

Review Article

Exosomes: A Paradigm in Drug Development against Cancer and Infectious Diseases

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Extracellular vesicles are small single lipid membrane entity secreted by eukaryotic and prokaryotic cells and play an important role in intercellular signaling and nutrient transport. The last few decades have witnessed a plethora of research on these vesicles owing to their ability to answer many hidden facts at the supramolecular level. These extracellular vesicles have attracted the researchers because they act as shuttle agents to transfer biomolecules/drugs between cells. Recently, studies have shown the application of exosomes in tumor therapy and infectious disease control. The present review article shows the importance of exosomes in cancer biology and infectious disease diagnoses and therapy and provides comprehensive account of exosomes biogenesis, extraction, molecular profiling, and application in drug delivery.

1. Introduction

Extracellular vesicles are cell membrane-derived small entity. In general, these are ubiquitously found in all organisms and scattered in all types of biofluids. These are multifunctional units that act as a carrier of cellular communication as well as help in removing cellular garbage. Extracellular vesicle cargo protein, lipid, nucleic acids, or RNA from mother cells to

distant tissue cells through the support of biofluids and transfer the information [1]. Earlier, extracellular vesicles were considered as an insignificant bioentity generated by the cell during its lifetime. However, in the last few decades, extracellular vesicles have generated significant interest among the scientific community as they have been found to be associated with many diseases. Recent research works demonstrate that the extracellular vesicles have potential to act as naturally

occurring drug-delivery vehicles and can find application in several diseases including cancers. This is due to their innate biocompatibility, unique capability for targeted delivery, and ability to reach remotely located recipient cells. Number of the findings demonstrated that membrane-derived vesicles are important for diagnostic and therapeutic purposes for different diseases, including cancer, infectious diseases, cardiovascular diseases, neurodegenerative diseases, and pregnancy [2]. Its application depends on the biogenesis and size of the entities; it could be classified into exosomes, microvesicles, apoptotic bodies, and oncosomes (Figure 1). Most of the exosomes are small size extracellular vesicles, ranging between 40 and 120 nm and are created by the fusion of an intracellular multivesicular body with the cell membrane. However, less than 40 nm vesicles were also detected by subdiffraction imaging and fluorescent probe during a real-time study of exosomes trafficking in living cells [3, 4]. Microvesicles are generated by normal cell and its size ranges from 100 to 1000 nm and apoptotic bodies' sizes up to 5 μm are generated by apoptotic cell. Similarly, oncosomes are the largest vesicles, their size ranges from 1 to 5 μm , and they are secreted by cancer cells [1, 5].

Among all above-mentioned vesicles, exosomes have attracted more interest among the researchers, owing to their intriguing capabilities like mediating cell-to-cell communication. Intercellular communication is an important process to maintain homeostasis in multicellular systems. The dysregulation of communication pathways have been associated with the cancer development and progression [48]. Therefore, the development of novel anticancer treatments will strongly depend on improving our understanding of the cellular interactions between cancer cells and other cells. Cellular communication takes place between cells through gap junctions, adhesion molecules, and nanotubes; also, it can be via soluble communication signals like growth factors, cytokines, tumor, and non-tumor-derived hormones. To date, several researches have showed a positive correlation between carcinogenesis/metastasis/drug resistance/bacterial infection and the concentration of exosomes [49, 50]. Logozzi et al. have also quantified the exosomes of plasma of melanoma patient and healthy donors by housekeeping proteins and caveolin-1 marker and found significant higher exosome in plasma of melanoma patient and concluded that exosomes have direct relation in malignant progression [51]. Recently, VanDeun et al. have developed a knowledge base EV-TRACK for enhancement of transparency and reproducibility of vesicles research road map [52]. Based on these notions, we herein present a brief review on the importance of exosomes in drug delivery in both cancerous and noncancerous ailments. Additionally, we have also discussed the basic function of exosomes in the human body, molecular profiling, and its characteristics.

2. Exosomes Uniqueness and Natural Existence

Initially, researchers observed human plasma fraction has coagulant properties as "platelet dust," and, later on, the fraction was characterized as microparticles [53]. Trams et al. made the first description of exosomes in 1981 [54]. Johnstone

and coauthors have also termed the extracellular vesicles as exosomes. They had isolated exosomes from sheep reticulocytes during reticulocytes maturation into erythrocytes [55]. Whenever cells enter apoptosis, irregular shaped apoptotic vesicles are generated with size 50–5000 nm [56]. These vesicles may contain histones, DNA, and RNA molecules and are immediately cleared by the immune system (macrophages) in response to their apoptotic signaling [57]. In contrast, microvesicles originate by budding and fission of the cellular plasma membrane into extracellular space. These vesicles are more homogeneously shaped, smaller than the apoptotic bodies in size ranging from 50 to 1000 nm and rich in specific proteins and lipids [58]. The major population of extracellular vesicles is identified as exosome by size less than 100 nm in diameter. Most of the mammalian cells like neurons and immune cells secrete exosomes and cancer cells also secrete greater amounts of exosomes. Interestingly, Gram-negative bacteria also produce outer membrane vesicles during normal growth, which contain bioactive proteins and it has diverse biological functions. The bacterial outer membrane vesicles are small ball-shaped structures with a size range from 20 to 250 nm in diameter. Mayrand and Grenier have described the formation, growth conditions, isolation, composition, and biological activities of bacterial outer membrane vesicles [59]. Similarly, Kulp and Kuehn investigated the natural sources, characteristics, biological functions, and biogenesis of vesicles from bacteria and revealed the vesicle secretion through the distal effect of membrane molecules during environmental interaction [60]. Moreover, it has been reported that bacteria can also secrete outer membrane vesicles during their normal activities. Gram-negative bacteria that are able to secrete outer membrane vesicles during normal growth include *E. coli*, *N. meningitis*, *Sh. flexneri*, *P. aeruginosa*, and *H. pylori* [61–64]. In addition, it has been reported that fungi and eukaryotic parasite can also secrete extracellular vesicles [65].

Exosomes are membrane-derived vesicles actively secreted by mammalian cell particularly immune cells, such as macrophages [66], dendritic cells, T cells [67, 68], and B cells [69]. Exosomes are also secreted by mesenchymal stem cells [70], epithelial [71] and endothelial cells [72], and cancer cells [42]. Li et al. have reported the secretion of exosomes from intestinal epithelial cells, fibroblasts, mastocytes, antigen presenting cells, platelets, hepatocytes, and lymphocyte [73]. In addition, exosomes have also been identified in different body fluids, including human saliva, serum, breast milk [74], CSF [75], urine [76], and semen [77].

3. Structure and Contents

In 2014, the International Society for Extracellular Vesicles (ISEV) has reported the presence of exosomes-associated surface markers and the absence of nonexosomal proteins for the characterization of exosomes. The exosomes-associated surface markers include Alix, TSG101, tetraspanins (CD9, CD63, and CD81), flotillin 1, cell adhesion molecules (CAM), and integrins [79]. Exosomes consist of a lipid bilayer membrane and are characterized by a size of 50–100 nm in diameter, and they have "cup" or "dish" shaped morphology when

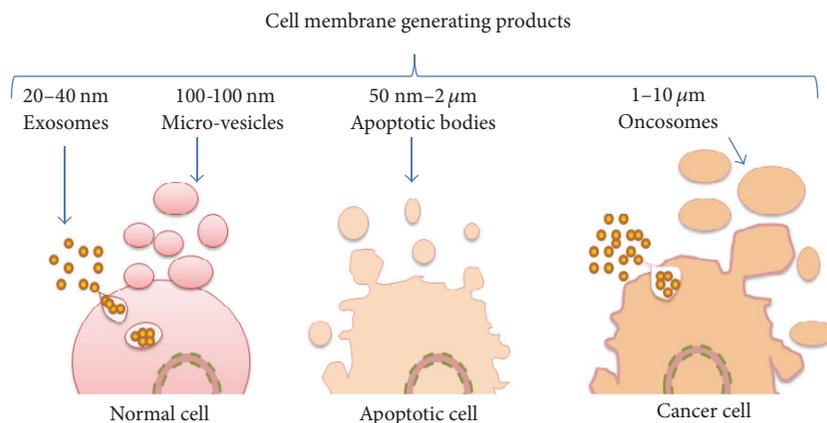


FIGURE 1: Different types of extracellular vesicles (EVs) produced by normal and diseased cells.

analyzed by electron microscopy and are enriched with certain protein markers such as tetraspanins [42]. Moreover, its presence is also determined by density gradient, it has density $\sim 1.13\text{--}1.19\text{ g/ml}$ and membrane rich in lipids like ceramide, cholesterol, and sphingolipids [80]. ExoCarta is an exosomes database, which was created in 2008 to collect and classify the identified exosomal proteins and RNA molecules. The resource is web-based (<http://www.exocarta.org>) and freely available to the scientific community [81]. To this date, they have identified 9769 proteins, 3408 mRNAs, and 2838 miRNAs by independent examinations in exosomes from different species and tissues. In addition, the membrane structure of Gram-negative envelope consists of two membranous structures: the inner one (IM) and the outer membrane (OM) and these two membranes are separated by the periplasm. The outer layer of the OM is composed of lipopolysaccharide (LPS), whereas the inner layer and both layers of the IM are composed of phospholipids. The periplasm is a gel-like layer that contains a thin layer of peptidoglycan (4 nm thick) [60]. Therefore, the secreted vesicles are composed of lipopolysaccharide, periplasm, and phospholipids [82].

4. Biogenesis, Release, and Uptake

Membrane vesicles play diverse roles in cellular communications in both prokaryotic and eukaryotic cells, while Gram-negative bacteria secrete outer membrane vesicles and eukaryotic cells secrete microvesicles for cellular contact. Mechanisms of bacterial vesicle biogenesis and the pathological roles are still not defined clearly. However, vesicles secreted by different types of cells may have several similarities in the biogenesis and functions in different biological systems. Zhou et al. [83] have suggested that the biogenesis of Gram-negative bacteria derived vesicles could be because of cell wall turnover during growth, which causes a turgor on the outer membrane, eventually causing the outer membrane to bulge and then bleb. It is excised and removed from the peptidoglycan layer of the cell wall. Independent experimental studies also demonstrated that membrane vesicles are generated as a result of cell wall turnover in Gram-negative bacteria [83, 84]. However, Wensink and Witholt

[85] have described a hypothesis for the biogenesis of Gram-negative bacteria derived vesicles. They suggested that the blebbing of outer membrane might occur in response to a high rate outer membrane synthesis in comparison to peptidoglycan.

On the other hand, the process of exosome biogenesis in human samples has been clearly defined in several studies, which includes four sequential stages: (1) initiation, (2) endocytosis, (3) multivesicular bodies, and (4) exosomes secretion [86]. Previous studies have suggested that exosomes are derived from the multivesicular bodies sorting pathway. Exosomes originate by inward budding into large multivesicular bodies in the cell cytoplasm, and then these bodies fuse with the plasma membrane leading to exosome secretion into the extracellular space. Moreover, either the endosomal-sorting complex is required for transport signaling or the sphingolipid ceramide pathway [87] regulates this process. In response to vesicular accumulation, the multivesicular bodies will be either sorted to be degraded by lysosome or released into the extracellular space for exosome secretion through the process of exocytosis. The exosomal cargo, including proteins, lipids, and RNA/DNA molecules, is all packed into the exosomes during this process; the exosomal contents may vary according to the parent cell type. Previous studies have reported that a number of proteins play a role in regulating exosomes secretion pathway. Ostrowski et al. have shown that Rab GTPase machinery can regulate exosome secretion, in which Rab27a and Rab27b proteins can affect the size and localization of multivesicular bodies [88]. Other studies have demonstrated that the exosome secretion can be affected by different factors. For instance, the secretion will be increased in response to intracellular Ca^{+2} accumulation [89]. Another factor that would affect the exosome release is the cellular pH. When the pH of the microenvironment is low, exosomes secretion and uptake by target cells increase as well. In a recent study, it was observed that low pH conditions have significant effect on the exosomes expression of the cancerous cells. When prostate cancer cell lines were cultured at both low pH and 7.4 pH, then exosomes were more predominant at low pH cultured condition. Similarly, tumor-released exosomes are able to transfer their content to target cells by

membrane-to-membrane fusion and this is much favored by microenvironmental conditions such as low pH. The delivery of exosomal cargo for uptake of their target cells can occur by one of the following ways: (1) receptor-ligand interaction; (2) direct fusion of exosomes with the plasma membrane of the recipient cell, which leads to releasing the exosomal content into the cellular cytoplasm; (3) endocytosis by phagocytosis.

5. Extraction of Exosomes

The abnormality in proteins/enzymes or nucleic acid function is an indication of cellular dysregulation or diseases. Identification of such dysfunctions could significantly change the outcome and condition of a patient. The biophysical characterization/screening of exosomes in biofluids are an emerging area of research, as they contain information from the mother cells. It has been demonstrated that the concentration of exosomes is directly related to the health condition of a person [90]. In such situations, the development of novel methods for isolation, molecular profiling, and concentration determination of exosomes would not only enhance the knowledge at supramolecular level but also help in delineating many hidden questions related to deadly diseases like cancer. For this purpose, several classical and modern methods for extraction, isolation, and characterization from different biomatrices are available. It must be noted that exosomes isolation methods to date only enable enrichment but not distinct separation of these extracellular vesicles subpopulations [1]. Table 1 depicts some general approaches and mechanisms used for the isolation of exosomes [6–13].

Recently, ultracentrifugation was used for exosomes isolation in combination with the sucrose gradient and immune-dependent isolation such as Magnetic Activated Cell Sorting (MACS) [91]. Deun et al. have suggested a comparative evaluation of exosome isolation protocols: (i) OptiPrep™ density gradient centrifugation which outperforms ultracentrifugation and (ii) ExoQuick and total exosomes isolation and precipitation in terms of purity, quantity, and purity and their impact on downstream omics approach for biomarker development [91].

Differential ultracentrifugation involves applying different levels of centrifugal force on a solution containing exosomes such as biological fluids or conditioned cell culture media [92]. Starting from low speed centrifugation, which is necessary initially to remove cells and large cellular debris, then, the resulting supernatant is centrifuged at 10,000–20,000 ×g in order to remove large debris and intact organelles. Lastly, the supernatant is again subjected to a high-speed centrifugation (100,000–150,000 ×g) in order to achieve a pellet exosomes. Therefore, this method precipitates not only the exosomes but also other membrane vesicles, proteins, and/or protein-RNA aggregates. In general, the density of exosomes is different from the contaminants; it can be separated by using sucrose density gradient with centrifugation. This technology is more efficient than ultracentrifugation while it is requiring more centrifugation time up to 62 to 90 h [52]. In addition to this, immune-affinity chromatography and size exclusion chromatography are also normally used for the extraction of exosomes. In the antibody-dependent

method, it is covalently attached to exosomal surface markers like TSG 101 or tetraspanins and nontarget particles remain unbound. When the unbounded particles are removed then washing the stationary phase with a low pH buffer may collect the bounded particles. This process is efficient and provides pure exosomes compared to other size/density-dependent methods. Likewise, Kalra et al. have confirmed OptiPrep density gradient method was more efficient to isolate exosomes without plasma proteins [94].

Size exclusion chromatography contains different size components which separate solutions according to their size. In addition, the SEC is using a gravity flow for separation, to maintain the vesicle structure, integrity, and biological activity of exosomes [12]. This technology has high sensitivity and excellent reproducibility, because it is using gravity flow for separation which makes it time-consuming if it could be combined with ultracentrifugation; then high isolation rate can be achieved in less time [94]. Moreover, commercial kits are also available for exosomal extraction process. Besides these two techniques, polymer precipitation and microfluidic technologies, are also effective methods to be used for exosomal isolation as an alternative to ultracentrifugation; these methods were described in detail by Batrakova et al. [93]. ExoQuick-TC kit is the most common commercial polymer precipitation-based method for exosomes extraction. This method is employed to isolate viruses and other macromolecules by using polyethylene glycol (PEG). On the other hand, microfluidic-based techniques use smaller volumes of starting solution and provide more pure exosomal isolate within a short time [95]. Moreover, these technologies have been used for diagnostic purposes because of their low yield and high sensitivity. This method depended on one of the following techniques: (a) immunoaffinity, (b) sieving, and (c) trapping exosomes, which was described by Batrakova et al. [93].

6. Molecular Profiling of Exosomes

Molecular profiling of exosomes obtained from different sources is an important step. Exosomes are characterized according to their biochemical properties (size, protein, and lipid content) using different methods [96] like Western Blotting, transmission electron microscope (TEM), nanoparticle tracking analysis (NTA) [9], dynamic light scattering (DLS), mass spectrometry (MS), flow cytometry [95, 96], tunable elastomeric pore sensing, [97] and microfluidics [98] but these are not limited. The detection and profiling are often hindered by the requirements of high purity and large sample amount. To circumvent these issues, several new, sensitive, and selective methodologies based on surface plasmon resonance (SPR), fluorescence fluctuation spectroscopy (FFS), and so forth have been applied in several reports in Table 2 [14–19]. Among these, SPR is the most popular label-free, real-time sensing technique [99]. Vogel and coworkers have developed a label-free SPR based methodology for the detection of exosomes derived from breast cancer cell lines (MCF-7, BT-474, and MDA-MB-231) [15]. They reported immunosensor surface has the ability to identify various exosomes as well as exosomal biomarkers [98, 100]. The molecular profiling results of exosomes have indicated the selective and

TABLE 1: Exosomes isolation methods and mechanisms with specificity and demerits.

Methods	Steps	Mechanism	Specificity	Demerit	Ref.
Differential centrifugation	(i) 300 ×g (10 min) (ii) 1000 ×g to 20000 ×g (30 min) (iii) 100,000 ×g (60 min)	Based on centrifugal force	Common method to isolate exosomes from biological fluids	Yield lower when sample is viscous	[6]
Density gradient	(i) 30% sucrose gradient (ii) Differential centrifugation	Based on centrifugal force and density gradient	Separate low-density exosomes from high density contaminants and vesicles	Sensitivity high with centrifugation time	[7]
Size exclusion chromatography	(i) Sample applied on column packed with specific designed porous beads that allow elation only exosomes, without centrifugal force	Based on porosity of materials	Centrifugal force sensitive vesicles isolate this method and specific beads used for specific size. multiple biological samples can run together in this method	Long time taking procedure	[8]
Filtration	(i) Exosomes separate from the high molecular weight proteins and fatty acids	Based on membrane materials and porosity	Easily separate the soluble molecules and small particles from exosomes	Exosomes attached with membranes and lost the yield and original size	[9]
Polymer-based precipitation	(i) Biological fluid mixing with polymer (ii) Incubation till precipitation (iii) Centrifugation at low speed. (vi) Resuspend in PBS	Based on polymer materials and precipitation	The advantages of precipitation include the mild effect on isolated exosomes and usage of neutral pH	Polymer-based precipitation and co-isolation of contaminants, like lipoproteins. In the presence of polymer material, not compatible with downstream analysis	[10, 11]
Immunological separation	(i) Magnetic beads bound to the specific antibodies Example: ELISA-based separation method	Based on antibody receptor interaction	Methods for characterization and quantification of protein involve in selective subtypes of exosomes	Method is not applicable for large volumes	[7]
Isolation by sieving	(i) Sample sieving via a membrane (ii) Perform filtration with pressure (iii) electrophoresis	Based on sieving size and pressure	Short separation time with high purity of exosomes	Low recovery rate	[12]
Cell sorting	(i) Sample incubation 4 h with magnetic beads (ii) centrifugation 100,000 ×g (60 min)	Based on centrifugal and magnetic force	Short separation time with high purity of exosomes	Expensive and hectic	[13]

effective discrimination between the antigen generated by three different types of cell lines. In previous studies, the same research team has reported the molecular and dimensional profiling of exosomes by using the FFS method [100, 101]. Another SPR based method has been reported recently to determine the concentration of exosomes in solution [98]. Using surface functioned sensor with anti-CD63 antibodies, the authors detected exosomes selectively obtained from human mast cells. By this method, total mass (lipids, proteins, and nucleotides) could also be determined using a small

sample volume. Despite the fact that exosome deformation hindered the study, the authors reported high accuracy ($\pm 50\%$) in concentration determination. An easy, efficient, and novel label-free SPR imaging (SPRi) technique in combination with antibody microarrays was reported for the quantitative determination of exosomes in cell culture supernatant (CCS) [102]. The study also showed a positive association between exosome secretion and metastatic potential in hepatocellular carcinoma cells (highly metastatic cell line secreted more exosomes than poorly metastatic ones).

TABLE 2: Recent reported methods for molecular profiling of exosomes.

Method	Source	Application	Explanations	Ref.
Combined colloidal gold nanoplasmonics and SPR	Serum	Oncology	Detection at molar level, Showed presence of 4-fold exosomes in MM patients than normal	[14]
Microfluidic	Plasma	Cancer as well as non-cancerous diseases	Enhanced and minimal invasive detection,	[15]
SPR	Serum	Oncology	Quantification of the proportion of CREs within the bulk exosome population	[16]
NTA	Whole blood sample	Oncology	Enriched level of MDR-1, MDR-3, endophilin-A2, and PABP4 in resistance prostate cancer cells (DU145)	[17]
Metal Nanoparticle	Serum	Oncology	Microsome and exosome detection, detection of two surface markers on exosome	[18]
Dual-wavelength SPR	Synthetic	Drug development	Vesicles carrying marker CD63 link to not greater than 10% of the vesicles in sample.	[19]

Zhu et al. developed a label-free nanoplasmonic exosome (nPlex) assay for quantitative analysis of exosomes [105]. The method, which was based on transmission mode SPR through functionalized nanohole arrays (Figure 2(a)), has the ability to detect proteins present on the surface as well as in the lysate of exosomes. They found that a large amount of exosomes with an average diameter of 100 nm was secreted by the ovarian carcinoma cell lines. The selective identification of ovarian carcinoma exosomes (detection of 12 potential exosomal markers within 30 mins) dictates the potential of the method for diagnostic purposes (Figure 2(b)).

Proteomic analyses have been used to identify and decode the proteins associated with outer membrane vesicles. Although bacterial outer membrane vesicles are more abundant and easier to obtain than human vesicles, only few proteomic profiling instances of native outer membrane vesicles derived from bacterial strains have been reported [78, 104–107]. Previous studies described the process of Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membranes and vesicle polypeptides to identify their protein profiles [108]. In addition, exosomal RNA and proteins were also identified using RT-PCR, nucleic acid sequencing, Western Blot, or ELISA [42]. Sambrook et al. have also described the conventional agarose electrophoresis in the presence of ethidium bromide in order to detect nucleic acids in samples of vesicles derived from bacterial strain [111].

7. Exosomes Role in Normal and Cancer Cells

A number of studies have demonstrated the tumor-derived exosomes, which serve as biologic messengers for immune suppression and other pro-cancer activities [64, 110, 111]. Cancer-derived exosomes play diverse roles in the tumorigenesis, tumor growth, angiogenesis, metastasis, and drug resistance [42]. During the process of tumorigenesis, normal cells are transformed into cancerous cells and subsequently secrete exosomes, which have the potential to transform the healthy cells to cancerous cells. As an example, neoplastic transformation of adipose-derived stem cells (ASCs) could be induced in response to prostate cancer cell-associated exosomes, which

deliver the oncogenic proteins and mRNA molecules to recipient cells and subsequently induce tumor formation [112]. Sometimes tumor-released exosomes, expressing a reporter-gene, travel through the blood of xenografts ending within the germ line that in turn expressed the exosomes-delivered gene, thus supporting the idea of a key role of extracellular vesicles in somato-to-germ-line transmission of nucleic acids. In addition, it has been widely reported that tumor-derived exosomes show promoting effect on tumor growth. During tumor formation, exosome containing cell survive because it has the ability to inhibit apoptosis and promote proliferation and metastasis [113]. Similarly, in human tumor cell line derived exosomes may induce tumor-like transformation of human mesenchymal stem cells, supporting a key role of exosomes in tumor metastasis [114]. Consequently, angiogenic factors are usually present in tumor-derived exosomes, which is necessary for angiogenesis and tumor proliferation [115]. Exosomes also contain factors required for metastasis, thus enhancing migration and invasiveness of cancerous cells [116–118]. Moreover, exosomes also play a role in the development of drug resistance via different mechanisms. For example, cancer cell-derived exosomes transmit multidrug resistance (MDR) associated proteins and miRNAs to recipient cells leading to the development of resistance [119]. Another mechanism by which exosomes induce resistance is an exosomal drug efflux, in which drugs can be affixed from the cancerous cells by exosomes [120]. During cancer therapy development of intrinsic resistance against drugs happens due to acidic microenvironment and chemo resistance impairment in drug delivery. In addition to these, several studies have shown the relationship between exosome and immune system function [121]. They suggested that exosomes can interact through signaling and the exosome comes from immune cells. While melanoma cell releases extracellular vesicles expressing FasL that efficiently induces Fas-mediated apoptosis in target T cells, suggesting the role of tumor exosomes in tumor immune escape. Extracellular vesicles released by human colon cancer cells express that both FasL and Trail are able to induce cell death of target T cells through the specific pathways and that exosomes expressing these

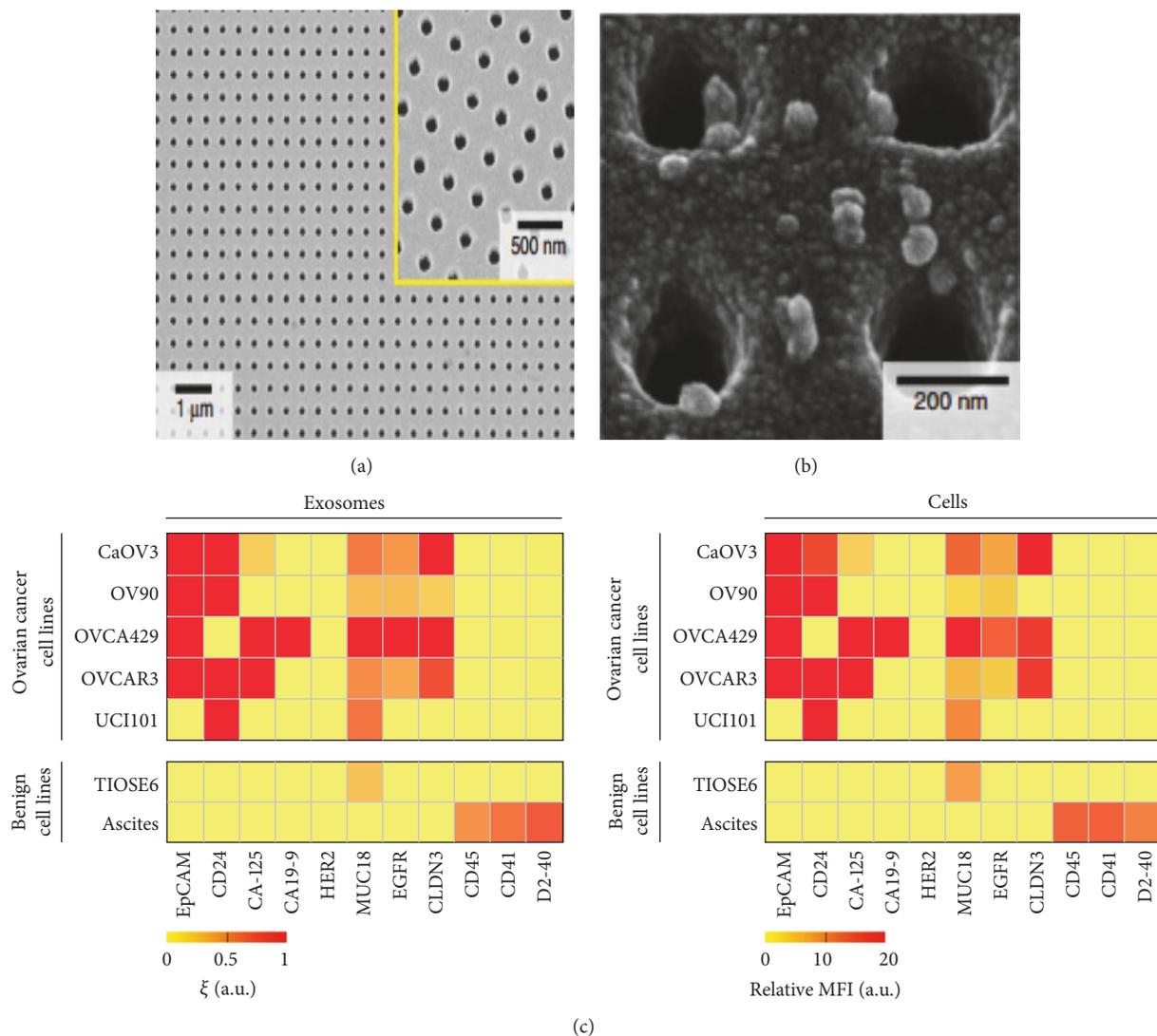


FIGURE 2: Scanning electron microscopy image of (a) nPLEX sensor with periodic nanoholes in the range from 200 to 450 nm in diameter, and here (2b) exosome captured by functionalized nPLEX. (2c) EpCAM, CD24, CA19-9, CLDN3, CA-125, MUC18, EGFR, and HER2, a list of putative ovarian cancer markers; CD41 and CD45 for immune host cell markers and D2-40 a mesothelial marker for exosomes profiled left in nPLEX sensor and their parental ovarian cell lines filled in right for flow cytometer, according to Im et al. [78].

two molecules are detectable in the plasma of colon cancer patients and entirely functional in inducing T cell death [122]. In mammalian cells, exosomes have pro- and anti-inflammatory properties depending on the type of cell origin [80]. For example, NK cells release exosomes expressing functional molecules associated with NK-cell function and the same exosomes are detectable in the plasma of healthy humans, suggesting a key role of NK-released exosomes in the control of our body homeostasis [114, 123]. However, similar findings have been reported with single cell eukaryotic-derived vesicles. Zhang et al. have demonstrated that exosomes are involved in cell-to-cell contact during immune responses for tumorigenesis, infectious diseases, allergies, and autoimmune diseases [42], for example, during myocardial infarctions in the presence of clusterin in exosomes obtained from pericardial fluids of patients [124]. Currently,

there are two main groups of exosomes that are involved in infectious biological: (1) single-celled eukaryotic exosomes and (2) exosomes derived from infected cells. The eukaryotic single-celled pathogens such as the pathogenic fungus *Cryptococcus neoformans* and the protozoan parasites *Leishmania major* and *donovani* [125] secrete exosomes, which may influence the host immune system. The second group is exosomes released by mammalian cells infected with pathogenic bacteria, prion protein, and viruses [126, 127]. Previous studies have reported that bacteria can secrete outer membrane vesicles; fungi and eukaryotic parasite can also secrete extracellular vesicles [128]. Pathogen-derived exosomes carry specific virulence factors like proteins or RNA molecules, which can either spread or limit the infection depending on the pathogen and its target cells, Table 3 [20–27]. Furthermore, the potential roles of pathogen-derived vesicles

TABLE 3: Examples of infectious disease where pathogen-derived exosomes play a role in pathogenesis.

Disease causing agents	Name of disease	Exosomes role	Ref.
<i>Leishmania</i> spp.	Leishmaniasis	Spread virulence factors	[20]
HIV	AIDS	CD4+ T cells transinfection and delivery of Nef to by stander cells	[21]
Prion protein	Transmissible spongiform encephalopathies	Virulent factors delivery to normal cells	[22]
<i>Cryptococcus neoformans</i>	Cryptococcosis	Virulence factors spread and polysaccharide capsule formation	[23]
<i>Histoplasma capsulatum</i>	Ascomycota opportunistic fungal pathogens	Spread virulence factor in intracellular and extracellular space and promote virulence and provide stress response and fungal growth	[24]
<i>C. Albicans</i> , <i>C. parapsilosis</i> <i>S. schenckii</i> <i>S. cerevisiae</i>	Oral, vaginal, and systemic infections Candidiasis RTI Rarely pathogenic	Help in growth, Spread virulence and enhance pathogenicity	[25]
<i>Trypanosoma cruzi</i>		Surface antigens spread during pathogenicity with membrane vesicles	[26]
HCV	Chronic HCV	v RNA transfer in normal cells	[27]

in infection biology are illustrated in Table 3 [20]. However, during infection pathogen derived vesicles formation is much less recognized about the molecules involved in exosomes biogenesis and secretion. In mammalian cells, the molecules that are involved in exosomes biogenesis and secretion include ESCRT protein [27], ceramide [87], Rab27 [88], Rab11 [89], and Rab35 [129]. Although a similar subset of these proteins (ESCRTs, Rab11, and Rab27) has been identified in and *Leishmania* exosomes [125], their role in single-celled eukaryotic exosome biogenesis and release is not clear yet. In another experimental study, Silverman et al. have suggested that HSP100 plays an important role in packaging of proteins into *Leishmania* exosomes [135]. Interestingly, exosome has been associated with prion related disease. Berrone et al. observed pathological prion protein from the blood of animals with a prion related disease only associated with exosomes and this was in turn associated with plasma infectivity as well [136].

8. Exosomes from Bacteria and Its Function

The outer membrane vesicles associated proteins have significant biological activities as mentioned in previous studies [132, 133]. In addition, the outer membrane vesicles can mediate the secretion of both soluble and insoluble compounds (such as bacterial lipids and membrane proteins). For instance, then it allows the secretion of adhesion molecules of pathogenic bacteria. Adhesins are insoluble proteins that mediate coaggregation and these proteins are important for host tissues colonization. Another type of insoluble molecule like quinolone was secreted in outer membrane vesicles by *Pseudomonas* as a signal molecule, which is important for cellular communication [134]. Moreover, the bacterial exosomes are also mediating the secretion of soluble proteins in a protective complex, in which soluble molecules are part of

the lumen or attached to their surface. The surface-associated soluble proteins and the periplasmic molecules within the vesicle lumen are resistant against extracellular degradations by proteases. This complexity provides the ability of membrane vesicles to protect the secreted proteins and to allow less stable molecules, to reach further destinations during their transport [135]. Kadurugamuwa and Beveridge [137] have proposed two mechanisms for delivering the soluble content of membrane vesicles to its target site. The content could be delivered either through spontaneous lysis of outer membrane vesicles and consequently content diffuses or attachment of vesicles to their target followed by proximal lysis, internalization, or fusion to deliver the content.

The outer membrane vesicles secreted by bacterial strain might contribute significantly to bacterial survival and virulence factor existence. For instance, membrane vesicles could act as a defense and resistance mechanism against both internal or external damaging agents, and thus the vesicles can eliminate these toxicants instantly [136]. In addition, vesicles are loaded with lytic enzymes and receptors, which are important for bacterial nutrient acquisition [137]. Moreover, these vesicles are also important for nucleation and mediating the interactions of biofilm [138]. All the above-mentioned survival-related functions are important to enable the pathogenic organism to survive inside the host and eventually cause diseases. In addition, it also contains virulence factors such as an active toxin, which can be delivered into host cells by different mechanisms [139, 140].

9. Exosomes-Mediated Drug Delivery

Recently, evidences of natural exosomes-mediated drug delivery are increasing for cancer and infectious disease treatment [70, 141, 142]. In current opinion, two approaches are

considered to develop drug carriers for in vivo drug-delivery system based on cell-derived membrane vesicles which include the following: (1) modification and engineering of natural cell membrane vesicles primed with therapeutic compounds to target certain cell types and (2) using the essential characteristics of membrane vesicles in order to design nanoscaled drug vehicles [141]. Lately, exosomes have gained considerable interests for being used as a vehicle for either cell-derived materials or therapeutic drug-delivery systems. Exosomes are ideal candidates for drug delivery because they are bioavailable vehicles, which are known to be well tolerated, bioactive, specific to their target cells, and resistant to metabolic processes and have the ability to easily penetrate through impermeable biological barriers like blood-brain barrier (BBB). Increased evidence suggested that the natural membrane vesicles have more advantages than the synthetic nanoscaled drug systems due to their natural specificity to their target cells [143], their natural stability in blood, and their ability to tolerate the patient's immune response. However, exosomal purification is difficult because mammalian cells usually release small quantities of exosomes; thus exosome-mimetic nanoscaled vesicles were developed when overproduction is needed [38].

10. Exosomes as Carrier for Therapeutic Agents

There are so many approaches that can be used in order to load exosomal carriers with therapeutic agents and all were described in detail by Batrakova et al. [93]. There are advantages and disadvantages for each approach, and also this may be restricted to the type of drug, the targeted disease, and the conditions required for a specific type of exosomal cargo. The first one is ex vitro loading of naïve exosomes that are purified from parental donor cells and then incorporated with a therapeutic agent. The other approach is by loading parental cells with a drug, which is subsequently released in the exosomes. The final approach is by transfecting or infecting donor cells with drug-encoding DNA, which is eventually released in exosomes.

Therefore, exosomes serve as effective vehicles for many molecules that would be otherwise rapidly degraded before approaching their target like drugs, proteins, and microRNA/silent interfering RNA (siRNA). Alvarez-Erviti et al. have provided the first demonstration of biotechnological exploitation of cell membrane vesicles [29]. The authors showed a successful in vivo delivery with low or no toxicity or immunogenicity of exosomes-mediated siRNA to the mouse brain through injection of targeted exosomes. In order to ensure a successful delivery of the injected exosomes to their target cells in vivo and to avoid the exosomal removal of tissues of drug clearance, a novel targeting strategy was suggested. Therefore, exosomal surface protein lamp2b was used to display a targeting peptide to bind (AChR) receptor present on neurons and the vascular endothelium of the blood-brain barrier (BBB). The BBB was the major obstacle in the macromolecular drug delivery to the CNS [144]. On the other hand, artificial cell membrane vesicles mimic is also an alternative approach to obtain membrane vesicles and subsequently provides a controlled and clean drug-delivery system.

Currently, specific lipid and protein compositions are used in liposomal drug formulations to create CMV mimics that have similar properties of the natural ones [145]. Gao et al. have presented a novel strategy to generate neutrophil cell membrane-derived nanovesicles to target inflamed vasculature and significantly reduce acute lung inflammation by using nitrogen cavitation. Nitrogen cavitation was described in this study [148], which is a novel approach to fracture cells with no chemicals or long-term physical stress that could disrupt the biological functions of cellular membrane antigens.

11. Therapeutic Application

11.1. As Anticancer Drug Delivery. Recently, in vitro and in vivo experimental findings have shown that extracellular vesicles can be used as vehicles for several therapeutic agents for cancer treatment [147]. Previous clinical studies have been conducted in order to assess the efficacy of dendritic cell-derived exosomes (dexosomes) as cancer vaccines. The authors obtained promising results in phase I clinical trial following vaccination of metastatic melanoma patients [28] and in patients with advanced non-small cell lung cancer [29]. In a recent study, the effect of exosome-delivered siRNA on target cancer cells was observed. These experimental findings have shown that exosome-mediated siRNA can induce posttranscriptional gene silencing and apoptotic cell death of targeted cancer cells [30]. Smyth et al. have found that tumor-derived exosomes have been used as an effective vehicle for drug delivery [31]. Moreover, experimental studies have shown that exosomes can be used as carriers for therapeutic agents with low molecular weight [35, 149]. Other studies have demonstrated that when exosomes or exosome-mimetic nanovesicles loaded with various chemotherapeutic agents, Dox or PTX, they were able to target tumor cells in mice and significantly inhibit tumor growth without observing any side effects [38, 150, 151]. Jang et al. [28] have found that both Dox-loaded exosomes and Dox-loaded nanovesicles demonstrated similar antitumor activity, but Dox-loaded liposomes were not efficient in diminishing tumor growth. Similarly, mesenchymal stromal cells (MSCs) are an efficient mass producer of exosomes, which makes them ideal for drug delivery [61]. Pascucci et al. [33] have shown that MSCs have the ability to pack and then release drug such as Paclitaxel through their microvesicles, suggesting that MSCs can be used for drug-delivery system development with high specificity and more detail is provided in Table 4 [28–38]. Moreover, there are recently published papers showing and commenting on the ability of exosomes in delivering photodynamic drugs, possibly useful in the therapeutics of tumors.

11.2. Exosome Removal as Cancer Therapy. Cancer cells ubiquitously secrete exosomes, which transport oncoproteins and other immune suppressive molecules in order to promote tumorigenesis as well as metastasis. Therefore, several attractive therapeutic strategies have been suggested for targeting their cancer activities. Previous studies have suggested the removal of exosomes from the circulation as a strategy to attenuate the exosomal metastatic effect. Researchers have

TABLE 4: Exosomes used for cancer therapy.

Exosomal cargo	Secreting cell	Recipient cell/patient	Activity	Ref.
DC-exosomes pulsed with functional MHC/peptide molecules	Dendritic cell	Metastatic melanoma patients	Cancer immunotherapy strategy (cancer vaccine)	[28]
DC-derived exosomes loaded with MAGE tumor antigens	Dendritic cell	Advanced non-small cell lung cancer	Cancer immunotherapy strategy (cancer vaccine)	[29]
exosome-delivered siRNA	Deliver tumor suppressors to their target sites by exosomes	Cancer cells	Posttranscriptional gene silencing and apoptotic cell death of targeted cancer cells	[30]
CMV-delivered miRNA-128		Glioblastoma	Can affect cancer cell behavior Inhibits Glioma proliferation and self-renewal	[31]
CMV-delivered miRNA-7		Glioblastoma	Inhibits the epidermal growth factor receptor and down-regulate the Akt-pathway	[32]
Aex in combination with GM-CSF	Ascites	Advanced colorectal cancer	Used as immunotherapy –tumor specific antitumor cytotoxic T lymphocyte	[33]
mmnResistant anticancer drug-treated HepG2 cells -derived exosomes	Resistant anticancer drug-treated HepG2 cells	Hepatocellular carcinoma	Elicit effective NK-cell antitumor responses in vitro; Vaccine for Hepatocellular carcinoma immunotherapy	[34]
Brain endothelial cell-derived exosomes	Brain endothelial cell	Brain cancer	Can deliver anticancer drugs across the BBB	[35]
List of various types	Refer to the reference	Various	Nanoscale cancer vaccine	[36]
List of tumor suppressive miRNA	Refer to the reference	Various	Tumor suppressive	[37]
Dox-loaded exosomes and Dox-loaded nanovesicles	Cell-derived exosomes (monocytes/macrophages)	Malignant cells	Antitumor	[38]

TABLE 5: Novel strategies/devices of exosomal removal for cancer and infectious disease therapy.

Mechanism of exosomal removal	Disease	Effect	Ref.
Extracorporeal hemofiltration of exosomes	Hepatitis C	Minimize viral titers in patients	[39]
Exosome enervation by Dimethyl amiloride (DMA) in mice	Colorectal cancer	Restore the cyclophosphamide (CTX) anti-tumor effect through the inhibition of MDSC functions	[40]
Extracorporeal filtration of exosomes (The Hemopurifier)	Advanced stage of cancer patients	To remove exosomes from the blood	[41]
Adjunct therapeutic method HER2osome	Breast cancer	Decrease the tumor secreted HER2 containing exosomes in circulation and afterward impede HER2 positive breast cancer progression	[42]

TABLE 6: Exosomes used for infectious diseases treatment.

Exosomal cargo	Secreting cell	Biological activity	Ref.
Exosomes pulsed with <i>Toxoplasma gondii</i> antigens	Dendritic cells	A defensive immune response against <i>Toxoplasma gondii</i> infection	[43, 44]
Exosomes pulsed with <i>Leishmania major</i> antigens	Dendritic cells	A defensive immune response against <i>L. major</i> infection	[45]
Exosomes containing M. tuberculosis antigens	Macrophage infected with M. Tuberculosis	Tuberculosis vaccine, activate innate/acquired immune responses	[46]
shRNA against HCV replication-loaded exosomes	Transfected into several cell types	Decrease in HCV infection of liver cells	[47]

suggested a wide range of methods to inhibit exosome production, which involve targeting microtubules assembly and stability and endosomal-sorting pathway and by using inhibitors of the proton pump [67, 88]. Marleau et al. have suggested a novel mechanism involving the use of extracorporeal hemofiltration of exosomes from circulation in order to minimize viral titers in patients [157]. Further, more information regarding exosomes removal from cancer and infected cells is listed in Table 5 [39–42].

11.3. Exosomes as Cancer Diagnostic Biomarkers. Depending on the type of tumor and location, exosomes can be isolated from almost all body fluids, including human saliva, serum, breast milk, CSF, urine, and semen [74–77]. Exosomes are novel sources of biomarkers because they contain bioactive molecules, which can help to assess the pathological state of the originated cells [40]. In addition, exosomes can be used to predict or monitor a patient's response to the given treatment. More importantly, exosomes provide noninvasive and continuous access to the required information to be used for tumor progression assessment [40]. It has been documented that the level of exosomes increased in some cancer patients in comparison to healthy individuals and has been correlated with poor prognosis [41]. Recent studies have shown that most of the circulating microRNAs detectable serum and saliva are concentrated in tumor-derived exosomes [152]. Extensive studies have shown that tumor-derived exosomes from either tumor cells or extracellular fluids of cancer patients can be used as biomarkers because they have a unique molecular signature on their biocontents (proteins, DNA/RNA molecules) [153–155]. A list of exosomes isolated

from biofluids of cancer patients used as biomarkers were prepared with details in previous studies [33, 40].

12. Exosomes Used for Infectious Disease Treatment

Bukong et al. have provided a novel mechanistic strategy for HCV transmission that can compromise immune-based therapies for HCV infection and thus suggested potential therapeutic strategies in order to block exosome-mediated transmission of HCV infection [132]. The clinical application of extracellular vesicles is discussed in detail: (i) therapeutic application and both (ii) diagnosis and therapy together [156]. Exosomes are recognized as a novel therapeutic tool for anti-tumor therapy, immune-modulation regenerative therapy, pathogen vaccination, and drug delivery but there is still a need for high level cooperation between researchers and expert clinicians for approval from recognized authorities [157]. Table 6 illustrates a number of studies that provided evidence for exosomes used for infectious disease treatment [43–47].

13. Benefits and Future Prospects

Exosomes are cell-derived membrane vesicles secreted by different cell types and present in body fluids. Proteomic profiling of EVs demonstrated that these vesicles play a valuable role in cellular communications and they act as natural vehicles for cell signaling proteins and genetic molecules. These findings indicated that it is possible to explore the vesicles as novel drug-delivery systems for many therapeutic agents that targeted different diseases such as cancers, infectious

diseases, and cardiovascular disease. European Cooperation in Science and Technology and International Society for Extracellular Vesicles (ISEV) are also constructing a network on Microvesicles and Exosomes in Health and Disease (ME-HaD) for ensuring the safety aspect and future application of exosomes in drugs.

In some cases exosomes not only are an ideal vehicle for therapeutic agents but also have been used as diagnostic and prognostic indicators for various cancers. Further studies of exosomes in the pathogenesis of cancer will open new avenues to explore novel diagnostic and therapeutic strategies. Isolation of vesicles of specific size and properties from the bacterial cells and application in drug delivery will be most fascinating research area in future because they will be cheaper and safe from cancers cell exosomes. Yield optimization and purification still require a single technique which is not available, and hopefully in future research will help in developing a single step device for development, purification, and characterization. Furthermore, the findings discussed in this review demonstrated that membrane-derived vesicles play a valuable role in infection biology. This applies to all organisms with different range of complexity from prions to eukaryotic pathogens. Further studies are needed to investigate more pathogens-derived exosomes that play a role in infection transmission and pathogenesis and to explore other novel therapeutic strategies to block exosome-mediated infection transmission. Further studies are also needed to explore novel bacterial species that are able to secrete outer membrane vesicles during their normal growth. More investigations are also needed to study the biogenesis of membrane vesicles in Gram-negative bacteria, which will help to generate more ideal outer membrane vesicles and improve human health. It is also important to study the mechanism of outer membrane vesiculation and to identify the essential envelop compositions that may play a role in vesicles generation. Therefore, a novel antibiotic could be designed to target these virulence components, which will help to inhibit bacterial growth and pathogens.

Novel insights for exosome-mediated drug-delivery systems were illustrated, which is important for the development of novel therapeutics and vaccines. In addition, there is an urgent need to develop advanced technologies to generate more controllable and homogenous membrane vesicles to be used for drug development. Finally, the findings discussed in this article showed the therapeutic effects of exosomes when loaded with drugs for the targeted diseases. The newly discovered drug-loaded exosomes should go for further animal testing and clinical trials to make the formulations ready in the market for clinical applications and to improve the therapeutic index of established drugs.

14. Conclusion

Taken together, these findings have shown that there is a relation between exosomes and cancer or infection biology, which is important for the development of novel therapeutics and vaccines. Exosomes are ideal candidates for drug delivery and further studies are needed to explore novel strategies of exosome-mediated therapies particularly for cancer

and infectious diseases. We have also discussed the major obstacles of exosome-mediated drug development and the most common methods used for exosomes generation and purification. We also confer the need for developing advanced technologies to generate more ideal and controllable exosomes for drug-delivery systems.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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