RESEARCH PAPER

Extracellular Vesicles as a Cargo in Therapeutics

Debangshi Dasgupta[1], Satyam Ravi Dwivedi[2], Dhiraj Kumar[2], Lokesh Chandra Mishra[2*], Gauri Mishra[3*]

[1] School of Life Sciences, Jawaharlal Nehru University - 110067, India
[2] Department of Zoology, Hansraj College, University of Delhi - 110007, India
[3] Department of Zoology, Swami Shraddhanand College, University of Delhi - 110036, India
***Corresponding Authors:** Gauri Mishra, Email: gaurimishra@ss.du.ac.in & Lokesh Chandra Mishra, Email: lcmishra@hrc.du.ac.in

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ABSTRACT

EVs (Extracellular vesicles) function as lipid-based nanoparticles that act as therapeutic vehicles. EVs release has been observed in both unicellular and multicellular organisms. EVs function is under exploration due to high loading capacity, low toxicity, immunogenicity, and bio-functioning. EVs function as essential for conveying genetic information to the cells of both healthy individuals and those with diseases. EVs act as vehicles for transporting proteins, lipids, nucleic acids, and other cellular metabolites. Numerous biologically active EVs derived from plants exhibit strong protective responses against diseases. The levels of EVs found in biological fluids are often studied for biomarker research in contexts of prognosis and medicative approaches. We herein discuss the classification of extracellular vesicles, their surface molecular composition, EVs serving as biomarkers, drug operators, synthetic mimetics, and their interaction with the immune system.

Keywords: EVs (Extracellular vesicles), SiRNA, Therapeutics, Biomarkers, Drug delivery.
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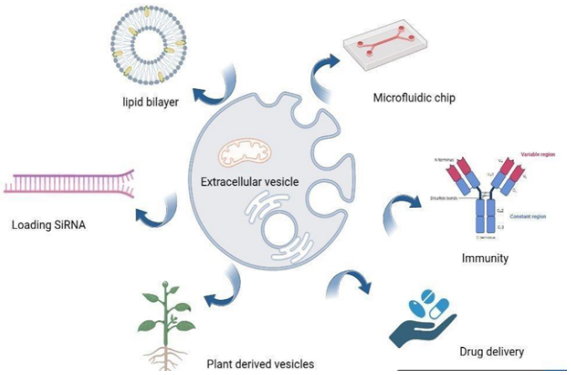
Graphical Abstract



INTRODUCTION

Cells release membrane-bound particles into the extracellular space, which make up the class of nanoscale structures known as extracellular vesicles (EVs). Microparticles, microvesicles, nanovesicles, calcifying matrix vesicles, oncosomes, prostasomes, secretomes, exosomes, exovesicles, exosome-like vesicles, ectosomes, and many more function as examples of cell-derived vesicles that function as EVs. Through a variety of mechanisms, including direct fusion, endocytosis, or receptor-mediated absorption as a type of intercellular communication, they contribute to the movement of numerous biomolecules, including DNA, RNA, lipids, and protein cargo, between cells and have an impact on a number of biological processes.

Numerous bodily fluids, including amniotic fluid, ascites, bile, blood, breast milk, cerebrospinal fluid, saliva, semen, and urine, are often discovered to contain EVs. Since EVs are often connected to several physiological and pathological problems, such as inflammation, cancer, neurodegeneration, infection, and immunological regulation, they function as acknowledged as a biomarker for the detection of disease conditions. [Sil et al. 2020; Février and Raposo 2004; Mathivanan, Ji, and Simpson 2010; Raposo and Stoorvogel 2013; Colombo, Raposo, and Théry 2014; Zaborowski et al. 2015].

EVs were previously believed to be unnecessary cellular debris, but major developments in nanomedicine have demonstrated their usefulness, particularly in the field of theranostics, which is based on nanostructured systems that contain medications for diagnosis and treatment. The route of entry and the chemical, physical, anatomical, and pathophysiological properties of the target also determine how a therapeutic agent is introduced and distributed throughout the body.

Depending on the context and the nature of the cargo, EVs can have various effects on target cells, such as modulation of gene expression. EVs can deliver nucleic acids to target cells and alter their gene expression profile. For instance, cancer cell exosomes have the ability to transform normal cells by transferring oncogenic miRNAs to them. Conversely, exosomes from immune cells can transfer anti-inflammatory miRNAs to inflamed cells and suppress their activation. EVs can deliver proteins to target cells and modulate by either activating or inhibiting signalling pathways.

For example, exosomes from mesenchymal stem cells can transfer growth factors to injured tissues and promote their regeneration. Conversely, exosomes from tumour cells can transfer inhibitors of apoptosis to immune cells and evade their killing. EVs can deliver metabolites to target cells and affect their metabolic state. For example, exosomes from hypoxic cells can transfer lactate to normoxic cells and induce glycolysis. Similarly, exosomes from adipocytes can transfer fatty acids to muscle cells and impair their insulin sensitivity.
This are potentially utilized as a drug-targeting delivery system that differs in composition, structure, and rate of drug release. This includes liquid crystals, nanocapsules, nanospheres, micelles, multifunctional dendritic polymers, phospholipid-based vesicular systems (liposomes), and polymer-based microparticulate systems. EVs are potentially synthesized from such artificial materials and loaded with desired cargo and surface molecules. Synthetic EVs can mimic the properties and functions of natural EVs but with improved control over their composition, size, shape and targeting [Wilczewska, Niemirowicz, and Markiewicz 2012; Yingchoncharoen, Kalinowski, and Richardson 2016]. The surface molecular composition of EVs is important for their recognition, uptake, and interaction with target cells. EVs function as heterogeneous in size, origin, and biogenesis [Raimondo et al. n.d.; Karamched et al. 2019].

EVs may find use in a number of biomedical domains, including immunomodulation, drug delivery, therapeutics, and diagnostics. Information about the existence, kind, stage, and prognosis of illnesses, as well as how well they respond to treatment, are potentially obtained from EVs. EVs are potentially designed to target particular cells or tissues and deliver particular medications. Additionally, EVs can get around some of the drawbacks of traditional drug delivery methods, like their toxicity, immunogenicity, and poor stability. For a variety of biological processes, including gene therapy, vaccination, and tissue engineering, EVs act as artificial mimetics. By reprogramming immune cells, generating tolerance or immunity, or increasing or decreasing immune responses, EVs are potentially used to modify the immune system in a desired way. Thus, EVs function as a promising field of research that offers new insights into the biology of cells and new opportunities for diagnosis and therapy.

Here, we will discuss Extracellular Vesicles (EVs) as an emerging and promising approach as a newer diagnostic and therapeutic strategy, their structural and molecular configuration, the diversified role of EVs in immunity, plant-based therapeutics, and synthetic mimetics. In this article, current knowledge on all the aspects of extracellular vesicles and applications of EVs is roofed.

1. Classification of extracellular vesicles

EVs function as a class of membrane-protected bioactive agent that facilitates signal transduction.

EVs function as generally divided into exosomes, microvesicles, and apoptotic bodies according to their capacity for biogenesis [Figure 1]. These vesicles function as studied based on transmission and immune microscopy, generic approaches, fluorescence imaging and other biochemical methods. Among the three types of EVs, exosomes have received the most importance.

Three categories of EVs are potentially distinguished based on the size and biogenesis process:
Exosomes function as 50-100 nm in size and composed of heat shock proteins, actin, tubulin, miRNA, mRNA, MHC molecules, and tetraspanins (CD63, CD81, CD82, CD9).
They originate from multivesicular endosomes bud through lumen and function as discharged through fusion [Fonseka, Marzan, and Mathivanan 2021].
Microvesicles Actin, tubulin, β1 integrin, VAMP3, and miRNA function as all found in microvesicles, which range in size from 100 to 1000 nm. Formed by budding off from the plasma membrane.
Apoptotic bodies function as 100-5000 nm in size and made up of Annexin V, thrombospondin, and other cellular components [Heijnen et al. 1999].

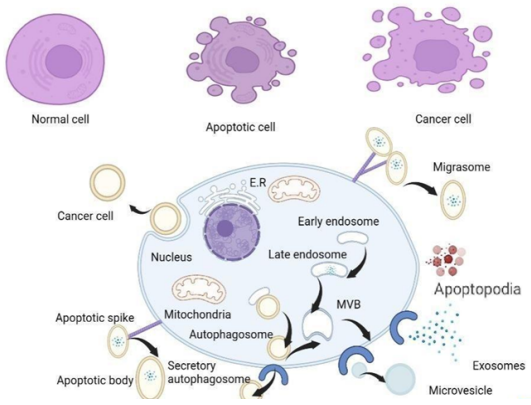


Figure 1: Types of Extracellular Vesicles

Large EVs, known as oncosomes, which function as mainly produced by malignant cells and range in size from 1 to 10 μm, can also develop from protrusions from the membrane's surface [Andaloussi et al. 2013].
EVs are released into the extracellular space as lipoproteic vesicles. EVs are potentially separated into populations with varying compositions using anion-exchange chromatography (AEC). By inserting field flow fractionation, tiny particles known as exomeres are often discovered in EVs. Exomeres function as aggregated, non-vesicular protein molecules that play a vital part in metabolism. Supermeres, which function as glycolytic enzymes with high levels of extracellular RNA, function as unique extracellular nanoparticles that have recently been discovered in the supernatant of exomeres [Mishra et al. 2022].

2. Molecular Composition

Lipids, proteins, nucleic acids, and glycans function as among the many compounds that make up extracellular vesicles. These molecules function as both essential and peripheral to the EV membrane.
The EVs' surface increases their signaling ability and serves as a biomarker, enabling the molecular classification of illnesses.

2.1 EV Membrane Lipids

Lipids function as the most critical component in the membrane of EVs. It constitutes about 50% of the mass of all eukaryotes and includes glycerophospholipid, sphingolipid, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, etc. [Alberts et al. 2002; Van Meer, Voelker, and Feigenson 2008].
Mass spectrometry of these lipids reveals that EVs originating from adipocytes exhibit uneven outer leaflet distribution of sphingomyelin and phosphatidylcholine [Figure 2], while the inner leaflet shows phosphatidylethanolamine and phosphatidylinositol [Durcin et al. 2017; Pook et al. 2014; Skotland et al. 2020].

Lipid orientation impacts EV membrane curvature, protein aggregation, and form. Diffraction and spectroscopy indicate EV membranes function as derived from JurkaT cells, yet recent research finds that 70% of EVs from complex fluids like plasma function as primarily lipoproteins and chylomicrons. They function as size and density with EV contaminants, and Dual-mode chromatography is employed to separate them [Mihály et al. 2017; Sódar et al. 2016].

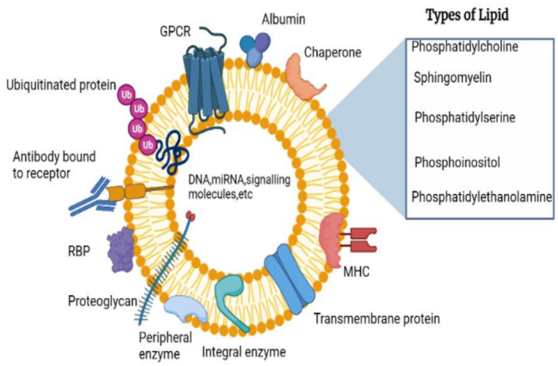


Figure 2: Molecular Composition of EVs

2.2 EV surface proteins

It is made up of several protein classes that function as both essential and auxiliary to the EV membrane. Peripheral or extrinsic proteins interact with the EV membrane through electrostatic interactions with hydrophilic head groups or non-covalent interactions with integral membrane proteins via their 16 α-helical transmembrane domains. [Yuana and others, 2014] The proteins on the surface of EVs provide information about their evolutionary history. According to one theory, these proteins perform similar tasks to those of their plasma membrane counterparts. Belov et al. (2016); R Xu et al. (2019) Numerous other studies have demonstrated that the protein or peptide present in exosomes serves a specific function for targeted therapy. A reversed, non-conventional membrane is seen in one-third of EV-associated transmembrane and lipid-anchored proteins, according to mass spectrometry of EV surface peptides from mast cells [Castillo et al. 2018].

2.3 EV surface glycoprotein and glycans

Numerous post-translational modifications, such as glycosylation, acetylation, phosphorylation, oxidation, methylation, and ubiquitination, function as known to be carried out by EVs. Nonetheless, covalently attaching to protein amino acid chains in one of four ways glycosylates 85% of human extracellular proteins are: N linked to asparagine, O connected to tyrosine, serine, and threonine, Tryptophan-related C and Glypiation.

According to Cvjetkovic et al. (2016), glycosylation aids in protein folding, stability, adhesion, transport, metastasis, signaling, and more. Glycoproteins function as filamentous and entangled structures that form a coronal layer, where glycans give the EV surface a negative charge, according to TEM imaging. EVs from patients with polycystic kidney disease have potential differential N-linked glycosylation. Similarly, EVs from metastatic colorectal cancer cells exhibit noticeably higher levels of O-glycosylation modification [Kesimer and Gupta 2015; Gerlach et al. 2013].

2.4 Nucleic acid on EV surface

The EV surface's nucleic acid facilitates intercellular communication. The presence of several DNA binding proteins on the EV surface indicates that different DNA sequences might attach to the surface. Its negative charge and aggregative property function as caused by the presence of DNA molecules [Chaiyawat et al. 2016; Yuana et al. 2014]. Systemic lupus erythematosus (SLE) and other autoimmune diseases function as associated with EV surface DNA.

The hallmark of SLE is 1L3 deficiency, which is typically generated by macrophages and dendritic cells and breaks down chromatin that is attached to the surface [Németh et al. 2017]. The EV surface also contains a large number of RNA nucleoproteins and RNA binding proteins (RBP). It is possible to genetically modify EVs to deliver siRNA. Numerous pieces of evidence support the use of EV-based therapies for the delivery of nucleic acids [Sisirak et al. 2016; Sódar et al. 2016].

3. EVs in Immune System

3.1 Innate immunity and inflammatory responses

Inflammatory immune cells release EVs with diverse roles in inflammation. EVs utilize arachidonic acid-dependent biologically active lipid mediators like eicosanoids for crucial behaviours [Hallal et al. 2022; Boilard 2018]. Neutrophil-derived EVs transfer arachidonic acid to platelets, which use cyclooxygenase 1 to generate thromboxane A2, triggering neutrophil discharge [Esser et al. 2010]. Platelet stem EVs transport arachidonic acid for enzymatic processing by platelet type 12 lipoxygenase (12LO). This results in the production of 12-hydroxyeicosatetraenoic acid, stimulating neutrophil internalization of platelet-derived EVs—an event examined in inflammatory arthritis [Rossaint et al. 2016]. EVs

can bind to extracellular matrix molecules [Duchez et al. 2015] potentially leading to stable chemotactic gradients or "trails" created by EVs from moving inflammatory cells, influencing other cellular layers [Buzás et al. 2018].

Both pro- and anti-inflammatory reactions function as shown by EVs [Sung and Weaver 2021]. EV-associated cytokines and damage-associated molecular patterns (DAMPs) like histones, high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), and mitochondrial DAMPs function as among the pro-inflammatory effects. These DAMPs promote leukocyte chemotaxis, T helper cell differentiation from naive T cells, and macrophage polarization to an M1-type phenotype and cytokine secretion. However, some EVs have anti-inflammatory properties, such as lowering serum levels of pro-inflammatory cytokines, reducing leukocyte chemotaxis, downregulating acute phase signaling and complement factors, and lowering the expression of adhesion molecules on endothelial cells. [Burgelman, Vandendriessche, and Vandenbroucke 2021].

3.2 Adaptive immunity

This section examines the development of T and B cells as well as how antigens function as presented to lymphocytes.

3.2.1 T and B cell development

It is well known that EVs derived from thymic epithelial cells transport tissue-restricted antigens to the thymus's traditional dendritic cells (DCs) for antigen presentation. T cells that function as specific for self-antigens may be negatively selected by EVs [Tu et al. 2020]. Furthermore, by containing proteins essential for their maturation and thymic withdrawal, such as sphingosine-1-phosphatase lyase 1 (SGPL1), Rho GDP-dissociation inhibitor 1 (GDIR1), dedicator of cytokinesis protein 2 (DOCK2), and p21 protein activated kinase (PAK), thymic epithelial cells derived from EVs play a part in promoting the maturation of single-positive (CD4+ or CD8+) thymocytes. [Skogberg et al. 2015].

EVs derived from CD24+ plasma membranes function as known to be released by immature primary bone marrow B cells during B cell development when CD24 is engaged by an antibody. It is hypothesized that EVs may have an impact on differentiating B cells because CD24 is known to play roles in bone marrow B cell development and selection [Lundberg et al. 2016].

3.2.2 Antigen presentation

The first significant finding demonstrating that EVs can play significant roles in adaptive immunity was the discovery of antigen-presenting EVs [Ayre et al. 2015]. Attaching the pMHC-carrying EVs to the surface of dendritic cells (DCs) increases the effectiveness of antigen presentation [Raposo et al. 1996]. For immune synapse formation and T-cell activation, it is assumed that "cross-dressed" DCs concentrate a large number of EV-associated pMHCs [Figure 3]. The "cross-dressed" DCs control this process, and exosomes attached to beads also promote greater T cell activation. Additionally, EVs present antigens through these mechanisms in a direct and semi-direct manner (mediated by cross-dressing). Antigen-presenting cells (APCs) can examine and uniformly process vesicles containing intact antigens and pMHC for indirect antigen presentation [Vincent-Schneider et al. 2002; Buzas 2022].

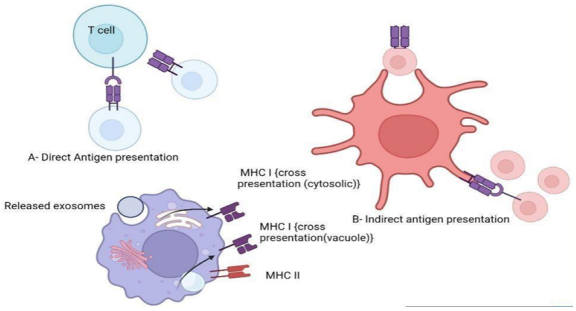


Figure 3: Immune Response by EVs

4. EVs as Biomarkers

EVs play a remarkable role in clinical molecular biology. Developing resilient EVs for liquid biopsies focuses on pathology. This involves targeting EV surface molecules and isolating them from complex bio-fluids [Table 1]. Recent investigations explore Micro-fluid and Microarray techniques due to their reliance on EV surface properties. In cancer patients' body fluids, EVs function as found to contain oncogenes, tumour suppressor genes, signature proteins, RNAs, and mutated DNA. DNA, a vital EV component, exists on the surface or within EVs, measuring around 200 bp. EVs contribute to pre-metastatic niche formation by stimulating angiogenesis, stroma and matrix remodelling, and immune response modulation [Théry et al. 2002].

By enhancing an isolation protocol for EVs derived from cerebrospinal fluid (CSF), EVs function as thought to be important in the detection of Parkinson's disease [Goričar, Dolžan, and Lenassi 2021]. They also contribute to the enrichment and increased detection of particular PD-associated mutations in LRRK2 (leucine-rich repeat kinase 2). By examining the expression of the HSP70 protein, isolated EVs from cancer patients' blood and urine are potentially used to detect malignant tumors early. [Anon. n.d.; Ciferri, Quarto, and Tasso 2021] .

The degree of protein expression of EV components are potentially ascertained using standard protein analysis methods like western blots, ELISA, and FCM. Additionally, EV RNA analysis are potentially conducted using nucleic acid analysis methods like RT-qPCR, digital droplet PCR, PCR arrays, RNA-sequencing, and microarray. Numerous methods based on microfluidic devices and chips are often developed for processing EVs. Utilizing fluids through micro-channels, micro-scaled EV isolation is the foundation for the design of microfluidic devices. Comparing this method to other isolation techniques, it processes fluids in incredibly small volumes (10 to 18 litres). Additionally, dielectrophoretic enrichment (DEP), ultrasonic purification, immunoaffinity beads, and microporous filtration systems can all be applied to microfluidic devices [Anon. n.d.; Pang et al. 2020].

Table 1: EVs as Biomarkers and its Application

Types of Samples	Uses	Reference
BAL fluid	Cause of smoking cigarette on EV miRNA profiles	[Sunkara et al. 2019]
Cerebrospinal fluid	Tumour-associated hypoxia	[Furuta et al. 2016]
Serum	Specific PD-associated mutations in LRRK2	[Qin et al. 2016]
Urine and blood	Detection of the HSP70 in cancer affected people	[Qi et al. 2016]

5. EV-based drug delivery system – an engineering approach

Although most of the research is focused on cancer treatment, EVs have the potential to deliver therapeutic drugs in a variety of animal disease models [Figure 4]. EVs derived from the cell line HEK-293 was used in the first study on EVs as drug-delivery agents. They were loaded with the gene for cytosine deaminase fused to uracil phosphoribosyl transferase, which caused cell death when prodrug 5-fluorocytosine was added.

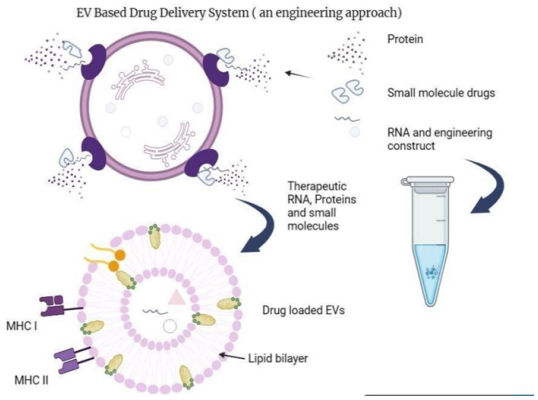


Figure 4: EV-based Medication Administration System

5.1 Adequate cytotoxicity and immunogenicity

EVs' inherent ability to have lower cytotoxicity and immunogenicity gives them a competitive edge [Kang, Kim, and Park 2017]. Synthetic liposomal nanoparticles, or liposomes, function as currently the mainstay for the delivery of small molecules and nucleic acids. However, their toxic immune response in vivo causes them to accumulate in the liver and perform their intended function less effectively.

EVs function as very biocompatible and natural. siRNA that targets the KRAS mutant protein was transported by exosomes. The results showed that, without triggering an immune response, siRNA exosomes delivered intravenously suppressed pancreatic cancer in mice more successfully than siRNA-loaded lipid nanoparticles [Armstrong and Stevens 2018; Johnsen et al. 2018; De Jong and Borm 2008; Zolnik et al. 2010].

5.2- Circulation Stability

EVs have a limited half-life of 2–20 minutes, which is shorter than liposomes [Armstrong, Holme, and Stevens 2017], despite their high stability in vivo [Fernandez-Fernandez, Manchanda, and McGoron 2011; Bell et al. 2016]. According to Wiklander et al. (2015), EVs function as frequently PEGylated, which extends their circulating time to over 60 minutes. Furthermore, PEGylation may increase IgM antibodies against it with repeated doses

[Kooijmans et al. 2016]. There have also been reports of several other changes to improve EV stability. For instance, in vivo stability is improved by EVs made from membrane-bound complement regulators CD55 and CD59 [Børresen et al. 2018]. Furthermore, EVs' duration in circulation is extended by the do-not-eat-me signal that is expressed by CD47-guided defense of EVs from single nuclear phagocytic systems [Clayton et al. 2003].

5.3 Properties of cell targeting

The heterogeneity of EVs, such as EV-derived hypoxic tumor cells, is dependent on various conditions [Kamerkar et al. 2017; Théry et al. 1999; Ludwig and Giebel 2012]. CNS-derived EVs function as specific to a group of neurons and have the ability to cross the blood-brain barrier. [Jung and others, 2018] Additionally, EVs made from microglia cells can target chronic inflammatory diseases of the central nervous system and multiple sclerosis [Shi et al. 2019].

However, scientists function as working to alter EVs by adding ligands that can attach to particular cells. To date, three approaches are often attempted in this regard:

1. Receptor-ligand binding
2. Antibody-antigen binding
3. The binding of molecules specific to the microenvironment [Casella et al. 2018]

A fusion protein of Lamp2b with neuron-specific rabies viral glycoprotein (RVG) peptide and muscle-specific peptide on the surface of EVs is used to bioengineer targeting of the brain and muscles [Limoni et al. 2019]. Additionally, by engineering anti-CD3 and anti-EGFR on the exosome surface, a second type of modification was discovered. This modification explains a cross-linkage between T cells and EGFR-cancer cells, resulting in strong antitumor immunity [Alvarez-Erviti et al. 2011]. Thirdly, hyaluronidase-engineered exosomes can target EVs and potentially degrade the [Cheng et al. 2018].

5.4 Drawbacks in EV-based drug delivery

5.4.1 Standardized isolation and purification methods function as inadequate

For the separation of EVs, there is no standard [Hong et al. 2018; P Li et al. 2017]. As messengers of cellular constituents, EVs function as abundant in blood, urine, saliva, and other biological fluids [Merchant et al. 2017] [Ruivo et al. 2017]. Therefore, it is difficult to develop efficient extraction and purification methods for EVs [Ferguson and Nguyen 2016; Nonaka and Wong 2017]. There function as five techniques for EV isolation: (i) isolation methods based on ultracentrifugation; (ii) isolation methods based on size (iii) methods based on immunoaffinity capture (iv) precipitation, and
(v) isolation methods based on microfluidics [Hong and others, 2018]

The most popular of these is ultracentrifugation [Street et al. 2017]. EVs function as grouped based on purification procedures, isolation strategies, and separation guidelines.

5.4.2 Inadequate drug loading capacity

EVs have a lower loading efficiency [Vergauwen et al. 2017]. The reason might be that there isn't much room for exogenous drug loading into EVs because EVs themselves function as components of their parent cells. Accordingly, a major obstacle is the loading of exogenous medications into EVs [Luan et al. 2017; Lai et al. 2013; Van Der Meel et al. 2014]. Numerous loading techniques are often created, including – (i) techniques for pre-loading such as transfection [SP [Batrakova and Kim 2015; Pascucci et al. 2014] co-incubation [Li et al. 2018; Akao et al. 2011; Ohno et al. 2013]. (ii) post-loading techniques such as electroporation [Sun et al. 2010], sonication [Tian et al. 2014], and incubation [Lee et al. 2015]. etc. (iii) Microfluidic synthesis of biomimetic lipid nanoparticles, which function as therapeutic platforms with inherent biological properties, and engineered to function as additional loading techniques.

Drug loading are potentially up to 11 times higher with saponin or hypotonic dialysis than with techniques like extrusion, electroporation, and incubation. Consequently, hypotonic dialysis and saponin show high loading efficiency [MS Kim et al. 2016]. Additionally, the application of RBC membrane-capped magnetic nanoparticles has improved cancer diagnostic techniques. [Z Li et al. 2019].

5.4.3 Clinical grades in production

These nanostructures must ensure both significant quantity and superior quality in a clinical setting [Rao et al. 2017; Lamparski et al. 2002]. GMP-grade EVs derived from antigen-presenting cells are often developed as a possible cancer vaccine [Rao et al. 2017]. Additionally, a large-scale production protocol for clinical-grade exosomes that complies with GMP standards was documented using a bioreactor. Sterile exosome generation with therapeutic contents, sufficient amounts for clinical trials, and consistent efficacy across batches function as all requirements of an ideal GMP-grade EV production process [Momen-Heravi et al. 2013; W Meng et al. 2020].

6. Loading of siRNA molecules

Because of its size, which prevents passive diffusion, and vulnerability to RNase conciliate degradation, siRNA delivery is difficult. On the other hand, the EV membrane's high capacity and protection aid in its delivery.

Electroporation, a technique that uses an electric field and conductive solution to create tiny holes in the EV membrane, was used to first incorporate siRNA into EVs. The penetration of siRNA is facilitated by these pores. EVs help deliver siRNA to lymphocytes and monocytes, preventing mitogen-activated protein kinase from 1. EVs loaded with siRNA were sequentially administered to mice that suppressed Brain expression of beta-site APP-Cleaving Enzyme 1 mRNA and proteins. HTT mRNA and protein in primary cortical neurons can also be targeted by siRNA-loaded EVs. The versatility of arrestin domain-containing protein 1 [ARRDC1]-mediated microvesicles (ARMMs) as an intracellular macromolecule delivery platform was recently highlighted by the discovery that ARMMs, a type of membrane-shed ectosomes, can package and deliver active siRNAs to recipient cells in a selective and efficient manner [Mendt et al. 2018].

Northern blotting, flow cytometry, and fluorescent microscopy were used to determine that the electroporation results in the encapsulation of siRNA. Another study demonstrates that even when EVs function as not present, electroporation causes large siRNA aggregates to form. However, membrane stabilizers, such as Trehalose Pulse Media (TPM), can also reduce the possibility of fusion between EVs, which is another potential consequence of electroporation [Q Wang et al. 2018].

7. Plant-based bioactive EVs

According to Bobo et al. (2016), there function as two distinct pathways for protein secretion in eukaryotic cells: the conventional protein secretion pathway and the unconventional protein secretion pathway. The alternative Golgi pathway and three pathways controlled by particular organelles—Multivesicular Bodies (MVB), Exosome-Positive Organelles (EXPO), and Vacuolar Bodies—function as among the unconventional protein secretion methods used to create plant EVs [Chung and Zeng 2017; Tse et al. 2004; Ding and Wang 2017]. These biologically active substances are potentially transported from in vitro to in vivo, then to the lesion tissue, and finally to cells by means of the numerous proteins, lipids, and miRNAs found in plant EVs [X Wang et al. 2018; Yim and Choi 2016].

Therefore, certain trans-boundary cellular or tissue responses function as mediated by plant EVs [Xiao et al. 2018; Zhang et al. 2012]. Among these, miRNAs derived from plants are potentially utilized for defensive responses in RNAs that function as important for the growth and development of plants [Chin et al. 2016]. For instance, broccoli miR159 can stop breast cancer cells from growing. [Zhang and others, 2012] Transfection of small RNA, which functions similarly to human miR34a, can reduce the protein expression of hsa-miR34a mRNA targets across borders and increase cell apoptosis in various tumors to slow their proliferation [Z Xu, Chen, and Hu 2022]. Additionally, HeLa cell proliferation are potentially considerably inhibited by certain small RNAs derived from maize [Potestà et al. 2020]. Because of their similar molecular makeup to animal EVs, plant EVs function as a delicate natural therapeutic agent that are potentially used to treat disease [Minutolo et al. 2018; Luo et al. 2017]. For instance, during wound healing and skin regeneration, exosomes derived from wheat, asparagus, and grapefruit function as antioxidants, improve cell viability, and encourage the proliferation of skin cells [Akers et al. n.d.; Rutter and Innes 2018; Şahin et al. 2019].

In many European nations, melanoma, one of the terrible skin conditions, is a major public health

issue. The incidence of melanoma is thought to be increased by UV radiation [MK]. Jackett and Scolyer 2019; Kim et al. 2021). In this context, ginseng is used as a traditional Chinese medicine in Southeast Asian countries to treat melanoma. By downregulating melanin-producing proteins, ginseng EVs, which function as isolated from ginseng cell culture supernatants, improve cellular melanosis brought on by UV-B radiation [Keim et al. 2021; Zhou and Yang 2015; L Meng et al. 2019]. Asparagus EVs have achieved new breakthroughs in the treatment of liver cancer [Cho et al. 2021]. Through immune pathways, plant EVs can also prevent cancer cells from proliferating and invading, as well as encourage apoptosis. Additionally, three routes of administration—oral, transdermal, and blood transport—have demonstrated the safety and efficacy of plant EVs [Zhang et al. 2021].

8. Synthetic EV mimetics for drug delivery

According to Abdul Rahman, Xiang Lian, and Mohana-Kumaran (2020), traditional EV engineering involves either directly synthesizing purified EVs or producing cells that secrete EVs. It are potentially difficult to use specific EV transport properties for drug delivery, such as isolating particular subtypes, because of the unknown degree of EV heterogeneity within a single cell. Large-scale standardized therapies function as hampered by the fact that EV characteristics differ depending on cell batches and passage numbers. Furthermore, widespread clinical production is hampered by the low yields from EV isolation. Designing artificial EV mimetics for drug delivery systems (DDSs) has become more popular as a result of this [Table 2]. In this regard, microfluidic systems provide easily accessible and repeatable settings for economical production scaling up.

8.1 Fully artificial EV mimetics production by Bottom-up synthesis

The bottom-up approach combines nanoscale components to create larger structures in order to create EV mimetics or lipid nanoparticles (LNPs). These liposome-like hyper structures function as made of proteins, nucleic acids, or medications. These EV mimetics are potentially developed using a variety of microfluidic techniques, such as droplet formation, hydrodynamic focusing, micro vortices, and chaotic mixing.

8.1.1. Hydrodynamic focusing

In order to create LNPs, a central fluid stream containing lipids dissolved in an organic solvent is focused using a sheath flow, usually water. By altering the flow rate and the ratio between the sheath and central flow, the size of the formed LNPs are potentially readily ascertained. For instance, LNPs of controllable size between 100 and 300 nm could be produced using T-shaped microfluidic geometry [Jahn et al. 2004]. Furthermore, it is most appropriate to use flow-focusing microfluidic geometry that is 3D printed and has an exceptionally high aspect ratio.

8.1.2. Micro vortices

By employing a three-inlet microfluidic platform to create symmetric micro vortices, micro vortices are potentially created in microfluidic systems and used for LNP formation at relatively high Reynolds numbers [Z Chen et al. 2019]. Doxorubicin and gold nanocrystals were combined to create these nanoparticles, which were then administered to mice as cancer treatment. [Y Kim and others, 2012] This demonstrates how this technology may be used in medical imaging and drug delivery. Additionally, as compared to bulk vortex mixing, counter-rotating vortices do quick substance swirling and DNA incorporation into LNPs result in noticeably higher production [Mieszawska et al. 2013].

8.1.3. Chaotic mixing

One of the most widely used microfluidic techniques for producing LNP is chaotic mixing. Gene silencing experiments use a similar microfluidic mixer to add siRNA to LNPs [Huang et al. 2019].

8.1.4. Droplet microfluidics

These instruments have proven to be very successful in creating reliable microparticles in the modern era and are employed in biological research. A hydrophilic agent (doxorubicin hydrochloride) is added to the aqueous core of core-shell emulsions, while a hydrophobic agent (paclitaxel) is added to the lipid shell [D Chen et al. 2012]. Nonetheless, the creation of complex, tiny multilayer particles highlights the range of potential drug delivery methods [Windbergs et al. 2013].

8.2. Top-down cell membrane-derived EV mimetics

For cells that function as far from EV mimetics, it involves the use of nanometer-sized structures. Silicon nitride blades function as widely used to create nanovesicles that contain polystyrene latex beads [Windbergs et al. 2013]. In EV mimetics, uncovered beads function as delivered around the recipient cells' plasma membrane, while beads cannot pass through the recipient cells' plasma membrane. It are potentially further purified by removing cell debris through a series of centrifugation steps. Few studies have recently addressed the use of microfluidic technologies to create EV mimetics derived from cell membranes. As a result, the cell membrane is a trustworthy source of EV mimetics, and new methods will probably be developed soon [Y Meng et al. 2021].

Table 2: Origin, Method and Application of EVs

Drug loading method	**Types of EVs**	**Drug/Agent**	**EVs origin**	***In vivo* or *in vitro***	**Disease target**	**Outcome**	**References**
Incubation	EVs	Paclitaxel	Prostate cancer cell	*In vitro*	Cancer	Inhibition of cancer	[Batrakova and Kim 2015]
	EVMs	Doxorubicin*	Monocytes	*In vitro*	Cancer	Inhibition of cancer	[Saari et al. 2015]
	EVMs	Doxorubicin**	Monocytes	*In vitro*	Cancer	Loading was successful	[Saari et al. 2015]
Sonication	EVs	siRNA/miRNA/ssDNA	Kidney cells	*In vitro*	cells	Knockdown of gene expression	[Goh et al. 2017]
Electroporation	EVs	i-126b-ASO/Cas9 mRNA/gRNA ii-Porphyrins iii-siRNA,miRNA, ssDNA	RBCs Endothelial, cancer and stem cells Kidney cells	*In vitro* or *In vivo* (mice) *In vitro* *In vitro*	Cancer Cells Cells	Inhibition of cancer Cellular uptake was higher than liposomes Knockdown of gene expression	[Lamichhane et al. 2016] [Usman et al. 2018] [Goh et al. 2017]
Freeze thaw	EVMs	Doxorubicin	Monocytes	*In vitro*	cells	Loading was successful	[Saari et al. 2015]
Saponification	EVs EVMs	Porphyrins Doxorubicin	Endothelial, cancer and stem cells Monocytes	*In vitro* *In vitro*	Cells Cancer	Cellular uptake was higher than liposomes Loading was successful	[Usman et al. 2018]) [Goh et al. 2017]

Extrusion	EVMs Hybrid EVMs EVs	i-Paclitaxel ii-LncRNA-H19 silencer VEGF siRNA Porphyrins	MSCs Cells of kidney Lipid composition of exosomes Stem, cancer and endothelial cells	*In vitro* or *in vivo* (mice) *In vitro* or *In vivo* (rat) *In vitro* *In vitro*	Cancer Diabetic wound model Cancer cells	Inhibition of cancer Accelerate the healing processes Inhibition of cancer Cellular uptake was higher than liposomes	[Kalimuthu et al. 2018] [Lu et al. 2018] [Tao et al. 2018] [Usman et al. 2018]
Drug treatment of parent cells	EVMs	Doxorubicin	Macrophage	*In vivo* or *In vitro* (in mice)	Cancer	Increased targeting and inhibition of cancer	[Jang et al. 2013]

LUSION

In the rapidly evolving landscape of biomedicine, Extracellular Vesicles (EVs) have emerged as remarkable lipid-based nanoparticles with immense therapeutic potential. These nanoscale entities, ubiquitous in both unicellular and multicellular organisms, represent a tempting frontier for biomedical innovation.

Once relegated to the status of cellular debris, EVs have transcended this perception to assume pivotal roles in intercellular signalling. Celebrated for their substantial cargo capacity, minimal toxicity, and immunogenic properties, these vesicles act as molecular carriers, shuttling a rich assortment of proteins, lipids, nucleic acids, and metabolites between cells. Notably, EVs function as conduits for the transfer of genetic information, exerting profound influence over gene expression and cellular behaviour.

The taxonomy of EVs encompasses a diverse array of subtypes, including exosomes and microvesicles, each with distinct attributes. These tiny messengers function as vital as disease detection biomarkers since they are often found in a wide range of body fluids and connected to a wide range of physiological and pathological processes. In the function of theranostics, EVs have etched a niche as versatile drug delivery vehicle. Their inherent advantages, encompassing precise control over composition, size, and target specificity, position them as a promising alternative to conventional drug delivery modalities. The exploration of synthetic EV mimetics heralds new pictures in tailored drug release.

Plant-derived EVs, characterized by unconventional protein secretion pathways, manifest potent therapeutic promise. Their multifaceted utility, spanning domains such as cancer therapy and wound healing, underscores the versatility of these natural agents.

The advent of microfluidic methodologies has revolutionized the isolation of EVs, enhancing both specificity and yield. These innovations bear profound implications for drug delivery and medical research, enabling the customization of EV mimetics endowed with tailored properties.

In summation, extracellular vesicles function as glorifying a transformative era in diagnostics and therapeutics. Their capacity to furnish insights into the presence, staging, and treatment responses of diseases, coupled with their potential for drug delivery and immunomodulation, gives them

burgeoning research prospects. As we plumb the depths of the structural and molecular intricacies of EVs, we unlock new vistas in diagnosis and therapy, illuminating a resplendent future for biomedicine.

Abbreviations-

AEC Anion Exchange Chromatography
APC Antigen Presenting Cells
ARMM ARRDC1 Mediated Microvesicles
ARRDC1 Arrestin Domain Containing protein 1
BAL Bronchoalveolar lavage fluid
BBB Blood Brain Barrier
Cas9 CRISPR associated protein 9
CD Cluster of Differentiation
CNS Central Nervous System
CSF Cerebrospinal Fluid
DAMPs Damage Associated Molecular Patterns
DC Dendritic Cells
DDSs Drug Delivery Systems
DNA Deoxyribonucleic acid
DOCK2 Dedicator of cytokinesis protein 2
EGFR Estimated Glomerular Filtration Rate
ELISA Enzyme linked immunosorbent assay
EVs EVs (Extracellular vesicles)
EXPO Exosome positive organelles
FCM Ferric Carboxymaltose
GDIR1 Rho GDP-Dissociation Inhibitor 1
GMP Guanosine monophosphate
GPCR G protein coupled receptor
gRNA guide Ribonucleic acid
HEK Human Embryonic Kidney
HMGB1 High Mobility Group Box 1
HMW High molecular weight
HSPs Heat Shock Proteins
IgM Immunoglobulin M
KRAS Kirsten Rat Sarcoma Virus
Lamp 2b Lysosome associated membrane protein 2
12LO Lipoxygenase
LncRNA Long non coding RNA
LNPs Lipid Nanoparticles
LRRK2 Leucine Rich Repeat Kinase 2
MHC Major Histocompatibility Complex
miRNA microribonucleic acid
mRNA messenger Ribonucleic acid
MVB Multivesicular Bodies
PAK p21 Protein Activated Kinase
PEG Polyethylene Glycol
RBC Red blood cell
RBP RNA binding protein
RNA Ribonucleic acid
RNase Ribonuclease
RVG Rabies Viral Glycoprotein
SGPL1 Sphingosine-1-Phosphate Lyase 1
siRNA small interfering RNA
SLE Systemic Lupus Erythematosus
ssRNA single stranded Ribonucleic acid
TEM Transmission Electron Microscope
TPM Trehalose Pulse Media
VAMP3 Vesicle Associated Membrane Protein 3
VEGF Vascular Endothelial Growth Factor A

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