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Review

Exosomes: Multiple-targeted multifunctional biological nanoparticles in the diagnosis, drug delivery, and imaging of cancer cells

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ABSTRACT

Exosomes are biological nanoparticles (30 − 150 nm) secreted in the extracellular area from all of cells, that mediate intercellular message. Exosomes act as the carriers for numerous proteins, DNAs, RNAs and cell-signaling molecules. Therefore, exosomes secreted by the tumor cells are useful for diagnostic purposes because of their persistent presence in the blood and their provision of genetic cargo similar to those in tumor. Due to the risks of aggressive activity and ambiguity of biological activity in other tissues, the use of exosomes in drug delivery and imaging has been limited. However, their high loading, stability and longer circulation time, excellent targeting, high cell penetration performance, and optimal biodegradability have made them potential agents in targeted cancer treatment. Therefore, in addition to examining methods for isolating and loading exosomes, this paper discusses the applications of exosomes in biological measurement, imaging, and therapeutic activities. Also, this review describes the challenges of using exosomes compared to conventional methods and shows that it is very useful to use them due to less aggressive activities. Finally, this review attempts to provide an appropriate incentive by showing the performance of exosomes in cancer therapy through targeted drug delivery, gene therapy, imaging and diagnosis.

1. Introduction

Nanotechnology has provided new insights for the early detection and cancers therapy based on nanocarriers such as extracellular vesicles and organic or non-organic mesoporous nano membranes. Since extracellular vesicles provide strong potential for application in therapeutic interventions, with a safe and accurate treatment method compared to other nanoparticles, they have been highly regarded as potential drug carriers in recent studies. Extracellular vesicles are mainly composed of cell membranes derived from cell destruction, and they can act as chemical messengers based on the proteins and RNAs being carried to establish communications between cells [1]. They are categorized into three groups according to the origin, synthesis and the size of the particles, namely, (1) exosomes (30 − 150 nm), (2) ectosomes (50nm-1 μm), and (3) apoptotic bodies (50nm-5 μm) [2–4]. Exosomes may be extracted *via* various extracellular fluids, such as urine, blood, and cerebrospinal fluid. They were first identified by Trams, et al. [5] and then examined by Johnstone, et al. [6]. Protein or peptide compounds within the exosomes can indicate the presence of cancers types, inflammation, viral infections, and neurodegenerative problems [7]. However, based on the cellular source, the exosomes encompass many proteins and lipids. Because, the results illustrate that the exosomes are used by the cells as the main pathway for removing unused or harmful proteins, if there are no lysosomal degradation systems [8]. The most public proteins in exosomes contain fusion proteins, heat shock proteins, membrane proteins (monoclines, Rab GTPases, flotine), multivesicular bodies proteins (TSG101, alixes, proteins) and tetraspanins [9], while the most common lipids include sphingolipids, cholesterols,

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and ceramides [10]. Also, one of the inherent powers of the exosomes is the development of cancer in other parts through the collaboration of the receptor-ligand at the cells and the transmission of metastatic messages into the cells [11,12]. Therefore, based on the mechanism above, exosomes can transfer the drug to cancerous tissues and other cells [13]. Recently, researchers have payed strong consideration in transferring pharmaceutical compounds to exosomes for potential application as drug carriers [8,14–16] for targeted therapies. This is because of the large loading space resulting from two-layer membrane, suitable size, high biocompatibility and biodegradability, excellent targeting, good modification, sustainability and simple transfer of biological barriers, as well as non-toxicity of exosomes.

In this literature, we present an outline of exosome based delivery systems for anticancer compounds in targeted tumor therapy. We discuss the formation, structure, natural functions and appropriate sources of exosomes for the treatment of cancers. We also discuss the mechanisms of loading the therapeutic compounds and modification of exosome surfaces to increase the targeting. Finally, we review the recent advances in the nano-complexes produced from exosomes and drug compounds, and the results of their therapeutic applications. The overall benefits and challenges of using exosomes and its promising pathways in medical development have also been discussed.

1.1. Facts

- Exosomes are carriers with a diameter of 30 − 150 nm, made by a two-layer membrane. Since these carriers are secreted by different cells, therefore, based on the intrinsic nature, the exosomes structure, their function and distribution are highly variable.
- The presence of various proteins in exosomes can lead to various activities with positive or negative effects, which ultimately cause problems in their homogeneous production as well as difficulties in their clinical activity.
- The proteins present in the Golgi system, endoplasmic reticulum, mitochondria or nucleus of cells in exosomes are not observed. Therefore, it can be considered that exosomes are one of the pathways for eliminating undegraded endosomal or lysosomal proteins.

1.2. Opening question

- What are the most common methods for synthesizing tumor-derived exosomes, purification, surface modification and their effects on cancer treatment activities despite metastatic activities?
- Based on the unique status of the exosomes due to protein differences, size, *etc.*, how can the therapeutic activities of the exosomes be controlled and improved?
- Based on the results, the anti-tumor activity of the exosomes generally returns to the detection and transmission of anti-tumor compounds. While, the direct effect of exosomes on tumor activity is confusing. Based on the origin of the produced exosomes, is the use of exosomes in treating cancer cells can have negative effects on non-target cells and even induced disease development?

2. Structure and biogenesis of the exosomes

As determined, exosomes with dimension 30 − 150 nm are a two-layer membrane with compounds such as proteins, fats, and sometimes organelles of the original cells, which are formed by most cells in mammals for intercellular communication (Fig. 1**A**). Since the synthesis of an exosomes from a cell is done randomly or intuitively from a cell's part, they are faced with high heterogeneity even when they are produced from a type of cell or tissue [17]. In the same vein, cancer cells use exosomes for intercellular communication, which called to as metastasis. Therefore, exosomes can be considered as profiles for detecting and even treating cancers [18]. For this activity, it is necessary to provide accurate definitions of biography, physiological function, and empirical events of exosomes. Hence, by providing definitions of biogenesis, the above strategy can be achieved as much as possible. Despite the identification of the two ways for the development of exosomes, the mechanism of the biogenesis of exosomes is still unknown. In the following sections we will overview the exosomes biogenesis and related pathways.

2.1. Endosomal assorting collections necessary for shipping (EACNS) mediated corridor

The initial phase in the formation of exosomes is the creation of intraluminal vesicles (ILVs). Despite the presence of various complexes in the ILVs production, the most important is the EACNS that consisting of two dozen protein with 5 collections [19]. Briefly, the procedure for the synthesis of exosome begins with the EACNS-0 through interaction with the phosphatidylinositol 3-phosphate and connects to the ubiquitinated vehicle through Zinc Finger Domains and Ubiquitin-interacting Motifs, respectively [20]. Then, an amplitude in the C-terminus of the Hrs subunit of EACNS-0 led to EACNS-I. In fact, EACNS-I with EACNS-II is a complex that induces endosomal budding from the cytoplasm. During the germinating, EACNS-0 cargo is transmitted to them. After the formation of buds, the well-ordered multivesicular body protein 6 of the EACNS-III assembled nonstop attaches to EACNS-II. This protein acts as a ring around the bud's neck for isolating buds [20].

2.2. EACNS-independent corridor

Along with the formation of ILV, there is an independent corridor to EACNS based on lipids like a ceramides that may play a role in the formation of exosomes [21]. However, the second route is still unclear and could be with multiple sorting and regeneration apparatuses that are independent of the membrane germination.

2.3. Aspects inducing the biogenesis of exosomes

Despite the influence of exosome production techniques on the performance of exosomes, biological factors such cell type, cellular communication, *etc*. are also highly effective in the production of exosomes. For instance, undeveloped dendritic cells generate a restricted number of exosomes [22] compared with mesenchymal stem cells (MSCs) [23]. Also, shutdown of cells by blocking cellular communications reduces the production of exosomes. In this regard, the results of Kucharzewska, et al. [24], Savina, et al. [25], and Koumangoye, et al. [26] showed that the use of hypoxia, Ca^{2+} ionophors and cell detachment compounds increases the secretion of the exosomes, respectively. Therefore, it is required to pay consideration to the environment surrounding the cells, the type of cell, and the stimulant or inhibitor compounds in the production of exosomes as well as the methods used.

2.4. Appropriate source cells for engineered exosomes

Despite the widespread use of cell types in the production of exosomes, due to the different biological functions of the exosomes caused by various compounds of proteins, fats, and even different surface biomarkers in the exosomes (Fig. 1**B**), it does not seem possible to use any cells types. Therefore, determining the advantages and disadvantages of the cells used in the production of exosomes is the one of the main priorities. For example, exosomes from tumor cells simply carry cancer cell antigens that can form metastasis processes [27], while it is shown that the exosomes derived from cancerous cells in a tumor detection and targeted drug delivery are very appropriate [17]. In this field, Harris, et al. [28] showed that the proteases like a urokinase plasminogen activator and cathepsin D in exosomes stimulate the metastasis of breast cancerous tumors. Also, the lack of uniformity in the production of exosomes from cancerous tumors and their complex separation from tumors due to invasive sampling has made it

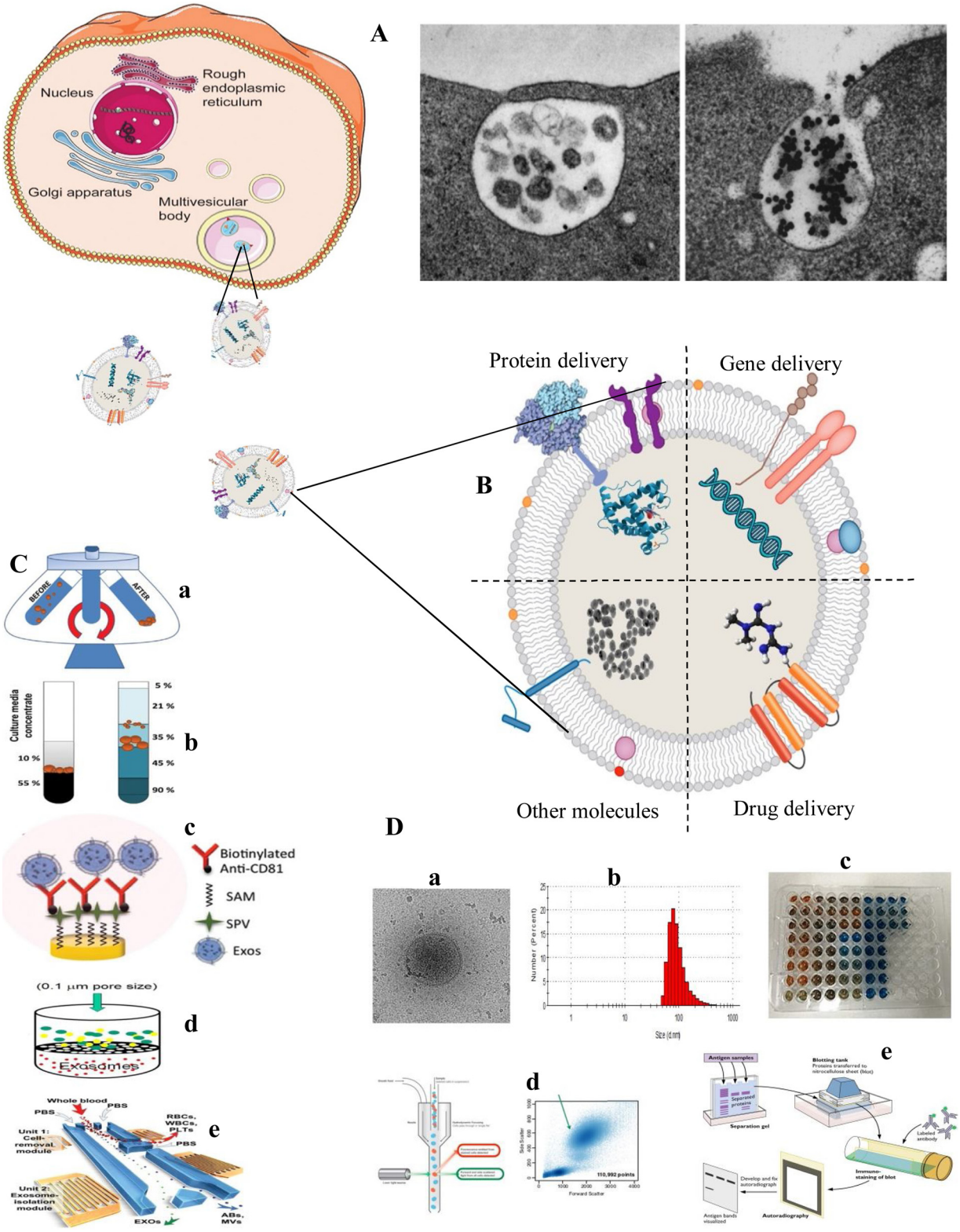

Fig. 1. (A) Biogenesis of exosomes, **(B)** Main classes of compounds contained in the exosome **(C)** Various techniques used for isolation: (a) Ultracentrifugation, (b) Precipitation, (c) Immunological (d) Filtration, (e) Microfluidic-based, and **(D)** characterization and analysis of exosomes: (a) TEM, (b) DLS, (c) Bradford assay (d) Flow cytometry, (e) Western blot.

difficult to develop this kind of exosomes [29]. Likewise, the use of immature dendritic cells is best option for the generating exosomes from human or animal resources due to less immune response of produced exosomes with the lowest of surface biomarkers like a MHC-I, MHC-II, CD40, and CD86 on their surface [23,30]. Nonetheless, as summerized in Table 1, the exosomes resulting from undeveloped dendritic cells are sometimes effective in the development and growth of cancer cells. Therefore, based on the information above, it seems that application of the exosomes derived from plant cells and then healthy mammalian cells, is much safer than tumor cells for clinical activity. In

Table 1
The function of MSCs-derived exosomes.

Source of exosomes	Performance	Ref.
Human bone marrow-derived MSCs	Enhanced breast tumor growth *in vivo*	[109]
Multiple myeloma-derived MSCs	multiple myeloma cell growth *in vitro*, Tumor growth *in vivo*	[110]
Glioma Associated-human MSC	Rise the tumorigenicity of glioma stem-like cells	[111]

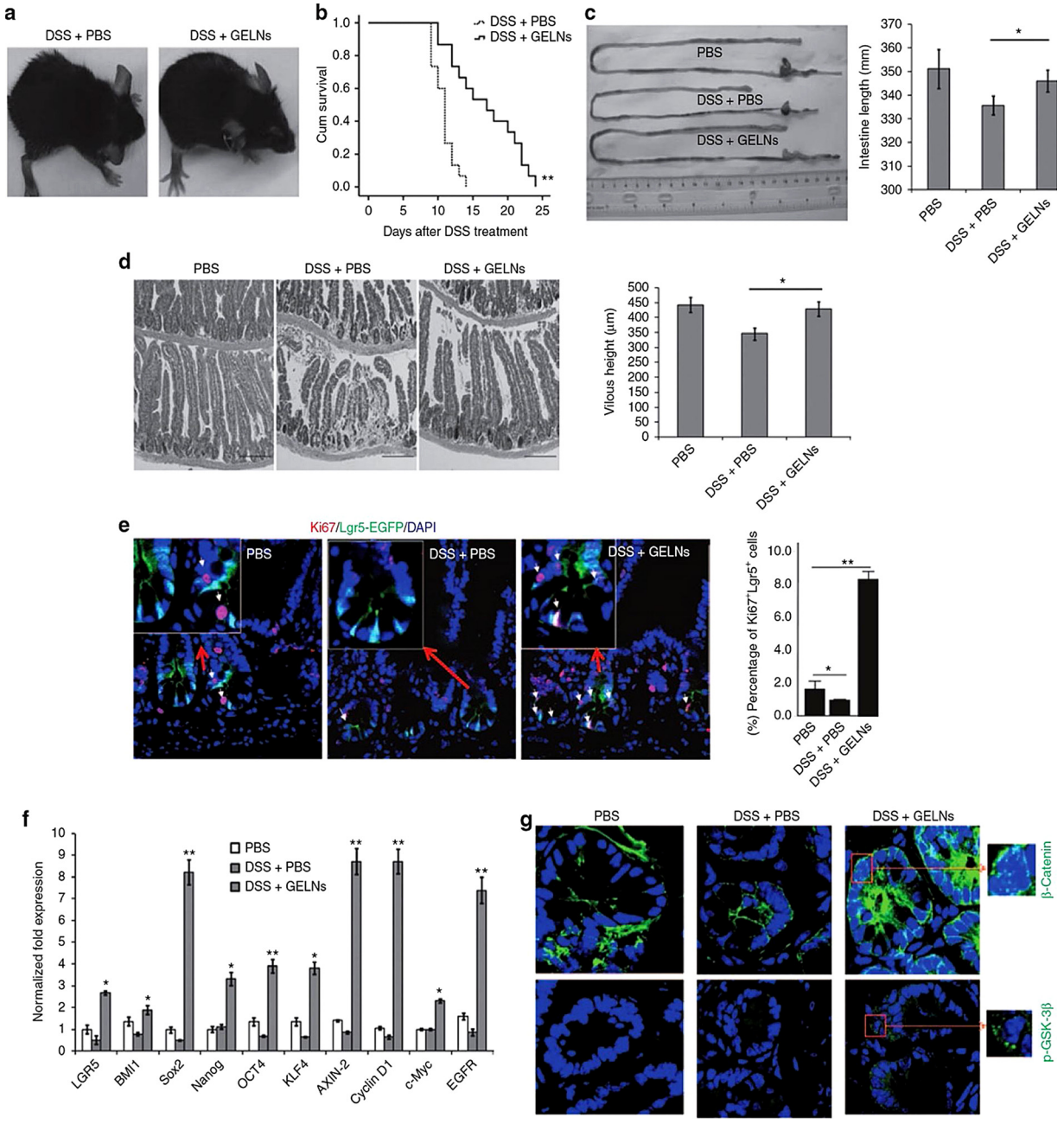

Fig. 2. Oral administration of grape exosome-like nanoparticles (GELNs) attenuates the severity of colitis induced by dextran sulfate sodium (DSS). Lgr5-EGFP-IRES-CreERT2 mice were treated for 7 days with 2 mg/mouse of GELNs in 200 μl PBS: (**a**) GELN-treated mice presented with a better body condition after 7-day of treatment. (**b**) Survival analysis. Intestinal morphometry of (**c**) intestinal length and (**d**) villus height. (**e**) $EGFP^+$ and $Ki67^+$ cells in frozen sections of intestine were examined by confocal microscopy and quantified (right, $n = 5$). The arrowhead indicates $EGFP^+Ki67^+$ cells. (**f**) Real-time analysis of mRNA expression of different genes in the intestinal crypts from C57BL/6 j mice. (**g**) Confocal analysis of nuclear translocation of β-catenin (top panels) and phosphorylation of GSK-3β (Ser 9) (bottom panels) in the crypt sections from DSS-treated C57BL/6 j mice [32]. Figures adopted and modified from refs [32] with permission from the American Society of Gene Therapy.

Table 2
The advantages and disadvantages of the exosome isolation techniques.

Methods	Mechanism	Advantage	Disadvantage
Ultracentrifugation	Based on centrifugal force	High purity, Isolation of low-density exosomes and high density, Isolation of very few samples, Suitable for protein analysis, Long lifespan.	Poor recovery of exosomes from highly viscous biofluids, Time consuming, Expensive, Multiple-steps, Change morphology.
Immunological isolation	Based on antibody receptor interaction	Rapid, Relatively simple, Can be tissue specific, High purity, No volume limitations.	Some of exosomes may not preserve biological functionality, Expensive, Not suited for large sample, Need expensive laboratory equipment, only cell-free sample used.
Filtration	Based on membrane materials and porosity	Preserves vesicle structure, Fast, Easy, Non-specific binding of vesicles, Scalable, Separation with high density.	Low purity (Contamination with other microvesicles), Low efficacy, Necessities extra steps such as ultracentrifugation, Isolation based on size only.
Precipitation	Based on polymer materials and precipitation	Rapid, Appropriate with cheap tools, Capability to detect biomarkers, Large samples can be used.	High heterogeneity, Not specific for exosomes, High impurity.
Chromatography	Based on porosity of materials	High efficacy, Determination of surface phenotype, Scalable, Can be tissue specific, High recovery.	Nonspecific bindings, Expensive, Need to high focus, Variable level of impurities, Chromatography column contamination, Time-consuming protocols.
Microfluidics-based	Based on microfluidic	Quick, Easy, Work with small sample, High sensitivity.	Needs to developed laboratories, Expensive, Contamination with microvesicles, Not matched with large samples, Lack of global protocol.

this regard, fruit- or food-derived exosomes due to their current consumption and cheapness have attracted much attention [31]. In a mice model, Ju, et al. [32] using grape exosome-like nanoparticles was able to increasing the maintenance, growth, and differentiation of intestinal SCs against colitis caused by dextran sulfate sodium (Fig. 2). Likewise, it was found that by modifying the exosomes surface derived from grapefruit and loading doxorubicin and curcumin drugs into them, in addition to improving targeted drug delivery, inflammatory activities of tumors in mice was significantly reduced [33].

3. Loading, isolation and surface modification of exosomes

3.1. Exosomes isolation

Access to exosomes due to their presence in the blood, milk, urine, saliva and other physiological fluids is simply possible. However, their isolation is always challenging, difficult, complex, and costly. The most important separation methods are ultracentrifugation, immuno-affinity, precipitation, and so on, each with advantages and disadvantages as reported in Table 2 (Fig. 1**C**). Based on the type of physiological fluid, the isolation of the exosomes is different, despite the common principles based on size, and biophysical characterization like an immuno-affinity [21,34]. After separation, the purity and characterization of the exosomes are then evaluated and quantified through common methods such as imaging, western blotting and flow cytometry (Fig. 1**D**).

3.2. Loading mechanisms

Although there are various strategies for loading drug compounds into exosomes, they may be separated into two passive and active loading categories (Table 3). Loading of drug compounds in exosomes has major challenges, most notably the control of membrane integrity, and the presence of bioactive compounds within the exosomes, which can greatly reduce the efficiency of the transfer. To this purpose, if the membrane integrity remains, the offloading of the exosomes content will be a good solution, while, some detail information is not available in this field. In this regard, plant-derived exosomes have been shown to have more bioactive compounds than animal-exosomes [35].

The common method of loading drug compounds on exosomes is incubation before (loading the drug in the cells of the exosomes source) and after their production, which has the least destructive effects on both of them [36]. While, some of studies suggest that the best way to load membrane-impermeable drugs, mi- or si-RNAs, and small DNA is *via* electroporation, which can cause pores on the membrane of two layers of exosomes to be loaded and cover the exosome [37].

3.3. Surface modification

Exosomes, like other particles, are absorbed and removed by the reticuloendothelial system after being secreted or injected into the bloodstream [38]. Besides, studies have exposed that exosomes act to transmit cellular signals or drug compounds loaded into them through a three-pitch receptor interplay, membrane fusion, and endocytosis mechanism [38,39]. Therefore, in order to more accurate detecting the target tissue or cells, increasing the stability of exosomes in body, and induction of intracellular penetration, comprehensive information must be provided from surface receptors and surface modification of exosomes. Differences in biological activity of exosomes, in addition to

Table 3
Methods of loading drug compounds in exosomes.

Classification	Sub-classification	Advantages	Disadvantage
Passive	Incubation of exosomes or donor cells with compound drugs	Easy, Has membrane integrity, Good stability, Lack of morphology change, Do not need extra tools.	Low loading efficacy, Cytotoxicity to the donor cells.
Active	Sonicating	High loading efficacy, Applicable for small RNAs, Rapid.	Low membrane integrity, Not efficient for hydrophobic drugs, Morphology change, Mechanical stress.
	Extrusion	High loading efficacy, Time conserving.	Low membrane integrity, Morphology change.
	Electroporation	Loading large molecules such as siRNA,	Low loading efficiency for hydrophobic molecules, Agglomeration. Morphology change, Mechanical stress,
	Antibody binding	Specific	Multi-step activities
	Saponin-assisted	Enhanced drug loading	Toxicity, Hemolysis, Lack of integrity, Presence of saponin in products.
	Click chemistry	Fast, Medium efficiency.	–
	Freezing	Medium loading	Agglomeration.

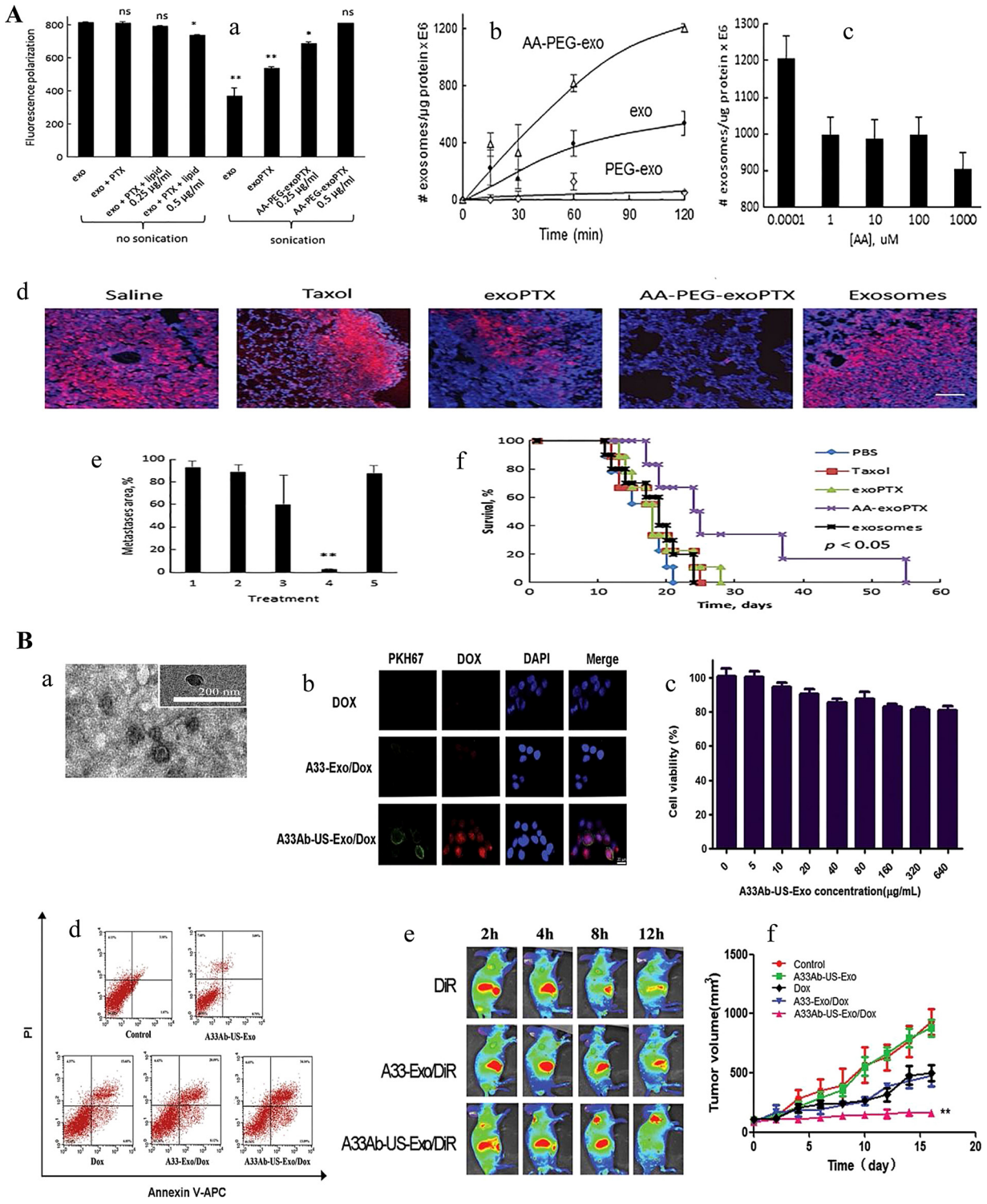

Fig. 3. (A): (a) Effect of AA-PEG-DSPE incorporation and paclitaxel (PTX) loading on fluidity of exosomal membranes, (b) 3LL-M27 cells were incubated with fluorescently-labeled AA-PEG-exo, or PEG-exo, or exo for various times, and accumulation levels were measured, (c) AA-PEG-exo formulation showed a dose-dependent response to competitive inhibition by AA, indicating that this formulation entered cells by receptor mediated endocytosis, (d) C57BL/6 mice with established Luc/mCherry-3LL-M27 lung metastases were i.v. treated with: saline, or Taxol, or exoPTX, or AA-PEG-exoPTX, or exosomes alone (no drug), (e) Quantitative assessment of metastases area on the lung slides of animals treated with: 1) saline, or 2) Taxol, or 3) exoPTX, or 4) AA-PEG-exoPTX, or 5) exosomes alone and (f) A survival of C57BL/6 mice with established metastases was recorded for four treatment groups: 1) saline (dimonds), or Taxol (squares), or 3) exoPTX (triangles), or 4) AA-PEG-exoPTX (crosses), or 5) exosomes alone (stars) [58]. **(B):** (a) Characteristics of A33Ab-US-Exo/Dox, (b) Confocal microscopy images of LIM1215 cells after incubation with Dox, A33-Exo/Dox, and A33Ab-US-Exo/Dox for 4 h, (c) Viability of LIM1215 cells was detected by CCK-8 assay after treated with A33Ab-US-Exo for 24 h, (d) Cell apoptosis and necrosis of LIM1215 cells treated with DOX, A33-Exo/Dox, and A33Ab-US-Exo/Dox for 24 h and measured by flow cytometry using annexinV-APC kit and PI staining, (e) *in vivo* fluorescence signals of mice bearing LIM1215 cell-derived tumors after intravenous injection of DiR, A33-Exo/DiR, and A33Ab-US-Exo/DiR and (f) Tumor volume of tumor-bearing mice treated with groups [59]. Figures adopted and modified from refs [58] and [59] with permission from Nanomedicine: Nanotechnology, Biology and Medicine, respectively.

cellular source, return to their surface molecules [40]. So, surface modification of the exosomes by cellular engineering or chemical modification is widely used to improve pharmacokinetic behavior [41]. Since exosomes intrinsically absorb some of the protein, fat, and other ligands from the original cells, it is based on the hypothesis that exosomes with known surface markers can be provided by injection of encoded genes into the original cell. For this purpose, using a vector of gene transfer like a lentiviral, fusion cassette is stated in parental cells and then transmitted to exosomes. For instance, recently Limoni, et al. [42] were able to track the breast tumor cells and inject drug compounds by applying exosomes modified with HER2 that constructed *via* encoding the LAMP2b-DARP in G3 chimeric gene in HEK293 T cells. Besides, chemical modification in the exosomes is considered as a method of quick, simple, efficient, and the presentation of one or more specific site to make changes [41]. Despite the introduction of different methods, procedures that prevent from the destruction and accumulation of exosomes membranes during temperature and pressure changes should be used. Based on this method, it is possible to loading a variety of small and large molecules, target molecules, imaging molecules, *etc*. on the exosomes by various chemical groups such as alkyne, carboxyl, and so on [43]. In this field, Tian, et al. [44] using a group of alkyne in bio-orthogonal copper-free azide, were able to produce modified exosomes with c(RGDyK) peptide containing the curcumin for the treatment of cerebral ischemia.

4. Exosomes as a therapeutic application

Because organic and non-organic nanoparticles used for therapeutic application especially drug delivery and detection are faced with different cytotoxicity, non-targeting and immunogenicity [45], the using exosomes in the transfer of therapy compounds such as drug, imaging labels, *etc*. is an ideal solution for crossing biological barriers, reducing cytotoxicity and immunogenicity activities [46,47]. Furthermore, their two-layer membranes are a natural protector and an ideal capsule for drug release. Also, in resistant drug-resistant cancerous cells, the use of exosomes from a cancer cells and loading drugs or other compounds on their surface could be a great way to control growth and stop cancer cells [15]. In this regard, in a mice model, Munagala, et al. [48] by applying the space of exosomes and its advantages in drug loading, were able to provide potential remedial affects by transferring the drug to lung tumor cells. Likewise, Morishita, et al. [49] using exosomes derived from B16BL6 murine melanoma cells, were able to provide specific responses in tumor. It was also previously determined that the toxic concentrations of doxorubicin and its negative effect on the heart were significantly reduced when exosomes were used for drug delivery, in addition to suppressing the growth of breast cancer tumors [50]. However, in order to obtain the promising therapeutic effect on tumors, in addition to choosing the potential loading method and generating the exosome, there must be an appropriate correlation between the carrier, type of exosomes and cancer suppressor compounds such as small molecules, nucleic acids, RNA and proteins [51]. It seems that the appropriate and desirable relationship between exosomes, drug compounds, and tumor type can greatly reduce the major problems of the use of exosomes in therapeutic activities such as drug loading, the kind of cargo, therapeutic loading and the type of surface charge.

4.1. Exosomes as a drug delivery

4.1.1. Exosomes as drug delivery vehicles for small molecules

Various studies have shown that the use of exosomes for the delivery of small molecules is an appropriate process. In this field, Dhillon, et al. [52] and Sun, et al. [53] were able to transfer the curcumin composition to a much higher efficiency to the target tissue. Also, in the mice model, Sun, et al. [53] using curcumin loaded on the exosome, in addition to the targeting of curcumin, they were able to induce reduced lung inflammation and higher survival of the mice compared to the only curcumin. In the same way, it was determined in the mice model that paclitaxel loaded onto exosomes not only increased the transfer of drug to the lung tumor tissue and increased cytotoxicity by up to 50 times the control, but also severely reduced drug toxicity in other tissues [54]. Furthermore, in mice model with cancer drug resistance Srivastava, et al. [55] by loading gold nanoparticles containing doxorubicin in exosomes, in addition to targeted drug delivery to lung tumor cells, were able to increase cellular uptake of the doxorubicin and also to carry out synchronous medical activities by gold nanoparticles. At the same time, in a mice model it was found that exosomes containing doxorubicin in comparison with the free drug decreased drug resistance in ovarian and breast cancer cells, reducing drug cytotoxicity in the heart, and increasing drug concentration in cancer tissue [50]. Likewise, it was determined that loading paclitaxel and curcumin in the exosomes, in addition to improving the targeted transfer of them to the lung tumors, increase the drug's sustainability in the blood due to the increased hydrophilicity [56,57]. In the following, Kim, et al. [58] showed that the use of paclitaxel-containing exosomes with amino-ethyl-anisamide-polyethylene glycol to bind to sigma receptors increases the drug's uptake and the penetration depth of the drug to the lung tumor (Fig. 3**A**). In an animal model, it was also determined that exosomes resulting from LIM1215 cells containing doxorubicin functionalized with superparamagnetic iron oxide nanoparticles and A33 antibodies, in addition to reducing drug cytotoxicity in the heart, increased drug loading in colon cancer cells along with simultaneous therapeutic activities such imaging or photodynamic therapy (Fig. 3**B**) [59]. Moreover, Li, et al. [60] with Sorafenib loaded on siGRP78-modified exosomes that are isolated from bone-marrow-derived MSCs, were able to prevent the development of cancer cells and metastatic activities, in addition to enhancing drug loading in drug resistant hepatocellular carcinoma. Recently, Das, et al. [61] in a MDA-MB-231 and MCF-7 cellular model, exhibited that the using exosomes isolated from embryonic SCs containing doxorubicin not only increased the breast cancer cells death with low dose of drug, but also improved the performance of the drug more than the free drug. Likewise, Melzer, et al. [62] by using exosomes resulted from human MSCs-like cell containing Taxol, in addition to increasing the cell death of lung cancer (A549), ovarian cancer (SK-OV-3), and breast cancer cells (MDA-hyb1), reduced their metastatic activity up to 50 % in the lung, spleen, liver, and kidney tissues, and a decrease of Taxol concentration up to 7.5-fold of free drug. Overall, the results of the studies exhibit that the use of exosome as a vehicle of small molecules reduces the cancer cells drug resistance, reduces the toxicity of the drug in other non-target tissues, increases the efficacy of stopping the development of cancerous cells, and even prevents metastases.

4.1.2. Exosomes as drug delivery vehicles for peptides and proteins

Exosomes, like organic and non-organic nanoparticles, are used to transport large molecules like a protein. For example, various studies displayed that exosomes loaded with catalase and superoxide dismutase enzymes [63], various antigens and nanobodies [64], proteasomes [65], transferrin and lactoferrin [66] for targeted therapy were investigated.

Although drug treatment for progressive pancreatic cancer has been challenged, Aspe, et al. [67] by using exosomes collected from a cell line of melanoma containing the Survivin-T34A protein, in addition to increasing the apoptotic activity of cancerous cells, increases the susceptibility of cancerous cells to Gemcitabine. In this regard, it was determined that HEK293 T cells-exosomes derived modified with a fragment of Interleukin 3 containing Imatinib protein reduced the activity of chronic myelogenous leukemia by inhibiting tyrosine kinases (Fig. 4**A**) [68]. Furthermore, a study by Koh, et al. [69] found that the use of HEK293 T cells-exosomes derived containing a signal controlling protein α (SIRPα) by blocking the CD47 receptor on Raji human B cell lymphoma, HT29 human colon adenocarcinoma, and CT26.CL25 mouse colon carcinoma caused tumor stoppage in mouse model and cell

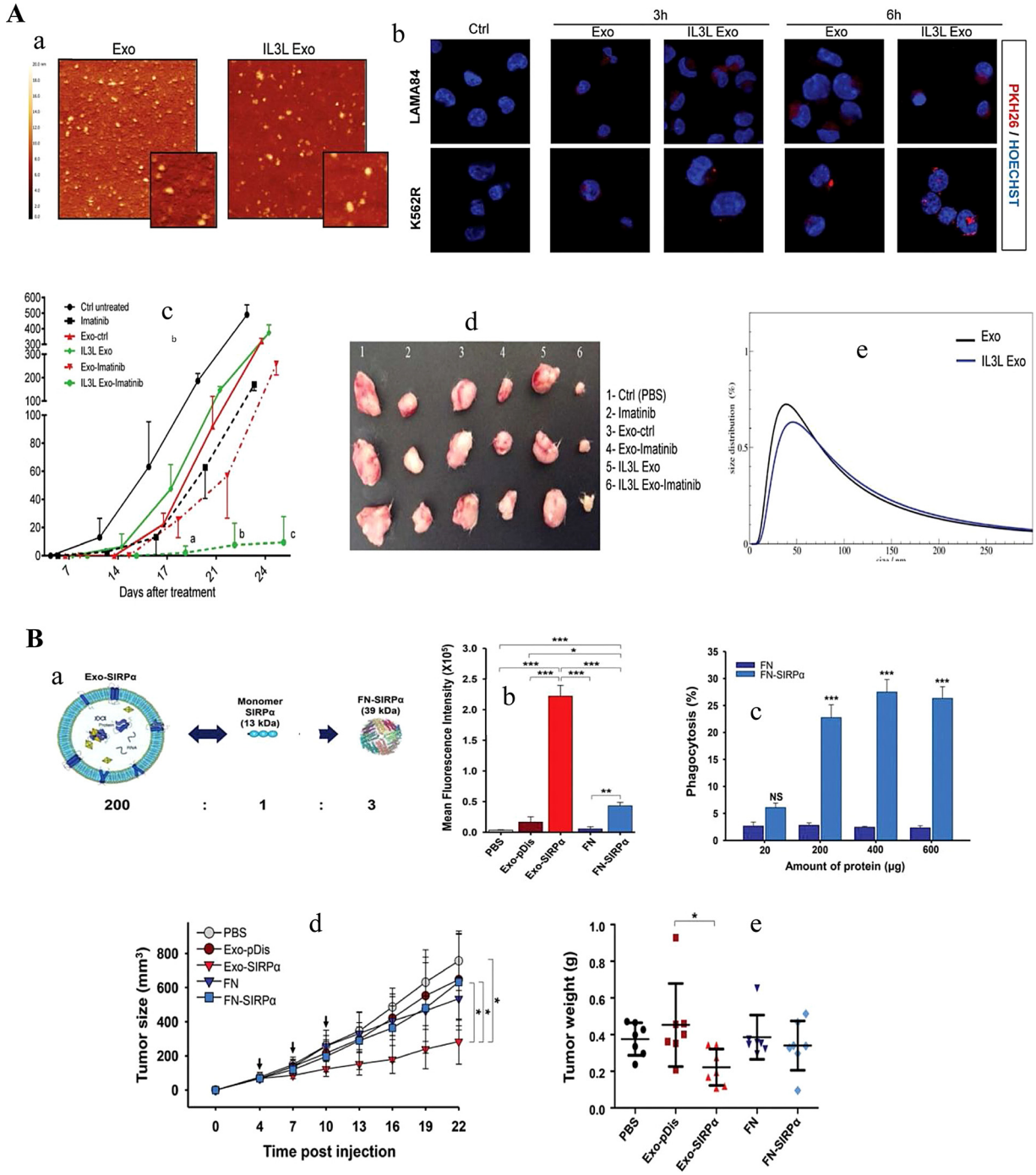

Fig. 4. (A): (a) Exosome particles were visualized by Atomic Force Microscopy (large square 2 μm, small square 500 nm), (b) Analysis at confocal microscopy of LAMA84 (upper panel) or K562R (lower panel) cells treated, for 3 h and 6 h, with 10 μg/mL of HEK293T-derived exosomes (Exo) and IL3L-HEK293T-derived exosomes (IL3L Exo). Nuclei were stained with Hoechst (blue); exosomes were labeled with PKH26 (red), (c) Comparison of the median tumor volume was an index of the antitumor efficacy of Imatinib loaded exosomes, (d) *in vivo* effects of Imatinib loaded IL3L-exosomes, (e) Exosome size distribution was determined by DLS analysis [68]. **(B):** (a) The ratio of protein concentration (g) for Exo-SIRPα, monomeric SIRPα, and FN-SIRPα corresponding to an equal amount of SIRPα, (b) The ability of FN-SIRPα and Exo-SIRPα to bind CD47-expressing HT29 cells, assessed by flow cytometry, (c) Analysis of HT29 cell phagocytosis by BMDMs in the presence of a high concentration of FN-SIRPα, (d) Antitumor effect of Exo-SIRPα in HT29 tumor-bearing BALB/c immuno-deficient mice, (e) Weights of excised tumors at experimental endpoints for HT29 mouse models [70]. Figures adopted and modified from refs [68] and [70] with permission from Theranostics and Journal of Controlled Release, respectively.

death in cellular models through binding macrophage and T cells to cancerous cells. Accumulation of CD47 on cancerous cells keeps them from macrophage immune activities. Similarly, Cho, et al. [70] revealed that exosomes isolated from a HEK293 T cells containing a SIRPα along with protein-scaffold-based ferritin nanocage, by blocking CD47 receptors on the tumor cell, inhibited HT29 human colon adenocarcinoma cells growth, and increased immune function (Fig. 4**B**).

4.1.3. *Exosomes as drug delivery vehicles for nucleic acids*

Exosomes can easily transfer nucleic acids like a DNA or RNA to targeted cells, compared with other carriers, and can cause genetic

changes in pathogenic cells and even healthy cells [71]. Exosomes can then be used to alter gene expression in gene therapy activities. Despite the high instability of nucleic acids like siRNA, miRNA, *etc*. [72], this compounds are used to destroy various genes. Although exosomes are capable of improving nucleic acids stability in the blood, further research and understanding is needed to incorporate nucleic acids into exosomes and utilize of the exosomes-nucleic acids complex in genetic therapy. In this field, Song, et al. [73] by immobilizing miRNA-7-5p in exosomes increased the autophagy activity in human bronchial epithelial cancer cells by inducing EGFR/Akt/mTOR signaling path for the treating lung cancer. At the same time, it was found that miRNA-302b in the exosomes of derived tumor from the lung tumor caused the suppression of creation, movement, and attack of lung cancerous cells by controlling the TGFβRII/extracellular signal-regulated kinase path [74]. Also, in cellular and mice models, Bellavia, et al. [68] using BCR-ABL siRNA loaded on exosomes modified with a fragment of Interleukin 3 that is overexpressed in chronic myeloid leukemia not only accumulated siRNA in cancerous cells based on targeting delivery and reducing the barrier to cancer drug resistance, but also they significantly decreased the activity of cancer cells compared to conventional methods. At the same time, by using exosomes isolated from normal fibroblast cells containing siRNA or shRNA special of oncogenic KRASG12D by blocking the CD47 receptor on the cancerous cells surface, Kamerkar, et al. [75] were able to suppress advanced pancreatic cancer. Another study determined that exosomes isolated from MSCs containing locked nucleic acid-modified anti-miR-142-3p in cellular 4T1 and animal models decrease tumorigenicity of breast cancer cells compared to common methods (Fig. 5**A**) [76]. Recently, in a cellular model, Bai, et al. [77] revealed that the siRNA loaded with highly targeted tLyp-1 exosomes derived from *Escherichia coli* with a dimension of 100 nm could easily suppress lung tumor A549 cells *via* knocking-down SOX2 gene. It was also found that microRNA-145 loaded on exosomes isolated from pancreatic cancer cells *via* precipitation method, in addition to decreasing cell drug resistance, effectively suppresses tumorigenesis activities including proliferation, migration, and invasion [78,79].

4.2. Exosomes as delivery of imaging molecules

One of the major problems with the imaging of tumor tissues is the lack of complete accumulation of cancer cells in one area, which causes strategic errors in the diagnosis and surgical treatment of tumors [80]. Since the exosomes provide the ability to detect cancerous cells at extremely high levels, even up to a few hundred cells, their application in cancerous imaging, especially lung cancers, was considered. This means that by attaching an optical reporter in the nanoscale dimension to the exosomes, in addition to optical imaging, in comparison with fluorescence dyes, it provides a higher optical stability for imaging. In this field, Zong, et al. [81] and Jiang, et al. [82] gained a high-resolution image of the live cells and the metastatic activity of breast tumor cells with minimal cytotoxicity by loading silicon quantum dots (30 nm) and gold-carbon quantum dots onto the outer membrane of the exosome (50 − 100 nm), respectively. Likewise, Srivastava, et al. [55] with the use of iron oxide nanoparticles (5 − 10 nm) and doxorubicin in exosomes derived from Lung fibroblast cells, in a dual activity, in addition to treatment activity in lung cancers cells (H1299 and A549), were able to capture a high-resolution tumor. Besides, In addition, using a dioctadecyl-tetramethylindodicarbocyanine-4-chlorobenzenesulfonate salt label on exosomes extracted from CaL62/Rluc cells, Gangadaran, et al. [83] in addition to providing a tissue and tumor edges imaging system for reducing surgical errors of thyroid cancer, were able to visualize the development of tumor in the body (Fig. 5**B**). Recently, in a mice model, Shi, et al. [84] using exosomes modified with fluorescence radiolabeled and PEG, were capable to imaging higher resolution from 4T1 breast cancer cells along with providing a visual examination of cellular uptake.

4.3. Exosomes as cancer diagnostic biomarkers

Based on the origin of the exosomes, it is possible to evaluate the cancerous cells by detecting them in physiological fluids [85]. In other words, exosomes as biomarkers of tumor types can be detected by nanobiosensors and other diagnostic methods. Based on this hypothesis, exosomes can be exploited to monitor and even predict a patient's response to a given treatment [86]. So, the use of exosomes in the diagnosis and control of remedial activities is recommended as a very successful and safe strategy. On the other hand, due to differences in the pattern of sampling compared to conventional methods, invasive activity causing tumor metastatic activity will not be observed. Moreover, a reasonable number of exosomes (at least 10^9 per ml) with the cancer cell origin compared to cancerous cell proteins that are very diluted in the early stages of cancer will allow more accurate and faster detection of cancers in the early stages [87].

4.3.1. Exosomal proteins as biomarker

As mentioned, exosomes contain a variety of proteins, such as the annexin, flotillin, Hsp70, Hsp90, CD9, CD37, CD53, CD63, Alix, Tsg101, EpCam, *etc.*, which reflect parental cells. These proteins can be identified as cancer biomarkers by immunoassay, western blot analysis, and so on (Table 4). In this line, Zhao, et al. [88] using microfluidic chip based on using immunomagnetic beads with detection of CA-125, EpCAM, CD24 exosomal proteins, were able to sense ovarian cancer at an early period with similar accuracy to the standard Bradford method (area under the curve (a.u.c.) = 1.0, p = 0.001 vs. a.u.c. = 1.0, p = 0.0009) (Fig. 6**B**). Likewise, in a human model, Sandfeld-Paulsen, et al. [89] showed that exosome proteins were not equally capable of detecting lung cancer, and that the strongest ones were CD151 (a.u.c. = 0.68, p = 0.0002), CD171 (a.u.c. = 0.60, p = 0.0002), and tetraspanin 8 (a.u.c. = 0.60, p = 0.0002). Nevertheless, in the multi-biomarker model (only covered 10 biomarkers), they increased the ability to detect all of lung cancer kinds several times (all cancer: a.u.c. = 0.74 [95 % confidence interval: 0.70 − 0.80]; adenocarcinoma only: a.u.c. = 0.76 [95 % confidence interval: 0.70 − 0.83]). In addition, the use of exosomes-derived from pancreatic ductal adenocarcinoma with glypican-1 and CD63 biomarkers not only improved the rate of cancer detection to under 30 min, but also increased the detection limit of cancer with a sensitivity of 99 % to over 82 % compared to conventional methods (Fig. 6**A**) [90]. Recently, based on the diagnosis of exosomal protein CD82, Wang, et al. [91] were able to detect breast cancer at all stages of cancer development and in different tissues. Their results exhibited that exosomal protein CD82 is extremely low in healthy tissues and initial tumor and highly high in malignant and metastatic cancers.

4.3.2. Exosomal nucleic acids as biomarker

Numerous studies have shown that nucleic acids of RNA and single-stranded or double-stranded DNA, like a protein, are an integral part of exosomes, the most important of which are: microRNAs (miRNAs), messenger RNAs (mRNAs), handover RNAs (tRNAs), and long non-coding RNAs (lnc RNAs) [92–94]. Among them, exosomal miRNAs have taken the most consideration due to their stability against degradation [95]. Hence, after year 2007, various studies have been done to evaluate them as markers of cancer cells (Table 4). In this regard, Tanaka, et al. [96] found in a human model that miRNAs-21 is one of the appropriate markers to determine esophageal squamous cell cancer (Fig. 6**D**). They demonstrated that with the growth and development of the tumor along with aggressive activities, the level of miRNAs-21 increased significantly. Likewise, in a cellular model, it was found that using great segments of double stranded genomic DNA (> 10 kb), in addition to diagnosing pancreatic cancer with high sensitivity, were able to predict the therapeutic resistance and type of cancer treatment based on the mutations in KRAS and p53 [97]. In addition, Jin, et al. [98] were capable to detect high-precision adenocarcinoma and

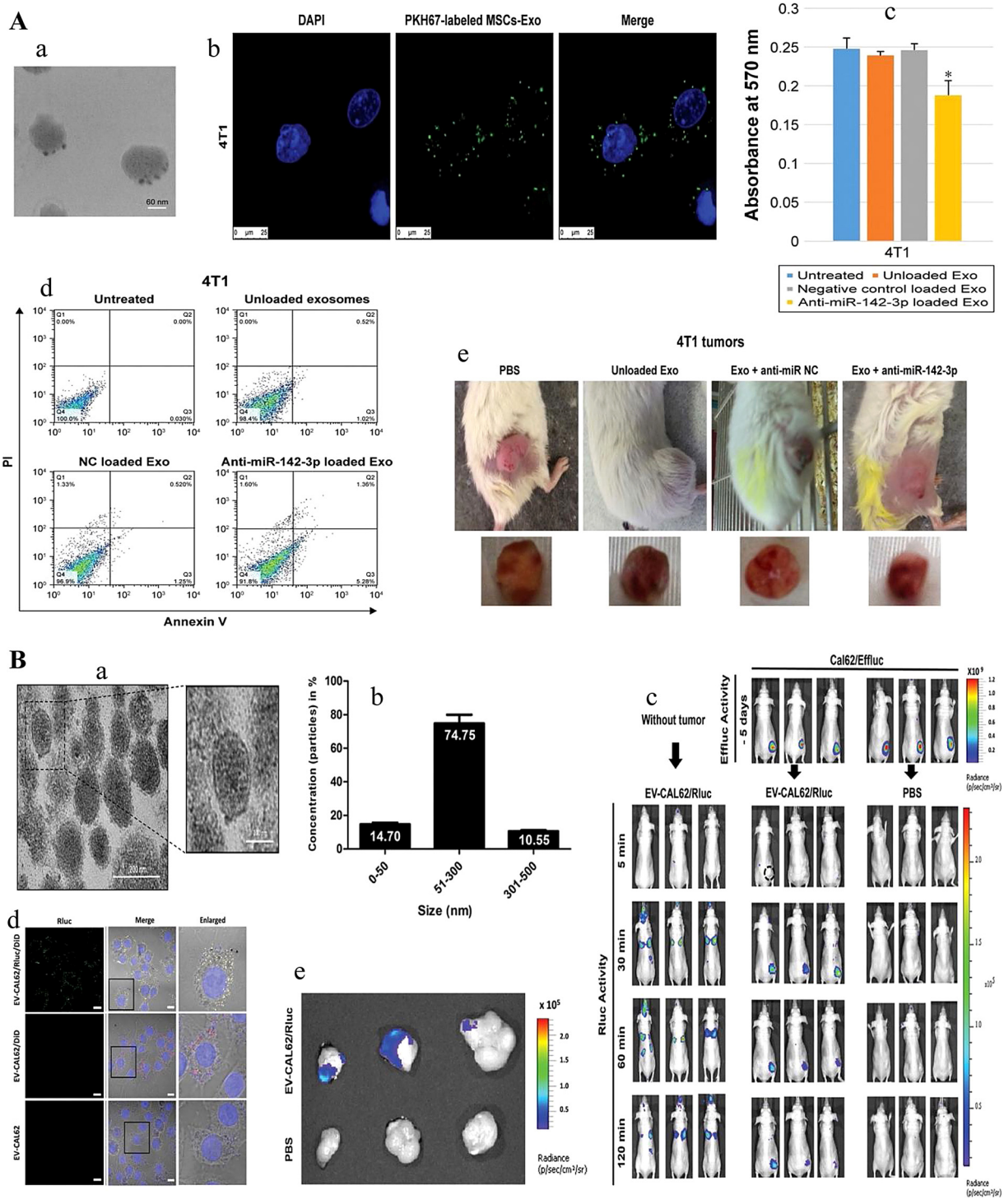

Fig. 5. (A): (a) Transmission electron micrograph of negatively stained exosomes with a diameter of 30–150 nm, labeled for CD63, (b) Cellular uptake of PKH67-labeled MSCs-Exo by 4T1 cell lines, (c) Evaluation of the cytotoxicity effect of exosome-based LNA-anti-miR-142-3p delivery on 4T1 cells, (d) Representative dot plots of 4T1 cancer cells, (e) Representative images of female BALB/c mice bearing 4T1 breast tumor and excised tumor tissues at the end of day 36 [76]. **(B):** (a) Examination of EVs from CAL62/Rluc cells using electron microscopy, (b) EV size and concentrations were determined by DLS (Avg. 93.1 ± 21.3 nm)., (c) *in vivo* imaging of EV-CAL62/Rluc intravenously administered to naïve or CAL62/Effluc tumor-bearing mice, (d) Representative confocal images in EV-CAL62/Rluc/DiD or EV-CAL62/DiD or EV-CAL62 in CAL62 cells., (e) Representative e*x vivo* bioluminescent imaging of EV-CAL62/Rluc excised tumor from EV-CAL62/Rluc or PBS intravenously administered to CAL62/Effluc tumor-bearing mice [83]. Figures adopted and modified from refs [76] and [83] with permission from International Journal of Nanomedicine and Scientific Reports, respectively.

squamous cell carcinoma (SCC) without invasive activity using exosomes containing miR-181-5p, miR-30a-3p, miR-30e-3p, and miR-361-5p for adenocarcinoma-specific; and miR-10b-5p, miR-15b-5p, and miR-320b for SCC-specific. They also showed that in a multi-miRNAs model the diagnosis of adenocarcinoma (AUC value of 0.936) and SCC (AUC value of 0.911) was more accurate [98]. Furthermore, the results of Xu, et al. [99] displayed that the simultaneous use of ENSG00000258332.1, LINC00635 and AFP exosomal as a lnc RNAs is a

Table 4
Exosomal protein biomarkers in cancer detection.

Tumor	Exosomal Protein	Stage	Detection method	Ref.
Exosomal protein				
Prostate	Survivin	Early diagnosis	ExoQuick	[112]
	PCA-3	–	PCR analysis	[113]
Colorectal	CD147, CD9	Metastatic	photosensitizer-beads	[114]
Lung	Annexin, Ras family proteins, CEACAM5	Monitoring	Chromatography-mass spectrometry	[115]
Glioblastoma	EGFR, EGFRvIII, CD63	Monitoring and prediction	μNMR system	[116]
Melanoma	HSP90	Early diagnosis	Western blot	[117]
Pancreatic	Glypican-1	Early diagnosis	FACS	[118]
Ovarian	TGF β1, MAGE 3/6	Prognosis/therapy monitoring	Ultracentrifugation	[119]
Breast	EDIL3	Diagnosis/monitoring	ELISA	[120]
	Fibronectin	Early diagnosis	ELISA	[121]
Gastric	GRN	–	Chromatography-mass spectrometry	[122]
Exosomal nucleic acid				
Colorectal	miRNA-4772-3p	Prognosis for recurrent stage II	ExoQuick/qPCR	[123]
	miRNA-1229, miR-150, miRNA-21,	Diagnosis	Ultracentrifugation/qPCR	[124]
Breast	miRNA-101, miRNA-372,	Diagnosis	ExoQuick/qPCR	[125]
Prostate	miRNA141, miRNA-375	Diagnosis	ExoMiR extraction kit /qPCR	[126]
	PCA3, TMPRSS2-ERG	Diagnosis/monitoring	qPCR	[127]
Glioblastoma	Snc RNA (RNU6-1) miRNA-320,	Early diagnosis	ExoQuick/qPCR	[128]
Pancreatic	Mutated KRAS DNA	Diagnosis/prognosis	Ultracentrifugation and Filtration /PCR	[94]
bladder	GALNT1, LASS2	Diagnosis	Microarray	[129]
Melanoma	miRNA-216a, miRNA217,	Metastatic	qPCR	[130]
Hepatocellular	miRNA-718	Tumor size	qPCR	[131]
Lung	miR-17-3p, miRNA-20b	Diagnosis/monitoring	Microarray	[132]

very promising and reliable method in the diagnosis and prognosis of carcinogenesis of human cancers. Recently, Zhu, et al. [100] by determining exosomal tRNAs levels among patients with liver cancer and healthy individuals, presented that four types of tRNAs including tRNA-ValTAC-3, tRNA-GlyTCC-5, tRNA-ValAAC-5 and tRNA-GluCTC-5 were significantly increased in plasma of patients with liver cancer (Fig. 6**C**). Therefore, examining their levels as a biomarker provides high hopes for accurate diagnosis of liver cancer.

5. Major advantages and disadvantages

A lot of drug compounds in the *in vivo* conditions are very unstable and have many challenges like a drug resistance, low access to target tissue and so on for successful treatment. On the other hand, due to the problems associated with many drug delivery systems such as cytotoxicity of organic and non-organic nanoparticles [101], the development of delivery systems based on biological patterns is very important. Because of the minor size of the exosomes between 30–150 nm along with their hydrophilicity, biocompatibility and cheapness compared to nanomaterials, this vehicle can progress the constancy of drugs by loading the drug in the interior space and even on its outer surfaces through preventing macrophage phagocytosis [102]. Also, exosomes increase the capability to surrender the nucleic acids like an mRNA or siRNA to the cytoplasm and even the nucleus of cancer cells by stopping phagocytosis and preventing lysosomal degradation [103]. Whereas, exosomes also increase the cellular uptake in drug resistance by cancer cells. For instance, in a murine model, Kim, et al. [104] exposed that macrophage-isolated exosomes can transduce Paclitaxel and Taxol into the lung cells several times more than other carriers such as liposomes. Moreover, exosomes can reduce the cytotoxicity of drug by decreasing accumulation of drugs in non-target tissues [14]. However, the lack of a complete understanding of the nature of exosomes due to their heterogeneity resulted from undesirable separation and lack of purity in production, as well as the possibility of exacerbation of the disease due to the presence of exosomes that have the same activity as metastatic cancer exosomes, has been confronted with uncertainties and fear in clinical practices.

6. Challenges of exosome-based nanocarriers for drug delivery

Despite the high loading efficiency of exosomes, abundance of original materials for them construction, optimal targeting, and scalable and repeatable manufacturing methods, they could operate as a double-edged sword in the development and spread of cancerous cells as metastases [105], or the diagnosis of tumor cells for treatment [106], because the exosomes act naturally as a coded and non-encoded messenger. For example, the immune responses from exosomes in the treatment of cancers are unclear. Because exosomes are allogeneic sources and induce immune responses [107]. Moreover, Zhang, et al. [108] illustrated that EGFR-containing exosomes isolated from gastric cancer cells can promote the growth of hepatic specific metastasis based on hepatocyte growth factor activation. Therefore, despite widespread attention to exosomes, the complexities and ambiguities of the exosomes in the treatment of cancers are still not fully understood. In order to overcome the existing ambiguities, it is necessary to take the appropriate and urgent attention to the following.

Most techniques and approaches for the isolation of cancerous exosomes are complicated and difficult. However, it is vital that the complexity of the manufacturing method such as the producing exosomes, drug loading, or biomarker binding, must be diminished. Furthermore, we must pay close attention to the nature of the cancer cells to produce the exosomes, as well as the type of cargo that is packed. Because, the high heterogeneity of exosomes even in one type of cancer is due to the different types of composition on the exosomal surface. One of the recommended strategies for controlling the loading of pharmaceutical compounds without destruction of exosomes, rapid production and reduction of potential immune response activities in exosomes is the application of cellular engineering techniques. However, it must be assured that the important structural ingredients of parental cells, along with the induced compounds on the produced exosomes, are retained to maintain organotropism.

Besides, the role of exosomes in the diagnosis and treatment of cancer cells in animal and cell models has been confirmed. However, large-scale clinical trials need to confirmation and validation the use of exosome and its associated efficacy in the treatment of cancer cells. Artificial exosomes or exosomes-inspired liposomes can be used to prevent possible safety problems associated with exosomes. Autologous sources can draw more attention in this regard. Furthermore, the

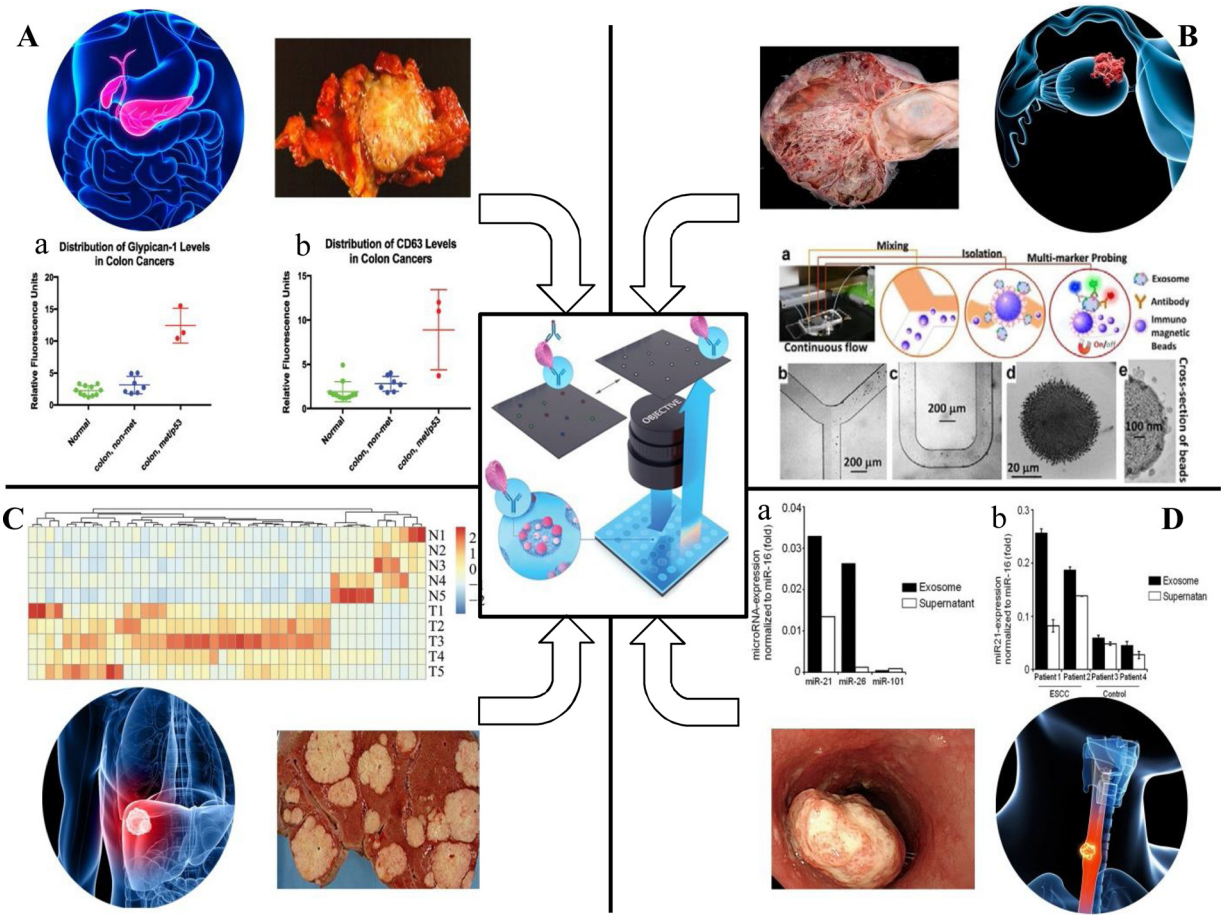

Fig. 6. Exosomal biomarker for cancer diagnostic. (A): A set of 10 colon cancer patient samples was assessed by ACE immunoassay for glypican-1 (a) and CD63 (b) levels [90]. **(B):** (a) Workflow of the ExoSearch chip for continuous mixing, isolation and (b) *in-situ*, multiplexed detection of circulating exosomes, (c) Bright-field microscope images of immunomagnetic beads manipulated in microfluidic channel for mixing and isolation of exosomes, (d) Exosome-bound immunomagnetic beads aggregated in a microchamber with on/off switchable magnet for continuous collection and release of exosomes, and (e) TEM image of exosome-bound immunomagnetic bead in a cross-sectional view [88]. **(C):** Heatmap of differentially expressed tsRNAs between liver cancer patients and healthy donors. N1-N5: five healthy controls; T1-T5: five liver cancer patients [100]. **(D):** The expression of microRNA-21 (miR-21) is illustrated in serum exosomes from patients who had esophageal squamous cell carcinoma (ESCC) and from those who had benign diseases without inflammation [96]. Figures adopted and modified from refs [90], [88] [100], and [96] with permission from Lab on chips, ACS Nano, Cancer and Molecular cancer, respectively.

identification and selection of exosome-based biomarkers in response to cancer therapies requires further studies and a change in their satisfaction pattern. Because, the diagnosis and use of drugs by exosomes containing biomarker could lead to a variety of responses resulting from the personalization of the experimental or therapeutic process. Other challenges such as the lack of a precise instruction for the maintenance of exosomes, the lack of a safe standard with high operation in the production of exosomes, and even the lack of a proper instruction for loading multiple drugs in the exosomes, is also observable.

7. Conclusions

Based on the vast variety of activities in the medical field and the amazing applications of exosomes in it, it is necessary to continually evaluate the progress made and their challenges. Despite some progresses in the generation, separation and loading of pharmaceutical compounds into exosomes, as described in this literature, there are some serious ambiguities and challenges in using exosomes. The most important leading challenges include the heterogeneity of exosomal products, the unclear biological activities of exosomes, the presence of uncontrollable genomic or protein constituents on their surface that can induce unwanted activities. However, the function of exosomes in therapeutic activity is very stimulating and can provide great hope for controlling the activity of cancer cells. For example, it has been shown in this review that the using exosomes can improve drug delivery based on the reduced drug resistance of cancer cells along with enhancing the imaging quality and prognosis of all types of cancer. On the whole, to reduce the ambiguity of the use of exosomes in therapeutic activities, it seems that a wide range of experiments should be performed in cellular, animal and ultimately human models, so that different immune and therapeutic responses from these experiments can provide a pattern of approvable for using exosomes by the medical community.

Declaration of Competing Interest

The 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.

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