is least affected. Irrespective of the method of incorporation, the size and surface properties of the nanoparticles seem to influence the formation of nanoparticle-loaded exosomes, with smaller nanoparticles exhibiting higher loading efficiency [182]. Most preclinical studies exploring the theranostic potential of exosomes were aimed at monitoring cancer therapy and are discussed in the following section.

Theranostic applications of exosomes in cancer

Jia et al. incorporated "superparamagnetic iron oxide nanoparticles" (SPION) and curcumin into exosomes isolated from RAW264.7 macrophage cells using electroporation. Furthermore, these exosomes were conjugated with RGERPPR peptide by click chemistry to enable targeting to Neurolipin-1, a transmembrane glycoprotein overexpressed in glioma cells. These exosomes could penetrate the blood−brain barrier and aid in targeted theranostics of glioma. SPION enabled magnetic hyperthermia and magnetic resonance imaging (MRI), while curcumin showed a potent anticancer effect [183]. Pan et al. fabricated multifunctional nanocomposites of amphiphilic polymer-coated gold nanoparticles (AuNPs) and a Ce6 photosensitizer trapped in bovine serum albumin (BSA) networks. These nanocomposites were further incorporated within urinary exosomes *via* electroporation, which could generate temporary nanopores in the exosome plasma membrane. The high surface area of AuNPs enabled the encapsulation of large amounts of Ce6 into exosomes. Upon injection into tumor-bearing nude mice, they showed deep penetration and retention in tumors, enabling fluorescent detection 72 h postinjection and higher efficacy in photodynamic therapy [184]. Recently, radiolabeled exosomes were engineered to incorporate the Fc portion of lgG2b to serve as a theranostic system that accumulates in the tumor and augments antibody-dependent cell-mediated cytotoxicity (ADCC). Exosomes isolated from HEK293 cells transfected with a plasmid encoding the Lamp2b construct were modified with precision peptide for CD206-positive M2 macrophages. The *in vivo* targeting ability of the engineered exosomes labeled with DiI was studied in 4T1 tumor-bearing Balb/c mice using tissue immunofluorescence staining. These exosomes were labeled with indium-oxine (^{111}In-oxine), and a further biodistribution study using *in vivo* SPECT and CT imaging revealed their accumulation in the tumor, lungs, spleen, lymph nodes, and bones of tumor-bearing mice. In addition, the Fc portion of IgG2b expressed by these exosomes successfully induced ADCC and resulted in decreased tumor growth and prolonged survival of treated animals [140]. In another study, macrophage-derived exosomes could be successfully incorporated with acridine orange (AO), an acidophilic photodynamic dye with strong tumoricidal action. The uptake and cytotoxic effect of the AO-incorporated exosomes could be monitored using cytofluorimetry. They were rapidly internalized and retained for longer than free dye in melanoma cells and 3D spheroids. Furthermore, the exosomal delivery system preserved the mechanism of action of AO and enhanced its tumoricidal effect by increasing the exposure time of the biological targets[83,185,186].

Another strategy would be nanoparticle incorporation *via* a biological pathway where functionalized nanoparticles are internalized

within exosome-secreting cells following incubation for a certain period under stressed culturing conditions. Furthermore, during exosome formation, they are spontaneously incorporated into secreted exosomes [141]. In one of the earlier studies, Silva et al. reported endothelial cell-derived hybrid nanovesicles loaded with multiple components, including iron oxide spherical nanoparticles and nanocubes, quantum dots, AuNPs, and gold/iron oxide nanodimers, which conferred theranostic potential in terms of magnetic targeting, MRI-based tracking, and magnetic hyperthermia therapy. These nanomaterials were first internalized into human umbilical vascular endothelial cells (HUVECs), followed by the induction of vesicle release and magnetic sorting of the vesicles from the conditioned medium. All these nanoparticle-loaded vesicles could be guided using an applied magnetic field gradient and tracked *via* MRI imaging. In the case of the encapsulation of quantum dots, individual nanovesicles could be detected *in vitro via* fluorescence. Furthermore, the authors established a proof-of-concept for magnetic hyperthermia by noting the increase in temperature at different magnetic field frequencies [142]. Similarly, they developed macrophage-derived microvesicles incorporated with iron oxide nanoparticles and different therapeutic agents, including Dox, tissue-plasminogen activators, and photosensitizers. They further demonstrated the *in vitro* magnetic targeting, MRI detection, and modulated therapeutic effect of these microvesicles [143]. Incorporating SPIONs in exosomes also enabled magnetic particle imaging (MPI), an emerging noninvasive tomographic technique with submicromolar sensitivity, high spatial resolution, and lack of ionizing radiation. Exosomes were generated from MDA-MB-231 breast cancer cells incubated with SPIONs in exosome-depleted medium using an exosome purification kit (ExoQuickTM, System Bioscience, Mountain View, CA, USA). The authors observed that the generation of exosomes was higher when the cells were exposed to hypoxia and X-ray ionizing radiation. Furthermore, the generated exosomes showed higher internalization in hypoxic cancer cells. They were incubated with lipophilic tracers DiO and DiI to enable fluorescence imaging and flow cytometry, while a therapeutic drug, olaparib, was incorporated *via* electroporation. The intratumorally injected hypoxic exosomes could be detected *in vivo via* MPI imaging, while the intravenously administered exosomes did not accumulate enough to yield a detectable signal from the tumor [144].

The biogenic method of nanoparticle incorporation in exosomes has yielded higher loading efficiency than incubation or physicochemical methods. For instance, Sancho-Albero et al. compared the efficiency of internalization of 45 nm PEGylated AuNPs in murine melanoma cell (B16F10 cell)-derived exosomes using different methods. Approximately 50% internalization of PEGylated AuNPs was observed *via* the exosome biogenesis pathway compared to less than 20% internalization *via* diffusion, thermal shock, sonication, and saponin-assisted loading methods. In addition, the PEGylated AuNP-incorporated exosomes exhibited reflective optical properties, enabling *in vitro* cell tracking and generating localized heat when irradiated with NIR light, thereby establishing their theranostic potential [187]. The same group had previously shown that these PEGylated AuNP-loaded exosomes generated *via* the biogenesis pathway showed preferential uptake by the parent cell line (human placental mesenchymal stem cells, MSCs), even under coculture conditions, suggesting the use of exosomes as targeted delivery vehicles in hyperthermia management. They also demonstrated a selective *in vitro* photothermal effect of these PEGylated AuNP-loaded exosomes in MSCs on cocultures of MSCs, B16-F1 and B16-F10 melanoma cells, and monocytes [188].

In another study, Lara et al. used folic acid-conjugated AuNPs (approximately 26 nm) to ensure enhanced uptake in malignant B16F10 melanoma cells and further isolated EVs with nanoparticles labeled within and on the outer side of the vesicular membrane. These labeled EVs showed preferential uptake in B16F10 melanoma
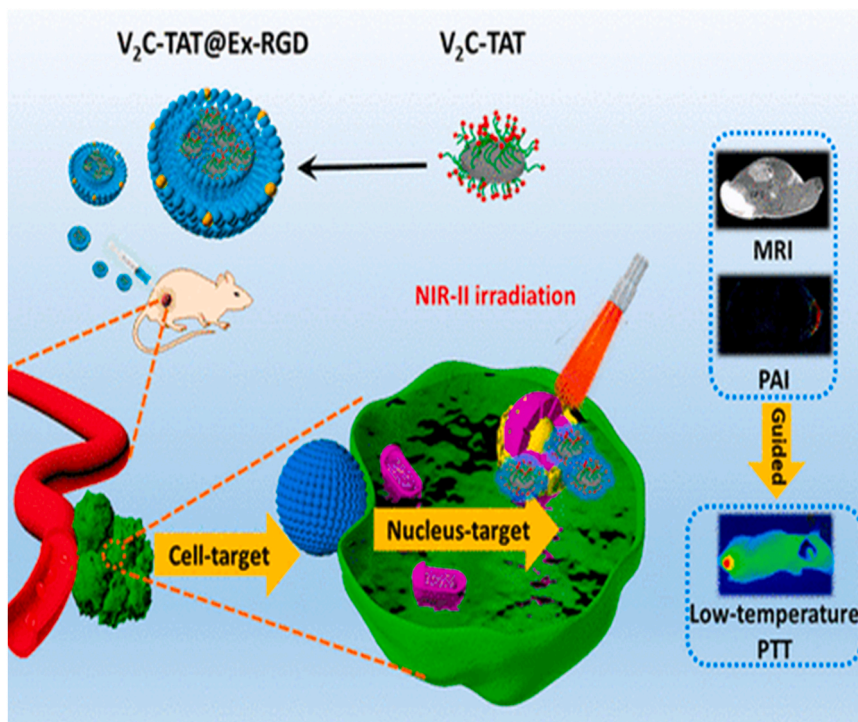
Fig. 6. Schematic diagram depicting the incorporation of TAT peptide-modified vanadium carbide quantum dots (V2C QDs) in RGD peptide-functionalized exosomes. These exosomes enabled the targeting of cancer cells and further nuclear targeting upon internalization. They could also aid multimodal image-guided photothermal therapy by absorbing NIR-II wavelengths at low temperatures.
Printed with permission from ref [190].

cells in metastatic tumors, enabling increased delivery of Au NPs, which could benefit theranostic applications [189]. Finally, Cao et al. developed multifunctional vanadium carbide quantum dots (V2C QDs) that showed an intense photothermal effect in the NIR-II region (1000–1350 nm) and enabled multimodal imaging, including fluorescent imaging, photoacoustic imaging, and MRI. These QDs were functionalized with cell nuclei-targeting TAT peptide and encapsulated within RGD peptide-modified exosomes (V2C-TAT@Ex-RGD), which could specifically recognize cancer cells (Fig. 6) [190].

Bose et al. explored tumor-derived EVs as multifunctional theranostic platforms. They demonstrated the loading and delivery of antisense miRNA targeting oncogenic miR-21 (anti-miR-21) over-expressed in cancer cells and further functionalized these EVs with gold-iron oxide nanoparticles. Gold-iron oxide nanoparticles have been investigated as contrast agents for multimodal imaging techniques, including CT, MRI, photoacoustic imaging, and surface-enhanced Raman spectroscopy. In addition, they facilitate the selective thermal ablation of cancerous cells [191,192]. Anti-miR-21 delivery *via* extracellular vesicles attenuated Dox resistance in cancer cells and showed threefold higher cell death when delivered along with DOX. The *in vivo* biodistribution of these multifunctional EVs was studied using ICG dye-mediated NIR imaging and T2-weighted MR imaging. At the same time, the anticancer therapeutic efficiency of the same could be monitored *via* bioluminescence imaging in 4T1 syngeneic tumor mouse models (Fig. 7) [193].

Another category of nanoparticles extensively studied for drug delivery, bioimaging, and theranostic applications includes luminescent porous silicon nanoparticles (PSiNPs) [194,195]. Dox-loaded PSiNPs (150 ± 11 nm) were incubated with human hepatocarcinoma Bel7402 cells, and the exocytosed Dox-loaded PSiNPs (260 ± 15 nm) were collected by centrifugation. These exosome-sheathed Dox-loaded PSiNPs showed enhanced cellular uptake and cytotoxicity against cancer stem cells and significantly attenuated P-gp expression, thereby reducing drug resistance. They also showed enhanced

tumor accumulation and anticancer efficacy in different tumor-bearing mouse models [196]. Lessi et al. found that exo-acridine orange (AO) had a longer drug delivery time to melanoma cells than free AO, which improved AO cytotoxicity [185]. Their research indicates that Exo-AO has high potential for real-world use as a novel theranostic strategy against malignancies based on AO given by exosomes.

Challenges for exosome-based theranostics

To summarize, the exosome-mediated delivery of therapeutics, radionuclides, and imaging agents can act as a promising theranostic platform. However, a few challenges remain owing to the use of different cell lines as sources of exosomes, various types of hybrid nanosystems, difficulty in the large-scale generation of exosomes and lack of standardized modification strategies. Furthermore, clinical trials exploring either diagnostic or therapeutic applications of exosomes have only been reported to date. Most studies examining the theranostic potential of exosomes are limited to preclinical evaluations. In addition, there is a need to develop reproducible and standardized protocols for studies involving engineered exosomes, and the long-term safety of such systems after *in vivo* administration needs to be evaluated. Thus, several technical hurdles still exist before cost-effective, less time-consuming, large-scale production and clinical translation of theranostic exosomes become a reality.

Future prospects

The unique morphological/structural/compositional peculiarities of exosomes from the nanoscale effect, their intrinsic biological features as important conveyers for intercellular communications, and their multiple functions at different levels have been determined recently, promoting rapid development in exosome-based science. Exosomes have now been implicated in many biological and
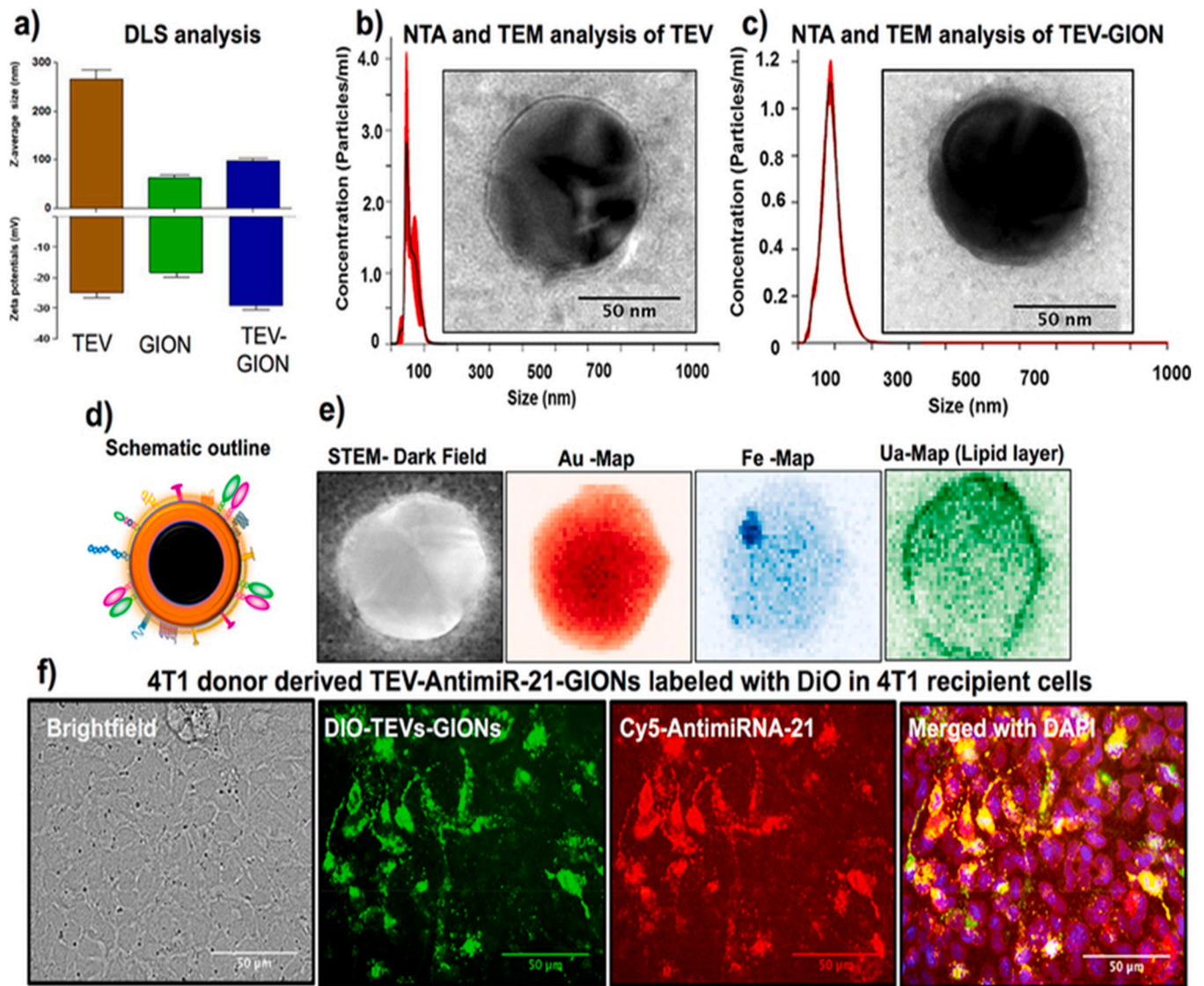
Fig. 7. Characterization of tumor-derived exosomes incorporating gold-iron oxide nanoparticles for anti-miR-21 delivery and theranostic imaging.
(a) Size and surface charge of tumor-derived exosomes (brown), gold-iron oxide nanoparticles (green), and exosomes incorporating gold-iron oxide nanoparticles (blue); nanoparticle-tracking analysis and transmission electron microscopy image of (b) gold-iron oxide nanoparticles and (c) tumor-derived exosomes incorporating gold-iron oxide nanoparticles; (d) schematic outline shows the shape of exosomes incorporating gold-iron oxide nanoparticles; (e) STEM−EDX results showing the hybrid bimetallic core of gold−iron oxide within tumor-cell-derived EVs (lipid layer); and (f) transfection of gold−iron oxide nanoparticle-incorporated (labeled with DiO) for Cy5-anti-miR-21 delivery in 4T1 recipient cancer cells Printed with permission from ref [193].

pathological processes. They have also shown great potential as diagnostic biomarkers, therapeutic targets, or efficient delivery platforms in nanotechnology-enabled enhanced synergistic therapy. To date, significant progress has been achieved in exosome-based science, and many ongoing clinical trials associated with exosomes have achieved impressive outcomes. However, despite many advances in exosome-based biology and related applications based on its properties, explorations of all exosome-based science still need to be developed. Hence, we expound on several key challenges to further developing exosome-based methods.

Due to the high heterogeneity of exosomes, including the source, size, content and functional heterogeneity[197], mass exosome production is difficult, costly, and nonscalable. To date, the main obstacle to deeper development is exosome production efficiency, which involves isolation, extraction, and purification. Different isolation methods are adopted based on the applications; integrated microfluidic techniques and some commercial kits are among the frequently used methods for exosome isolation [198,199]. However, the differences in the isolation methods, operational processes, and

storage conditions result in significant differences in the amount and quality of produced exosomes and make the final purpose discontinuous or worse. Furthermore, the purification of exosomes from other EVs could induce a further decrease in exosome production, which is why beyond the source of exosomes, the status of the source should be ignored[4]. Therefore, beyond the source of exosomes, the status of the source should be ignored. Therefore, there is an urgent need to develop scalable, standard, and cost-effective methods for mass exosome production to overcome low productivity and costly and complicated procedures.

More endeavours are needed to explore the underlying biological mechanisms of exosome-based science that hamper more therapeutic applications, including biogenesis, constituent parts, long-distance delivery and communication, overcoming the blood−brain barrier (BBB), and so on. For example, exosomes are believed to be intermediates within the endosomal system, where the intraluminal vesicles formed by the inward budding of the endosomal membrane during maturation of multivesicular endosomes are then secreted upon fusion of multivesicular endosomes with the cell surface[200],

which is regulated by their contents[201]. Exosomes are also secreted in several cell types, including immune cells, endothelial cells (ECs), MSCs, fibroblasts, neurons, and epithelial cells. However, the detailed conditional mechanisms of exosome biogenesis are still unknown. In addition, the mechanisms by which exosomes overcome biological barriers, such as the blood−brain barrier, blood tumor barriers, and oppositive microenvironments in specific diseases, still require further exploration. Based on the undiscovered mysteries, understanding the biological mechanisms is expected to provide deeper insight and bring a bright future for treating various diseases.

From a theranostic viewpoint, exosomes possess huge potential in disease diagnosis and therapeutics. Exosomes have been proposed as diagnostic biomarkers in tumors and nontumor tissues. For instance, saliva-derived exosomal small RNAs (tRNA-GlyGCC-5) have been established as predictive biomarkers for esophageal carcinoma, and Parkinson's disease is indexed with increased DJ-1 and α-synuclein in plasma neural-derived exosomes [202]. For therapeutic applications, exosomes from MSCs and DCs are well studied and are likely to be used due to their immunomodulatory properties. More exosomes that act as delivery platforms or vaccines have been developed recently[203]. Engineered neutrophil-derived exosomes loaded with Dox decorated with SPIONs exhibit excellent tumor targeting and inhibition[204]. Moreover, owing to the natural anti-inflammatory activity of exosomes from regulatory T cells, rEXS-cLaV synergistically inhibits inflammation and the formation of blood vessels to relieve ocular neovascular disease[205]. In the clinic, there are several engineered exosome-based drug delivery (proteins, small molecule drugs and DNA/RNA) therapeutics in phase I/II clinical trials in tumors or rare diseases. At present, the content and function of exosomes are affected by the extraction methods, the cell types, disease status, the culture medium, or even the culturing conditions (mainly 2D, 3D, and suspension culture). Thus, a standard operation of achieving exosomes for therapeutic applications and precision medicine is necessary [167].

For potential applications with exosome-based labeling, excellent and stable signals are important, and more effective technologies are required to track their fate *in vitro* or *in vivo*. An engineered exosomal marker-fused protein developed for *in vivo* fluorescent labeling of exosomes could not specifically provide information on the correct location, concentration, and transportation of exosomes[206]. In addition, the combination of fluorescent labeling and high-resolution flow cytometry is time-consuming. Other imaging technologies, such as bioluminescence imaging (BLI), nuclear, fluorescence, and magnetic resonance imaging (MRI), have also been applied for *in vivo* imaging and have achieved good imaging results [207]. However, the low sensitivity, low spatial resolution (mm), temporal resolution (s-min), and side effects on biological bodies are still limitations of exosome imaging. Thus, more effective exosomal labeling technologies, which expediently promote the tracking of exosomes, biodistribution, and therapeutic targets, are urgently needed.

With their abundant content and varied functions, exosomes act as a double-edged sword for disease inhibition and progression; thus, exosomes' potential toxicity or side effects should be addressed and investigated in detail. For example, exosomes from Alzheimer's patient brain tissues contained an increased content of amyloid-beta oligomers, which induced cell-to-cell transfer of such toxic species in recipient cells [208]. In another study, miR-192–5p in exosomes induced lipotoxic injury and promoted the activation of M1 macrophages, which led to hepatic inflammation and the progression of nonalcoholic fatty liver disease[209]. Similarly, the miR-30d-5p of neutrophil exosomes promoted the activation of M1 macrophages in sepsis to induce related acute lung injury[210]. Hence, therapeutic exosomes must be further evaluated in several relevant preclinical models to assess biological effects, including chronic and acute safety/toxicology and metabolism profiles. The exploration of potential toxicity or side effects will make a great step toward supporting clinical applications.

From a long-term perspective, the exosomes generated from natural sources are more suitable than synthetic exosomes for therapeutic applications or delivery needs because they contain a wide spectrum of bioactive and most nucleic acids but not empty bubbles (such as liposomes), which is ideal for nanodelivery tools based on their high loading efficiency, biocompatibility, biosafety, and disease targeting capability, especially exosomes from plants, fruits and vegetables of organic agriculture. Furthermore, preclinical and clinical studies have also proven that plant-derived nanovesicles are safe and have intrinsic therapeutic activities that can improve pathological conditions in numerous disease conditions [64,211,212]. These findings suggest that these naturally derived exosomes could effectively deliver therapeutics to treat human diseases, and more attention should be given to this topic in the field.

Concluding remarks

With an ever growing research and information on exosome biology, rapid and significant breakthroughs in various aspects of exosome functionality and applications have been reported. To date, intrinsic exosome properties have been widely explored and proposed as therapeutic targets for multiple diseases. The present review serves as a comprehensive summary of recent advances in the field of exosome-based science, including the classification, isolation/extraction/purification of exosomes, exploring the composition and genetic modifications, summarizing the potential of exosomes as diagnostic biomarkers and therapeutic targets, and the delivery of various therapeutic agents in different diseases. The present review will provide comprehensive instructions for exosome-based development. On the other hand, key scientific challenges were also proposed. Future collaborations between researchers in different fields are required to solve the current limitations and promote the development of exosome-based applications.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Author contributions section

Vivek P Chavda has prepared the backbone of the manuscript. All authors have contributed to the original draft of the manuscript. Lalitkumar Vora, Yanhong Duo, and Vivek P Chavda refined the first draft. Vivek P Chavda and Lalitkumar K. Vora critically revised the

manuscript for intellectually correct content. Ben Zhong TANG supported the project. All authors approved the submitted version.

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